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Advances in Brief

The SLUG Zinc-Finger Protein Represses E-Cadherin in Breast Cancer[1]

Karen M. Hajra, David Y-S. Chen, and Eric R. Fearon[2]

Program in Cellular and Molecular Biology [K. M. H., E. R. F.], Department of Molecular, Cellular, and Developmental Biology [D. Y-S. C.], Comprehensive Cancer Center [E. R. F.], and Departments of Internal Medicine, Human Genetics, and Pathology [E. R. F.], University of Michigan Medical School, Ann Arbor, Michigan 48109

Abstract

Loss of expression of the E-cadherin cell-cell adhesion molecule is important in carcinoma development and progression. Because previous data suggest that loss of E-cadherin expression in breast carcinoma may result from a dominant transcriptional repression pathway acting on the *E-cadherin* proximal promoter, we pursued studies of *cis* sequences and transcription factors regulating *E-cadherin* expression in breast cancer cells. E-box elements in the *E-cadherin* promoter were found to play a critical negative regulatory role in *E-cadherin* gene transcription in breast cancer cell lines lacking *E-cadherin* transcription. The E-box elements had a minimal role in *E-cadherin* transcription in breast cancer cell lines expressing E-cadherin. Two zinc-finger transcription factors known to bind E-box elements, SLUG and SNAIL, repressed *E-cadherin*-driven reporter gene constructs containing wild-type promoter sequences but not those with mutations in the E-box elements. Additionally, both SLUG and SNAIL repressed endogenous E-cadherin expression. These findings suggest SLUG and SNAIL are potential repressors of *E-cadherin* transcription in carcinomas lacking E-cadherin expression. Analysis of the expression patterns of *SLUG*, *SNAIL*, and *E-cadherin* in breast cancer cell lines demonstrated that expression of *SLUG* was strongly correlated with loss of *E-cadherin* transcripts. Taken together, the data indicate the E-box elements in the proximal *E-cadherin* promoter are critical in transcriptional repression of the *E-cadherin* gene, and SLUG is a likely *in vivo* repressor of *E-cadherin* in breast cancer.

Introduction

E-cadherin is a cell-cell adhesion molecule that participates in homotypic, calcium-dependent interactions to form the epithelial adherens junction. This function is critical in the development and maintenance of a polar epithelium. Loss of E-cadherin expression has been demonstrated in carcinomas arising in many tissues, and E-cadherin loss is believed to contribute to both cancer development and progression (1). Work in our laboratory, based on the analysis of somatic cell hybrids, demonstrated the loss of E-cadherin in breast cancer cell lines was because of dominant transcriptional defects, suggesting the presence of a transcriptional repression pathway that extinguishes *E-cadherin* expression (2). We narrowed the critical region of the *E-cadherin* promoter responsible for *E-cadherin* expression defects in breast cancer to bp −108 to +125 (2). Other groups have also implicated the proximal *E-cadherin* promoter and E-box elements contained therein in transcriptional inactivation of *Ecadherin* in some cancer cell lines (3–5). Recent studies of the *E-cadherin* promoter have additionally supported the critical role of the proximal promoter region in regulating *E-cadherin* expression. The work has also highlighted specific transcription factors that may function in repression of *E-cadherin* in cancer. These factors include the zinc-finger transcription factors SNAIL (4, 6), δEF1/ZEB-1 (7), and SIP1/ZEB-2 (5), and the basic helix-loop-helix factor E12/E47 (8). The specific factors that repress *E-cadherin* likely vary depending on cell type and context. Additionally, the various *E-cadherin* repression factors described to date may act alone or in concert, and there may be other currently undefined factors required for transcriptional silencing of *E-cadherin* in cancer cells. The goal of the studies described here was to determine the specific promoter element(s) and factor(s) critical for repression of *E-cadherin* in breast carcinomas. We found both SNAIL and its family member SLUG to be capable of repressing *E-cadherin* in epithelial cells via the E-box elements in the proximal *E-cadherin* promoter. However, *SLUG* expression showed a much stronger correlation with loss of E-cadherin in breast cancer cell lines than did *SNAIL* expression, suggesting SLUG is a likely *in vivo* repressor of *E-cadherin* expression in breast carcinoma.

Materials and Methods

Cell Culture. The RK3E cell line was provided by J. M. Rupert (University of Alabama, Birmingham, AL). The amphotropic Phoenix retrovirus packaging line was provided by G. Nolan (Stanford University, Stanford, CA). Both RK3E cells and Phoenix cells were grown in DMEM (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen Corp.) and 1% penicillin/streptomycin (Invitrogen Corp.). All of the other cell lines were acquired from the American Type Culture Collection (Manassas, VA) and maintained as recommended. For the generation of stable RK3E clonal lines expressing ER[3] chimera constructs, selection was carried out in medium supplemented with 1 μg/ml puromycin (Sigma Chemical Co., St. Louis, MO). After selection, the cell lines were maintained in medium containing 0.5 μg/ml puromycin. Designated cell lines were treated with medium supplemented with 0.5 μM 4-OHT (Sigma Chemical Co.) dissolved in 100% ethanol or mock treated with medium supplemented with 100% ethanol for the indicated time periods. SLUG- and SNAIL-expressing retroviruses were generated by transfecting plasmids into Phoenix packaging cells using FuGene6 as per the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). After transfection (24 h) into the packaging line, the medium was changed and an additional 24 h later virus-containing supernatant was harvested. The supernatant was filtered and diluted 1:1 with fresh medium. It was then supplemented with 4 μg/ml Polybrene (Sigma Chemical Co.) and used to infect MDA-MB-468 cells. After infection of MDA-MB-468 (48 h), selection was initiated in 0.5 mg/ml Geneticin (Invitrogen Corp.). Total protein lysates were prepared 2 weeks after commencing drug selection.

Plasmids. *Luciferase* reporter gene constructs containing wild-type *E-cadherin* promoter sequences were described in detail previously (2, 9). Briefly, *E-cadherin* promoter sequences were amplified by PCR and cloned into the pGL2-Basic vector (Promega Corp., Madison, WI) upstream of firefly *Luciferase*. In all of the reporter gene constructs, the endogenous initiating methionine of the *E-cadherin* gene, located at bp +125, has been destroyed, and an additional 33 bp of flanking sequence separate *E-cadherin* promoter

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[2] To whom requests for reprints should be addressed, at Division of Medical Genetics, University of Michigan Medical Center, 4301 MSRB3, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0638. Phone: (734) 764-1549; Fax: (734) 647-7979; E-mail: fearon@umich.edu.

[3] The abbreviations used are: ER, estrogen receptor; 4-OHT, 4-hydroxytamoxifen; Ecad+, intact *E-cadherin* transcription; Ecad−, defective *E-cadherin* transcription; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione *S*-transferase.

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sequences from the *Luciferase* initiating methionine. PCR-based site-directed mutagenesis was used for the generation of reporter gene constructs with E-box mutations. All of the mutant constructs were made within the context of the reporter gene construct Ecad(-108)-Luc, which contains *E-cadherin* promoter sequences from −108 to +125 of the endogenous *E-cadherin* gene upstream of firefly *Luciferase*. E-box elements were mutated from 5′-CANNTG-3′ to 5′-AANNTA-3′ (sense strand). Full-length cDNAs for human *SLUG* and *SNAIL* were amplified from cell line RNA by reverse transcription-PCR, and a COOH-terminal flag epitope tag was added by PCR. Constructs were subcloned into the retroviral expression vector pPGS-CMV-CITE-neo (gift of G. Nabel, NIH, Bethesda, MD). For the generation of vectors encoding SLUG- and SNAIL-ER fusion proteins, *SLUG* and *SNAIL* cDNAs were cloned into the pBabePuro plasmid (gift of A. Friedman, Johns Hopkins University, Baltimore, MD) upstream of a modified mouse ERα ligand-binding domain. The identities of all plasmid inserts and vector boundary regions were confirmed by sequence analysis. The pCH110 plasmid contains a functional *lacZ* gene expressed under the control of the SV40 early promoter (Amersham Biosciences, Piscataway, NJ).

Reporter Gene Assays. Cell lines growing at ~70% confluence in six-well plates were transfected using FuGene6 (Roche Molecular Biochemicals) according to the manufacturer's protocol. For experiments assessing activation of *E-cadherin* reporter gene constructs by endogenous factors, 0.8 μg *E-cadherin* reporter gene construct and 0.8 μg pCH110 were transfected per well. For experiments to determine the effects of SLUG and SNAIL on *E-cadherin* reporter gene activity, 1 μg effector plasmid (empty expression vector, SLUG expression vector, or SNAIL expression vector), 0.5 μg *E-cadherin* reporter gene construct, and 0.5 μg pCH110 were transfected per well. Experiments on the dose-dependent repression of *E-cadherin* reporter gene constructs by SLUG and SNAIL used 0.15 μg reporter construct, 0.35 μg pCH110, and increasing amounts of effector plasmid. In these dose-response studies, the total amount of transfected DNA was kept constant by adding empty expression vector as necessary. Cell extracts were prepared 36–40 h after transfection using reporter lysis buffer (Promega Corp.) followed by determination of luciferase and β-galactosidase activities. β-Galactosidase activity was used to normalize for transfection efficiency.

Northern Blotting. Total RNA was isolated from cells using TRIzol reagent (Invitrogen Corp.). Electrophoretic separation and membrane transfer of RNA were carried out by standard methods. For use as probes, *E-cadherin*, *SLUG*, *SNAIL*, and *GAPDH* cDNA fragments were labeled with [^{32}P]dCTP (Amersham Biosciences) by random priming with the RediPrime II kit (Amersham Biosciences). Prehybridization and hybridization were carried out in Rapid-Hyb buffer (Amersham Biosciences), the membrane was washed, and the blot was exposed to BioMax MS film (Kodak, Rochester, NY).

Antibody Production. GST fusion proteins were generated by subcloning cDNA sequences corresponding to the amino half of either SLUG (amino acids 1–151) or SNAIL (amino acids 1–146) into the vector pGEX-2T (Amersham Biosciences). Plasmids were introduced into the *Escherichia coli* strain BL21, and a large-scale preparation of recombinant protein was performed. After sonication of the bacteria, the fusion protein-containing supernatant was collected by centrifugation. GST fusion proteins were purified from this supernatant on a glutathione Sepharose 4B (Amersham Biosciences) column. Purified recombinant GST-SLUG protein was used directly as antigen for antibody production. Purified recombinant GST-SNAIL protein was separated by electrophoresis on a SDS-polyacrylamide gel, and the band corresponding to full-length GST-SNAIL was excised and used as antigen. Rabbit injection and serum collection were carried out by Covance Research Products Inc. (Richmond, CA). Serum was ammonium sulfate precipitated and then used either directly (anti-SNAIL antibodies) or purified (anti-SLUG antibodies) against a recombinant maltose-binding protein (MBP) fusion protein using the AminoLink Plus Immobilization kit (Pierce, Rockford, IL). Recombinant MBP-SLUG was generated by subcloning cDNA sequences corresponding to the amino half of SLUG (amino acids 1–151) into the plasmid pMAL-c2 (New England Biolabs, Inc., Beverly, MA), inducing recombinant protein expression and purifying the MBP-SLUG on a column of amylose resin (New England Biolabs, Inc.).

Immunoblotting. Whole cell lysates were prepared in radioimmunoprecipitation assay buffer [150 mM NaCl, 0.5% deoxycholic acid, 0.1% SDS, 1% NP40, 50 mM Tris (pH 8.0)] with Complete protease inhibitors (Roche Molecular Biochemicals). Approximately 35 μg of total protein per sample were separated by electrophoresis on SDS-polyacrylamide gels and transferred to Immobilon P membranes (Millipore Corp., Bedford, MA) by semi-dry electroblotting (Transblot; Bio-Rad Laboratories, Hercules, CA). Primary antibodies and the dilutions used were as follows: mouse monoclonal anti-flag M2 (Sigma Chemical Co.), 1:5,000; mouse monoclonal antibody HECD-1 against E-cadherin (Zymed Laboratories, Inc., San Francisco, CA), 1:5,000; rabbit polyclonal antibody A2066 against β-actin (Sigma Chemical Co.), 1:1,000; rabbit polyclonal anti-SNAIL, 1:2,000; and rabbit polyclonal anti-SLUG, 1:800. Secondary antibodies and the dilutions used were as follows: horseradish peroxidase-conjugated goat antimouse IgG antibody (Pierce), 1:20,000; horseradish peroxidase-conjugated donkey antirabbit IgG antibody (Pierce); 1:20,000 for antiactin blots; and 1:80,000 for anti-SNAIL and anti-SLUG blots. Antibody complexes were detected using Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Boston, MA) followed by exposure to X-OMAT AR film (Kodak).

Results and Discussion

Repression of E-cadherin Is Mediated via E-Box Elements in the Proximal Promoter. The proximal *E-cadherin* promoter contains multiple characterized elements including three E-boxes, a CCAAT box, and a GC-rich element. To assess the role of distinct elements in the regulation of *E-cadherin* gene transcription, promoter elements were mutated within the context of the reporter gene construct containing *E-cadherin* promoter sequences extending to −108 of the *E-cadherin* gene [Ecad(−108)-Luc]. This region was chosen because it is the minimal portion of the *E-cadherin* promoter demonstrating strong activity in breast cancer cell lines with intact *E-cadherin* transcription (Ecad+) and greatly reduced activity in breast cancer cell lines defective for *E-cadherin* transcription (Ecad−; Ref. 2). The three E-box elements in the proximal *E-cadherin* promoter were mutated, either singly or in combination, from their consensus sequence 5′-CANNTG-3′ in the sense strand to 5′-AANNTA-3′ (Fig. 1*A*). Mutations to the outer nucleotides of the 6-bp sequence were selected, because they have been reported previously to abolish factor binding (3). The mutant constructs were then tested in a panel of breast cancer cell lines of known *E-cadherin* transcription status, and the reporter activity compared with that of the wild-type *E-cadherin* promoter-driven reporter gene construct Ecad(−108)-Luc. As shown in Fig. 1*B*, mutation of the most 3′ E-box, EboxC, resulted in an increase in *E-cadherin* reporter gene construct activation in Ecad− lines with minimal effect on reporter activity in Ecad+ cell lines. Mutation of all three E-box elements in the proximal *E-cadherin* promoter additionally increased reporter gene activity in the Ecad− lines, again with minimal effect in Ecad+ lines (Fig. 1*B*). These findings suggest the E-box elements negatively regulate *E-cadherin* transcription in Ecad− lines, potentially via transcriptional repressor(s) binding to one or more of the E-box elements.

Of the three proximal E-box elements, EboxC appeared to play the most significant role in the repression of *E-cadherin* gene transcription in breast cancer cells (Fig. 1*B*; data not shown). Mutation of EboxA alone resulted in an ~2-fold derepression of *E-cadherin* reporter gene activity in Ecad− breast cancer cell lines, with no effect on activity in Ecad− breast cancer cell lines (data not shown). EboxB, the central of the three E-box elements, did not appear to significantly modulate *E-cadherin* gene transcription in either Ecad− or Ecad+ breast cancer cell lines (Fig. 1*C*). Our results differ from those reported by other investigators on the relative importance of the E-box elements in the proximal *E-cadherin* promoter. Specifically, a previous report suggested mutation of EboxB alone resulted in clear derepression of *E-cadherin* reporter gene activity in carcinoma cell lines (3). Additionally, whereas other studies have also implicated

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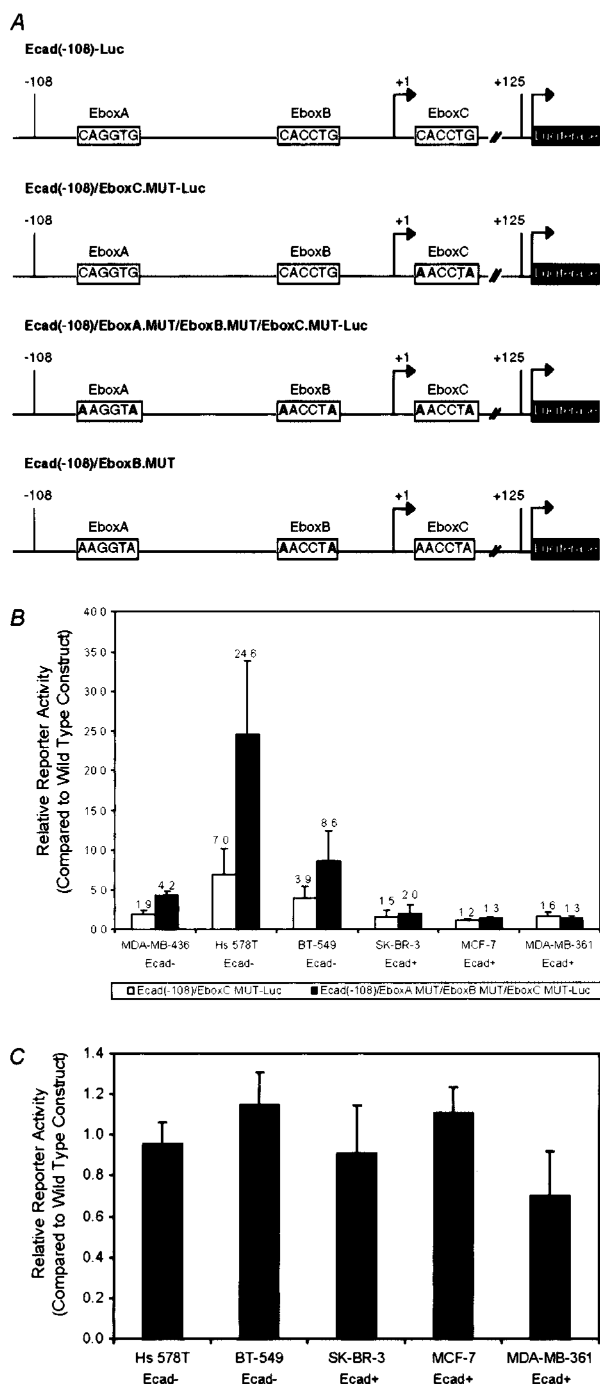

Fig. 1. Repression of *E-cadherin* expression in breast cancer cell lines is mediated via E-box elements in the proximal promoter. *A*, schematic of *E-cadherin* proximal promoter elements and the mutations created. The proximal *E-cadherin* promoter contains three E-box elements. Ecad(−108)-Luc contains wild-type promoter sequences from −108 to +125 of the endogenous *E-cadherin* promoter upstream of a *Luciferase* reporter gene. +1 indicates the transcription start site. The reporter gene constructs Ecad(−108)/EboxC.MUT-Luc, Ecad(−108)/EboxA.MUT/EboxB.MUT/EboxC.MUT-Luc, and Ecad(-108)/EboxB.MUT-Luc contain mutations in the most 3′ E-box, all three E-boxes, and the central E-box, respectively. Mutant residues are shown in **bold**, and for all E-box mutations the first and sixth nucleotides of the 6-bp E-box consensus sequence (5′-CANNTG-3′) have been mutated to adenines in the sense strand. *B*, repression of *E-cadherin* gene transcription is mediated via E-box elements in the proximal promoter. Reporter gene assays were carried out in three breast cancer cell lines defective for *E-cadherin* gene transcription (Ecad−) and three breast cancer cell lines with intact *E-cadherin* gene transcription (Ecad+). All experiments were performed in duplicate on at least two occasions. The average of the ratio of the activity of the mutant reporter construct to that of the reporter with wild-type sequence, Ecad(−108)-Luc, are shown; *bars,* ±SD. *C*, EboxB is not critical in regulating *E-cadherin* expression in breast cancer cell lines. Reporter gene activity of the Ecad(−108)/EboxB. MUT-Luc construct relative to that of the Ecad(−108)-Luc construct was assessed in a panel of breast cancer cell lines. All experiments were performed in duplicate on at least two occasions, and the average of the ratio of the reporter activities are shown; *bars,* ±SD.

E-box elements in the proximal *E-cadherin* promoter in regulating *E-cadherin* transcription in carcinoma cell lines (3–5), our results offer some new insights. For instance, the most 3′ E-box, EboxC, was the single largest contributor to repression of *E-cadherin* expression in breast cancer cells (Fig. 1*B*).

The three E-box elements in the proximal *E-cadherin* promoter are of a specific subclass of E-boxes; all contain the sequence 5′-CAC-CTG-3′. The sequence 5′-CACCT-3′ is known to bind members of the δEF1 zinc-finger transcription factor family, including δEF1/ZEB-1 and SIP1/ZEB-2. Both δEF1/ZEB-1 and SIP1/ZEB-2 have been proposed to repress *E-cadherin* transcription (5, 7). These factors are characterized by a protein domain structure in which a central homeodomain is flanked by NH_2- and COOH-terminal clusters of zinc-fingers, so that one monomer can bind to bipartite DNA elements (10). The SIP1/ZEB-1 protein has been proposed to repress *E-cadherin* transcription by simultaneously interacting with both EboxA and EboxB in the proximal promoter (5). Thus, our finding that EboxB is not critical in regulating *E-cadherin* transcription in breast cancer cell lines (Fig. 1*C*) may be of some significance. Given the likelihood that the EboxB mutation we created abolished SIP1/ZEB-1 binding, either SIP1/ZEB-1 is not a critical factor in the repression of E-cadherin in breast cancer or EboxB is not a necessary target for the binding of one zinc-finger of SIP1/ZEB-1 to the *E-cadherin* promoter.

Both SLUG and SNAIL Repress E-Cadherin *in Vitro*. We next sought to identify and characterize specific proteins that may bind to the E-box elements in the proximal E-cadherin promoter and repress transcription in breast cancer. In light of the established roles of Slug and Snail in the down-regulation of E-cadherin during epithelial-mesenchymal transitions in development (11, 12) and the recent suggestion that SNAIL represses *E-cadherin* transcription in carcinomas (4), we focused on the role of these factors in the repression of *E-cadherin* in breast cancer. SLUG and SNAIL belong to the larger Snail family of proteins, and contain an NH_2-terminal repression domain and a COOH-terminal zinc-finger DNA-binding domain (13).

Constructs expressing full-length, flag epitope-tagged SLUG and SNAIL were generated (Fig. 2*A*), and the effects of these proteins on *E-cadherin* reporter gene activity were assessed. When compared with the effects of the empty expression vector on *E-cadherin* reporter gene activity, both SLUG and SNAIL repressed the wild-type *E-cadherin* reporter construct in Ecad+ breast cancer cell lines (Fig. 2*B*). However, neither SLUG nor SNAIL could repress a construct in which all three of the E-box elements were mutant (Fig. 2*C*). Additionally, both SLUG and SNAIL demonstrated dose-dependent repression of the wild-type *E-cadherin* reporter gene construct Ecad(−108)-Luc in the Ecad+ breast cancer cell line MCF-7 (Fig. 2*D*). Taken together, these data show that SLUG and SNAIL are capable of repressing *E-cadherin* transcription *in vitro*, and this repression is mediated via the E-box elements in the proximal *E-cadherin* promoter.

***In Vivo* Repression of *E-Cadherin* by SLUG and SNAIL.** We chose to use a regulatable system to assess the effects of SLUG and SNAIL expression on endogenous E-cadherin expression in epithelial cell lines. For this purpose we used ER chimeric proteins, in which sequences from the protein of interest were fused to a modified ligand-binding domain from the murine ER α. The ligand-binding domain contains a mutation that renders it resistant to binding by endogenous estrogens yet capable of binding to the synthetic ligand 4-OHT (14). Chimeric proteins are constitutively expressed but inactive in the absence of ligand and activated after the exposure of cells to 4-OHT. Constructs expressing SLUG-ER and SNAIL-ER fusions were generated. Stable clones of rat kidney epithelial RK3E cells expressing one of the chimeric proteins were obtained, and expression of the chimeric proteins was confirmed (Fig. 3*A*). After 4-OHT treatment for 48 h, *E-cadherin* transcript levels were decreased in both

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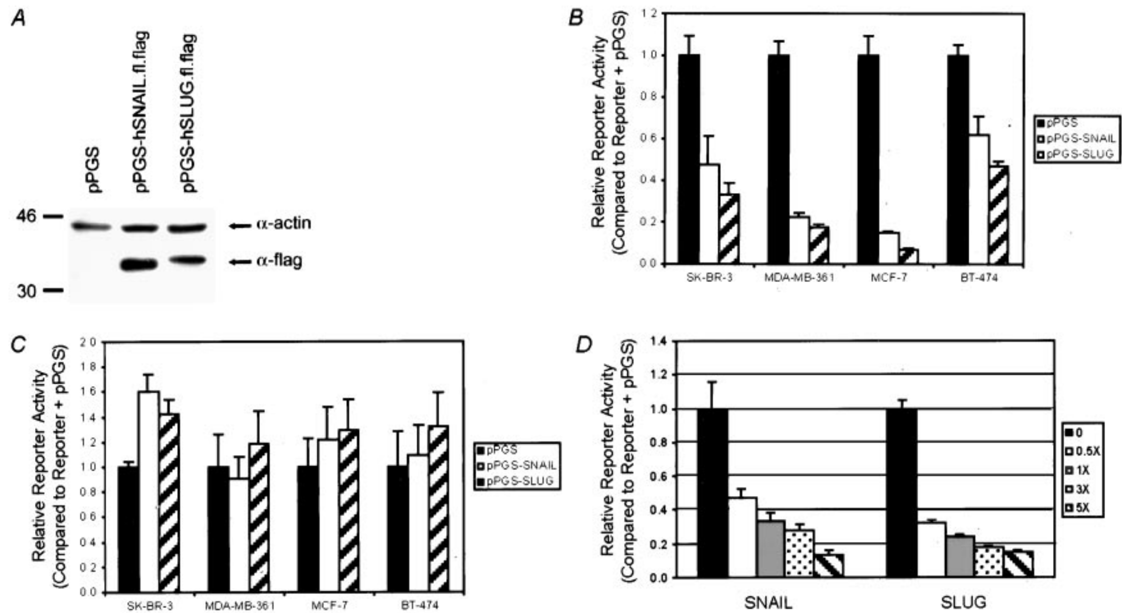

Fig. 2. SLUG and SNAIL repress *E-cadherin* transcription via the E-box elements in the proximal *E-cadherin* promoter. *A*, expression of flag epitope-tagged SLUG and SNAIL proteins. Empty vector, SNAIL, and SLUG constructs were transfected into 293T cells, lysates were prepared, and immunoblotting was performed. The antiflag signal indicates the expression of SNAIL and SLUG proteins, and the antiactin signal confirms equivalent loading of the lanes. The mobility of prestained molecular weight standards of specified sizes in kilodaltons is shown at the left. *B*, SLUG and SNAIL repress *E-cadherin* reporter gene activity. Empty expression vector or a vector encoding SNAIL or SLUG was cotransfected with the wild-type *E-cadherin* reporter gene construct Ecad(−108)-Luc into the indicated Ecad+ breast cancer cell lines, and reporter gene activity was assessed. *C*, SLUG and SNAIL do not repress an *E-cadherin* reporter gene construct with mutant E-box elements. The experiment was performed as in *B*, except the reporter gene construct Ecad(−108)/EboxA.MUT/EboxB.MUT/EboxC.MUT-Luc was used. For *B* and *C*, experiments were performed in duplicate on one or more occasions and the average of the ratio of luciferase values for the reporter plus SNAIL or SLUG *versus* the reporter plus empty vector are shown. *D*, SLUG and SNAIL demonstrate dose-dependent repression of an *E-cadherin* reporter gene construct in MCF-7 breast cancer cells. For each transfection, 0.15 μg of the *E-cadherin* reporter gene construct Ecad(−108)-Luc was used plus the indicated relative amount of expression vector encoding the candidate effector protein (SNAIL or SLUG). A constant amount of total DNA was used in each transfection, with increasing amounts of candidate effector protein. Each experiment was performed in duplicate and the mean of the luciferase activity of the reporter plus the indicated amount of effector protein relative to that of the reporter plus empty vector are shown; *bars,* ±SD.

the SLUG-ER and SNAIL-ER lines, whereas no change in *E-cadherin* expression was observed in parental RK3E cells (Fig. 3*B*).

Given the ability of SLUG and SNAIL to repress endogenous *E-cadherin* expression in RK3E cells, we sought to study repression of endogenous *E-cadherin* in E-cadherin-expressing breast cancer cell lines. Attempts to generate stable lines with constitutive expression of SLUG, SNAIL, or stable lines with 4-OHT-regulated SLUG-ER or SNAIL-ER chimeric proteins failed. These data suggest that in the breast cancer cell lines studied, SLUG and SNAIL may have deleterious effects on breast cancer cells when overexpressed. An alternate approach was pursued in which SLUG and SNAIL were introduced into the E-cadherin-expressing breast cancer cell line MDA-MB-468 by retroviral transduction, followed by a brief (2-week) G418 selection to eliminate nontransduced cells. Lysates were then made, and protein expression patterns were assessed. As shown in Fig. 3*C*, in this setting, both SLUG and SNAIL decreased E-cadherin protein levels. Thus, both SLUG and SNAIL can repress endogenous *E-cadherin* in the rat kidney epithelial line RK3E and the human breast cancer cell line MDA-MB-468.

Endogenous SLUG Expression Is Correlated with a Loss of E-Cadherin Transcription. Because both SLUG and SNAIL were capable of repressing *E-cadherin* promoter activity and endogenous E-cadherin expression, we sought to characterize expression of SLUG and SNAIL in breast cancer cell lines by Northern blot analysis. Expression of *SLUG*, rather than that of *SNAIL*, was strongly correlated with the absence of *E-cadherin* transcripts (Fig. 4). The data imply SLUG is the more likely *in vivo* repressor of *E-cadherin* transcription in breast cancer. In cell lines analyzed for their ability to activate *E-cadherin* reporter gene activities, *E-cadherin* promoter activities were reduced in lines with *SLUG* expression (Fig. 1*B*; data not shown). However, a tight correlation between the relative levels of endogenous *SLUG* transcripts and *E-cadherin* promoter activity was not observed in the three Ecad− cell lines studied, perhaps because SLUG protein levels may not be strictly tied to transcript levels, and differences in the expression of other proteins from one line to another may affect the ability of SLUG to repress *E-cadherin* transcription. A fraction of the cell lines studied showed seemingly discordant results in regard to *SLUG* and *E-cadherin* expression patterns (*i.e.,* SK-BR-3, MDA-MB-468, and BT-20; Fig. 4). Therefore, additional studies were carried out to clarify the relationship between SLUG and E-cadherin in the three lines.

SK-BR-3 has been shown to harbor a homozygous deletion of a large portion of the *E-cadherin* gene (15). Additionally, it has been demonstrated that SK-BR-3 retains all of the necessary *trans*-acting factors for *E-cadherin* transcription and, in fact, activates *E-cadherin* reporter gene constructs (9). These data are consistent with the view that if a *cis* genetic mechanism inactivates *E-cadherin* in SK-BR-3, defects in *trans*-acting pathways regulating *E-cadherin* gene transcription would not be expected. The lack of SLUG up-regulation in SK-BR-3 is consistent with this hypothesis. MDA-MB-453 showed *E-cadherin* and *SLUG* expression like that seen in SK-BR-3 (Fig. 4). Akin to SK-BR-3, we found that MDA-MB-453 activated *E-cadherin* reporter gene constructs (data not shown). Thus, the cell line retains the necessary *trans*-acting factors for *E-cadherin* gene transcription (Ecad+). A potentially subtle *cis* genetic alteration at the *E-cadherin* locus may underlie loss of detectable *E-cadherin* transcripts, because gross deletions or rearrangements of *E-cadherin* gene sequences were not seen on Southern blot analysis (data not shown). Alternatively, other mechanisms, such as promoter hypermethylation (16), could contribute to loss of E-cadherin in MDA-MB-453. In the BT-20 cell line both *SLUG* and *E-cadherin* transcripts were seen (Fig. 4), suggesting the *E-cadherin* gene may somehow be resistant to repression

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by SLUG in this cell line. No mutations were found in the E-box elements or elsewhere in the proximal *E-cadherin* promoter in the BT-20 cell line (data not shown). Possible explanations for the apparently discordant data are that the SLUG protein is unstable in this cell line, or necessary cofactors for SLUG-mediated repression are not expressed in the BT-20 line. The lack of insights into the identity of such cofactors precluded studies to address this hypothesis.

In closing, we would emphasize that our findings demonstrate the

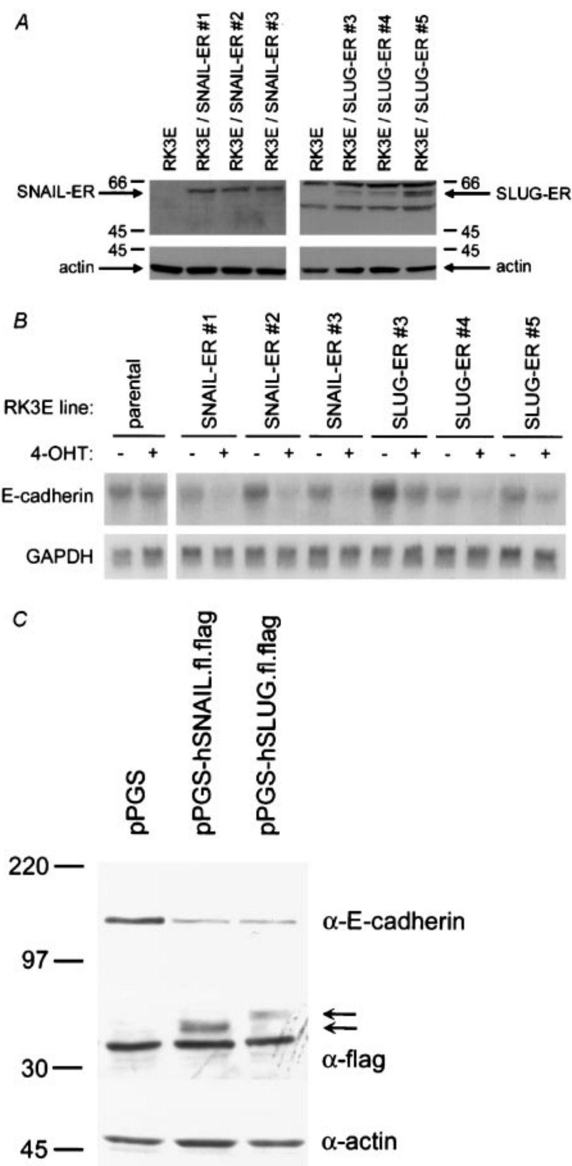

Fig. 3. SLUG and SNAIL repress endogenous *E-cadherin* expression. *A*, expression of ER fusion proteins in RK3E clonal lines. SNAIL-ER chimeras are detected with an anti-SNAIL antibody, and SLUG-ER chimeras are detected with an anti-SLUG antibody. The antiactin blot confirms equivalent loading of the lanes. The mobilities of prestained molecular weight standards of specified sizes in kilodaltons are shown at the sides. *B*, activation of ER fusion proteins decreases *E-cadherin* transcript levels. Cell lines were either treated with 4-OHT (+) or mock treated (−) for 48 h, then RNA was collected and Northern blot analysis was carried out. Treatment of parental RK3E cells does not decrease *E-cadherin* transcript levels, whereas all SNAIL-ER and SLUG-ER lines show a decrease in *E-cadherin* transcript levels after treatment with 4-OHT. The membrane was stripped and probed with a *GAPDH* cDNA fragment to demonstrate equal loading of the lanes. *C*, SLUG and SNAIL decrease endogenous E-cadherin levels in MDA-MB-468. MDA-MB-468 cells were infected with a control retrovirus (pPGS), a virus expressing SNAIL, or a virus expressing SLUG. Cells underwent antibiotic selection for 2 weeks to eliminate noninfected cells, followed by the preparation of total protein lysates. The antiflag blot demonstrates expression of SNAIL and SLUG proteins, indicated by the *arrows*. The antiactin blot serves as a loading control. The mobility of prestained molecular weight standards of specified sizes in kilodaltons is indicated at the *left*.

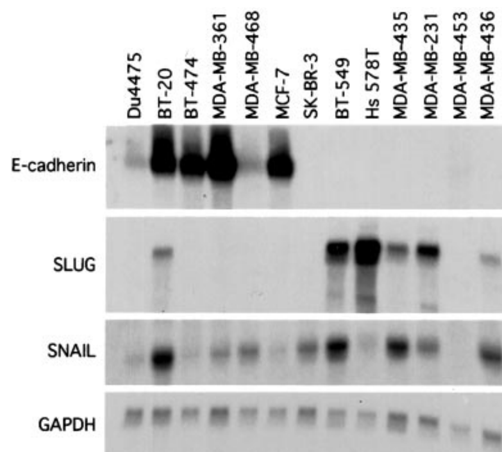

Fig. 4. SLUG expression is correlated with the absence of *E-cadherin* transcripts. Total RNA was collected from a panel of breast cancer cell lines, separated on an agarose-formaldehyde gel, and subjected to Northern blotting. The same membrane was hybridized sequentially with probes to *E-cadherin*, *SLUG*, *SNAIL*, and *GAPDH*. The *GAPDH* blot confirms roughly equivalent loading of RNA samples.

E-box elements, specifically EboxA and EboxC, contained in the proximal *E-cadherin* promoter appear critical in repression of *E-cadherin* gene transcription in breast cancer. Whereas SLUG and SNAIL were found to be capable of repressing *E-cadherin* gene transcription via these E-box elements, our data indicate that SLUG is a more likely *in vivo* repressor in breast cancer. Given the sizable number of potential transcriptional repressors thus far implicated in the repression of *E-cadherin* gene transcription (4, 5, 7, 8), it is possible that different factors function in the repression of *E-cadherin* in different settings. Alternatively, multiple factors may collaborate in mediating repression *in vivo*. Additional studies should help in resolving the remaining uncertainties about the mechanisms by which SLUG and other factors repress *E-cadherin* expression in breast and other cancers.

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