PEA-15 Inhibits Tumorigenesis in an MDA-MB-468 Triple-Negative Breast Cancer Xenograft Model through Increased Cytoplasmic Localization of Activated Extracellular Signal-Regulated Kinase

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Abstract

Purpose: To determine the role of PEA-15 in breast cancer.

Experimental Design: A reverse-phase protein array was used to measure PEA-15 expression levels in 320 human breast cancers; these levels were correlated with clinical and tumor characteristics. PEA-15 was overexpressed by an adenovirus vector or by stably expressing PEA-15 in different breast cancer cell lines. The effects on breast cancer cell survival and on the downstream apoptotic signaling pathway were measured in terms of cell proliferation (trypan blue for cell viability, bromodeoxyuridine incorporation for DNA synthesis), anchorage-independent growth (soft agar colony formation), and apoptosis (fluorescence-activated cell sorter analysis). The preclinical efficacy of Ad.PEA-15 given intratumorally was evaluated in nude mice bearing tumors from s.c. implanted human MDA-MB-468 triple-negative breast cancer cells.

Results: In human breast cancers, low levels of PEA-15 expression correlated with high nuclear grade (P < 0.0001) and with negative hormone receptor status (P = 0.0004). Overexpression of PEA-15 in breast cancer cells resulted in growth inhibition, reduction in DNA synthesis, and onset of caspase-8-dependent apoptosis. In athymic nude mice bearing MDA-MB-468 xenografts, tumor volumes were significantly smaller in mice treated intratumorally with Ad.PEA-15 than in control mice (P < 0.0001). Tumors from mice treated with Ad.PEA-15 had increased levels of activated (phosphorylated) extracellular signal-regulated kinase and reduced levels of Ki-67 compared with tumors from nontreated or control-adenovirus-treated mice.

Conclusion: PEA-15 has therapeutic potential in breast cancer. Further preclinical and clinical exploration of PEA-15 as a druggable target is warranted.

Breast cancer is the most common cancer in women in the United States and most of the Western world (1). In 2008, 182,460 cases are expected to be diagnosed (26% of all cancers in women), and 40,480 women are expected to die of the disease (accounting for 15% of all cancer deaths in women, making this disease the second most common cause of cancer death among women). Breast cancer that is negative for estrogen receptor, progesterone receptor, and HER-2 is known as triple receptor-negative breast cancer. It is the only major type of breast cancer for which no specific targeted therapy is available to improve patient outcomes (2). A deeper understanding of the molecular mechanisms responsible for breast cancer is imperative if more effective treatments are to be discovered.

PEA-15 (also called PED) is an acidic, serine-phosphorylated, 15-kDa phosphoprotein that contains a death effector domain and is associated with microtubules (3). In primary astrocytes (the first cells in which this protein was identified), PEA-15 was shown to regulate the nuclear localization of extracellular signal-regulated kinase (ERK) and consequently affect the transcription of ERK-dependent targets such as transcription factor Elk. This function was shown to be important in slowing astrocyte proliferation (4). We previously showed that the antitumor activity of the adenovirus protein E1A was dependent on PEA-15 (5) and that women with ovarian tumors that expressed high
levels of PEA-15 survived longer than did women with tumors that expressed low levels of PEA-15 (6).

A study of PEA-15 suggested that overexpression of PEA-15 led to the inhibition of both Fas-mediated and tumor necrosis factor receptor-1 (TNFR1)–mediated apoptosis in MCF-7 breast cancer cells; this function was thought to involve the displacement of FADD-FLICE binding through MCF-7 breast cancer cells; this function was thought to involve the displacement of FADD-FLICE binding through

Correspondingly, in athymic nude mice bearing human MDA-MB-468 xenografts, tumor volumes were significantly smaller in mice treated intratumorally with Ad.PEA-15 adenovirus than in control mice. Combined, these results contribute to the rationale for evaluating PEA-15 as a therapeutic gene in breast cancer.

The objectives of this project were to look at the correlation of PEA-15 with clinical and tumor characteristics in human breast cancers and to study the in vitro and in vivo antitumor effects of PEA-15 in breast cancer cell lines. In vitro, we used adenovirus Ad.PEA-15 and breast cancer cells that stably express PEA-15 to study its biological function in breast cancer. We found that overexpression of PEA-15 in breast cancer cells resulted in growth inhibition (both ERK and pERK were largely confined to the cytoplasm), reduction in DNA synthesis, and onset of caspase-8–dependent apoptosis. In nude mice, intratumoral injection of adenoviral PEA-15 led to effective delivery and subsequent expression of PEA-15 at the tumor site, which correlated with suppressed tumor growth compared with that in mice treated with control virus or nontreated mice.

**Materials and Methods**

**Cell lines and culture conditions.** The human breast adenocarcinoma cell lines SKBR-3, MDA-MB-231, MCF-7, MDA-MB-468, and MDA-MB-453 were obtained from the American Type Culture Collection. The cells were grown in DMEM/nutrient mixture F12 (DMEM/F12; Life Technologies) supplemented with 10% fetal bovine serum and penicillin/streptomycin, and maintained in a humidified incubator at 37°C containing 5% CO2.

**Western blot analysis.** Cells were washed thrice with PBS (pH 7.4; 1×) and then lysed in lysis buffer [20 mmol/L Na2PO4 (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 100 mmol/L NaF, and 2 mmol/L Na3VO4] as previously described (11). PEA-15 was extracted with NP40 lysis buffer as previously described (12). The antibodies used were rabbit anti–PEA-15 polyclonal antibody (Cell Signaling Technology; used in a 1:1,000 dilution), rabbit anti–p-PEA-15 (S116) (Invitrogen; 1:1,000), rabbit anti–p-PEA-15 (S104) (1:1,000; Cell Signaling Technology), ERK (1:500; Santa Cruz Biotechnology), pERK (1:500; Cell Signaling Technology), actin (1:5,000; Sigma-Aldrich Chemical Co.), poly (ADP-ribose) polymerase (PARP; 1:1,000; BD Biosciences), α-tubulin (1:5,000; Sigma-Aldrich), and caspase-8 (1:500; EMD Chemicals). Secondary rabbit (1:5,000) and mouse (1:5,000) fluorescent antibodies were from Molecular Probes and were detected with an Odyssey imaging system (Li-Cor Biosciences).

**Colony formation assay.** Three human breast cancer cell lines (MDA-MB-468, MCF-7, and MDA-MB-453) were transfected in 100-mm plates with 10 μg of either a vector containing HA-tagged PEA-15 (4) or a control vector (pcDNA3; Stratagene) by means of FuGENE transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. Both plasmids contained a neomycin resistance gene. At 48 h after transfection, cells were trypsinized, diluted at a ratio of 1:5 or 1:10, and then plated on 100-mm plates and grown in DMEM/F12 containing 500 μg/mL of the neomycin analogue G418. Three weeks later, colonies were stained with 0.5% crystal violet in 20% ethanol and counted.

**Transfection conditions.** The PEA-15 transfectants were established as follows. MDA-MB-453 human breast cancer cells were seeded on 100-mm plates and transfected with 10 μg of either a HA-PEA-15 vector (4) or a control vector (pcDNA3; Stratagene) by means of FuGENE transfection reagent. Both plasmids contain a neomycin resistance gene. At 48 h after transfection, cells were trypsinized, diluted 1:5 or 1:10, plated on 100-mm plates, and grown in DMEM/F12 containing 500 μg/mL of G418. Three weeks later, individual neomycin-resistant colonies were cloned and expanded to mass culture.

**In vitro growth assays.** For the in vitro growth experiments, MDA-MB-453 cells (2 × 10⁵ cells) were plated in...
trypan blue, and the numbers of dead and live cells were counted. For the in vitro growth experiments with adenovirus, MDA-MB-468 cells (2 × 10^5) were plated and exposed the next day to Ad.Control or Ad.PEA-15 in serum-free medium for 1 h, which was followed by the addition of DMEM/F12 and incubation for 24, 48, or 72 h. Cells were then harvested for Western blotting and trypan blue exclusion.

**Evaluation of anchorage-independent growth.** To assess anchorage-independent growth, an indicator of in vitro tumorigenicity (13), we mixed cells at 37°C with 0.5% agarose in complete medium and poured the mixture over a layer of 1% agarose in complete medium in six-well plates. The top layer was allowed to gel at 4°C for 15 min, and the plates were then incubated at 37°C for 3 wk. After 3 wk, the plates were stained by adding p-iodonitrotetrazolium violet to each well and incubated at 37°C for 24 h, after which the colonies were photographed with a Zeiss microscope and counted by using the software associated with the microscope.

**Evaluation of DNA synthesis.** DNA synthesis was measured with bromodeoxyuridine (BrdUrd) labeling and detection kit (Roche Diagnostics) as follows. Cells were incubated with culture medium containing BrdUrd for 30 min. Direct immunofluorescence staining was done according to the manufacturer's protocol (Becton Dickinson), and the samples were analyzed on a BD FACScan flow cytometer.

**Evaluation of cell viability following exposure to caspase inhibitor.** MDA-MB-468 cells (2 × 10^5) were plated in triplicate and exposed the next day to Ad.Control or Ad.PEA-15 in serum-free medium for 1 h. Cells were washed with PBS, and DMEM/F12 or DMEM/F12 containing cell-permeable caspase-8 inhibitor Z-IETD-FMK (R&D Systems, Inc.) was then added to achieve a final concentration of 50 μmol/L. The medium was changed after 24 h, and the caspase-8 inhibitor was added again. Forty-eight hours after infection, cells were stained with trypan blue, and the numbers of dead and live cells were counted.

**Fluorescence-activated cell sorting analysis.** MDA-MB-468 cells (2 × 10^5) were plated and exposed the next day to Ad.Control or Ad.PEA-15 in serum-free medium for 1 h, which was followed by the addition of DMEM/F12 and incubation for 48 or 72 h. Apoptotic cells were analyzed by flow cytometry as previously described (11).

**Nuclear/cytoplasmic fractionation.** MDA-MB-468 cells were plated at 1 × 10^6 cells per well in DMEM/F12 medium containing 10% fetal bovine serum and incubated at 37°C for 24 h, after which they were exposed the next day to Ad.Control or Ad.PEA-15 in serum-free medium for 1 h. This step was followed by the addition of DMEM/F12 and incubation for 48 h. Cells were pelleted, and the nuclear and cytoplasmic fractions were separated with a nuclear/cytoplasmic fractionation kit according to the manufacturer's protocol (BioVision). Protein concentrations in the cytoplasmic and nuclear fractions were measured with a protein assay kit from Bio-Rad Laboratories.

**Apoptosis detection by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay.** The presence of apoptotic cells within the tumor sections was evaluated by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay as previously described. (6). Percentage of apoptosis was determined by counting the number of apoptotic cells and dividing by the total number of cells in the field. Nine high-power fields per slide were evaluated, and the average was determined.

**Athymic nude mice.** Four- to six-week-old female athymic BALB/c nu/nu mice were purchased from The Jackson Laboratory. Animal care and use were in accordance with institutional and the NIH guidelines.

**MDA-MB-468 breast cancer xenograft model.** To establish breast cancer xenografts in nude mice, MDA-MB-468 cells in log-phase growth were trypanized and washed twice with PBS. Cell viability was determined by trypan blue exclusion, and cells were resuspended in PBS. Cells (2 × 10^6) in 0.2 mL of PBS were injected under aseptic conditions into the mammary fat pads of nude mice. The mice that developed tumors were randomly assigned to three groups: group 1, nontreated; group 2, Ad.Control; and group 3, Ad.PEA-15. After tumors reached a volume close to 100 mm^3, treatment was initiated. For intratumoral injection, 1 × 10^8 plaque-forming unit viruses per treatment were administered with a treatment schedule of weekly for 6 wk. Tumor size was measured once weekly. Tumor volume (mm^3) was calculated, and changes in tumor volumes were tested for statistical significance with the Mann-Whitney test or Student’s two-tailed t test.

**Immunohistochemical analysis.** For immunohistochemical analysis (IHC), paraffin blocks were sliced in 4-mm sections and deparaffinized. The expression of PEA-15, pERK, and Ki-67 protein in the tumor tissue sections was detected with the labeled streptavidin-biotin detection kit (DAKO) as previously described (14). Expression of PEA-15, pERK, and Ki-67 protein levels in established MDA-MB-468 tumors after treatment with Ad.PEA-15 was evaluated by IHC staining. Briefly, tumors were harvested, and sections were incubated with anti-PEA-15 polyclonal antibody (SynPep) diluted 1:200, pERK monoclonal antibody (Cell Signaling) diluted 1:100, or Ki-67 antibody (Lab Vision) diluted 1:200.

**Human tumor samples.** We obtained 488 primary solid breast tumors from the breast tissue frozen-tumor banks at the M.D. Anderson Cancer Center and the Hospital Clínico Universitario de Valencia. Chart review, use of tissue blocks in the creation of the reverse-phase microarrays, and use of the microarrays for the studies described here were approved by the institutional review boards of M.D. Anderson and Hospital Clínico Universitario de Valencia.

We only included in our retrospective analysis patients who had invasive ductal or invasive lobular histologic findings, as well as a disease stage of I, II, or III (n = 320). The mean, median, minimum, and maximum values are reported for age at diagnosis, number of affected...
nodes, and PEA-15 level. The numbers and percentages of patients for each tumor histologic type, nuclear grade, estrogen receptor (ER) and progesterone receptor (PR) status, and disease stage were summarized.

**Reverse-phase protein lysate microarray.** Protein was extracted from human tumors, and a reverse-phase protein lysate microarray was created as previously described (15). Before reverse-phase protein array (RPPA) lysates were prepared from breast core tissue, the minimal percentage of tumor tissue in these samples was confirmed by a pathologist to be at least 70%. Briefly, the tumors were lysed by using lysis buffer and homogenization. Tumor lysates were normalized, and the supernatants were manually diluted in six or eight 2-fold serial dilutions with lysis buffer. An Aushon Biosystems 2470 arrayer created 1,056 sample arrays on nitrocellulose-coated FAST slides (Schleicher & Schuell BioScience, Inc.) from the serial dilutions. Each slide was then probed with a validated primary PEA-15 antibody (SynPep), and the signal was amplified with a DakoCytomation catalyzed system. The level of PEA-15 in each sample was expressed as a log-mean centered value after correction for protein loading with the use of the average expression levels of >50 proteins as previously described (15).

**Statistical analysis.** The statistical analysis was based on the subset of 320 patients with invasive ductal or invasive lobular stage I, II, or III tumors. For demographic and clinical factors, descriptive statistics were provided. For continuous variables, mean, SD, median, and range were summarized, and the two-sample t test or ANOVA method was used to compare the continuous variables between two or more than two groups. For categorical variables, the number of patients in each level and their corresponding frequency were provided.

**Results**

**PEA-15 correlated with hormone receptor status and nuclear grade of tumors of patients with breast cancer.** We first examined the clinical relevance of PEA-15 in samples from 320 breast cancer patients. Patient characteristics are summarized in Table 1. The median age is 64 years (range, 27-89 years). Most patients had stage II disease (61%), invasive ductal carcinoma (93%), modified tumor nuclear grades of 2 and 3 (79%), and positive hormone receptor status (68%).

We examined correlations between PEA-15 expression and clinical factors such as hormone receptor status,

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**Table 1. Summary statistics for PEA-15 by clinical and tumor characteristics**

<table>
<thead>
<tr>
<th>Clinical factor</th>
<th>Sample number (%)</th>
<th>Mean (SD)</th>
<th>Median</th>
<th>Range (min, max)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (y)</td>
<td>320</td>
<td>62.58</td>
<td>64</td>
<td>(27, 88.9)</td>
<td></td>
</tr>
<tr>
<td>Histopathologic diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>297 (92.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive lobular carcinoma</td>
<td>23 (7.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease stage</td>
<td>320</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>78 (24.4)</td>
<td>0.151 (0.374)</td>
<td>0.110</td>
<td>(0.550, 1.327)</td>
<td>0.59*</td>
</tr>
<tr>
<td>II</td>
<td>196 (61.2)</td>
<td>0.103 (0.377)</td>
<td>0.093</td>
<td>(−0.112, 1.184)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>46 (14.4)</td>
<td>0.089 (0.447)</td>
<td>0.055</td>
<td>(−0.706, 1.277)</td>
<td></td>
</tr>
<tr>
<td>Nuclear grade</td>
<td>232</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>47 (20.3)</td>
<td>0.246 (0.315)</td>
<td>0.222</td>
<td>(−0.367, 1.062)</td>
<td>&lt;0.0001†</td>
</tr>
<tr>
<td>2</td>
<td>92 (39.7)</td>
<td>0.134 (0.350)</td>
<td>0.100</td>
<td>(−0.629, 1.327)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>93 (40)</td>
<td>−0.107 (0.363)</td>
<td>−0.106</td>
<td>(−1.112, 0.917)</td>
<td></td>
</tr>
<tr>
<td>ER/PR status</td>
<td>320</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DN</td>
<td>103 (32.2)</td>
<td>0.0021 (0.421)</td>
<td>−0.070</td>
<td>(−1.112, 1.277)</td>
<td>0.0004†</td>
</tr>
<tr>
<td>Positive for ER, PR, ER and PR</td>
<td>217 (67.8)</td>
<td>0.165 (0.358)</td>
<td>0.152</td>
<td>(−0.706, 1.327)</td>
<td></td>
</tr>
<tr>
<td>No. of positive nodes</td>
<td>320</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>190</td>
<td>0.142 (0.405)</td>
<td>0.103</td>
<td>(−1.112, 1.327)</td>
<td>0.43*</td>
</tr>
<tr>
<td>1-3</td>
<td>84</td>
<td>0.076 (0.340)</td>
<td>0.074</td>
<td>(−0.667, 1.149)</td>
<td></td>
</tr>
<tr>
<td>4-9</td>
<td>34</td>
<td>0.053 (0.385)</td>
<td>0.043</td>
<td>(−0.706, 0.779)</td>
<td></td>
</tr>
<tr>
<td>≥10</td>
<td>12</td>
<td>0.077 (0.391)</td>
<td>0.020</td>
<td>(−0.548, 0.917)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: For nuclear grade, because overall ANOVA showed a significant P value, pairwise comparisons were done at an α level of 0.05 using the Bonferroni comparison procedure. We detected a significant difference between grades I and III and between grades II and III. The number of patients with known nuclear grade is lower as 88 patients have the grade variable missing.

Abbreviation: DN, double negative for both ER and PR.

*P values from ANOVA test.
†P value from two-sample t test.
number of nodes affected, stage of disease, and nuclear grade of breast cancer (Table 1). There was an inverse correlation between PEA-15 expression and nuclear grade ($P < 0.0001$). Low levels of PEA-15 expression correlated with negative hormone receptor status ($P = 0.0004$). The Pearson correlation coefficient for the correlation between PEA-15 and proliferating cell nuclear antigen, a cell proliferation marker, was $-0.37$, and the $P$ value associated with it was $<0.0001$. This indicated that high PEA-15 expression was significantly correlated with low proliferation. There was no significant correlation evident between PEA-15 and tumor-node-metastasis disease stage or number of involved axillary lymph nodes. We confirmed that there was good correlation between PEA-15 expression measured by RPPA and by IHC. To do this we analyzed the correlation between ER positivity by IHC and expression level of ER by RPPA using receiver operating characteristic curve to confirm the accuracy and found that the area under the curve was 0.919.

The results proved that the expression level of ER by RPPA correlated well with ER positivity by IHC.

**PEA-15 overexpression inhibited human breast cancer cell growth in vitro.** Because of low PEA-15 expression breast tumor had undifferentiated features and high proliferation in clinical samples, we hypothesized that high PEA-15 expression inhibits cell proliferation in human breast cancer. We first assessed PEA-15 expression in five human breast cancer cell lines. MDA-MB-468, MCF-7, and MDA-MB-453 cells expressed low levels of PEA-15, and MDA-MB-231 and SKBR-3 cells expressed high levels of PEA-15 (Fig. 1A).

To determine whether PEA-15 overexpression could suppress cell growth, we transfected PEA-15 into MCF-7, MDA-MB-453, and MDA-MB-468 cells (which all expressed low basal amounts of PEA-15) and did a colony formation assay. We observed inhibition (range, 30-60%) of the number of G418-resistant colonies in the PEA-15–transfected cells in all three cell lines (Fig. 1B). This result suggested that PEA-15 possesses strong

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**Fig. 1.** Ad.PEA-15 inhibited cell proliferation and anchorage-independent growth in breast cancer cells. A, Western blot analysis showed heterogeneous PEA-15 protein expression in five breast cancer cell lines. Equal loading was confirmed by blotting with an anti-actin antibody. The PEA-15 levels was low in MDA-MB-453, MDA-MB-468, and MCF-7 cells. B, high PEA-15 expression correlated with suppression of colony formation. Three human breast cancer cell lines were transfected with the control pcDNA3 vector or HA-PEA-15 vector. The shown percentages of inhibition after transfection are averages of two different experiments. C, PEA-15 overexpression produced by transfection with HA-PEA-15–induced pERK expression in MDA-MB-453 cells. D, the MDA-MB-453-PEA-15 stable transfectant showed growth inhibition after 3 d ($P < 0.001$). E, soft agar colony formation showed a reduction in number of colonies for the MDA-MB-453-PEA-15 stable transfectant.
growth-inhibitory activity in breast cancer cells. To confirm the antitumor activity of PEA-15, we established a stable, PEA-15–overexpressing transfectant in MDA-MB-453 cells, which, in addition to expressing high levels of PEA-15, also expressed high levels of p-ERK (Fig. 1C). Cell proliferation was slower in the wild-type PEA-15 transfected than in control cells that did not overexpress PEA-15 (parental and control vector–transfected cells; Fig. 1D).

Next, we assessed the anchorage-independent growth of the PEA-15 transfectants in soft agar as an indirect test of their tumorigenicity. We observed that the PEA-15 stable transfectants formed fewer colonies (i.e., were less tumorigenic) than did the parental cells or the control vector–transfected cells (Fig. 1E).

To study the biological function of PEA-15 and to generate a therapeutic agent for efficacy study in a breast cancer xenograft model, we used the adenoviral vector Ad. PEA-15 in our previous studies (6). We infected MDA-MB-468 triple-negative breast cancer cells, known to be tumorigenic in mice (16), with Ad.Control or Ad.PEA-15 and confirmed by Western blotting that PEA-15 was expressed in the cytoplasm of Ad.PEA-15–infected cells and not in nontreated or Ad.Control-infected cells (Fig. 2A). Ad.PEA-15 sequestered pERK predominantly in the cytoplasm (Fig. 2A) and increased pERK expression level, consistent with the findings of a previous study (4).

We then assessed cell viability by trypan blue exclusion (72 hours) and DNA synthesis by BrdUrd incorporation assay (24 and 48 hours). We observed suppression of growth at 72 hours of Ad.PEA-15–infected MDA-MB-468 cells compared with that of Ad.Control-infected cells (P < 0.005; Fig. 2B). Ad.PEA-15 infection also significantly inhibited DNA synthesis at 48 hours (Fig. 2C). These results indicated that Ad.PEA-15 had a cytotoxic effect on breast cancer cells, resulting in growth inhibition.

**Ad.PEA-15 induced apoptosis in breast cancer cells.** To determine whether the cytotoxicity of PEA-15 in MDA-MB-468 cells was attributable to an increase in apoptosis or autophagy, we used propidium iodide staining and flow cytometry. Because PEA-15 has been shown to induce...
enhancement of TNFR1-induced apoptosis (8), we tested if PEA-15 could induce apoptosis in MDA-MB-468 cells. The sub-G1 fractions ranged from 4% to 9% in Ad.Control-treated cells and from 5% to 17% in Ad.PEA-15–treated cells, indicating that Ad.PEA-15 induced apoptosis (Fig. 2D) but not LC-3 cleavage, which is indicative of autophagy (data not shown). We observed that when PEA-15 was overexpressed in MDA-MB-468 cells, there

![Image](74x178 to 526x652)

**Fig. 3.** PEA-15 gene therapy inhibited tumor growth in a breast cancer xenograft model. A, IHC staining of a representative tumor tissue sample from mice implanted with MDA-MB-468 cells. B, female nude mice (seven per group) were given injections of MDA-MB-468 cells and, after tumor formation, divided into groups for intratumoral therapy. Representative mice from each group (left, nontreated; center, Ad.Control; right, Ad.PEA-15) show that mice treated with Ad.PEA-15 had smaller tumors than did control mice. C, the average tumor size was significantly smaller in PEA-15–treated mice (P < 0.001). D, IHC stains of representative tumor tissue samples from mice implanted with MDA-MB-468 cells and treated with Ad.PEA-15. Total PEA-15, p-PEA-15 (S116), p-PEA-15 (S104), and pERK were highly expressed in the PEA-15–treated group. Ki-67 (a hallmark of proliferation) expression was significantly reduced in these mice. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (depicting apoptosis) was marginally increased in these mice.
was cleavage of caspase-8 (Fig. 2E), resulting in caspase-8–dependent apoptosis. In addition, cells treated with Ad.PEA-15 showed increased cleavage of PARP (Fig. 2A and E). Furthermore, the addition of a caspase-8 inhibitor reduced cell death induced by Ad.PEA-15 by 7-fold (Fig. 2F) and inhibited cleavage of caspase-8, as seen in Western blot analysis (data not shown). These findings indicated that the enhanced apoptosis resulting from Ad.PEA-15 was a consequence of cleavage of caspase-8, suggesting that Ad.PEA-15 induced apoptosis at least in part through activating the death effector pathway.

**Ad.PEA-15 therapy inhibited tumor growth in a breast cancer xenograft model.** After confirming that PEA-15 reduced cell viability and enhanced apoptosis in vitro, we examined whether PEA-15 gene therapy would inhibit tumors in a xenograft model of breast cancer. When human MDA-MB-468 cells were injected into the mammary fat pads of nude mice, the tumors that developed following the injection of MDA-MB-468 cells showed features of a poorly differentiated tumor, consistent with a mammary tumor (Fig. 3A). Intratumoral administration of Ad.PEA-15 elicited a strong antitumor effect, significantly suppressing tumor growth after six weekly injections compared with that in mice that received the control vehicle (P < 0.001; Fig. 3B and C). IHC analysis of tumor tissue verified that the tumor cells had been transfected by Ad.PEA-15. As expected, PEA-15 was overexpressed in tumors from mice treated with Ad.PEA-15 (Fig. 3D). In addition, IHC staining of these tumors showed phosphorylation at both S104 and S116 (Fig. 3D), further suggesting that phosphorylation of PEA-15 at both S104 and S116 is required for PEA-15–induced apoptosis. Quantification of apoptotic cells within the tumor sections revealed a marginal increase in apoptosis in the tumors treated with Ad.PEA-15 compared with apoptosis in tumors treated with either control. The effects of PEA-15 on mitogen-activated protein kinase signaling and cell proliferation were also assessed by immunohistochemistry by measuring levels of pERK and Ki-67 staining, respectively, in the tumor samples. An increase in pERK and reduction in Ki-67 (50%) were seen in tumors treated with Ad.PEA-15 compared with the levels in tumors treated with either control. These results suggest that the antitumor activity of PEA-15 in breast cancer may be attributed to the inhibition of cell proliferation (as indicated by Ki-67) and of tumorigenicity through the modulation of ERK.

**Discussion**

Our results show that the PEA-15 gene therapy inhibits tumor growth in a xenograft model of breast cancer. We confirmed the importance of PEA-15 antitumor activity in vitro by showing that when PEA-15 is overexpressed, it inhibits cell proliferation, sequesters ERK in the cytoplasm, and induces apoptosis through the death effector pathway. Reverse-phase protein lysate microarray revealed that a loss of PEA-15 expression correlated with undifferentiated (high-grade) tumors and that low levels of PEA-15 are expressed in hormone receptor–negative tumors of patients with breast cancer. Through these findings, we have shown that, by inhibiting tumorigenicity through modulation of ERK, PEA-15 may serve as a therapeutic agent in breast cancer.

PEA-15 is a novel protein that is expressed in a wide range of normal tissues (3, 17). It has also been noted to be present in breast and ovarian cancer. PEA-15 may act as a master regulator of ERK by regulating the cellular localization of ERK. Specifically, PEA-15 can sequester ERK in the cytoplasm, thereby preventing its activation. Our group and others have shown that PEA-15 blocks the activity of ERK by inhibiting the transcription factor Elk-1, which regulates ERK-dependent transcription (4, 5, 18, 19). Thus, PEA-15 controls cell proliferation by preventing ERK accumulation in the nucleus. However, certain cancer and normal cells proliferate rapidly while expressing PEA-15 at high levels (3, 8). In contrast, we recently found that high levels of PEA-15 protein expression in women with ovarian cancer were independently associated with improved overall survival (5).

The structure of PEA-15 suggests that phosphorylation could regulate its binding to ERK. The COOH-terminal tail, which contains Ser104 and Ser116, is required for ERK binding (4, 18, 20–22). PEA-15 is an endogenous substrate for protein kinase C (S104; ref. 3), calmodulin–dependent protein kinase II (Ser116; ref. 23), and Akt (Ser116; refs. 3, 17, 23, 24). It has been shown that the phosphorylation of both Ser104 and Ser116 on PEA-15 is required to block its interaction with ERK to regulate the mitogen-activated protein kinase cascade (25). Thus, how PEA-15 modulates ERK activity in breast cancer cells may depend on the state of phosphorylation of PEA-15.

In breast cancer, low PEA-15 expression has been linked with aggressive forms of disease. Loss of PEA-15 expression is a marker of the transition from noninvasive (ductal carcinoma in situ) to invasive mammary epithelial tumors (9). Similarly, increased PEA-15 expression was associated with reduced invasion in breast cancer through effects on the ERK pathway (9). As reported in this article, we found that PEA-15 expression was lower in high-grade than in low-grade breast tumors. These findings led us to speculate that overexpressing PEA-15 in breast cancer cells could inhibit tumorigenicity. One interesting finding we noted is that high levels of PEA-15 were expressed in hormone receptor–positive tumors of patients with breast cancer. Furthermore, we did not observe a high incidence of axillary lymph node involvement when PEA-15 expression was low. Therefore, we speculate that low PEA-15 may contribute to high proliferation and aggressiveness of hormone receptor–negative breast cancer.

To our knowledge, we are the first to report that PEA-15 may be responsible for reduced tumorigenicity in a breast cancer xenograft model. In the present study, we evaluated the antitumor activity of Ad.PEA-15 in a xenograft breast cancer model and found clear evidence of apoptosis in vitro and of Ki-67 downregulation and pERK activation in vivo. However, we only observed marginal apoptosis in vivo. This discrepancy may be due to differences between the in vitro and in vivo conditions.
and in vivo experiments in the time points at which cells or tumors were harvested after adenoviral infection of cells or injection in mice. In addition, apoptosis in vivo is very difficult to study because apoptotic cells are rapidly removed by the neighboring phagocytic cells (26). We observed both nuclear and cytoplasmic pERK and PEA-15 in vivo, but only cytoplasmic pERK and PEA-15 in vitro. This may be because the cellular response to MAP/ERK kinase signaling is modulated by features of the tumor microenvironment in vivo, such as the extent of hypoxia, growth factor availability, and stroma-tumor interactions (27). Another potential reason for the difference between the results of cytoplasmic/nuclear fractionation and IHC staining is that nuclear localization of ERK is transient and harvest of cells in vitro and tumors in vivo was done at significantly different times. Our in vitro finding that Ad.PEA-15 was more cytotoxic as a result of apoptosis in breast cancer cells seems to contradict the previous finding in which PEA-15 was shown to be antiapoptotic. However, depending on the cell line, overexpression of PEA-15 led to either inhibition of both Fas-mediated and TNFR1-mediated apoptosis, as seen in MCF-7 breast cancer cells, or inhibition of Fas-mediated apoptosis but enhancement of TNFR1-induced apoptosis, as seen in NIH3T3 cells (8). The phosphorylation status of PEA-15 was found to be key in determining which pathway was activated in those cells; artificially phosphorylating Ser104 or Ser116 in PEA-15 blocked the inhibition of Fas-induced apoptosis but had no effect on TNFR1-mediated apoptosis (apoptosis was still enhanced; ref. 8). In other work, phosphorylation of PEA-15 at Ser116 by Akt led to an increase in the stability and the antiapoptotic function of PEA-15 in HEK293 cells; in other words, the unphosphorylated form was apoptotic, but the phosphorylated form was antiapoptotic (24). Others have shown that phosphorylating PEA-15 at both Ser104 and Ser116 blocked the interaction of PEA-15 with ERK 1 and 2 both in vitro and in vivo, resulting in the proliferation of cells that expressed high levels of PEA-15 (25). Finally, it was shown that phosphorylating PEA-15 at Ser104 blocked ERK binding in vitro and in vivo, but phosphorylation at Ser116 promoted binding to the proapoptotic protein FADD, resulting in inhibition of apoptosis (28).

A recent study has shown that phosphorylation of PEA-15 at Ser116 sequesters FADD, resulting in weak caspase-8 activation. In our study, the endogenous expression of Ser116-phosphorylated PEA-15 in MDA-MB-468 cells, which lack PTEN, was low. In our results, cell type specificity, phosphorylation status of PEA-15, and the fact that PEA-15 was exogenously overexpressed could explain, at least in part, the heterogeneity in the response of cancer cells to PEA-15 overexpression. Further studies are warranted to determine the mechanism by which PEA-15 induces apoptosis when PEA-15 is intentionally overexpressed in breast cancer.

In summary, PEA-15 may suppress the tumorigenicity of breast cancer through the regulation of cell proliferation. Our findings of induction of apoptosis and inactivation of ERK activity by PEA-15 and the inhibition of tumorigenicity in a MDA-MB-468 triple-negative breast cancer xenograft model, and of correlation of low PEA-15 expression with high nuclear grade and negative hormone receptor in breast cancer samples, justify the development of PEA-15 as a druggable target in hormone receptor-negative breast cancer. In particular, in the future, we propose to develop a new cellular localization-based targeted therapy in triple-negative breast cancer. We will design a molecularly targeted drug with a structure very similar to that of PEA-15 that will inhibit ERK and thus block the growth and spread of cancer. Instead of using gene therapy, we will work with peptides and small molecules, which have much faster approval processes for entering clinical trials because of their stability as drugs. In breast cancer cells, PEA-15, an ERK-binding protein, may represent a useful way of targeting ERK, a key signaling molecule involved in proliferation and invasiveness.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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