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EpCAM Is Overexpressed in Breast Cancer and Is a Potential Target for Breast Cancer Gene Therapy

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ABSTRACT

EpCAM (epithelial cell adhesion molecule) is a cell surface molecule that is known to be highly expressed in colon and other epithelial carcinomas. EpCAM is involved in cell-to-cell adhesion and has been the target of antibody therapy in several clinical trials. To assess the value of EpCAM as a novel target for breast cancer gene therapy, we performed real-time reverse transcription-PCR to quantify the level of EpCAM mRNA expression in normal breast tissue and primary and metastatic breast cancers. We found that *EpCAM* is overexpressed 100- to 1000-fold in primary and metastatic breast cancer. Silencing *EpCAM* gene expression with EpCAM short interfering RNA (siRNA) resulted in a 35–80% decrease in the rate of cell proliferation in four different breast cancer cell lines. EpCAM siRNA treatment decreased cell migration by 91.8% and cell invasion by 96.4% in the breast cancer cell line MDA-MB-231 *in vitro*. EpCAM siRNA treatment was also associated with an increase in the detergent-insoluble protein fraction of E-cadherin, α-catenin, and β-catenin, consistent with the known biology of EpCAM as a regulator of cell adhesion. Our hypothesis is that modulation of *EpCAM* expression can affect cell migration, invasion, and proliferation by enhancing E-cadherin-mediated cell-to-cell adhesion. These data provide compelling evidence that EpCAM is a potential novel target for breast cancer gene therapy and offer insights into the mechanisms associated with *EpCAM* gene silencing.

INTRODUCTION

The primary modality for breast cancer therapy remains surgery. Additional treatment modalities include radiotherapy, chemotherapy, and hormonal therapy. One of the main limitations of current treatment modalities is that systemic therapies for metastatic disease are not curative. In this context, gene therapy might prove a useful addition to, or a substitute for, conventional treatment modalities. Recent advances in molecular technologies have facilitated the search for genes that are overexpressed in metastatic cancers and could serve as novel targets for gene therapy. The advent of short interfering RNA (siRNA) provides the opportunity to rapidly determine whether these overexpressed genes are potential candidates for gene therapy.

EpCAM is a M_r 40,000, type I transmembrane glycoprotein that consists of two epidermal growth factor-like extracellular domains, a cysteine-poor region, a transmembrane domain, and a short cytoplasmic tail. It is encoded by the *GA733-2* gene located on the long arm of chromosome 4. EpCAM has been described by various names, including those associated with monoclonal antibodies specific for the cell surface antigen (MH99, AUA1, MOC31, 323/A3, KS1/4, GA733, and HEA125) and cDNA clones used to define the antigen [KS 1/4, EGP, EGP40, and GA733-2 (1–9)]. Litvinov *et al.* (4, 6) first suggested the name EpCAM, which more precisely reflects its function and tissue specificity.

EpCAM is detected at the basolateral membrane of the majority of epithelial tissues (all simple, pseudo-stratified and transitional epithelia), with the exception of the adult squamous epithelium and some epithelium-derived cells, such as hepatocytes, epidermal keratinocytes, gastric parietal cells, myoepithelial cells, and thymic cortical epithelium (2, 5, 7, 9). However, *de novo* expression of EpCAM can be observed for these cell types as well during active cell proliferation, whether normal or neoplastic (2, 3, 5, 10). Of particular interest, EpCAM appears to be overexpressed by the majority of human epithelial carcinomas, including colorectal, breast, prostate, head and neck, and hepatic carcinomas (1–3, 11–14). For this reason, EpCAM has attracted major attention as a target for monoclonal antibody-based immunotherapy to combat a spectrum of malignancies, most notably colorectal carcinoma (15–17). Indeed, the use of the EpCAM-specific monoclonal antibody has been successful in increasing disease-free survival in colon and breast cancer patients with minimal residual disease (15, 17). These antibodies contribute to tumor cell destruction by activating an array of endogenous cytotoxic mechanisms, including antibody-dependent complement-mediated cytotoxicity (18, 19). In addition to being a target of monoclonal antibody-based immunotherapy, there is evidence that EpCAM expression levels correlate with proliferative activity and contribute to neoplastic transformation (1, 3, 5, 7, 10). In fact, EpCAM overexpression in primary breast cancers is associated with poor disease-free and overall survival (20). These data suggest that EpCAM is a potential target for molecular intervention and that it requires further investigation.

In this study, we demonstrate that EpCAM is highly overexpressed in primary and metastatic breast cancer. In addition, we demonstrate that silencing *EpCAM* gene expression decreases the proliferation, migration, and invasion potential of breast cancer cell lines *in vitro*. To define the mechanisms associated with *EpCAM* gene silencing, we investigate the effect of EpCAM siRNA treatment on the gene expression and protein levels of the cell adhesion molecule E-cadherin and related molecules, α-catenin, and β-catenin. Based on the results of this study, we conclude that EpCAM is a potential novel target for breast cancer gene therapy.

MATERIALS AND METHODS

Tissue Specimens. Tissue specimens were obtained in accordance with federal and institutional guidelines as described previously (21). Primary breast cancer specimens and axillary lymph nodes were obtained from breast cancer patients undergoing surgical therapy for the treatment of breast cancer. The tissues were evaluated immediately by a surgical pathologist at the time of acquisition. For the purpose of real-time reverse transcription-PCR (RT-PCR) analysis, the specimens were identified and bisected. One portion was processed for real-time RT-PCR, and the other portion was sent for routine pathology analysis. All specimens for RT-PCR analysis were immediately snap-frozen in liquid nitrogen by the Hollings Cancer Center Tissue Procurement/Tissue Bank technician to prevent RNA degradation. They were then stored at −70°C in the Hollings Cancer Center Tissue Bank until total RNA processing could be performed.

Normal lymph nodes from patients without evidence of cancer were obtained from patients undergoing elective carotid endarterectomy. None of these patients had any history or clinical evidence of cancer. At the time of the procedure, a single cervical lymph node was removed and processed as noted above.

Normal breast tissue specimens were obtained from patients at Saint

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Vincent's University Hospital (Dublin, Ireland) undergoing breast surgery for benign and malignant breast disease. All tissue specimens were evaluated immediately at the time of excision by a surgical pathologist, and normal breast tissue was identified. In cases in which the procedure was performed for malignant disease, normal breast tissue was obtained from a part of the specimen at least 3 cm from the malignant disease. Tissues were then snap-frozen in liquid nitrogen and stored at −70°C. Specimens were shipped overnight to the Medical University of South Carolina in dry ice.

Cell Lines. All cell lines were obtained from American Type Culture Collection (Manassas, VA). Media and fetal bovine serum (FBS) were purchased from Gibco-BRL (Rockville, MD). Growth conditions of cell lines were as follows: MCF-7, RPMI 1640 with 10% FBS; MDA-MB-361, Leibovitz's L-15 medium with 10% FBS in a non-CO_2 environment; MDA-MB-453 and MDA-MB-231, Leibovitz's L-15 medium with 10% FBS in a non-CO_2 environment; and MCF-10A, serum-free mammary epithelial cell medium (MECM). For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells were plated in 96-well plates at 1.5×10^4 cells/well. For real-time RT-PCR, cells were seeded in 6-well plates at 1×10^5 cells/well.

RNA Isolation. Total cellular RNA was isolated from breast cancer cell lines, normal lymph nodes, and lymph nodes from breast cancer patients using a guanidinium thiocyanate-phenol chloroform solution (RNA STAT-60t; TEL-TEST, Friendswood, TX). Briefly, a single lymph node specimen was removed from −70°C storage and weighed as quickly as possible without allowing the tissue to thaw. Tissue (0.15 g) was then homogenized in 1.5 ml of RNA STAT-60 using a model 395, type-5 polytron (Dremel, Racine, WI). Total RNA was isolated as per the manufacturer's instructions, except that 1 μl of a 50 mg/ml solution of glycogen (Sigma, St. Louis, MO) was added to the aqueous phase immediately before the addition of isopropanol. Cells from the cancer cell lines listed above were harvested at near-confluence from one 75-cm^2 flask, pelleted, and resuspended in 1.5 ml of RNA STAT-60. Final RNA pellets were dissolved in 50 μl of diethyl pyrocarbonate-treated water. RNA yield was determined by spectroscopy. Complementary DNA was made from 5 μg of total RNA using 500 ng of oligo(dT). RNA was reverse transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in a reaction volume of 20 μl (10 min at 70°C, 50 min at 42°C, and 15 min at 70°C).

Real-Time Reverse Transcription-Polymerase Chain Reaction. Real-time RT-PCR analyses were performed on a Gene Amp 5700 Sequence Detection System (PE Biosystems, Foster City, CA). The standard reaction volume was 10 μl and contained 1× QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA), 0.1 unit of AmpErase UNG enzyme (PE Biosystems), 0.7 μl of cDNA template, and 0.25 μM of both forward and reverse primers. The initial step of PCR was 2 min at 50°C for AmpErase UNG activation, followed by a 15-min hold at 95°C. Cycles ($n = 40$) consisted of a 15-s denaturation step at 95°C, followed by a 1-min annealing/extension step at 60°C. The final step was a 60°C incubation for 1 min. All reactions were performed in triplicate. The data were normalized to an internal control gene, β_2-microglobin, to control for RNA preparation. Real time RT-PCR results were analyzed using Q-Gene software (22), which expresses data as mean normalized expression (MNE). MNE is directly proportional to the amount of RNA of the target gene (EpCAM) relative to the amount of RNA of the reference gene (β_2-microglobin). The SE is calculated by Q-Gene software using the differential equation of Gauss (22).

Primer Design. Primers for EpCAM, E-cadherin, α-catenin, β-catenin, and β_2-microglobin were designed using Perkin-Elmer Primer Express software. These primers were designed to be intron-spanning to preclude amplification of genomic DNA. Primer sequences were as follows: EpCAM, 5′-CGCAGCTCAGGAAGAATGTG-3′ (forward) and 5′-TGAAGTACAC-TGGCATTGACG-3′ (reverse); E-cadherin, 5′-TTCCGAAGCTGCTAGTCT-GAGC-3′ (forward) and 5′-GCCCCATTCGTTCAAGTAGTCA-3′ (reverse); α-catenin, 5′-AATTGCTGAGGCAGGATCCA-3′ (forward) and 5′-TGAC-CTTGCTGCAGATGTTCA-3′ (reverse); and β-catenin, 5′-TGGCCTGGTT-TGATACTGACCT-3′ (forward) and 5′- CTCTACAGGCCAATCACAAT-GC-3′ (reverse).

RNA Interference. The siRNAs were purchased from Qiagen (Santa Clarita, CA). Gene silencing of *EpCAM* expression was performed essentially as described previously using sequence-specific siRNA reagents (23).[3] Briefly, 100,000 cells were plated in each well of 6-well plates and allowed to grow for 24–36 h (until they were 40–60% confluent). Short interfering RNA was then transfected into cells at a concentration of 200 nM using Oligofectamine reagent (Invitrogen, Carlsbad, CA) and serum-free medium. After 4 h of incubation, serum-rich medium was added. Human EpCAM siRNA sequences were 5′-CUACAAGCUGGCCGUAAACdTdT-3′ and 5′-GUUUACGGC-CAGCUUGUAGdTdT-3′. Scrambled sequences were 5′-UUCUCCGAACG-UGUCACGUTdTd-3′ and 5′-ACGUGACACGUUCGGAGA ATdTdT3′. All sequences were evaluated for gene specificity using the National Institutes of Health Blast program.

MTT Assay. Cells plated in 96-well plates were grown in their respective media for 24, 48, or 72 h after the addition of siRNA. At each time point, cells were checked visually for growth and proliferation, and then MTT (Sigma) was added to the wells, and the cells were incubated at 37°C for 4 h. Next, MTT solubilization solution (10% Triton X-100 in acidic isopropanol, 0.1 N HCl) was added, and the cells were incubated overnight. Colorimetric measurements were made in a microplate reader (Molecular Devices) at 562 nm, and background was subtracted at 650 nm.

***In Vitro* Invasion/Migration Assay.** BioCoat Matrigel invasion chambers (12-well cell culture inserts containing an 8.0-μm PET membrane with a uniform layer of Matrigel matrix; Becton Dickinson, Bedford, MA) were used to assess cell invasion. Cell migration was assessed in BioCoat control cell culture chambers (12-well cell culture inserts containing an 8.0-μm PET membrane without a Matrigel layer). The membranes were rehydrated with warm serum-free Dulbecco's modified Eagle's medium (1.0 ml/chamber) for 2 h. The MDA-MB-231 breast cancer cell line was used for this experiment. The upper chamber was filled with 1×10^5 cells in L-15 medium containing 5% FBS. The lower chamber was filled with L-15 medium containing 25% FBS as a chemoattractant. The chambers were incubated for 24 h at 37°C in a 5% CO_2 atmosphere. Cells from the upper surface of the membranes were removed by scrubbing them with a cotton swab, and those on the lower surface of the membranes were fixed and stained with H&E. The number of cells that penetrated the filter was counted by a technician blinded to the experimental settings in four microscopic fields of each filter, under ×20 magnification, in both Matrigel and control membranes. The percentage of invasion is expressed as the ratio of the mean cell number from invasion chamber to the mean cell number from control chamber according to the manufacturer's recommendation. The percentage of migration was expressed as the ratio of the mean cell number in control inserts containing siRNA treated cells to mean cell numbers in control inserts containing untreated cells (untreated cells were given a value of 100%).

Cell Lysis and Protein Extraction. To prepare total cell lysates, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 300 μl of hot (100°C) 1% SDS/10 mM EDTA. The extraction of the detergent-soluble (not anchored to the cytoskeleton) and detergent-insoluble proteins (anchored to the cytoskeleton) was performed as described by Hinck *et al.* (24), with minor modifications. Cells were rinsed three times with cold PBS, and then 300 μl of cold extraction buffer [50 mM Tris-HCl (pH 7.0), 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, 300 mM sucrose, and 0.1% protease inhibitor mixture (Sigma)] were added to the cells. Cells were incubated for 45 min on a shaker at 4°C, detached with a scraper, collected, and spun down in an ultracentrifuge for 1 h at 100,000 × g at 4°C. The supernatant was collected, and the pellet was lysed with 300 μl of hot (100°C) 1% SDS/10 mM EDTA and then boiled for 5 min. The protein concentration was determined using the Bio-Rad DC protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

Immunoblotting. Aliquots from the total protein lysate, and soluble and insoluble protein fractions were resolved on 8.0% polyacrylamide gels (SDS-PAGE) under denaturing conditions and then transferred to 0.45 μm nitrocellulose membranes. After blocking with 5% nonfat milk in PBS/0.1% Tween 20 (PBST) and washing, membranes were incubated with primary antibodies for 3 h at room temperature and then washed three times with PBST for 5 min each. The blots were probed using specific primary antibodies to mouse anti-EpCAM-1 (Oncogene Research Product, San Diego, CA), mouse anti-E-cadherin, rabbit anti-α-catenin, and rabbit anti-β-catenin (Zymed Laboratories, Inc., San Francisco, CA.). The signal was detected using the appropriate

[3] http:www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html.

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horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescence immunoblotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical Analysis. Statistical analyses included the independent *t* test and analysis of variance. Statistical analyses were performed using SAS Version 9.0 Software (SAS Institute, Inc., Cary, NC).

RESULTS

***EpCAM* Is Highly Expressed in Primary Breast Cancers.** *EpCAM* expression levels were determined in nine normal breast tissues and nine primary breast cancers by real-time RT-PCR analysis (Fig. 1). In the normal tissues, the MNE ranged between 6.45E-06 and 4.22E-04 (mean = 1.43E-04; SE = 6.85E-05). For the cancer tissues, the MNE ranged between 1.26E-02 and 1.16E-01 (mean = 5.68E-02; SE = 9.10E-03). These results indicate that *EpCAM* expression is approximately 100-fold higher in primary breast cancers than in normal breast tissue ($P < 0.01$).

***EpCAM* Is Significantly Overexpressed in Lymph Nodes Containing Metastatic Breast Cancer.** Real-time RT-PCR analysis was performed on 24 normal lymph nodes and 24 lymph nodes containing metastatic breast cancer (Fig. 2). For the normal lymph nodes, the MNE ranged between 6.09E-6 and 4.03E-4 (mean = 7.76E-5; SE = 1.80E-5). For the lymph nodes containing metastatic breast cancer, the MNE ranged between 3.37E-5 and 4.2E-1 (mean = 6.26E-2; SE = 2.27E-2). The mean expression level in metastatic lymph nodes is therefore approximately 810-fold higher than the mean expression level in normal lymph nodes ($P < 0.01$).

To determine the diagnostic value of EpCAM as a molecular diagnostic marker, we performed a receiver operating characteristic curve analysis (data not shown). Receiver operator characteristic curve analysis is based on a plot of sensitivity as a function of 1 − specificity. The area under the curve is a measure of the diagnostic accuracy such that values between 0.5 and 0.7 indicate low accuracy, values between 0.7 and 0.9 indicate moderate accuracy, and values greater than 0.9 indicate high accuracy (25). For EpCAM, the area under the curve was 0.951 (95% confidence limit, 0.872–0.990), indicating that EpCAM can be used as a molecular diagnostic marker to detect metastatic breast cancer in axillary lymph nodes with high accuracy. Another way to define the potential value of EpCAM as a molecular diagnostic marker is to define a threshold and determine the sensitivity and specificity at this defined threshold level. In recent publications, we have consistently defined a threshold based on the expression levels in normal lymph nodes in an attempt to maximize specificity. The threshold value that we have consistently used in our recent publications is based on an expression level that is 3 SDs greater than the mean in normal lymph nodes (26). At this threshold level, 22 of 24 (92.7%) pathology-positive axillary lymph nodes have molecular evidence of metastatic breast cancer. This result underscores the potential value of EpCAM as a molecular marker for the detection of metastatic breast cancer and confirms the potential relevance of this gene as a target of gene therapy.

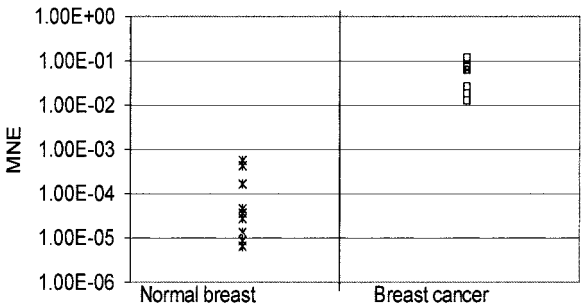

Fig. 1. Real-time RT-PCR analysis of nine normal breast and nine primary breast cancer samples. *EpCAM* gene expression levels in nine primary breast cancer specimens, obtained from breast cancer patients undergoing surgical therapy for the treatment of breast cancer, and nine normal breast tissue specimens, obtained from patients undergoing breast surgery for benign and malignant breast disease, were determined using real-time RT-PCR. The data are expressed as MNE, which is directly proportional to the relative amount of mRNA in a given tissue. The MNE of each sample was determined from triplicate reactions using the Q-Gene software ($P < 0.01$).

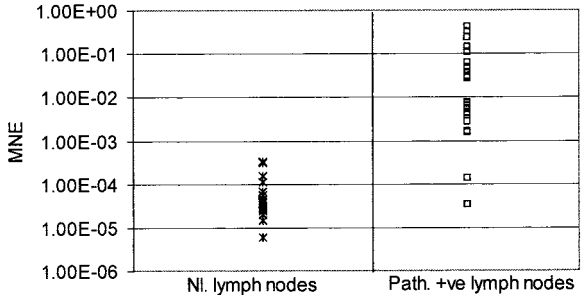

Fig. 2. Real-time RT-PCR analysis of normal control and pathology-positive lymph nodes. *EpCAM* gene expression levels in 24 normal control normal lymph nodes, obtained from patients undergoing elective carotid endarterectomy, and 24 pathology-positive axillary lymph nodes, obtained from breast cancer patients undergoing surgical therapy for the treatment of breast cancer, were determined using real-time RT-PCR. The data are expressed as MNE, which is directly proportional to the relative amount of mRNA in a given tissue. The MNE of each sample was determined from triplicate reactions using the Q-Gene software ($P < 0.01$).

EpCAM Short Interfering RNA Treatment Decreases *EpCAM* Gene Expression and Proliferation of Breast Cancer Cell Lines. Short interfering RNA is a relatively novel but well-recognized method for gene silencing in mammalian cells (27, 28). The action of siRNA relies on the fact that the presence of short, double-stranded RNA in the cell results in the rapid degradation of mRNA containing identical or nearly identical sequences (29). To determine whether EpCAM is a potential target for breast cancer gene therapy, four different breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-361, and MDA-MB-453) and one nontumorigenic breast epithelial cell line (MCF-10A) were treated with EpCAM-specific siRNA as well as with scrambled siRNA. The scrambled siRNA serves as a control for nonspecific effects of siRNA treatment on cell growth. EpCAM mRNA levels and the number of viable cells were determined at 24, 48, and 72 h using real-time RT-PCR and MTT assay, respectively (Fig. 3). The data show that EpCAM siRNA treatment results in an approximately 10-fold decrease in *EpCAM* gene expression in the breast cancer cell lines compared with control. The EpCAM siRNA treatment effect was maximal at 48 h and attenuated by 72 h (consistent with the known transient effects of siRNA in mammalian cells). Scrambled siRNA treatment had no effect on EpCAM mRNA levels, thus supporting the specificity of the EpCAM siRNA.

EpCAM siRNA treatment resulted in a significant decrease in cell proliferation in all of the breast cancer cell lines. MCF-7, MDA-MB-231, and MDA-MB-453 had more than a 50% decrease in the number of viable cells at all time points. MDA-MB-361 seemed to be relatively resistant to EpCAM siRNA treatment and had only a 30% decrease in the number of viable cells. The effect of EpCAM siRNA treatment on the breast epithelial cell line MCF-10A (which expresses EpCAM at normal levels) was even less significant, with a minimal decrease in cell proliferation observed at all time points. Control scrambled siRNA treatment had no effect on *EpCAM* gene expression or cell proliferation.

EpCAM Short Interfering RNA Significantly Decreases the Invasion and Migration Potentials of Breast Cancer Cell Lines *in Vitro*. Given the known role of EpCAM in the regulation of cell adhesion molecules E-cadherin, α-catenin, and β-catenin, we attempted to determine the ability of EpCAM siRNA treatment to affect

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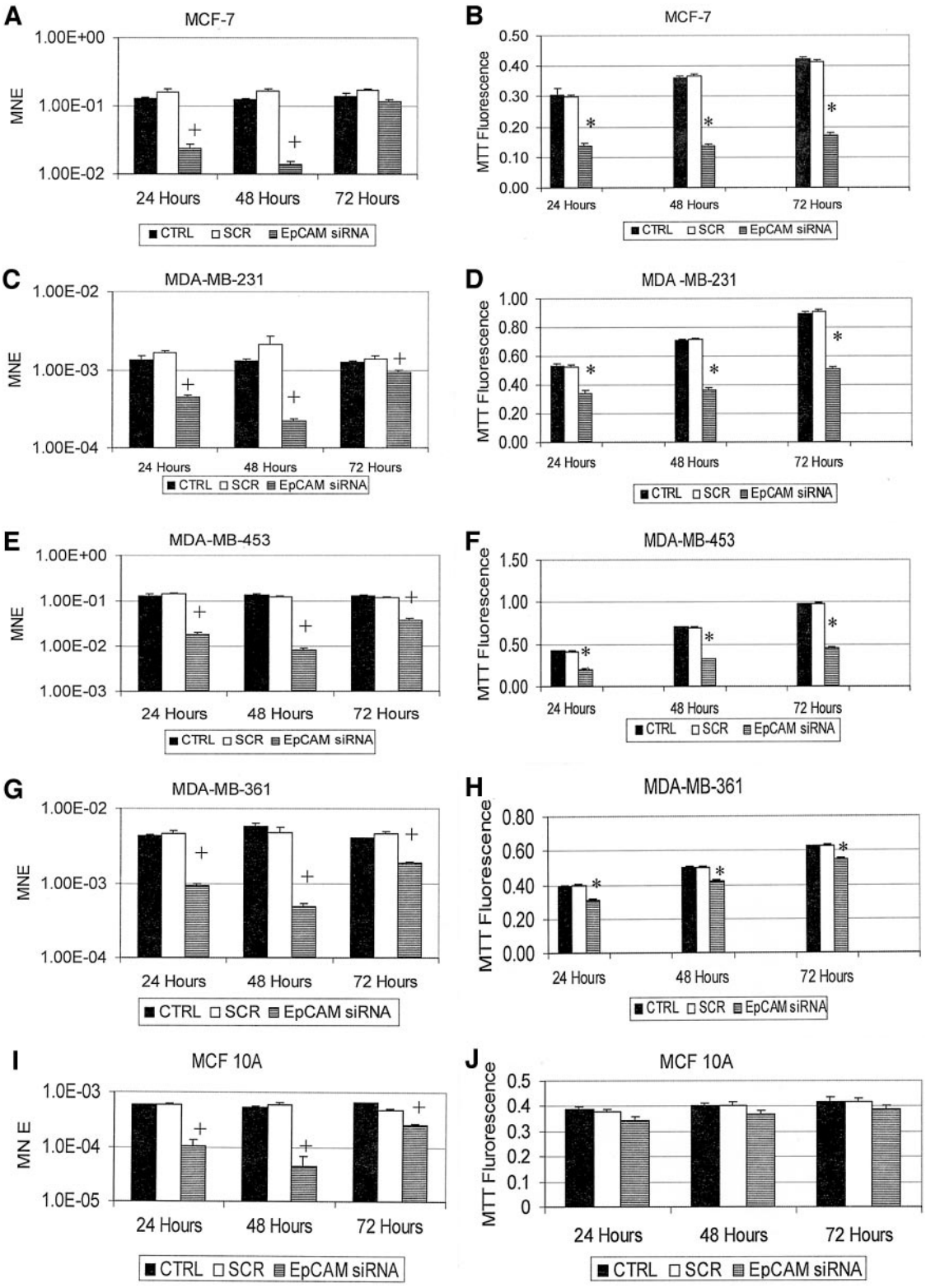

Fig. 3. EpCAM siRNA treatment decreases *EpCAM* gene expression and cell proliferation. A total of 100,000 cells were plated in each well of 6-well plates and allowed to grow for 24–36 h (until they were 40–60% confluent). Short interfering RNA was then transfected into cells at a concentration of 200 nM. Cell proliferation and *EpCAM* gene expression levels were assessed at 24, 48, and 72 h using MTT assay and real-time RT-PCR, respectively. ■, control cells that were not treated with siRNA. □, cells treated with scrambled siRNA. *Striped bars*, cells treated with EpCAM siRNA. ∗, statistically significant decrease in cell proliferation. +, statistically significant decrease in gene expression.

cell migration and invasion. Cell migration and invasion studies were performed using the Matrigel matrix assays. Matrigel matrix is a material that mimics the basement membrane. Cells that are characterized as invasive and metastatic *in vivo* are typically able to invade Matrigel matrix *in vitro* (30). Tumor cells require both migration and invasion properties to invade through the Matrigel matrix. Control inserts lack the Matrigel layer, so any cell movement across their membrane reflects only the migration potential of the cancer cells and

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is unrelated to the invasion potential. MDA-MB-231 cells from control cultures and cultures treated with either EpCAM siRNA or scrambled siRNA for 24 h were plated on Matrigel and control inserts. Four wells of each type were used for each condition. Two independent experiments were performed. The cells were counted after 24 h of incubation as described in "Materials and Methods." The results show that EpCAM siRNA treatment resulted in a dramatic inhibition of the migration and invasion potential of MD-MB-231 cells (Fig. 4). Cell migration was decreased 91.8%, and cell invasion was decreased 96.4%. Analysis of variance reveals that the differences between EpCAM siRNA treatment and treatment with control scrambled siRNA and untreated control cells are significant at a P of <0.0001.

EpCAM Short Interfering RNA Treatment Increases α-Catenin Gene Transcription. EpCAM is known to be involved in the regulation of E-cadherin, α-catenin, and β-catenin. One hypothesis is that EpCAM siRNA treatment decreases cell migration and invasion through regulation of these molecules, which are known to be involved in cell adhesion. EpCAM siRNA treatment may regulate these molecules at the level of gene transcription (addressed in this section) or at the functional level (addressed in the next section). To determine whether EpCAM siRNA treatment affects E-cadherin, α-catenin, and β-catenin gene expression, we quantitated gene expression using real-time RT-PCR. MDA-MB-231 cells were treated with EpCAM siRNA or scrambled siRNA and harvested at 36 h. RNA was extracted, and real-time RT-PCR was then performed using primers designed for EpCAM, E-cadherin, α-catenin, and β-catenin (Fig. 5).

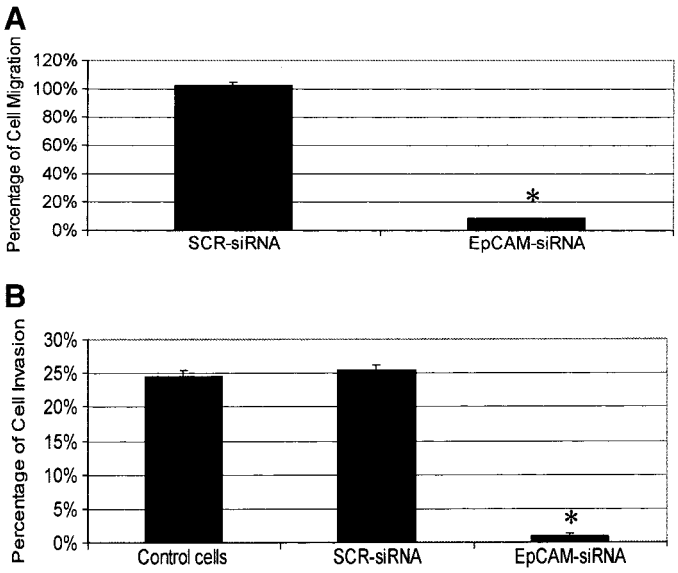

Fig. 4. EpCAM siRNA treatment decreases migration and invasion of breast cancer cells. The MDA-MB-231 breast cancer cell line was used in this experiment. Cell migration was assessed in BioCoat control cell culture chambers. Cell invasion was assessed in BioCoat Matrigel chambers. Control, scrambled siRNA-, and EpCAM siRNA-treated cells were added to control and Matrigel chambers. The upper chamber was filled with 1×10^5 cells in L-15 medium containing 5% FBS. The lower chamber was filled with L-15 medium containing 25% FBS as a chemoattractant. The chambers were incubated for 24 h at 37°C in a 5% CO_2 atmosphere. The number of cells that penetrated the filter was counted by a technician blinded to the experimental settings in four microscopic fields of each filter under ×20 magnification in both control and Matrigel membranes. *A*, percentage of migration was expressed as the ratio of mean cell number in control inserts containing siRNA-treated cells to mean cell number in control inserts containing untreated control cells. Control cells were arbitrarily given a value of 100%. The *left column* corresponds to cells treated with scrambled siRNA (*SCR*). The *right column* corresponds to cells treated with EpCAM siRNA. *B*, percentage of invasion was expressed as the ratio of mean cell number from invasion chamber to mean cell number from control chamber according to the manufacturer's recommendation. The *left column* corresponds to control cells that were not treated with siRNA. The *middle column* corresponds to cells treated with scrambled siRNA (*SCR*). The *right column* corresponds to the cells that were treated with EpCAM siRNA. *Error bars* correspond to the SE of four independent determinations.

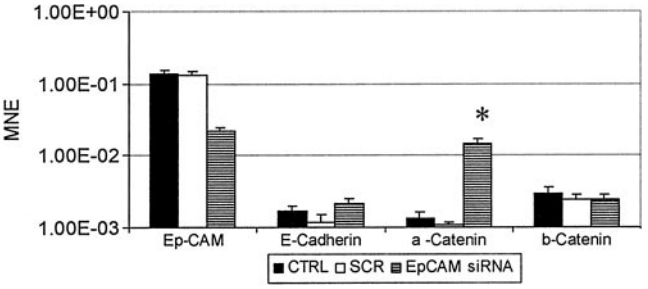

Fig. 5. EpCAM siRNA treatment is associated with increased transcription of α-catenin. The MDA-MB-231 breast cancer cell line was used in this experiment. Short interfering RNA was transfected into cells at a concentration of 200 nM. Cells were harvested after 48 h of siRNA treatment, and mRNA was isolated for real-time RT-PCR. ■, control cells that were not treated with siRNA. □, cells treated with scrambled siRNA. *Striped bars*, cells treated with EpCAM siRNA. Each reaction was done in triplicate. *Error bars* correspond to SE as calculated by Q-Gene software. *, significant increase in gene expression.

EpCAM siRNA treatment did not affect E-cadherin or β-catenin gene transcription but increased α-catenin gene transcription more than 10-fold. Treatment with scrambled siRNA did not affect gene transcription levels for any of the genes tested.

EpCAM Short Interfering RNA Treatment Improves the Anchorage of E-Cadherin with the Cytoskeleton. We assessed the effect of EpCAM siRNA treatment on the total cellular content and detergent solubility of the E-cadherin, α-catenin, and β-catenin proteins. E-cadherin interacts with the cytoskeleton through α- and β-catenins, and there is evidence that modulation of α-catenin gene expression can affect the anchorage of E-cadherin to the cytoskeleton. The detergent-insoluble fraction of EpCAM, E-cadherin, α-catenin, and β-catenin represents the amount of these proteins that is anchored to the cytoskeleton and involved in cell adhesion (31). MDA-MB-231 breast cancer cells were grown in two 6-well plates. Two wells from each plate were treated with EpCAM siRNA, two wells were treated with scrambled siRNA, and the last two wells served as controls and were not subjected to any treatment. One plate was used to extract the total protein content, and the other plate was used to extract the detergent-soluble and -insoluble fractions of the proteins. Consistent with the gene transcription data outlined above, there was a significant increase in total cellular α-catenin with no apparent change in the levels of total cellular E-cadherin and β-catenin (Fig. 6). However, EpCAM siRNA treatment resulted in a significant increase in the insoluble fractions of E-cadherin, α-catenin, and β-catenin proteins. These increases suggest that EpCAM siRNA treatment results in improved anchorage of the E-cadherin/α-catenin/β-catenin complex to the cytoskeleton and provide a mechanistic insight into how EpCAM siRNA treatment dramatically affects cell migration and invasion.

DISCUSSION

Breast cancer remains the most common cancer among American women and is the second leading cause of cancer death in American women. Despite the efficacy of current treatment modalities, 41,000 women will die from breast cancer in the United States in 2004. Clearly, additional treatment modalities are required to improve the outcomes of this disease. EpCAM is known to be overexpressed in epithelial cancers, and overexpression of this gene appears to be associated with enhanced proliferation and malignant potential (1, 2, 5). EpCAM has been targeted in clinical trials using monoclonal antibodies, and we believe that EpCAM represents a novel target for gene therapy in breast cancer. In support of this hypothesis, we show in this study that *EpCAM* is significantly overexpressed in primary

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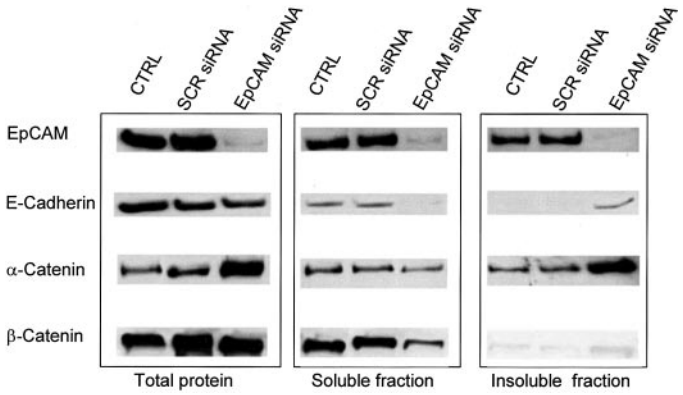

Fig. 6. EpCAM siRNA treatment is associated with an increase in the detergent-insoluble fraction of E-cadherin, α-catenin, and β-catenin. A total of 100,000 cells from the MDA-MB-231 breast cancer cell line were plated in each well of 6-well plates and allowed to grow for 24–36 h (until they were 40–60% confluent). Short interfering RNA was then transfected into cells at a concentration of 200 nM. Cells were harvested after 48 h of siRNA treatment. Total cell protein, detergent-soluble protein fraction, and detergent-insoluble protein fraction were extracted and analyzed by Western immunoblotting with antibodies to EpCAM, E-cadherin, α-catenin, and β-catenin. The column labeled *CTRL* corresponds to protein extracted from control cells that were not treated with siRNA. The column labeled *SCR siRNA* corresponds to protein extracted from cells treated with scrambled siRNA. The column labeled *EpCAM siRNA* corresponds to protein extracted from the cells treated with EpCAM siRNA.

and metastatic breast cancer tissues. Furthermore, silencing of the *EpCAM* gene significantly decreases the proliferative capacity and invasive potential of breast cancer cells. Finally, these studies provide a mechanistic insight into the role of EpCAM in the process of malignant transformation.

The mechanisms by which EpCAM enhances proliferation and increases invasive potential are still not fully understood. Adhesion molecules are known to play an important role in defining cell fate, differentiation, and other biological characteristics (32). EpCAM functions as a Ca^{2+}-independent homotypic intercellular adhesion molecule (6). It demonstrates adhesion properties when introduced into cells that are deficient in intercellular adhesion interaction (such as mouse fibroblast cell lines), mediating cell aggregation, preventing cell scattering, and directing cell segregation (4, 6, 31). Based on these adhesion properties, it might be assumed that EpCAM expression inhibits metastasis and is associated with an improved prognosis (33). However, many studies have demonstrated that EpCAM expression decreases adhesion mediated by cadherins (including E-cadherin in epithelial cells), a family of Ca^{2+}-dependent homophilic cell-to-cell adhesion molecules colocalized with EpCAM at the basolateral membrane in epithelial cells. The adhesion function of cadherins depends on their association with regulatory proteins, such as α- and β-catenin (32, 34, 35). Catenins link cadherins with the actin cytoskeleton and can also form complexes with other proteins (35–37). In epithelia, cadherins are crucial for the establishment and maintenance of epithelial cell polarity, morphogenesis of epithelial tissues, and regulation of cell proliferation and apoptosis (32, 38). The negative effect of EpCAM expression on cadherin-mediated adhesion may explain the association of EpCAM expression with invasion and metastasis in epithelial carcinomas (39). The data from this study confirm the importance of this interaction between EpCAM and the cadherins. After EpCAM siRNA treatment, we did not observe any significant increase in cell death or apoptosis in a standard annexin-V-phycoerythrin/7-amino-actinomycin D flow cytometry assay (data not shown). This indicates that EpCAM siRNA inhibition of cell proliferation is not due to an increase in cell death or apoptosis but is due to some other change in intrinsic cellular mechanisms. Data from this study show that down-regulation of EpCAM expression after siRNA treatment increases the cytoskeleton-anchored fractions of E-cadherin, α-catenin, and β-catenin. We believe that this is the primary mechanism responsible for the decrease in proliferation and invasion potential observed after EpCAM siRNA treatment.

Another potential mechanism involves the possible links between EpCAM overexpression and activation of the Wnt pathway. The Wnt pathway is well known to be associated with tumor development (40, 41). Wnt glycoproteins are a family of highly conserved signaling molecules that regulate cell-to-cell interaction during embryogenesis. Wnt proteins bind to receptors of the Frizzled family on the cell surface. Through several cytoplasmic relay components, the signal is transduced to β-catenin, which is stabilized, accumulates in the cytoplasm, and enters the nucleus, where it binds a lymphoid enhancer factor/T-cell factor transcription factor. Together, β-catenin and lymphoid enhancer factor/T-cell factor activate the expression of many target genes. Some of these genes, such as *c-Myc*, *vascular endothelial growth factor*, *cyclooxygenase 2*, and others, are known to be associated with cancer development (40). This pathway relies on the availability of cytoplasmic (not anchored to the cytoskeleton) β-catenin. We have shown that *EpCAM* gene silencing leads to a significant decrease in cytoplasmic β-catenin by causing an increase in its association with the E-cadherin adhesion complex. Consequently, EpCAM down-regulation might decrease the availability of β-catenin for the wnt pathway and thus shut down the activation of its target genes.

EpCAM down-regulation was associated with an increase in total cellular α-catenin. Other studies demonstrated an opposite phenomenon with EpCAM up-regulation (31). We have shown that this effect is, at least in part, due to a change in α-catenin gene transcription. These data suggest that EpCAM might participate in cellular signaling, a manifestation of which is to regulate α-catenin gene transcription. This signaling action may also be mediated by a change in the cytoplasmic β-catenin levels. By similar signaling mechanisms, it is likely that EpCAM affects other cellular events, such as growth, proliferation, and differentiation.

Gene therapy is emerging as a new method of therapeutic intervention at the level of cellular gene expression. Ongoing studies have shown some success in the treatment of several carcinomas in animal models including lung, pancreas, and gastric cancers (42–44). For breast cancer, *in vitro* studies targeting several genes, such as *maspin*, *cyclooxygenase 2*, *ErbB-2*, and others, have shown promising results concerning the possible use of gene therapy as a treatment modality (45, 46). Another potential prospect for therapeutic intervention is the possible use of siRNA technology in disease therapy. Many studies have proven that siRNA can be used successfully for gene silencing *in vivo* (47, 48). Recently, it has been shown that intravenous injection of siRNA or of plasmids expressing sequences processed to siRNA can inhibit infection by HIV-1, polio, hepatitis C, and malaria (49, 50). The application of siRNA technology to silence EpCAM expression in breast cancer patients might prove to be a valuable strategy in patients with advanced breast cancer.

In summary, targeting EpCAM for molecular intervention appears to be an attractive strategy. First, EpCAM is substantially overexpressed in primary and metastatic breast cancer. Furthermore, because EpCAM overexpression is associated with proliferation and neoplastic transformation (1, 2, 11), silencing of *EpCAM* gene expression is likely to dramatically alter the phenotype of cancer cells without significantly influencing normal or nonproliferating cells. Future studies targeting *EpCAM* gene expression *in vivo* will help to delineate the mechanisms associated with *EpCAM* gene function in neoplastic transformation and define the potential for EpCAM-based molecular intervention in breast cancer patients.

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