

# Identification of a Novel Inhibitor of Breast Cell Growth That Is Down-Regulated by Estrogens and Decreased in Breast Tumors<sup>1</sup>

Bryan M. Wittmann, Nancy Wang, and Monica M. Montano<sup>2</sup>

Department of Pharmacology, Case Western Reserve University, Cleveland, Ohio 44122 [B. M. W., M. M. M.], and Department of Pathology, University Hospitals of Cleveland, Cleveland, Ohio 44106 [N. W.]

## ABSTRACT

Lifetime exposure to estrogens is a major risk factor in breast cancer, but the mechanism for this action is not fully defined. To better determine this mechanism, the activation domain of estrogen receptor (ER)  $\alpha$  was used in yeast two-hybrid screenings. These screenings resulted in the identification of a novel antiproliferative protein, estrogen down-regulated gene 1 (EDG1), of which the mRNA and protein were shown to be down-regulated directly by estrogens. Our studies additionally suggested an important role for EDG1 in ER $\alpha$ -mediated breast cancer development. Analysis of 43 invasive breast cancer samples and 40 adjacent normal breast samples demonstrated EDG1 protein levels to be significantly higher in normal breast epithelial tissue as compared with breast epithelial tumor tissue. EDG1 expression levels were also correlated with the proliferation activity and ER $\alpha$  status of the tumors to examine the prognostic value of EDG1 in invasive breast tumors. EDG1 expression was more disassociated from proliferative activity as compared with ER $\alpha$  expression in tumor cells. A growth regulatory function for EDG1 is additionally indicated by studies wherein overexpression of EDG1 protein in breast cells resulted in decreased cell proliferation and decreased anchorage-independent growth. Conversely, inhibiting EDG1 expression in breast cells resulted in increased breast cell growth. Thus, we have identified a novel growth inhibitor that is down-regulated by estrogens and colocalizes with ER $\alpha$  in breast tissue. These studies support a role for EDG1 in breast cancer.

## INTRODUCTION

The steroid hormone estrogen is involved in the development, growth, and maintenance of many tissues. Estrogens exert their effects on these tissues by binding to their specific nuclear receptor, ER.<sup>3</sup> Once ER is bound to its ligand, it forms a homodimer and binds to DNA at specific sites termed ER elements. The binding of the ER homodimer to DNA causes the recruitment of coactivators and the rest of the transcriptional machinery. This process results in the expression of genes that somehow brings about the physiological actions of estrogen such as cell proliferation. Synthetic antiestrogens, like tamoxifen, can repress the transcriptional activity of ER (reviewed in Refs. 1–5).

Although estrogens play an important role in the initiation and development of breast cancer, the exact mechanism(s) by which estrogens regulate mammary epithelial cell proliferation is not well-defined (6). Some of the genes induced by estrogen, cyclin D1, c-myc, and prothymosin- $\alpha$ , may partially explain the ability of estrogen to stimulate cell proliferation (7–9). It is also possible that estrogen could

stimulate cell proliferation by down-regulating genes that may inhibit cell proliferation. Protein expression of *Hairy* and *Enhancer of Split* homologue-1 (of which the down-regulation appears to be necessary for estrogen-induced cell proliferation) is suppressed by estrogen in breast cancer cells (10). Although estrogen suppresses the expression of this protein, only autologous down-regulation of ER has been shown to be a direct effect of estrogen (11).

We have identified a protein that inhibits cell growth and is down-regulated directly by estrogens, termed EDG1. We also report on findings that suggest that altered expression (between normal and tumorigenic breast epithelial tissue) of EDG1 is involved in the initiation and/or development of breast tumors. Previous studies have demonstrated that breast tumors expressing ER $\alpha$  have better prognosis and are often responsive to hormonal therapies (Ref. 12; as reviewed in Ref. 13). Proliferative activity of breast tumors, as measured by Ki67 expression, appears to be an indicator of poor prognosis in node negative breast cancer patients (14). We show that breast tumor cells expressing EDG1 were more likely to express ER $\alpha$  than Ki67. This suggests a possible role for EDG1 as both an antigrowth and prognostic tool in breast cancer.

## MATERIALS AND METHODS

**Tissue Culture and Transfections.** Breast epithelial cells (MCF10A and MDA-MB-231) and PA317 amphotropic packaging cells were obtained from American Type Culture Collection (Manassas, VA) and maintained according to their recommended protocols. CHO and MCF7 cells were maintained and transfected as described previously (15).

**Plasmids.** The EDG1 clone, pAD-GAL4-2.1-EDG1, obtained from yeast two-hybrid screening contains the coding sequence cloned in frame with the activation domain of GAL4 in the pAD-GAL4-2.1 phagemid vector (Stratagene, La Jolla, CA). The human homologue clone for EDG1, HEXIM1, was obtained from American Type Culture Collection. The EDG1 coding sequence was released by an *NcoI/XbaI* digest. The *NcoI/XbaI*-digested and blunted EDG1 fragment was inserted into *EcoRI* digested and blunted pBD-Gal4-cam vector (Stratagene) to make pBD-EDG1. pET-15b-EDG1, which encodes the full length EDG1 in frame with six NH<sub>2</sub>-terminal His-tag sequences, was constructed by inserting the *EcoRI/SalI*-digested and blunted EDG1 cDNA from pBD-EDG1 into the *BamHI*-digested and blunted pET-15b vector (Novagen, Madison, WI). The *NcoI/XbaI*-digested and blunted EDG1 fragment was inserted into *BamHI*-digested and blunted pBPSTR1 retroviral vector (16) in the sense or antisense direction to make pBPSTR1-EDG1 or pBPSTR1-EDG1<sub>AS</sub>, respectively. pEGFP-EDG1, which encodes full-length EDG1 in frame with the coding sequence for GFP, was constructed by inserting *NcoI/XbaI*-digested and blunted EDG1 fragment into *HindIII*-digested and blunted pEGFP-C3 vector (Clontech, Palo Alto, CA).

**Yeast Two-Hybrid Screenings.** The yeast two-hybrid screenings used to identify ER $\alpha$ -interacting clones were described previously (17).

**Expression and Purification of Recombinant EDG1-His Tagged Fusion Protein.** pET15b-EDG1 was transformed into BL21 bacteria (Novagen), and expression of His-tagged EDG1 fusion protein was induced by 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (Promega, Madison, WI). His-tagged EDG1 fusion proteins were isolated by running the bacterial lysate over a Ni-NTA agarose column (Qiagen, Valencia, CA) and competed off with increasing concentrations of imidazole (10, 25, 100, 250, and 500 mM) containing buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub> and 300 mM NaCl). Eluted His-tagged EDG1 was resolved by SDS-PAGE and detected using the Qiaexpress Detection System (Anti-His

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<sup>2</sup> To whom requests for reprints should be addressed, at Case Western Reserve University School of Medicine, Department of Pharmacology, H.G. Wood Building W307, 2109 Adelbert Road, Cleveland, OH 44106. Phone: (216) 368-3378; Fax: (216) 368-3395; E-mail: mxm126@po.cwru.edu.

<sup>3</sup> The abbreviations used are: ER, estrogen receptor; E<sub>2</sub>, 17 $\beta$ -estradiol; EDG1, estrogen down-regulated gene 1; BR, Bloom-Richardson; LOH, loss of heterozygosity; CHO, Chinese hamster ovary; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMBA, hexamethylene-bis-acetamide; EDG1<sub>AS</sub>, estrogen down-regulated gene 1 antisense.

antibody chromogenic method) according to the manufacturer's protocol (Qiagen).

**Western Analysis.** Total protein was extracted from MCF7 cells using M-PER Mammalian Protein Extraction Reagent according to the manufacturer's protocol (Pierce, Rockford, IL). EDG1 was detected using the EDG1 (peptide 154–171) polyclonal rabbit antibody at a 1:1000 dilution (2.2  $\mu\text{g}/\text{ml}$ ) and an horseradish peroxidase-conjugated antirabbit IgG secondary antibody (Amersham Biosciences, Piscataway, NJ). Cytokeratin 18 or GAPDH were detected as recommended using the DC-10 monoclonal mouse anticytokeratin 18 IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or the 6C5 monoclonal mouse anti-GAPDH IgG antibody (Chemicon International, Inc., Temecula, CA), respectively, and an HRP-conjugated goat antimouse IgG secondary antibody (Amersham Biosciences). EDG1, cytokeratin 18, and GAPDH signals were detected using enhanced chemiluminescence Western Blotting Analysis System (Amersham Biosciences).

**Northern Blot Analysis.** RNA was extracted from breast epithelial cells using TRIzol (Life Technologies, Inc. Invitrogen, Carlsbad, CA) and was subjected to Northern Analyses as described previously (15).

**Retroviral-mediated Transfection.** Retroviruses were made by transfecting PA317 cells with the pBPSTR1 plasmid alone or pBPSTR1 containing EDG1 cDNA in the sense or antisense orientation. Breast epithelial cell lines were infected with retrovirus-containing supernatants in the presence or absence of 3  $\mu\text{g}/\text{ml}$  tetracycline. When tetracycline was added, expression of the viral gene was inhibited. Changes in EDG1 protein were verified by immunofluorescence staining or immunoblot analyses.

**Immunofluorescence Staining of Breast Tissue and Cells.** Breast tissue samples were obtained from the Cooperative Human Tissue Network-Western Division and University Hospitals of Cleveland, Department of Pathology. Breast tissue samples were fixed in formalin, embedded in paraffin, and sectioned at 5  $\mu\text{m}$  thickness. To unmask epitopes we used heat-induced antigen retrieval technique using 10 mM citrate buffer. After blocking with PBS (140 mM sodium chloride, 2.6 mM potassium chloride, 1.5 mM potassium phosphate monobasic, and 10 mM sodium phosphate) containing 10% normal goat serum and 0.3% Triton X-100, sections were incubated with EDG1 (peptide 154–171) polyclonal rabbit antibody (1:100 dilution; 22  $\mu\text{g}/\text{ml}$ ) and goat, antirabbit IgG Alexa 488 fluorescence secondary antibody (1:500 dilution; Molecular Probes, Eugene, OR). As a negative control, duplicate sections were immunostained with nonspecific rabbit IgG. To detect ER $\alpha$ , tissue was immunostained with ER $\alpha$  1D5 IgG monoclonal mouse antibody (1:100 dilution; Lab Vision, Fremont, CA) and goat, antimouse Alexa 594 secondary antibody (1:500 dilution; Molecular Probes). To detect Ki67, tissue was immunostained with Ki67 IgG monoclonal mouse antibody (1:100 dilution; Lab Vision) and goat, antimouse Alexa 594 secondary antibody (1:500 dilution; Molecular Probes). As a negative control, duplicate sections were immunostained with nonspecific mouse IgG.

Cells grown on coverslips were fixed in PBS containing 4% paraformaldehyde and nuclei were permeabilized using 0.3% Triton X-100. After blocking with serum, samples were incubated with EDG1, and Ki67 primary and secondary antibody as described above. Coverslips were mounted on slides using Vectashield mounting medium with 4',6-diamidino-2-phenylindole for nuclear staining (Vector Laboratories, Burlingame, CA).

**Proliferation Assays.** Breast epithelial cancer cells were infected with retrovirus-containing supernatant as described above and weaned off estrogen-containing medium as described previously (18). Cell proliferation was determined using Cell Titer 96 Aqueous One Solution Proliferation Assay as recommended by the manufacturer (Promega).

**Anchorage-independent Growth.** Four days after infection cells were detached and suspended at a concentration of  $1 \times 10^4$  in medium containing 0.3% agar and then plated onto a six-well plate precoated with 0.7% agar base layer. At 24 h and 21 days after plating, colonies  $>50 \mu\text{m}$  were counted.

**Quantification and Statistical Analysis.** Expression levels for EDG1 in Western and Northern blots were quantified as a percentage of loading control by densitometry using NIH image 1.62 software. To quantify EDG1 immunostaining levels, mean histogram readings of cell nuclei were averaged from  $\geq 15$  cells per sample using Adobe Photoshop 6.0. Five background readings were taken for each sample, averaged, and subtracted from the EDG1 reading. To analyze the tissue data,  $2 \times 2$  contingency tables were used to obtain  $\chi^2$  and  $P$ s. Significant differences were comparisons with  $P$ s  $< 0.05$ .

## RESULTS

### Identification, Purification, and Characterization of EDG1.

EDG1 was identified from an MCF7 breast cancer cell cDNA library by yeast two-hybrid screenings using the COOH-terminal EF domain of ER $\alpha$  as bait. Sequence analysis of the EDG1 cDNA clone indicated an open reading frame of 1077 bp (359 amino acids) encoding a  $M_r$  41,000 protein with a nuclear localization sequence at amino acids 150–177. His-tagged recombinant EDG1 was affinity purified and eluted with 100 mM imidazole, and observed on SDS-PAGE as a  $M_r$  41,000 protein, along with an additional larger  $M_r$  70,000 protein, suggesting either post-translational modifications or dimer formation (data not shown). To determine the specificity of our EDG1 (peptide 154–171) polyclonal rabbit antibody, the 10 mM imidazole eluate (containing high levels of bacterial proteins and no His-tagged EDG1) and the 100 mM imidazole eluate (containing low levels of bacterial proteins and high concentration of His-tagged EDG1) were subjected to Western analysis. No bands were observed in the 10 mM imidazole elute, whereas the  $M_r$  41,000 and  $M_r$  70,000 His-tagged EDG1 fusion proteins were detected by the EDG1 polyclonal antibody (data not shown). Fluorescence studies indicated that transfected EDG1 (in CHO cells) and endogenous EDG1 (in nontumorigenic MCF10A cells) localized to the nucleus (Fig. 1A).

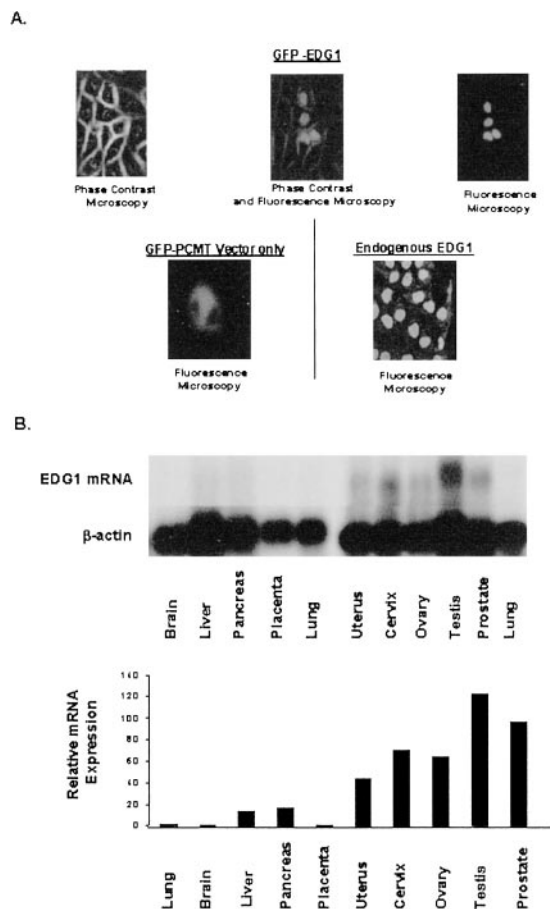


Fig. 1. Intracellular localization of EDG1 and tissue distribution of EDG1. A, CHO cells were transfected with 100 ng of pEGFP-C3-EDG1 vector or pEGFP-C3-PCMT (PCMT is a known nonnuclear protein). MCF10A cells were stained for endogenous EDG1 using EDG1 (peptide 154–171) polyclonal rabbit antibody and goat, antirabbit Alexa 488 secondary antibody. For fluorescence images a fluorescein filter was used (original images at  $\times 400$  total magnification). B, master human normal blots (Invitrogen) containing mRNA from different tissues was probed with random primer-labeled EDG1 cDNA. To control for RNA loading the same blot was re-probed with  $\beta$ -actin. EDG1 and  $\beta$ -actin mRNA levels were quantified using densitometry. EDG1 mRNA levels were normalized to  $\beta$ -actin levels and expressed relative to EDG1 expression in the lung.

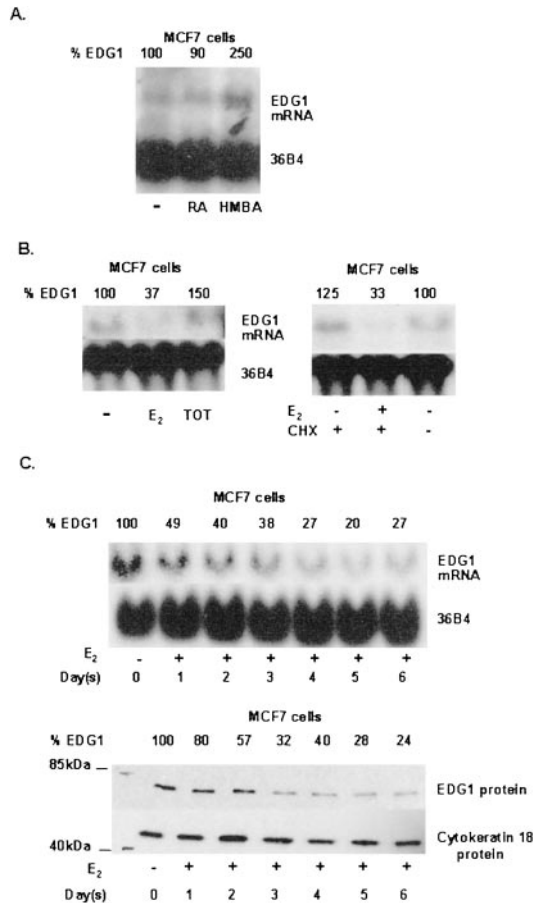


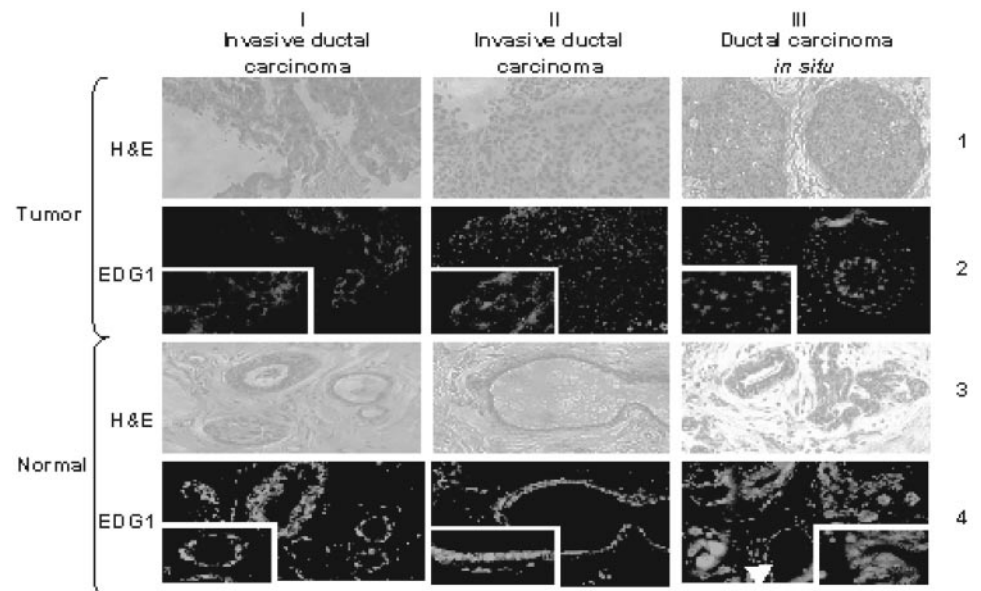
Fig. 2. EDG1 expression is directly down-regulated by estrogens and up-regulated by HMBA. Total RNA was collected from untreated MCF-7 cells (-), and cells treated for 24 h with A,  $10^{-6}$  M all-*trans*-retinoic acid (RA), or 5 mM hexamethylene bisacetamide (HMBA) or B,  $10^{-9}$  M E<sub>2</sub>,  $10^{-7}$  M *trans*-hydroxytamoxifen (TOT), or  $10^{-5}$  M cycloheximide (CHX), an inhibitor of protein synthesis. All blots were probed with random primer-labeled EDG1 cDNA. To control for RNA loading the same blot was reprobed with 36B4. EDG1 and 36B4 mRNA levels were quantified using densitometry. The autoradiographs are representative of three separate experiments. C, total RNA or total protein was collected from untreated MCF7 cells (-), and cells treated for 1–6 days with  $10^{-9}$  M E<sub>2</sub>. EDG1 mRNA was detected as in A and B. EDG1 protein was detected by Western analysis using the EDG1 (peptide 154–171) polyclonal antibody. To control for protein loading, the blot was stripped and cytokeratin 18 was detected. EDG1 and cytokeratin 18 protein levels were quantified by densitometry. The blot is representative of three separate experiments.

EDG1 cDNA shows homology to a human gene of unknown function, hexamethylene-bis-acetamide- inducible transcript in human vascular smooth muscle cells, *HIS1*, that is up-regulated in vascular smooth muscle cells by the differentiating agent HMBA (19). During preparation of this article, a report on the mouse homologue of EDG1, cardiac lineage protein-1, was published. Consistent with our results, cardiac lineage protein-1 also ran on SDS-PAGE at  $M_r \sim 70,000$  (20). EDG1 mRNA expression was also prevalent in other human endocrine tissues such as the ovary and prostate (Fig. 1B), which supports a role for EDG1 in endocrine signaling.

**EDG1 Expression Is Increased by the Differentiating Agent HMBA and Decreased by the Mitogen Estrogen.** Consistent with findings in vascular endothelial cells (19), up-regulation of EDG1 mRNA expression by HMBA was also evident in MCF7 breast cancer cells (Fig. 2A). It was shown previously that MCF7 cells treated with HMBA have morphological characteristics of a more differentiated cell (21). Viable MCF7 cells exhibit decreased cell growth and anchorage-independent growth, enlargement of cytoplasm, and increased expression of the epithelial differentiation marker, epithelial membrane antigen, after treatment with HMBA (21). Conversely E<sub>2</sub>, which induces breast cancer cell growth, had an inhibitory effect on EDG1 mRNA expression even in the presence of cycloheximide, suggesting a direct effect by ER (Fig. 2B). Endogenous EDG1 from breast cancer cells ran on SDS-PAGE exclusively as the  $M_r$  70,000 size protein. Treatment of breast cancer cells with E<sub>2</sub> resulted in a gradual decrease in EDG1 protein levels from a 20% decrease after 1 day and a 76% decrease after 6 days of E<sub>2</sub> treatment. The decrease in EDG1 protein coincided with, although at a slower rate, the decrease observed in the EDG1 mRNA levels from 1- to 6-day E<sub>2</sub> treatments (Fig. 2C).

**Preliminary Analysis of EDG1, ER $\alpha$ , and Ki67 Expression, and Colocalization in a Small Breast Tissue Set.** Because of the down-regulation of EDG1 expression by the mitogen E<sub>2</sub> and the up-regulation of EDG1 by the differentiating agent HMBA, we determined whether EDG1 expression was altered in breast tumors. We initially immunostained a small population of 16 breast tumor samples and adjacent normal breast samples for EDG1, ER $\alpha$ , and Ki67 (Figs. 3 and 4). Analysis of our initial breast tissue set suggested that some (5 of 16) invasive breast tumors (Fig. 4A, Patient II, row II) and (1 of 3) ductal carcinoma *in situ* (Fig. 3, Patient III, row 2) express EDG1. We

Fig. 3. Expressions levels of EDG1 in normal breast and breast cancer tissue. Sections obtained from breast tumor and adjacent normal breast tissue of 3 patients were stained for endogenous EDG1 as described in "Materials and Methods" section. Arrowhead indicates endothelial cells.



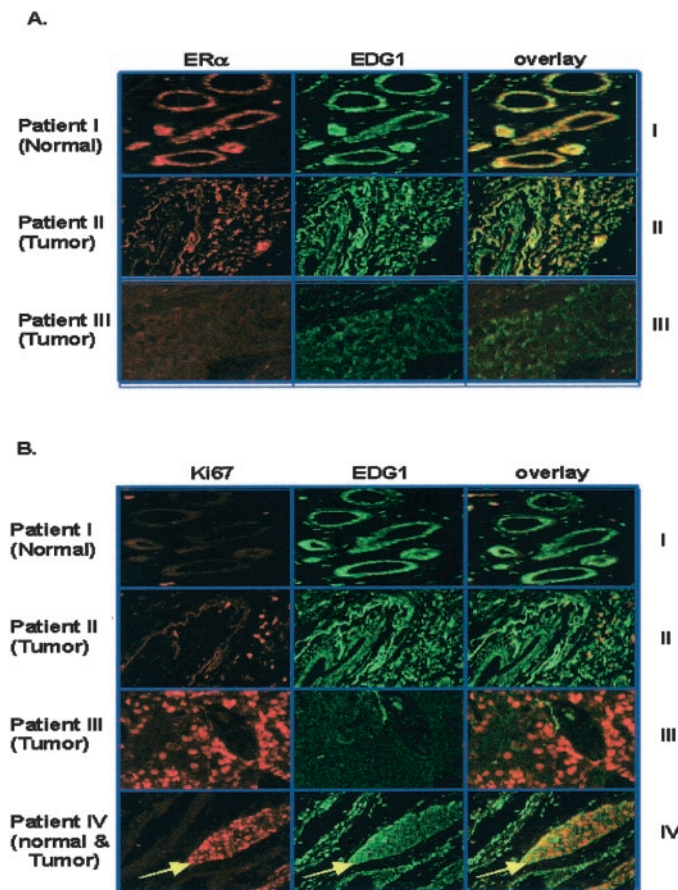


Fig. 4. EDG1 expression is associated with ER $\alpha$  expression and dissociated from proliferative activity. Sections obtained from breast tumor and adjacent normal tissue were stained for A, EDG1 and ER $\alpha$ , or B, EDG1 and Ki67 as described in "Materials and Methods."

also observed many (11 of 16) invasive tumors (Fig. 3, *Patient I* and *II*, row 2) and some (2 of 3) ductal carcinoma *in situ* (Fig. 4B, *Patient IV*, row IV; yellow arrow) that did not express EDG1. This was in contrast with the adjacent normal breast samples, in which all of the samples contained some epithelial cells that expressed nuclear EDG1 (Fig. 3, *Patient I, II, III*, row 4). These luminal epithelial cells give rise to the majority of breast tumors (reviewed in Ref. 22). Also, as expected, we observed EDG1 expression in the nuclei of endothelial cells (Fig. 3, *Patient III*, row 4, indicated by arrowhead).

This initial study also suggested that tumors positive for EDG1 expression correlated with tumors that were ER $\alpha$  positive (Fig. 4A, *Patient II*, row II and *Patient III*, row III). ER $\alpha$ -positive tumors are considered to be more differentiated and have better prognosis than ER $\alpha$ -negative tumors (12). We also observed colocalization of EDG1 within normal breast epithelial cells that expressed ER $\alpha$  (Fig. 4A, *Patient I*, row I).

Finally, although EDG1-positive tumors still contained proliferating cells, the amount of cells undergoing proliferation was low (Fig. 4B, *Patient II*, row II). Of note, highly proliferating tumors either did not express EDG1 or expressed low levels of EDG1 (Fig. 4B, *Patient III*, row III). We observed high Ki67 expression in both of the ductal carcinoma *in situ*, which did not express nuclear EDG1 (Fig. 4B, *Patient IV*, row IV; yellow arrow). Conversely, Ki67-positive cells were rarely observed in the 16 samples of normal breast epithelial ducts, which were highly positive for nuclear EDG1 (Fig. 4B, *Patient I*, row I and *Patient IV*, row IV). It has been shown previously that ER $\alpha$  and the proliferation antigen, Ki67, rarely colocalized in normal

breast epithelial cells, whereas in a proportion of human breast tumors there is increased colocalization between ER $\alpha$  and Ki67 (23). The data from this initial tumor study suggested that additional investigation of breast tumors was necessary because of the small sample size and under-representation of both BR grade I and grade II tumors. Therefore, we did a more in-depth analysis of a larger set of invasive tumors for each BR grade.

**Analysis of 43 Invasive Breast Tumors and Adjacent Normal Breast Epithelial Cells for EDG1, ER $\alpha$ , and Ki67 Expression.** Additional studies were conducted on a total of 43 invasive breast cancer samples and 40 adjacent normal breast samples (1 grade III tumor and 3 normal samples were unavailable for the study). Analyses were done on 15 BR grade I, 15 BR grade II, and 13 BR grade III breast tumors, and the adjacent normal breast epithelial tissue samples for 13 BR grade I, 13 BR grade II, and 14 BR grade III. Spatial expression of ER $\alpha$  and EDG1 or Ki67 and EDG1 was compared for each sample. At least 1000 epithelial cells per tumor sample and up to 500 epithelial cells per normal sample were counted (10 fields of view were randomly chosen for each sample). Positive expression for EDG1, ER $\alpha$ , and Ki67 were quantified as cells with complete nuclear staining (weak or strong) above background. The samples were then categorized as either negative (0%), low expressers (<10% of cells expressing antigen), moderate expressers (10–50% of cells expressing antigen), or high expressers (>50% of cells expressing antigen). The data are summarized in Fig. 5, A–E, and Table 1.

The majority of breast tumors and adjacent normal breast samples expressed ER $\alpha$  [53% (5% low expressers, 39% moderate expressers, and 9% high expressers) and 80% (45% low expressers, 35% moderate expressers, and 0% high expressers), respectively] and Ki67 [93% (33% low expressers, 58% moderate expressers, and 2% high expressers) and 62.5% (57.5% low expressers, 5% moderate expressers, and 0% high expressers), respectively]. As expected, tumor and normal samples were different in that the majority of ER $\alpha$ - and Ki67-positive tumors were moderate expressers (39% and 58%, respectively), whereas the majority of adjacent normal samples were low expressers (45% and 57.5%, respectively; data not shown). This resulted in the overall percentage of cells expressing ER $\alpha$  and Ki67 to be significantly higher in tumor cells as compared with the adjacent normal ducts (ER $\alpha$ :  $\chi^2 = 1023$ ,  $P < 0.001$ ; Ki67:  $\chi^2 = 2993$ ,  $P < 0.001$ ; Table 1).

Thirty-nine of 43 samples had tumors with adjacent normal samples. We observed a decrease in EDG1 expression in 37 of these 39 tumor samples as compared with their adjacent normal samples. The 2 samples in which a decrease was not observed were both BR grade I tumor samples (Fig. 5A). Nine of 43 tumors (21%) were negative for EDG1 expression, and 20 tumors (47%) were either EDG1 negative or EDG1 low expressers. The majority of tumors [20 of 43 (47%)] were EDG1 moderate expressers, and very low percentage [3 of 43 (6%)] were high EDG1 expressers (Fig. 5B). As was observed in the initial set of 16 samples, none of the 40 normal breast samples were negative or low expressers for EDG1, and the majority [32 of 40 (80%)] were high EDG1 expressers (>50% expression) with 20% (8 of 40) moderate EDG1 expressers (Fig. 5C).

When we compared the number of breast tumors that were 100% EDG1 negative (9 of 43) to the normal breast samples that were 100% EDG1 negative (0 of 40), the two sets of samples were significantly different ( $\chi^2 = 8.2$ ;  $P = 0.004$ ). These data supports the cell by cell counts where the majority of adjacent normal breast epithelial cells expressed EDG1 (63%), and the proportion was significantly lower in tumor cells (16%; EDG1:  $\chi^2 = 12,717$ ,  $P < 0.001$ ; Table 1).

No correlation between BR tumor grade and the level of EDG1 expression within the tumors was observed (Fig. 5B; Table 1). Sixty-seven percent of normal samples adjacent to BR grade I and 77%

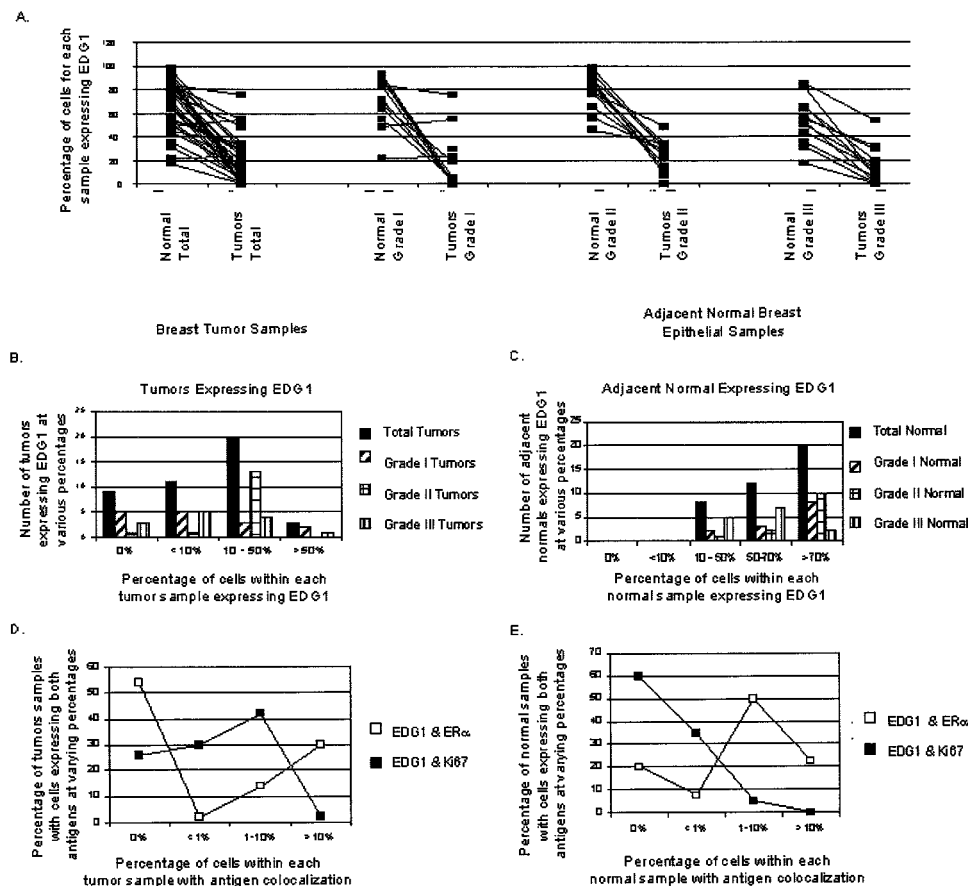


Fig. 5. Comparison of 43 invasive breast tumors and 40 adjacent normal breast epithelial samples for EDG1, ER $\alpha$ , and Ki67 expression and colocalization. A, percentage of cells expressing EDG1 for each breast tumor sample *versus* normal adjacent breast epithelial samples. The number of tumors (B) or normal samples (C) expressing EDG1 was compared for total samples and tumor grade, based on level of expression (percentage of cells expressing EDG1). The percentage of tumor samples (D) or normal samples (E) that contain cells expressing EDG1 and ER $\alpha$  or EDG1 and Ki67 within the same cells were compared based on the extent of colocalization (percentage of cells within each tumor or normal sample with antigen colocalization).

adjacent to BR grade II tumors contained >70% of their cells expressing nuclear EDG1. In comparison, only 14% of those normal samples adjacent to BR grade III tumors contained >70% of their cells expressing nuclear EDG1 ( $\chi^2 = 5.5$ ,  $P = 0.004$ ; Fig. 5C). This correlated with the cell by cell counts in which we observed that the difference for EDG1-positive expression in adjacent normal breast epithelial ducts, between grade I and II (66% and 78%, respectively;  $\chi^2 = 183$ ;  $P < 0.001$ ), was not as strong as the difference between grade I and II *versus* grade III (71% and 51%, respectively;  $\chi^2 = 386$ ;  $P < 0.001$ ; Table 1). This may also partially explain why the difference in the percentage of proliferating adjacent normal breast cells from grade I (0.5%) and II (0.8%) tumors was not as significant ( $\chi^2 = 4.0$ ;  $P > 0.025$ ) when compared with the difference in the percentage of proliferating adjacent normal breast cells from grade I and II (0.7%) *versus* grade III (2.0%;  $\chi^2 = 98$ ;  $P < 0.001$ ). These findings are consistent with our observation that a higher percentage of grade III adjacent normal breast epithelial cells were proliferating.

As we stated previously, our initial small sample of 16 breast tumors suggested that EDG1-positive breast tumors were correlated with ER $\alpha$ -positive breast tumors. Therefore, we analyzed the breast tumors from our larger sample to determine the percent of tumors that

contained cells expressing both ER $\alpha$  and EDG1 within the same cells (Fig. 5D), and the percentage of cells from all of the tumors expressing both EDG1 and ER $\alpha$  within the same cells (colocalization; Table 1). Forty-six percent (20 of 43) of tumors contained cells expressing both ER $\alpha$  and EDG1 within the same cells at various percentages. The majority of these tumors [65% (13 of 20)] had >10% colocalization (Fig. 5D). Because some tumors were either negative for both EDG1 and ER $\alpha$ , only EDG1 positive, or only ER $\alpha$  positive, it was necessary to consider these samples as a separate population to optimally determine whether EDG1-expressing and ER $\alpha$ -expressing tumors were correlated. Fifty-nine percent of the tumors that were EDG1-positive expressed ER $\alpha$  (20 of 34), and 86% (20 of 23) of the samples that were ER $\alpha$ -positive expressed EDG1 (data not shown).

As stated previously EDG1 expression was not completely dissociated from breast tumors undergoing proliferation in the initial 16 tumor samples. Therefore, we analyzed the larger tumor set for Ki67 and EDG1 expression within the same tumor cells as we did with ER $\alpha$  and EDG1. We observed 74% of the tumors to contain cells expressing both EDG1 and Ki67 within the same cells at various percentages, but in contrast to tumors expressing both ER $\alpha$  and EDG1, only 5% of EDG1- and Ki67-positive tumors had >10% of cells expressing both

Table 1 Analysis of 43 invasive human breast cancer tissue samples for EDG1, ER $\alpha$ , and Ki67 expression

Antigen	Total							
	Tumor cells	Normal cells	Grade I tumor cells	Adjacent normal cells	Grade II tumor cells	Adjacent normal cells	Grade III tumor cells	Adjacent normal cells
% ER $\alpha$	18%	8%	22%	10%	27%	8%	5%	6%
% EDG1	16%	63%	14%	66%	22%	78%	13%	51%
% EDG1 and ER $\alpha$	7%	6%	7.1%	8.2%	13.2%	6.9%	0.9%	4.5%
% Ki67	16%	1.3%	7%	0.5%	12%	0.8%	27%	2%
% EDG1 and Ki67	1.6%	0.3%	0.8%	0.08%	1.6%	0.2%	2.4%	0.53%

EDG1 and Ki67 within the same cells (Fig. 5D). This was observed although the majority of tumors were EDG1 (47%) and Ki67 (58%) moderate expressors (10–50% of the cells).

Although the difference between percentage of total tumor cells expressing ER $\alpha$  (18%) versus Ki67 (16%) was significant ( $\chi^2 = 32$ ;  $P < 0.001$ ; Table 1), it was not as significant as the difference between tumor cells expressing both ER $\alpha$  and EDG1 (7%) as compared with tumor cells expressing both Ki67 and EDG1 (1.6%;  $\chi^2 = 1396$ ;  $P < 0.001$ ; Table 1). This suggests that there is a stronger disassociation between proliferating breast tumor cells and EDG1-positive tumor cells as compared with ER $\alpha$ -positive and EDG1-positive breast tumor cells.

We also determined whether normal breast epithelial cells adjacent to tumors were also more likely to express ER $\alpha$  and EDG1 within the same cells as compared with EDG1 and Ki67. Eighty percent (32 of 40) of adjacent normal samples contained cells expressing both ER $\alpha$  and EDG1 within the same cells. In contrast, 40% of normal samples contained cells expressing both EDG1 and Ki67 within the same cells (Fig. 5E). This observation was supported by cell by cell counts in which a significantly higher percentage (6%) of total normal breast epithelial cells counted expressed both ER $\alpha$  and EDG1 within the same cells as compared with 0.3% of normal breast epithelial cells expressing EDG1 and Ki67 ( $\chi^2 = 1144$ ;  $P < 0.001$ ; Table 1). We also observed that 9% of EDG1-positive normal cells were ER $\alpha$  positive, and 83% of ER $\alpha$ -positive normal cells were EDG1 positive. In contrast, only 0.4% of EDG1-positive normal cells were Ki67 positive, and only 22% of Ki67-positive normal cells were EDG1 positive (data not shown). This study additionally supports decreased expression of EDG1 in breast tumors and a stronger disassociation with proliferating breast epithelial cells (normal and malignant) as compared with ER $\alpha$ -positive breast epithelial cells (normal or malignant).

**EDG1 Inhibits Breast Cell Proliferation.** Although the majority of proliferating and EDG1-positive breast epithelial cells were disassociated, it is not conclusive from these studies that EDG1 can regulate cell proliferation. Therefore, a self-contained tetracycline-regulated retroviral vector system (17) was used to modulate EDG1 expression in breast epithelial cell lines. Infection of MCF7 cells with EDG1 retrovirus resulted in increased nuclear expression of EDG1 and decreased expression of Ki67 as compared with control cells (Fig. 6A). In contrast, inhibiting EDG1 expression in MCF7 cells resulted in decreased nuclear expression of EDG1 and an increased number of cells expressing Ki67 as compared with control cells (Fig. 6A). Proliferation assays indicated that a minor increase (50%) in EDG1 expression resulted in an inhibition of growth of MCF7 cells, whereas a decrease (90%) in EDG1 expression enhanced growth rate (Fig. 6B). It appears that tetracycline did not fully repress EDG1<sub>AS</sub> retroviral expression (Fig. 6B). This could explain why an increase in proliferation was observed in EDG1<sub>AS</sub> cells with tetracycline, but to a lesser degree than EDG1<sub>AS</sub> cells without tetracycline. These findings support our results that breast tissue cells positive for nuclear EDG1 are less likely to express Ki67 as compared with breast tissue cells negative for nuclear EDG1. Studies presented in Fig. 6, A and B, also indicate that EDG1 inhibited E<sub>2</sub>-induced cell proliferation.

Anchorage-independent growth is a necessary requirement for tumor growth and is a well-established *in vitro* assay for the malignantly transformed cellular phenotype. Soft agar colony formation, a measure of anchorage-independent growth, was examined in control, MCF7-EDG1, and MCF7-EDG1<sub>AS</sub> cells. We observed a 72% decrease in colony formation as a result of increased EDG1 expression, whereas increased colony formation was observed in MCF7-EDG1<sub>AS</sub> cells (Fig. 6C).

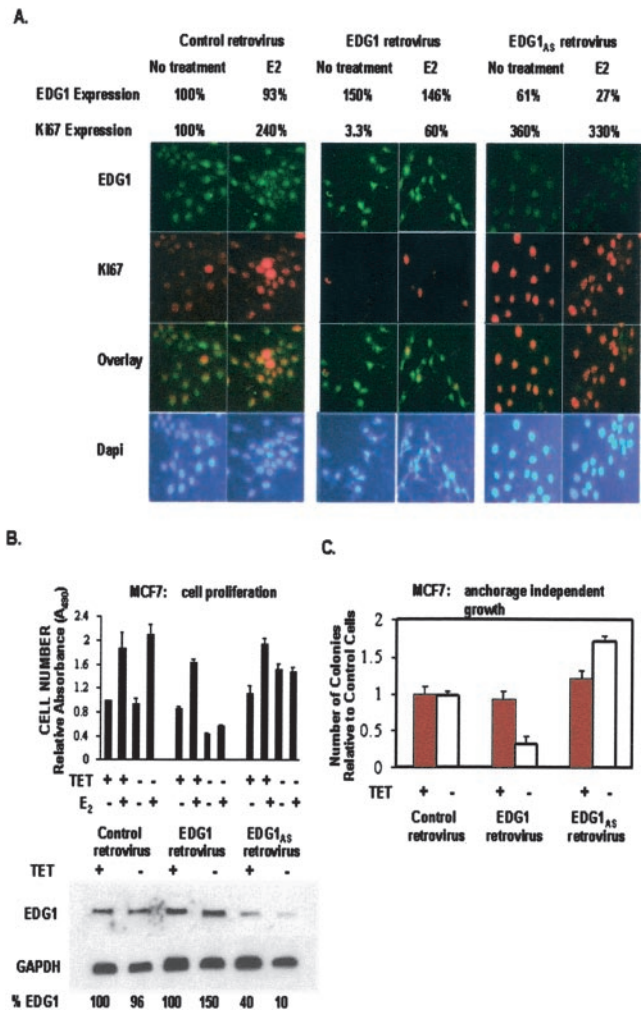


Fig. 6. Altering the expression levels of EDG1 affects proliferation and anchorage-independent growth of breast cancer cells. MCF7 cells were infected with control, EDG1, or antisense EDG1 retrovirus in the absence (–), or presence of tetracycline or  $10^{-8}$  M E<sub>2</sub>. A, 2 days after infection cells were stained for EDG1 and Ki67 as described in “Materials and Methods.” B, to determine the effects of EDG1 on cell numbers, MCF7 cells were infected as described in A and treated with or without E<sub>2</sub> for 5 days. Cell number was determined using the CellTiter 96 Aqueous One Solution Proliferation Assay. Values for cell number are expressed relative to the absorbance in control cells grown in the presence of tetracycline (which is set at 1). Values are the means from three separate experiments with triplicate wells for each group; bars,  $\pm$ SE. EDG1 protein was detected by Western analysis as in Fig. 2C. To control for protein loading, the blot was stripped and GAPDH was detected. EDG1 and GAPDH protein levels were quantified by densitometry. C, MCF7 cells were infected with control, EDG1 or EDG1<sub>AS</sub> retrovirus in the presence of 3  $\mu$ g/ml tetracycline. Twenty-four h later cells were given fresh medium  $\pm$  tetracycline. Two days later cells were detached and plated for anchorage-independent growth. Values are expressed as the number of colonies formed per number of cells plated  $\times$ 100. Values are expressed relative to the number of colonies/cells plated for cells infected with control retroviruses grown in the presence of tetracycline (which is set at 1). Values are the means  $\pm$  SE from two separate experiments with triplicate wells for each group.

To determine whether the effects of EDG1 on cell proliferation were cell line-specific, EDG1 and EDG1<sub>AS</sub> retroviruses were infected into a normal breast epithelial cell line (MCF10A) or another breast cancer cell line (MDA-MB-231). Decreased nuclear expression of EDG1 in MCF10A after infection with EDG1<sub>AS</sub> retroviruses was associated with 4.5-fold increase in proliferation, whereas a slight increase in EDG1 nuclear expression inhibited proliferation markedly (Fig. 7A). No significant effects on proliferation of MDA-MB-231 cells were evident after infection with EDG1<sub>AS</sub> retroviruses. However, after infection with EDG1 retroviruses we saw a 64% decrease in proliferation (Fig. 7B).

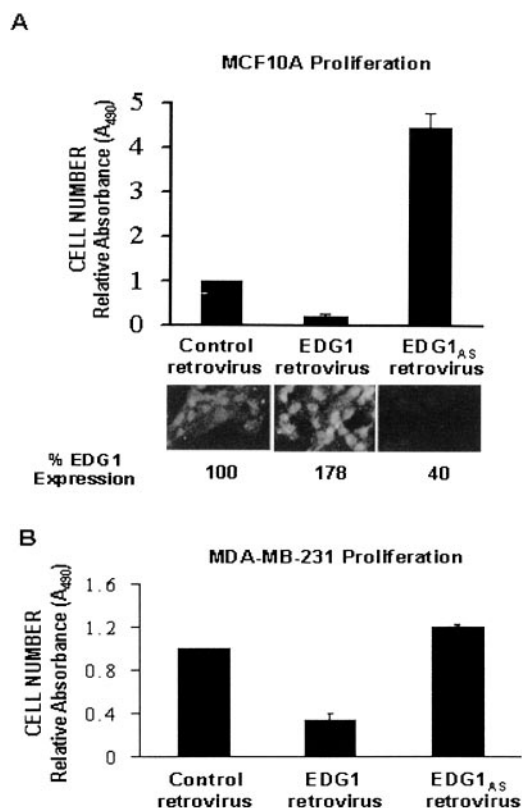


Fig. 7. EDG1 inhibits cell growth of both normal breast and breast cancer epithelial cells. *A*, MCF10A and *B*, MDA-MB-231 cells were infected with retroviruses and plated for proliferation or immunostaining as described in Fig. 6, *A* and *B*. Values are the means from two separate experiments with triplicate wells for each group; bars,  $\pm$ SE.

## DISCUSSION

We have identified an estrogen regulated gene, *EDG1*, that encodes a protein that functions as an inhibitor of cell growth. Differences in expression levels of EDG1 were observed between normal breast epithelial and breast tumor cells, and tissue. The decrease in EDG1 expression in the majority of breast tumor cells, the inverse correlation between EDG1 and Ki67 expression, and the ability of EDG1 to inhibit breast cell growth suggest a role for EDG1 in breast epithelial cell proliferation. The ability of EDG1 to inhibit cell growth, along with the decrease in EDG1 expression by estrogens, also suggest a role for EDG1 in estrogen-mediated initiation and development of breast cancer.

Tumors expressing EDG1 appeared to be correlated with tumors expressing ER $\alpha$ . Although this may appear to contradict what one would expect if estrogens directly down-regulate EDG1, there is a logical explanation as to why cells in which ER $\alpha$  is detected could still express EDG1. Numerous studies have demonstrated autoregulation of ER $\alpha$ , wherein estrogen induces a rapid decline in both ER $\alpha$  mRNA and protein. Although the mechanism is not well defined, studies support estrogen induction of ER $\alpha$ -mediated transcriptional down-regulation of ER gene transcription, and/or degradation of ER $\alpha$  protein via a proteasome-mediated proteolytic mechanism (11, 24–26). Regardless of the mechanism, ER $\alpha$  may not be detected by immunostaining in cells wherein EDG1 has been down-regulated by estrogens. The correlation between EDG1 and ER $\alpha$  expression in the same tumors provides some evidence that EDG1 expression can be correlated with better prognosis, suggesting correlative studies with other prognostic factors are necessary (Ref. 12; reviewed in Ref. 13).

A more thorough understanding of the role of estrogens in the initiation or promotion of breast cancer is relatively lacking. Some

have proposed ER $\alpha$ -independent pathways involving cytochrome P450-mediated metabolism of estrogens, which elicit direct genotoxic effects by increasing mutation rates (27). Whereas estrogen induces expression of growth factors, proto-oncogenes, proteases, and basement membrane receptors (reviewed in Refs. 3, 28), the exact mechanism(s) by which estrogens regulates mammary epithelial cell proliferation and the relative role of gene induction on the proliferative effects of estrogens is still not well-defined. More recent studies using Serial Analysis of Gene Expression technology have provided additional insights into estrogen gene regulation in normal ER breast cells and breast cancer cells (29, 30). Gene transcripts that have a putative role in cell proliferation were induced or down-regulated by estrogens. Two genes in particular, one encoding a secreted protein lipocalin 2 and another protein of unknown function, EIT-6, appear to be induced directly by estrogen, and enhance proliferation in normal breast cells and breast cancer cells, respectively (29, 30).

Our results support the down-regulation of EDG1 as being involved in estrogen-induced proliferation of breast cells. In particular, EDG1 expression is inhibited by estradiol and EDG1 overexpression (which bypasses the inhibitory effects of estradiol on EDG1 expression) attenuated estradiol-induced cell proliferation. We did not see enhancement of estradiol-induced cell proliferation upon down-regulation of EDG1 expression. It is possible that in this case maximal stimulation of cell proliferation by estrogens has already been achieved.

The fact that only 21% of the breast tumor samples in the larger study showed complete loss of EDG1, and the remaining tumors only showed a severe reduction in EDG1, suggests that the loss of EDG1 in the majority of tumors was not clonal in origin. It is possible that for some tumors, loss of EDG1 is a genetic or epigenetic event, warranting the investigation of possible genetic or epigenetic alterations of EDG1 in breast cancer patients. However, for the majority of the tumors, the decrease in EDG1 expression can be attributed to other factors.

As stated previously, for some tumors, the likely cause is estrogen down-regulation. Although this seems likely for some tumors, six tumors showing severe loss of EDG1 expression (<10% of the cells expressing EDG1) were ER $\alpha$  negative. Recent studies have shown that some breast tumors are not clonal in origin and are instead polyclonal (31, 32). Thus, it is possible that tumors that were 100% negative for ER $\alpha$  expression and only showed a severe reduction in EDG1 expression, instead of 100% loss, may have been tumors of polyclonal origin. It has been proposed that clones with certain somatic mutations may have a growth advantage over other clones (31). It could be speculated that clones which originally had or subsequently acquired alterations in normal EDG1 expression might have a growth advantage over other originating clones because of the ability of EDG1's to inhibit cell growth. This is reasonable to expect because of the loss of EDG1 expression in the majority of the cells within most tumors. Although we only screened a small set of samples, we found that 2 of 3 preinvasive ductal carcinoma *in situ* observed were 100% negative for EDG1. This suggests that in some tumors the loss of EDG1 occurs before tumor invasion, but additional investigation that is beyond the scope of this paper is necessary.

Database searches indicated that EDG1 is localized to chromosome arm 17q.<sup>4</sup> LOH in 17p (containing the *p53* locus) and in 17q (including loss of the whole 17q arm) is common in breast cancers (33, 34). Whereas the *BRCA1* locus is a common site of LOH in breast cancer there are other sites of LOH outside the *BRCA1* locus (35). Although we have not yet established whether the *EDG1* locus undergoes

<sup>4</sup> Internet address: <http://www.ncbi.nlm.nih.gov/cgi-bin/entrez/maps.cgi>.

genetic alterations in breast cancer patients, studies do indicate that *EDG1* is surrounded by markers that have been reported to frequently undergo LOH in breast tumors (36–38).

Regardless of whether or not *EDG1* undergoes LOH in breast cancer patients, the decreased expression of *EDG1* in the majority of breast tumor cells and the ability of *EDG1* to inhibit breast cell growth, strongly support a role for *EDG1* in breast cancer development. The normal function of *EDG1* in normal breast epithelial cells may be to inhibit cell growth and play a role in breast tissue differentiation, as suggested by the increase in *EDG1* expression by the differentiating agent HMBA. The decrease in expression of *EDG1* in breast tumors could be attributed to down-regulation by estrogens, LOH, hypermethylation of the *EDG1* promoter, a combination of these factors, or other unknown factors. Studies are under way to determine the mechanism(s) by which *EDG1* expression is lost in the majority of breast cancer cells and how *EDG1* inhibits cell growth. Understanding these mechanisms should provide additional insights into how estrogens play a role in the initiation of breast tumorigenesis.

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## Identification of a Novel Inhibitor of Breast Cell Growth That Is Down-Regulated by Estrogens and Decreased in Breast Tumors

Bryan M. Wittmann, Nancy Wang and Monica M. Montano

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