

# Phosphomimetic Mutants of Pigment Epithelium-Derived Factor with Enhanced Antiangiogenic Activity as Potent Anticancer Agents

Alexander Konson, Sunila Pradeep, and Rony Seger

## Abstract

Pigment epithelium-derived factor (PEDF) is an endogenous inhibitor of angiogenesis and a promising anticancer agent capable of suppressing solid tumor growth in animal cancer models. We have previously shown that PEDF can be phosphorylated and that distinct phosphorylation states of this factor differentially regulate its physiologic function. Here, we report that phosphomimetic mutants of PEDF, which possess significantly increased antiangiogenic activity, are much more efficient than wild-type (WT) PEDF in inhibiting growth and neovascularization in MDA-MB-231 (breast cancer), HCT116 (colon cancer), and U87-MG (glioblastoma) xenograft models. Importantly, the antitumor activity of the phosphomimetic mutants is comparable with that of the established antiangiogenic agent bevacizumab. However, unlike bevacizumab, these compounds act in a vascular endothelial growth factor (VEGF)-independent manner, as they do not affect the levels of VEGF-A mRNA and VEGF receptor 2 phosphorylation. Further immunohistochemical analysis revealed that PEDF mutants affect mainly tumor-residing endothelial cells and prevent the formation of intratumoral vascular network by facilitating endothelial cell apoptosis. It was also found that PEDF mutants reduce survival of endothelial cells in culture much better than WT-PEDF, an effect that is apparent even in the presence of VEGF or basic fibroblast growth factor, and promote much stronger endothelial cell apoptosis. On the other hand, PEDF and its mutants did not affect survival of cultured cancer cells, indicating that the antiangiogenic activity of these agents is the foremost element of the observed antitumor effect. These findings have specific implications on improving the properties of WT-PEDF, which is currently in preclinical development, and encourage the development of PEDF mutants as specific, neovascularization-targeting anticancer agents. *Cancer Res*; 70(15): 6247–57. ©2010 AACR.

## Introduction

Neovascularization is a well-recognized target for anti-cancer therapy. Numerous angiogenesis-targeted anticancer agents have already been approved, while others are in clinical trials nowadays (1). As such, the pigment epithelium-derived factor (PEDF) was first isolated from the conditioned medium of primary human retinal pigment epithelial cells, where it was shown to act as a neurotrophic and antiangiogenic factor (2). In particular, PEDF plays a key role in the reduction of abnormal neovascularization in the eye, and its natural antiangiogenic activity is far greater than that of any other known endogenously produced factor (3, 4).

Recently, it has become evident that PEDF is widely expressed throughout the human body and is persistent in systemic circulation (5, 6), thereby suggesting its possible involvement in angiogenesis-associated processes, including development and growth of solid tumors. Indeed, downregulation of PEDF mRNA and/or protein has been detected in a wide range of human malignancies (7–11). PEDF expression was shown to be inversely correlated with cancer progression (7), intratumoral microvessel density (MVD; refs. 11, 12), metastatic potential (12, 13), and less favorable prognosis (11, 12). In addition, it has been shown that PEDF-overexpressing cancer cells exhibit reduced growth rate *in vivo* (14, 15) and that PEDF therapy results in a profound inhibition of tumor growth in animal cancer models (8, 16–19). Yet, the exact molecular mechanism by which PEDF causes tumor suppression is not completely understood, and it has been proposed that PEDF may possess both indirect and direct antitumor effects (6). Indirectly, reduction in tumor growth is achieved through the antiangiogenic action of PEDF, in which the selective targeting of newly formed vasculature without harming the existing blood vessels is of a particular importance (20). This effect was suggested to involve inhibition of activity and/or expression of vascular endothelial growth factor (VEGF; refs. 6, 21), as well as the proapoptotic activity of

**Authors' Affiliation:** Department of Biological Regulation, The Weizmann Institute of Science, Rehovot, Israel

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

**Corresponding Author:** Rony Seger, Department of Biological Regulation, The Weizmann Institute of Science, POB 26, Herzl Str., 76100 Rehovot, Israel. Phone: 972-8-9343602; Fax: 972-8-9344116; E-mail: rony.seger@weizmann.ac.il

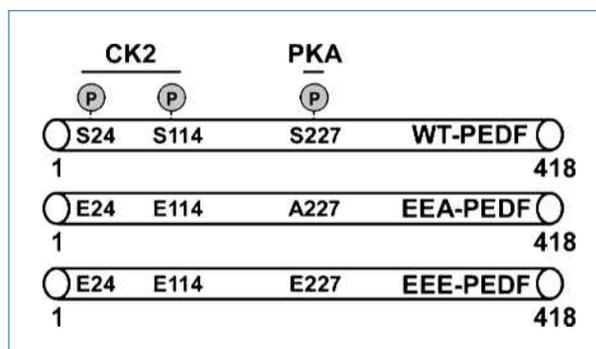
doi: 10.1158/0008-5472.CAN-10-0434

©2010 American Association for Cancer Research.

PEDF toward immature and migrating endothelial cells (6, 21, 22). Although still controversial, it has been reported that PEDF also exerts a direct antitumor effect, possibly by inducing either antiproliferative or prodifferentiation activities toward cancer cells (15, 21).

In a previous study, our group showed that plasma PEDF is a phosphoprotein that is phosphorylated by casein kinase 2 (CK2) on Ser24 and Ser114 and by protein kinase A (PKA) on Ser227, and that variable phosphorylation states of PEDF differentially regulate its physiologic function (23, 24). Using several phosphorylation site mutants that mimic either the phosphorylated (Ser to Glu) or nonphosphorylated (Ser to Ala) forms of PEDF, we showed that triply phosphorylated PEDF serves as a much stronger antiangiogenic factor than its wild-type (WT) form while retaining basal neurotrophic activity. On the other hand, the doubly CK2-phosphorylated PEDF also exhibits higher antiangiogenic activity than WT-PEDF, but has no detectable neurotrophic activity. Thus, we hypothesized that phosphomimetic mutants of PEDF, namely EEA-PEDF (S24E114E227A) and EEE-PEDF (S24E114E227E; schematically represented in Fig. 1), may serve as more efficient neovascularization-targeting agents than WT-PEDF and thus may have a stronger inhibitory effect on the growth of primary tumor, either with or without a neurotrophic activity.

Indeed, we report here that PEDF mutants inhibit tumor growth better than WT-PEDF, where EEE-PEDF exhibits superior antitumor activity, which is comparable with that of the established antiangiogenic agent bevacizumab (Avastin). The enhanced antitumor effect of PEDF mutants is associated with more profound reduction in MVD and increased ability to induce apoptosis of endothelial cells that build the tumor vasculature, acting in a VEGF-independent manner. In addition, we show that PEDF and more so its mutants efficiently induce apoptosis of cultured endothelial cells but do not affect the survival of cancer cells, suggesting that the antitumor effect of these agents is indirect and is achieved mainly through their antiangiogenic activity.



**Figure 1.** Schematic representation of WT-PEDF and its phosphomimetic mutants, EEA-PEDF and EEE-PEDF. EEA-PEDF (S24E114E227A): PEDF mutant in which Ser24 and Ser114 (CK2 phosphorylation sites) were replaced with phosphomimetic Glu, whereas Ser227 (PKA phosphorylation site) was replaced with nonphosphorylatable Ala. EEE-PEDF (S24E114E227E): all three phosphorylation sites mutated to phosphomimetic Glu.

## Materials and Methods

### Materials

Bevacizumab (Avastin) for injection was purchased from Roche. Recombinant VEGF and basic fibroblast growth factor (bFGF) were from Sigma. The following primary antibodies were used: anti-CD34 (Cedarlane), anti-CD31 (BD Pharmingen), anti-VEGF receptor 2 (VEGFR2)/Flk-1, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology), anti-phospho (Y951) VEGFR2, anti-cleaved caspase-3, anti-His-tag (Cell Signaling), and anti-tubulin (Sigma).

### Cell culture

All cancer cell lines used in this study were purchased from the American Type Culture Collection (ATCC) during the last 10 years. Cells were expanded by culturing them for less than two passages and stored frozen in liquid nitrogen according to explicit ATCC guidelines. Thawed low-passage cells were used for experiments within the period of 4 months. U87-MG were cultured in 4.5 g/L D-glucose DMEM (Invitrogen) supplemented with 2 mmol/L L-glutamine, antibiotics, and 10% fetal calf serum (FCS). HCT116 and MDA-MB-231 were grown in McCoy's 5A medium (Sigma) and RPMI medium (Invitrogen), respectively, with the same supplements plus 1 mmol/L sodium pyruvate. Endothelial cells [human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC)] were a gift of Prof. I. Vlodavsky (Cancer and Vascular Biology Research Center, Technion, Haifa, Israel) and were previously described and authenticated (25, 26). For the experiments, HUVEC and BAEC were used between passages 3 to 7. BAEC were cultured in 4.5 g/L D-glucose DMEM supplemented with 2 mmol/L L-glutamine, antibiotics, and 10% FCS. HUVEC were cultured on gelatin-coated plates in M199 medium (Sigma) with the same supplements, 0.1 mg/mL heparin, and 0.025 mg/mL ECGS (BT-203, Biomedical Technologies). All cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

### Animal studies

All animal experiments were approved by the Animal Care and Use Committee of the Weizmann Institute of Science (Rehovot, Israel). Female CD-1 nude mice (Harlan), 6 to 8 weeks of age, were inoculated s.c. in the flank region with  $3 \times 10^6$  MDA-MB-231,  $3 \times 10^6$  U87-MG, or  $2 \times 10^6$  HCT116 cells in 100  $\mu$ L PBS. Tumors were allowed to develop to the size of  $\sim 100$  mm<sup>3</sup> and then animals were randomly allocated to different treatment groups. Recombinant PEDF constructs and bevacizumab were administered by i.v. injection into the tail vein. Tumor dimensions were measured with a digital sliding caliper and, thereafter, tumor volume was calculated from the major dimension ( $L$ ) and minor dimension ( $S$ ) using the following equation: tumor volume ( $V$ ) =  $L \times (S)^2 / 2$ . To assess any signs of systemic toxicity, body weight was monitored three times per week. At the end point, weight of selected organs was recorded and vascularization was examined immunohistochemically using anti-CD31 antibody, as described below.

### PEDF production

WT-PEDF, EEE-PEDF, and EEA-PEDF were cloned from pBlueScript/PEDF, pCDNA3/EEE, and pCDNA3/EEA (23, 24), respectively, into pRSET(A) (Invitrogen), and expressed in *E. coli* BL21. Bacterial cells were grown at 30°C to OD<sub>600 nm</sub> = 0.5 to 0.6, and the expression of recombinant proteins was induced by 0.5 mmol/L isopropyl- $\beta$ -D-galactopyranoside for 4 to 5 hours. Pelleted bacterial cells were lysed, and purification of recombinant proteins was performed using ion metal affinity chromatography with Ni-NTA His-Bind resin (Novagen) according to the manufacturer's protocol. Elution fractions were resolved on SDS-PAGE followed by silver staining or Western blotting with anti-PEDF antibody developed by the Antibody Unit of the Weizmann Institute of Science. The identity of recombinant WT-PEDF and its mutants was verified by mass spectroscopy. Eluates that exhibited >90% purity were dialyzed against PBS.

### Immunohistochemistry

Excised tumors were fixed in either formalin or zinc buffer, embedded in paraffin, and sectioned. Deparaffinized and rehydrated tumor sections were subjected to antigen retrieval by microwave treatment in 10 mmol/L sodium citrate (pH 6.0; formalin-fixed tissues only). Sections were blocked with 2% normal horse serum and either underwent terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (as described below) or were immunostained with hematoxylin and eosin (H&E), 4',6-diamidino-2-phenylindole (DAPI; Jackson ImmunoResearch), anti-CD31, or anti-CD34 antibodies followed by incubation with corresponding secondary antibodies. MVD in the most intensive neovascularization areas ("hotspots") was assessed according to previously established methods (27). Individual microvessels were counted at  $\times 10$  field using DeltaVision OMX fluorescent microscope station supplied with a digital camera (Applied Precision). Quantification of the apoptotic rate in tumors was performed by calculating the percentage of apoptotic (TUNEL-positive) cells out of total number of either CD34-positive or CD34-negative cells in a field. Pictures were taken, deconvolved, and processed using SoftWorx software (Applied Precision).

### Immunoblotting

Cells were grown to subconfluency and then serum starved (0.1% FCS) for 16 hours. After incubation with indicated treatments, cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer and extracts were obtained by centrifugation at 15,000  $\times g$  at 4°C. Tissue homogenates from freshly harvested tumors were prepared using pestle homogenizer in RIPA buffer (2 mL/g tissue) followed by centrifugation. Aliquots of cellular extracts or tissue homogenates were subjected to SDS-PAGE and immunoblotting with the corresponding primary antibody, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Jackson), and developed using EZ-ECL kit (Biological Industries) in Bio-Rad ChemiDoc XRS imaging station. Each experiment was performed three times to test for reproducibility and obtain statistically significant data. Quantification of the

band intensities was performed using QuantityOne software (Bio-Rad).

### Reverse transcriptase-PCR

Total tissue mRNA was isolated using the RNA extraction kit (Qiagen). First-strand cDNA was synthesized from 2  $\mu$ g of total RNA using SuperScript (Invitrogen). The subsequent PCR reaction used specific human or mouse VEGF-A primers. PCR products were separated on 2% agarose gels and stained with ethidium bromide. Levels of VEGF-A mRNA expression were assessed by comparing band density using QuantityOne software (Bio-Rad). Amplification of the product representing GAPDH was used to check the efficiency of the PCR reaction. GAPDH PCR profiles were compared and equalized. To test for reproducibility, duplicate amplifications were performed for each sample.

### Cell survival assay

Cell survival was assessed over 48 hours using methylene blue assay. Following treatments, cells were fixed in 4% paraformaldehyde, washed once in 0.1 mol/L sodium borate buffer (pH 8.5), and thereafter incubated with 1% methylene blue for 10 minutes. Excess stain was washed out with double distilled water. Stain was extracted with 0.1 mol/L HCl, and OD<sub>595</sub> was determined using enzyme-linked immunosorbent assay reader.

### Apoptosis assay

Apoptosis was evaluated using TUNEL kit (Roche) according to the manufacturer's protocol. DeltaVision OMX fluorescent microscope station supplied with a digital camera and SoftWorx software (Applied Precision) was used to process the slides.

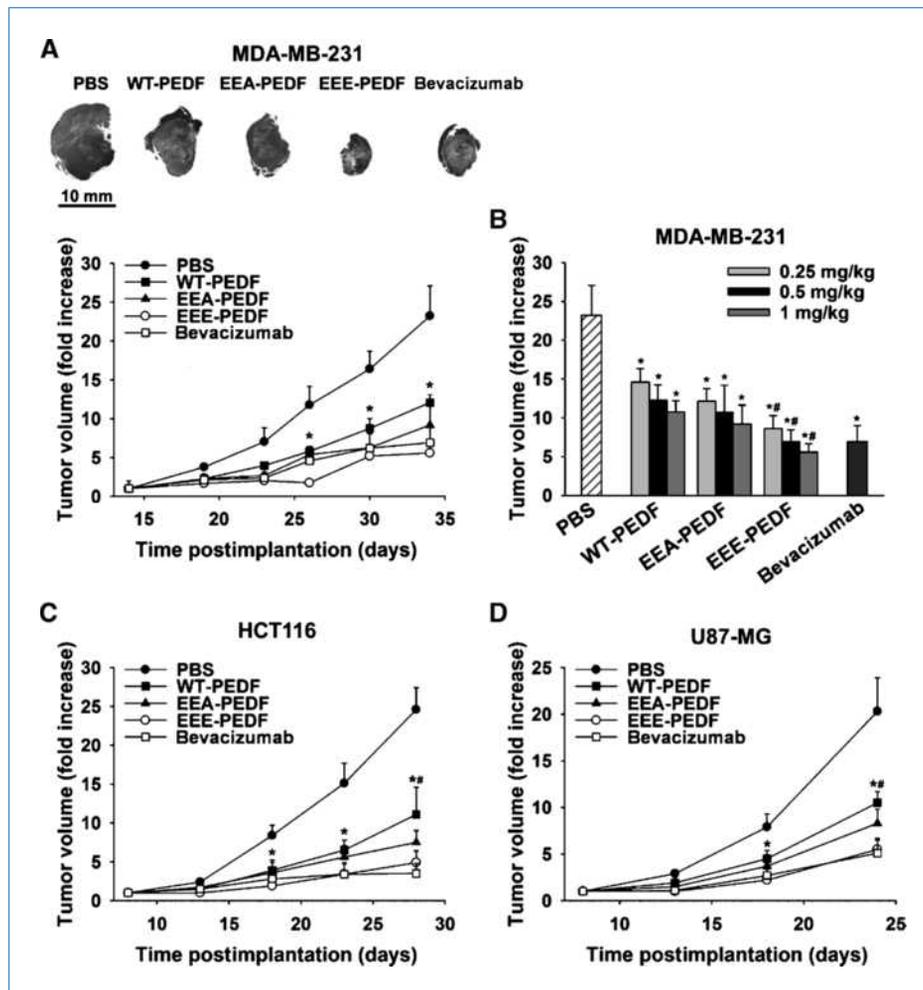
### Statistical analysis

*In vitro* data were expressed as mean  $\pm$  SD, whereas *in vivo* data were expressed as mean  $\pm$  SEM. To test for differences between the control and experimental results, statistical evaluation was carried out using either functional analysis (ANOVA) followed by Student's *t* test (two-tailed) or log-rank test (Mantel-Cox; for detailed description, see Supplementary Data). Values of *P* < 0.05 were considered statistically significant.

## Results

### Triple phosphomimetic PEDF exhibits enhanced antitumor activity *in vivo*

To compare the antitumor activity of WT-PEDF to that of its phosphomimetic mutants, EEA-PEDF and EEE-PEDF, we first used a xenograft model of human invasive breast carcinoma (MDA-MB-231), implanted s.c. into CD-1 nude mice. Upon establishment of visible tumors, mice were treated i.v. with WT-PEDF, EEA-PEDF, or EEE-PEDF at three different dosage regimens along with a bevacizumab-treated positive control group. In mock-treated (PBS) controls, progressive tumor growth occurred over a treatment period of 3 weeks, whereas significant and dose-dependent decrease in tumor growth was



**Figure 2.** Effect of WT-PEDF and its phosphomimetic mutants on tumor growth rate of MDA-MB-231, HCT116, and U87-MG xenografts. CD-1 nude mice were inoculated s.c. with tumorigenic dose of MDA-MB-231, HCT116, or U87-MG cells, and, upon the establishment of tumors, treated i.v. with WT-PEDF, EEA-PEDF, EEE-PEDF (three times a week), or bevacizumab (five times a week). In the MDA-MB-231 xenograft model, WT-PEDF and its phosphomimetic mutants were administered at three different dosage regimens as indicated. In the HCT116 and U87-MG models, all PEDF constructs were used at a single dosage of 1 mg/kg. Bevacizumab was administered at 5 mg/kg concentration. Control group received an initial volume of PBS. A, top, H&E-stained central sections (a section performed at the largest diameter of the tumor) of the representative tumors treated with WT-PEDF, EEA-PEDF, EEE-PEDF (1 mg/kg), or bevacizumab. Tumors were removed at the end point and sectioned. Pictures were taken at  $\times 1$  magnification. Bottom, tumor growth curves for MDA-MB-231 xenografts treated with WT-PEDF and its mutants at 1 mg/kg body weight. B, dose-dependent effect of WT-PEDF and its mutants on tumor growth of MDA-MB-231 xenografts. Bars indicate fold increase in tumor volume. C and D, tumor growth curves for HCT116 (C) and U87-MG (D) xenografts treated with WT-PEDF and its mutants at 1 mg/kg body weight. Data represent mean  $\pm$  SEM (MDA-MB-231:  $n = 5$ ; HCT116 and U87-MG;  $n = 4$ ). \*,  $P < 0.05$ , all treatments versus control; #,  $P < 0.05$ , EEE-PEDF versus WT-PEDF.

observed in all other experimental groups (Fig. 2A and B). When administered at 1 mg/kg body weight, EEE-PEDF caused profound attenuation in tumor growth already after 2 weeks of treatment, reducing the tumor volume by 85% compared with mock-treated animals (Fig. 2A). This effect was much stronger than that of WT-PEDF (52%), EEA-PEDF (56%), or bevacizumab (61%). At the end point, the antitumor effect of EEE-PEDF at all tested dosages significantly prevailed over that of WT-PEDF (Fig. 2B). The maximal final reduction of tumor volume achieved with EEE-PEDF was 76% compared with 57% with WT-PEDF, 61% with EEA-PEDF, and 70% with bevacizumab. Similar results were obtained in two additional

xenograft models, HCT116 (human colorectal carcinoma) and U87-MG (human glioblastoma). In both of these models, the antitumor effect of EEE-PEDF significantly exceeded that of WT-PEDF (Fig. 2C and D). To further compare the efficacies of treatments used, we performed statistical evaluation of tumor growth data using log-rank test, with tumor volume quadrupling time as time-to-end point (TTE), which thereby reflects relative delay in tumor growth. Data analysis showed that all treatments resulted in significantly prolonged TTE compared with PBS-treated controls (Supplementary Table S1). Importantly, TTE values following EEE-PEDF administration were significantly longer (1.27–1.33-fold, depending on

the model) than those observed in WT-PEDF-treated groups. Overall, these data indicate that EEE-PEDF possesses improved antitumor properties compared with WT-PEDF, and its anticancer effect is comparable with that of bevacizumab.

It should be noted that PEDF constructs were administered three times per week at dosages of up to 1 mg/kg body weight, whereas bevacizumab was injected at 5 mg/kg body weight five times per week. Toxicity studies indicated that phosphomimetic PEDF lacks any detectable systemically harmful effects even when injected at 5 mg/kg body weight (Supplementary Fig. S1A and B), and does not affect vascularization of major organs (Supplementary Fig. S1C). Following i.v. injection, phosphomimetic PEDF is retained within the vascular compartment (Supplementary Fig. S2A) and exhibits considerable stability in murine plasma over 48 to 72 hours (Supplementary Fig. S2B). These observations indicate a possibility of additional dose escalation, which may produce a further enhanced antitumor effect.

### Triple phosphomimetic PEDF inhibits tumor angiogenesis more efficiently than WT-PEDF

Because PEDF was reported to exert its anticancer effect mainly through inhibition of intratumoral neovascularization (6, 21, 22), we determined whether the antitumor activity of EEE-PEDF and EEA-PEDF correlates with their ability to disrupt establishment of tumor vascular network. For this purpose, MDA-MB-231 xenografts, excised at the end of the experiment, were sectioned and analyzed immunohistochemically using antibody to CD34, a well-known marker for newly formed blood vessels. In these studies, xenografts from the treated mice exhibited much lower vascular density than PBS-treated controls, where EEE-PEDF showed the most pronounced effect (Fig. 3A and B), leading to 87% reduction in MVD, compared with 54%, 66%, and 81% in WT-PEDF-, EEA-PEDF-, and bevacizumab-treated groups, respectively. A corresponding trend of reduction in MVD was observed in HCT116 and U87-MG xenografts (Fig. 3A and B).

### PEDF constructs act in a VEGF-independent manner

A number of reports have suggested that the antiangiogenic effect of PEDF arises, at least in a part, from a decline in VEGF expression (21, 28). To address this point, we examined xenograft tissues of the treated animals for the expression of VEGF-A and the phosphorylation of VEGFR2, a major signal transducing receptor for VEGF-A. Under our experimental conditions, none of the treatments caused significant fluctuations in the mRNA levels of either human VEGF-A, expressed by the MDA-MB-231 tumor cells, or mouse VEGF-A, expressed by the host-derived tumor milieu (Fig. 4A and B). Correspondingly, VEGFR2 phosphorylation was not affected by the PEDF constructs, whereas bevacizumab, being a humanized VEGF-A monoclonal antibody (29), almost completely inhibited VEGFR2 phosphorylation (Fig. 4C and D). Because bevacizumab does not neutralize mouse VEGF, this indicates that tumor VEGFR2 was activated mainly by human VEGF secreted by xenograft cells, rather than by mouse VEGF produced by the host. Thus, it is more likely that the anticancer effect

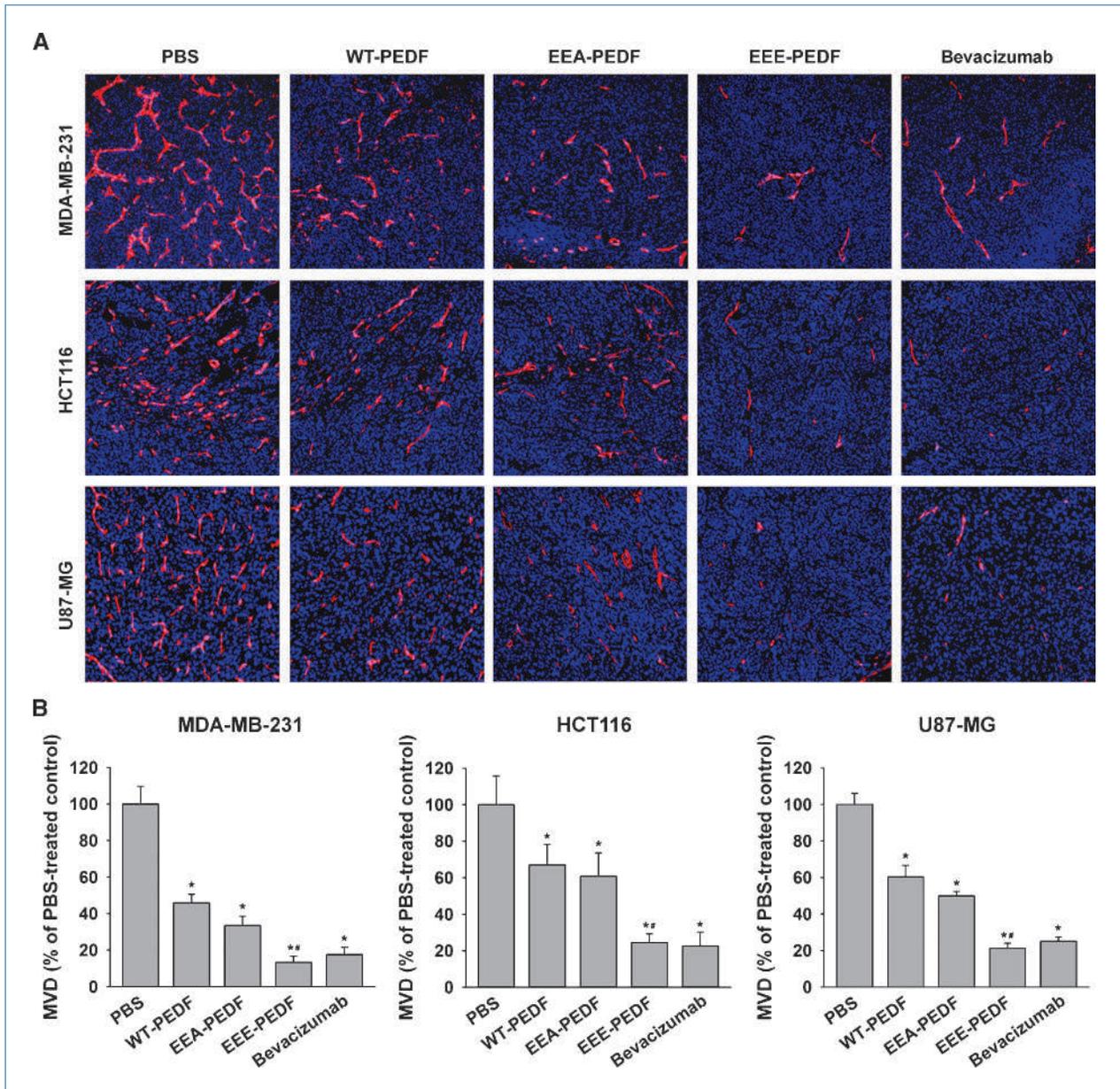
achieved by bevacizumab in our experiments reflects its maximal activity. These findings, reproduced also in HCT116 and U87-MG xenografts (Fig. 4A–D), clearly show that the antiangiogenic effect of PEDF and its mutants is achieved in a VEGF-independent manner in all tested models.

### Triple phosphomimetic PEDF facilitates endothelial cell apoptosis *in vivo*

Induction of endothelial and cancer cell apoptosis has been implicated in the mechanism of antiangiogenic and anticancer activity of PEDF (21, 22). Therefore, we evaluated the rate of apoptosis in the treated MDA-MB-231 xenografts. To discriminate between endothelial and tumor cell apoptosis, xenograft sections were costained with anti-CD34 and TUNEL to identify apoptotic cells. We found that all treatments targeted predominantly endothelial cells of the newly formed blood vessels, as the majority of TUNEL-positive cells colocalized with CD34 staining (Fig. 5A). On the other hand, other xenograft-residing tumor cells were affected to a much lesser extent. Depending on the treatment, as much as 22% to 60% of CD34-positive endothelial cells per field were identified as apoptotic, compared with only 4% to 14% of CD34-negative cells, the latter representing mostly xenografted cancer cells (Fig. 5B). Again, EEE-PEDF showed the highest activity, leading to 60% apoptosis in CD34-positive endothelial cells and 14% apoptosis in CD34-negative tumor cells compared with the respective 22% and 4% for WT-PEDF, 28% and 5% for EEA-PEDF, and 53% and 12% for bevacizumab (Fig. 5B). The low level of tumor cell apoptosis, which was comparable with that caused by bevacizumab, is likely to be derived from the lack of blood supply to nonvascularized tumor areas and/or production of apoptotic cytokines by the affected endothelial cells. Thus, the preferential induction of apoptosis in endothelial cells further implies that the antiangiogenic effect of PEDF and its mutants is the foremost element of their antitumor activity.

### PEDF constructs reduce survival and induce apoptosis of endothelial cells but do not affect cancer cells *in vitro*

We then studied the effects of WT-PEDF and its mutants in cultured endothelial and cancer cells. First, we examined whether WT-PEDF, EEA-PEDF, and EEE-PEDF affect survival of BAEC and HUVEC, and found that nanomolar concentrations of PEDF constructs efficiently reduce endothelial cell survival, with EEE-PEDF exhibiting a significantly stronger effect than WT-PEDF (Fig. 6A). This effect was apparent even in the presence of strong proangiogenic stimuli such as VEGF and bFGF, where EEE-PEDF prevented stimulus-dependent increase in BAEC proliferation much more efficiently than WT-PEDF (Fig. 6A). On the other hand, the same concentration of the PEDF constructs did not affect survival of cancer cells, including MDA-MB-231, HCT116, and U87-MG (Fig. 6B). TUNEL assay revealed that WT-PEDF and more so its mutants induce apoptosis of BAEC, but not of MDA-MB-231 cells (Fig. 6C). These results were supported by the ability of PEDF and its mutants to activate caspase-3, known to be involved in PEDF-induced endothelial cell apoptosis, as



**Figure 3.** Effect of WT-PEDF and its phosphomimetic mutants on intratumoral neovascularization. A, CD-1 nude mice were treated as described in Fig. 1. Tumors were excised, and intratumoral neovascularization was assessed by immunohistochemistry with anti-CD34. Shown are representative CD34-stained sections of MDA-MB-231, HCT116, and U87-MG xenografts treated with WT-PEDF, its mutants, or bevacizumab. Pictures were taken at  $\times 10$  magnification. Red, CD34; blue, DAPI. B, MVD quantification performed in five random hotspots per tumor. Results are expressed as percentage reduction in MVD compared with PBS-treated controls and represent mean  $\pm$  SEM ( $n = 5$ ),  $P < 0.05$ , treated versus control; #,  $P < 0.05$ , EEE-PEDF versus WT-PEDF.

manifested by the appearance of 17- and 19-kDa cleavage products in BAEC (Fig. 6D). Also in this case, the accumulation of caspase-3 cleavage products following treatment with PEDF mutants was faster than with WT-PEDF. In contrast to BAEC, PEDF constructs failed to induce caspase-3 cleavage in cultured MDA-MB-231 cells (Fig. 6D), suggesting again that the inhibitory effect of PEDF constructs on tumor growth is mostly indirect.

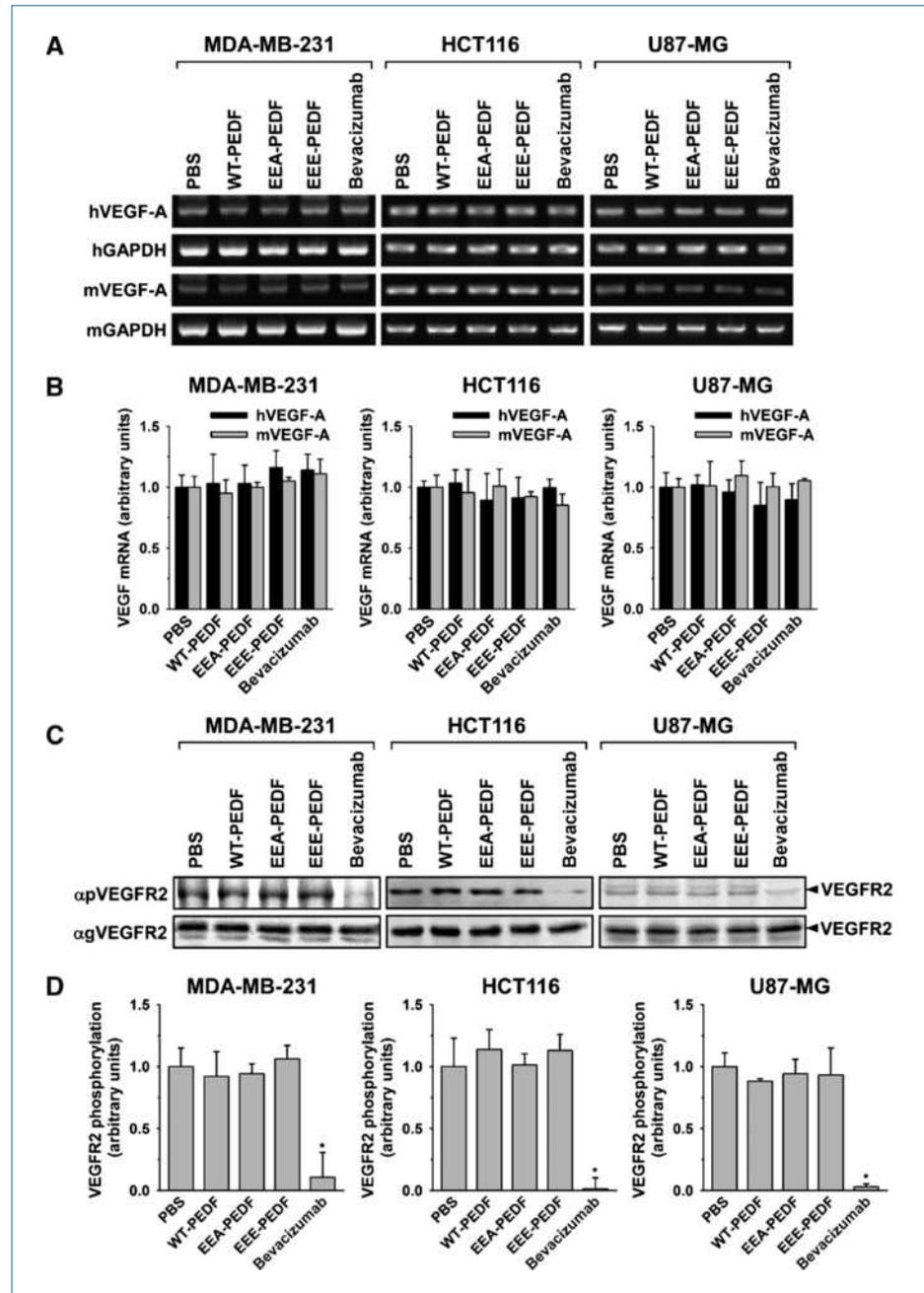
## Discussion

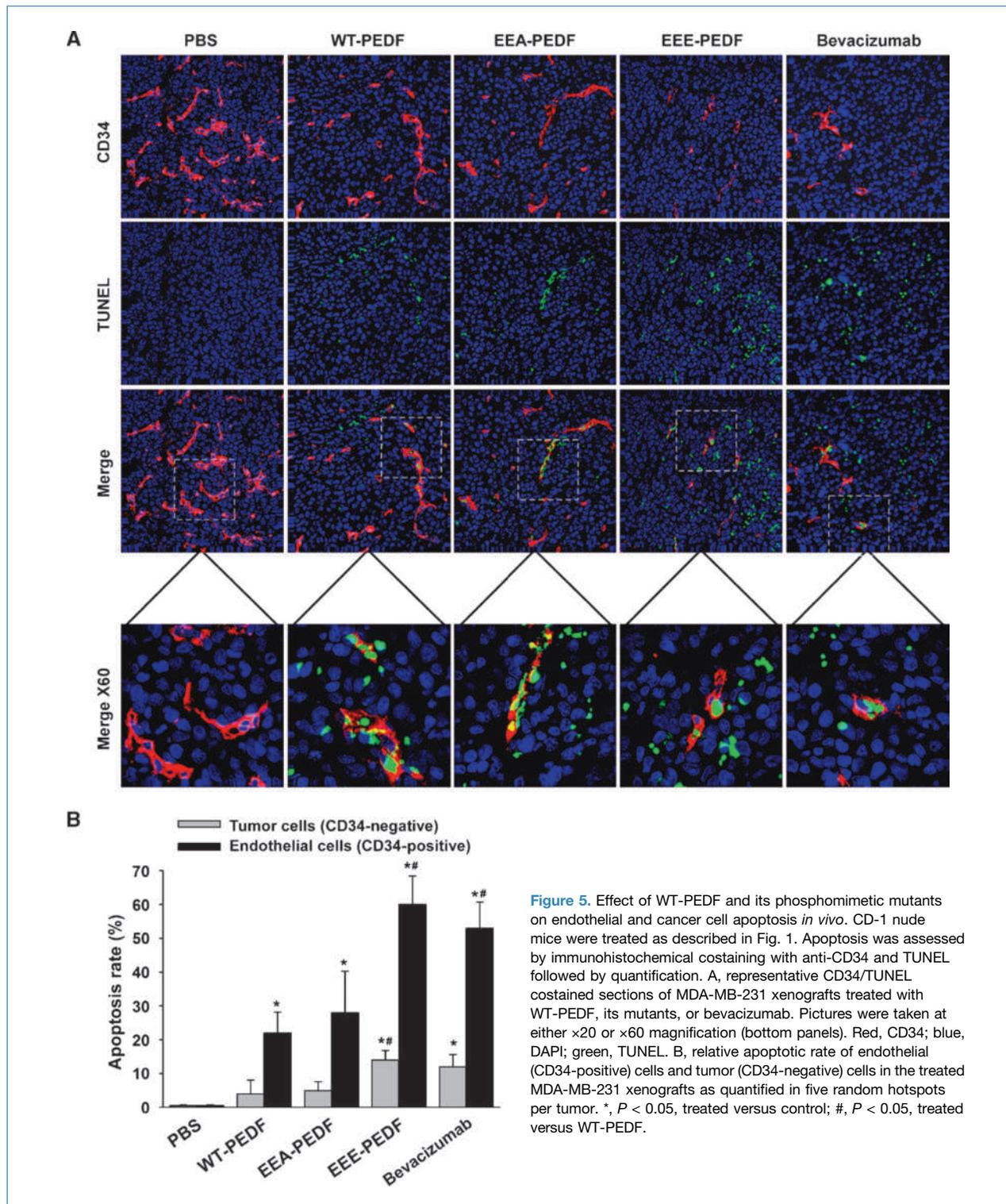
PEDF is a well-established antiangiogenic agent, with an increasing number of reports showing that it can be used as an anticancer drug (6, 8, 16–19). Our group has previously shown that physiologic activities of PEDF are differentially regulated by phosphorylation, and that triple phosphorylation site mutants mimicking different phosphorylation

states of PEDF (EEE-PEDF and EEA-PEDF) exhibit significantly elevated antiangiogenic activity compared with WT-PEDF (23, 24). We therefore hypothesized that these mutants can serve as much better angiogenesis-targeting anticancer drugs than WT-PEDF, which is currently in pre-clinical development. Indeed, we found that EEE-PEDF, but not EEA-PEDF, produces significantly better inhibition of xenograft growth and intratumoral angiogenesis than WT-PEDF in three different xenograft models, all of which are known to develop major neovascularization (30, 31). In these models, the extent of tumor growth inhibition

produced by EEE-PEDF was similar and sometimes slightly better than that achieved by the well-known and potent antiangiogenic agent bevacizumab, currently approved for use in metastatic breast and colon cancer. Most importantly, our studies reveal superiority of the phosphomimetic PEDF over WT-PEDF in its ability to reduce the survival and promote apoptosis of tumor endothelial cells, and, because PEDF does not affect quiescent endothelial cells of the existing vasculature (20), these properties represent an exceptionally effective mechanism for the inhibition of tumor growth.

**Figure 4.** Effect of WT-PEDF and its phosphomimetic mutants on VEGF-A mRNA expression and VEGFR2 phosphorylation in MDA-MB-231, HCT116, and U87-MG xenografts. CD-1 nude mice were treated as described in Fig. 1. A, CD-1 nude mice were treated as described in Fig. 1. Human and mouse VEGF-A mRNA expression in the treated MDA-MB-231, HCT116, and U87-MG xenograft tissues analyzed by reverse transcriptase-PCR. B, quantification of the relative VEGF-A mRNA expression in xenograft tissues as evaluated in three independent tumors from each experimental group. C, levels of VEGFR2 phosphorylation in MDA-MB-231, HCT116, and U87-MG tumor homogenates as analyzed by immunoblotting with anti-VEGFR2 or anti-phospho (Y951) VEGFR2 antibodies. D, quantification of the relative VEGFR2 phosphorylation/activity as evaluated in three independent tumors from each experimental group. \*,  $P < 0.05$ , treated versus control.





In contrast to VEGF-targeting antiangiogenic agents, which act by inhibiting VEGF signaling and thereby preventing recruitment of endothelial cells to form new blood vessels (32), PEDF and more so its phosphomimetic mutants affect

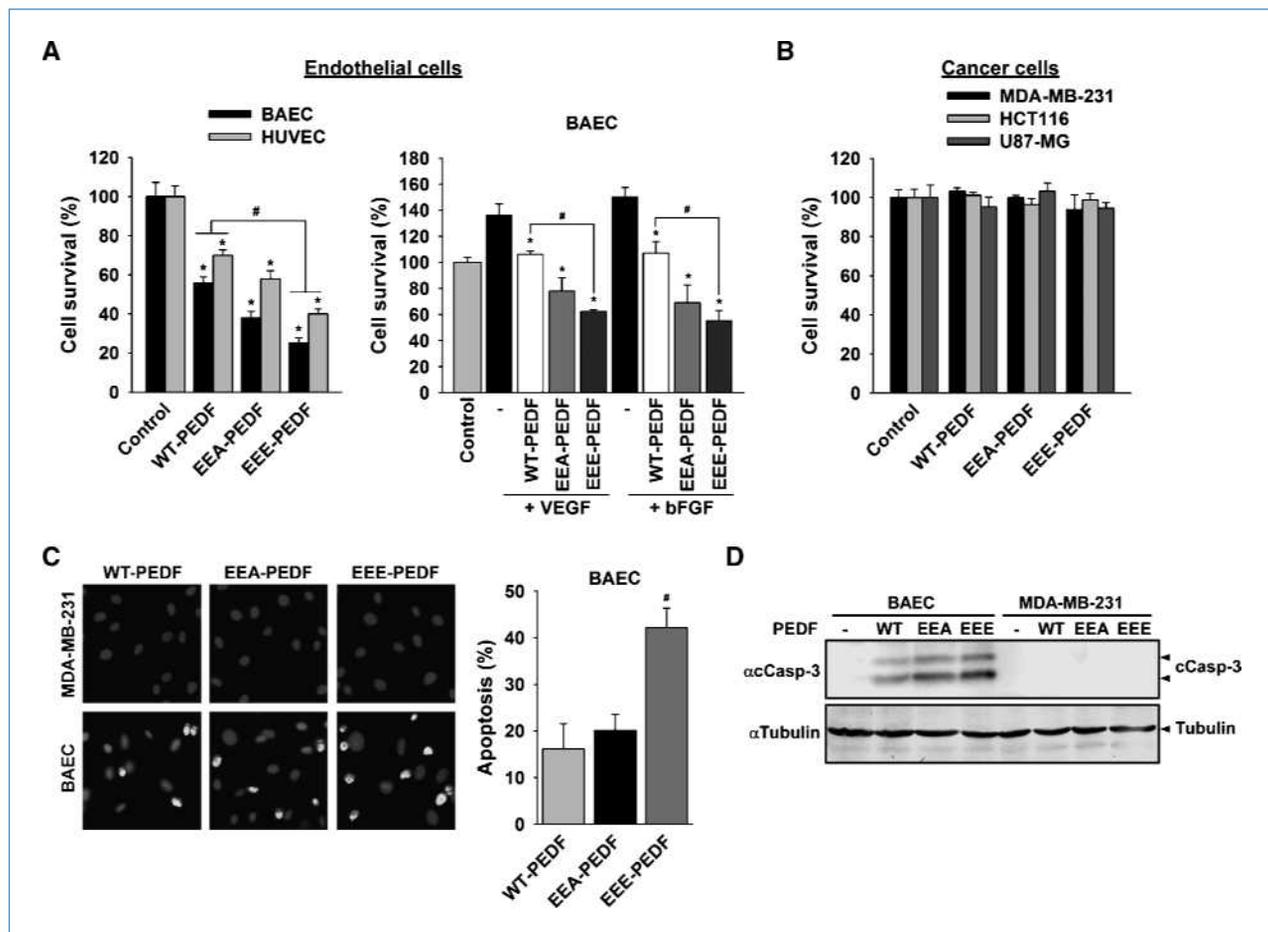
the recruited endothelial cells themselves, thereby directly interfering with human and murine angiogenic system. These distinct mechanisms of action suggest that PEDF mutants can serve as drugs of choice when VEGF-targeting agents

are not effective. The fact that PEDF and its mutants act in a VEGF-independent manner points to a disparity of the mechanisms of action and provides a rationale for the combined therapy with the PEDF mutants and VEGF-targeting drugs, which is expected to produce an additive effect. Combined therapy with other known chemotherapies should be taken into consideration as well. Furthermore, the apparent lack of systemic toxicity of PEDF and its mutants is an additional important advantage of these agents as anticancer drugs.

Several studies have shown that PEDF successfully regresses tumor growth in animal cancer models (16–18), although a study by Doll and colleagues suggested that, in some cases, PEDF administration does not produce significant tumor growth inhibition (10). Our preliminary experiments with other xenograft models have indicated that the extent of tumor growth reduction following PEDF treatment depends on angiogenic profile of a given model, suggesting

that PEDF affects mainly tumor-associated endothelium. Indeed, we showed that PEDF and its mutants preferentially induce apoptosis in endothelial and much less in cancer cells *in vivo* and do not affect cancer cell survival *in vitro*, while efficiently inhibiting the growth rate of xenografts originating from these cells. This is in agreement with another study that showed similar effects of PEDF in HeLa cells, although the effect of phosphomimetic mutants of PEDF was not examined there (33). These results suggest that the antiangiogenic activity of PEDF, for example, its ability to affect actively proliferating and migrating endothelial cells, is the main component of its antitumor action.

The exact molecular mechanism of action of PEDF is not fully elucidated yet. However, a number of studies in the past decade have shown the involvement of different signaling pathways in the biological activity of PEDF toward endothelial cells. It has been shown, for example, that PEDF-induced



**Figure 6.** Effect of WT-PEDF and its phosphomimetic mutants on survival and apoptosis of endothelial and cancer cells *in vitro*. A, left, effect of WT-PEDF and its mutants (20 nmol/L, 48 hours) on BAEC and HUVEC survival analyzed using methylene blue colorimetric assay. Right, BAEC survival following treatment WT-PEDF or its mutants (20 nmol/L) in the presence of VEGF or bFGF (both at 25 ng/mL). B, MDA-MB-231, HCT116, and U87-MG cell survival following treatment for 48 hours with WT-PEDF or its mutants (20 nmol/L). C, left, representative TUNEL-labeled  $\times 20$  fields of MDA-MB-231 and BAEC treated with WT-PEDF or its mutants (20 nmol/L) for 12 hours. White, TUNEL; gray, DAPI. Right, quantification of the relative apoptotic rate of BAEC treated with WT-PEDF and its mutants as assessed by TUNEL. D, effect of the PEDF constructs on caspase-3 cleavage in MDA-MB-231 and BAEC as assessed by immunoblotting with anti-cleaved caspase-3. Data represent mean  $\pm$  SD ( $n = 3$ ), \* $P < 0.05$ , treated versus control; # $P < 0.05$ , EEE-PEDF versus WT-PEDF.

apoptosis of endothelial cells can be mediated by the activation of the Fas-FasL system (22), p38 mitogen-activated protein kinase-dependent cleavage of multiple caspases (34), and by peroxisome proliferator-activated receptor- $\gamma$  signaling to p53 (35). The main obstacle on the way to full delineation of the signaling pathways involved in the antitumor activity of PEDF is the question of existence of a putative PEDF receptor, which is still obscure. A few candidates have been identified thus far, including extracellular matrix components (36, 37), a phospholipase-linked membrane protein (38), and a laminin receptor (39). Thus, the observed enhanced activity of the phosphomimetic PEDF toward endothelial cells can have several explanations. The effects of the PEDF mutants on endothelial cells can be regulated by the same mechanism and/or receptor as WT-PEDF, and their enhanced activity could be explained by a stronger affinity to receptor or a diverse time pattern of ligand-receptor interaction. Alternatively, PEDF mutants may affect the signaling component of such receptor in a different manner. Finally, PEDF mutants may exert stronger effects on downstream signaling pathways. Evidence that PEDF exerts diverse effects in endothelial, cancer, and neural cells (40, 41) may suggest expression of different PEDF receptors with similar binding characteristics and a distinct signaling component. As we show here, PEDF and its mutants fail to induce apoptosis in cancer cells, which may indicate the presence of a distinct, less-specific receptor, rather than excluding the existence of a PEDF receptor in these cells. Another possibility for the lack of the proapoptotic effect of PEDF toward cancer cells is that cancer cells could be more resistant to PEDF action by

virtue of the higher basal activity of their survival pathways. However, additional studies are needed to further clarify these points.

In summary, we show here that the antiangiogenic properties of PEDF can be considerably enhanced by mimicking the triply phosphorylated form of this factor, resulting in a significantly increased antitumor activity. Phosphomimetic PEDF mutants operate through the mechanism that does not involve VEGF inhibition, and therefore can be efficiently used either as a single therapy or in combination with either VEGF-targeting drugs, such as bevacizumab, or other chemotherapies. We believe that phosphomimetic mutants of PEDF provide a basis for the development of new and extremely potent antiangiogenic anticancer drugs, which will be superior to WT-PEDF and other antiangiogenic agents.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Grant Support

Israeli Ministry of Industry and Trade (Nofar program), Horowitz Foundation, Gurwin Fund for Scientific Advancement, and the EU 6th Framework Program (GROWTHSTOP, LSHC CT-2006-037731). R. Seger is an incumbent of the Yale Lewine and Ella Miller Lewine professorial chair for cancer research.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 02/03/2010; revised 05/04/2010; accepted 05/24/2010; published OnlineFirst 07/06/2010.

### References

- Folkman J. Endogenous angiogenesis inhibitors. *APMIS* 2004;112:496–507.
- Tombran-Tink J, Chader GG, Johnson LV. PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. *Exp Eye Res* 1991;53:411–4.
- Stellmach V, Crawford SE, Zhou W, Bouck N. Prevention of ischemia-induced retinopathy by the natural ocular antiangiogenic agent pigment epithelium-derived factor. *Proc Natl Acad Sci U S A* 2001;98:2593–7.
- Dawson DW, Volpert OV, Gillis P, et al. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. *Science* 1999;285:245–8.
- Petersen SV, Valnickova Z, Enghild JJ. Pigment-epithelium-derived factor (PEDF) occurs at a physiologically relevant concentration in human blood: purification and characterization. *Biochem J* 2003;374:199–206.
- Ek ET, Dass CR, Choong PF. PEDF: a potential molecular therapeutic target with multiple anti-cancer activities. *Trends Mol Med* 2006;12:497–502.
- Guan M, Yam HF, Su B, et al. Loss of pigment epithelium derived factor expression in glioma progression. *J Clin Pathol* 2003;56:277–82.
- Matsumoto K, Ishikawa H, Nishimura D, Hamasaki K, Nakao K, Eguchi K. Antiangiogenic property of pigment epithelium-derived factor in hepatocellular carcinoma. *Hepatology* 2004;40:252–9.
- Cai J, Parr C, Watkins G, Jiang WG, Boulton M. Decreased pigment epithelium-derived factor expression in human breast cancer progression. *Clin Cancer Res* 2006;12:3510–7.
- Doll JA, Stellmach VM, Bouck NP, et al. Pigment epithelium-derived factor regulates the vasculature and mass of the prostate and pancreas. *Nat Med* 2003;9:774–80.
- Zhang L, Chen J, Ke Y, Mansel RE, Jiang WG. Expression of pigment epithelium derived factor is reduced in non-small cell lung cancer and is linked to clinical outcome. *Int J Mol Med* 2006;17:937–44.
- Uehara H, Miyamoto M, Kato K, et al. Expression of pigment epithelium-derived factor decreases liver metastasis and correlates with favorable prognosis for patients with ductal pancreatic adenocarcinoma. *Cancer Res* 2004;64:3533–7.
- Halin S, Wikstrom P, Rudolfsson SH, et al. Decreased pigment epithelium-derived factor is associated with metastatic phenotype in human and rat prostate tumors. *Cancer Res* 2004;64:5664–71.
- Guan M, Pang CP, Yam HF, Cheung KF, Liu WW, Lu Y. Inhibition of glioma invasion by overexpression of pigment epithelium-derived factor. *Cancer Gene Ther* 2004;11:325–32.
- Abe R, Shimizu T, Yamagishi S, et al. Overexpression of pigment epithelium-derived factor decreases angiogenesis and inhibits the growth of human malignant melanoma cells *in vivo*. *Am J Pathol* 2004;164:1225–32.
- Abramson LP, Stellmach V, Doll JA, Cornwell M, Arensman RM, Crawford SE. Wilms' tumor growth is suppressed by antiangiogenic pigment epithelium-derived factor in a xenograft model. *J Pediatr Surg* 2003;38:336–42; discussion 336–42.
- Wang L, Schmitz V, Perez-Mediavilla A, Izal I, Prieto J, Qian C. Suppression of angiogenesis and tumor growth by adenoviral-mediated gene transfer of pigment epithelium-derived factor. *Mol Ther* 2003;8:72–9.
- Ek ET, Dass CR, Contreras KG, Choong PF. Inhibition of orthotopic

- osteosarcoma growth and metastasis by multitargeted antitumor activities of pigment epithelium-derived factor. *Clin Exp Metastasis* 2007;24:93–106.
19. Ek ET, Dass CR, Choong PF. Pigment epithelium-derived factor: a multimodal tumor inhibitor. *Mol Cancer Ther* 2006;5:1641–6.
  20. Bouck N. PEDF: anti-angiogenic guardian of ocular function. *Trends Mol Med* 2002;8:330–4.
  21. Takenaka K, Yamagishi S, Jinnouchi Y, Nakamura K, Matsui T, Imaizumi T. Pigment epithelium-derived factor (PEDF)-induced apoptosis and inhibition of vascular endothelial growth factor (VEGF) expression in MG63 human osteosarcoma cells. *Life Sci* 2005; 77:3231–41.
  22. Volpert OV, Zaichuk T, Zhou W, et al. Inducer-stimulated Fas targets activated endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor. *Nat Med* 2002; 8:349–57.
  23. Maik-Rachline G, Shaltiel S, Seger R. Extracellular phosphorylation converts pigment epithelium-derived factor from a neurotrophic to an antiangiogenic factor. *Blood* 2005;105:670–8.
  24. Maik-Rachline G, Seger R. Variable phosphorylation states of pigment-epithelium-derived factor differentially regulate its function. *Blood* 2006;107:2745–52.
  25. Yedgar S, Vlodaysky I, Panet A, Reisfeld N, Eldor A. Interferon enhances phospholipase A2 activity in bovine aortic endothelial cells. *Eicosanoids* 1990;4:225–9.
  26. Godder K, Vlodaysky I, Eldor A, Weksler BB, Haimovitz-Freidman A, Fuks Z. Heparanase activity in cultured endothelial cells. *J Cell Physiol* 1991;148:274–80.
  27. de Jong JS, van Diest PJ, Baak JP. Heterogeneity and reproducibility of microvessel counts in breast cancer. *Lab Invest* 1995;73:922–6.
  28. Browne M, Stellmach V, Cornwell M, et al. Gene transfer of pigment epithelium-derived factor suppresses tumor growth and angiogenesis in a hepatoblastoma xenograft model. *Pediatr Res* 2006; 60:282–7.
  29. Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov* 2004;3:391–400.
  30. Hendrix MJ, Seftor EA, Kirschmann DA, Seftor RE. Molecular biology of breast cancer metastasis. Molecular expression of vascular markers by aggressive breast cancer cells. *Breast Cancer Res* 2000;2:417–22.
  31. Xu L, Cochran DM, Tong RT, et al. Placenta growth factor overexpression inhibits tumor growth, angiogenesis, and metastasis by depleting vascular endothelial growth factor homodimers in orthotopic mouse models. *Cancer Res* 2006;66:3971–7.
  32. Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat Rev Cancer* 2008;8:579–91.
  33. Hosomichi J, Yasui N, Koide T, Soma K, Morita I. Involvement of the collagen I-binding motif in the anti-angiogenic activity of pigment epithelium-derived factor. *Biochem Biophys Res Commun* 2005; 335:756–61.
  34. Chen L, Zhang SS, Barnstable CJ, Tombran-Tink J. PEDF induces apoptosis in human endothelial cells by activating p38 MAP kinase dependent cleavage of multiple caspases. *Biochem Biophys Res Commun* 2006;348:1288–95.
  35. Ho TC, Chen SL, Yang YC, Liao CL, Cheng HC, Tsao YP. PEDF induces p53-mediated apoptosis through PPAR $\gamma$  signaling in human umbilical vein endothelial cells. *Cardiovasc Res* 2007;76:213–23.
  36. Becerra SP, Perez-Mediavilla LA, Weldon JE, et al. Pigment epithelium-derived factor binds to hyaluronan. Mapping of a hyaluronan binding site. *J Biol Chem* 2008;283:33310–20.
  37. Meyer C, Notari L, Becerra SP. Mapping the type I collagen-binding site on pigment epithelium-derived factor. Implications for its anti-angiogenic activity. *J Biol Chem* 2002;277:45400–7.
  38. Notari L, Baladron V, Aroca-Aguilar JD, et al. Identification of a lipase-linked cell membrane receptor for pigment epithelium-derived factor. *J Biol Chem* 2006;281:38022–37.
  39. Bernard A, Gao-Li J, Franco CA, Bouceba T, Huet A, Li Z. Laminin receptor involvement in the anti-angiogenic activity of pigment epithelium-derived factor. *J Biol Chem* 2009;284:10480–90.
  40. Hutchings H, Maitre-Boube M, Tombran-Tink J, Plouet J. Pigment epithelium-derived factor exerts opposite effects on endothelial cells of different phenotypes. *Biochem Biophys Res Commun* 2002; 294:764–9.
  41. Araki T, Taniwaki T, Becerra SP, Chader GJ, Schwartz JP. Pigment epithelium-derived factor (PEDF) differentially protects immature but not mature cerebellar granule cells against apoptotic cell death. *J Neurosci Res* 1998;53:7–15.

# Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

## Phosphomimetic Mutants of Pigment Epithelium-Derived Factor with Enhanced Antiangiogenic Activity as Potent Anticancer Agents

Alexander Konson, Sunila Pradeep and Rony Seger

*Cancer Res* 2010;70:6247-6257. Published OnlineFirst July 7, 2010.

<b>Updated version</b>	Access the most recent version of this article at: <a href="https://doi.org/10.1158/0008-5472.CAN-10-0434">doi:10.1158/0008-5472.CAN-10-0434</a>
<b>Supplementary Material</b>	Access the most recent supplemental material at: <a href="http://cancerres.aacrjournals.org/content/suppl/2010/07/02/0008-5472.CAN-10-0434.DC1">http://cancerres.aacrjournals.org/content/suppl/2010/07/02/0008-5472.CAN-10-0434.DC1</a>

<b>Cited articles</b>	This article cites 41 articles, 15 of which you can access for free at: <a href="http://cancerres.aacrjournals.org/content/70/15/6247.full.html#ref-list-1">http://cancerres.aacrjournals.org/content/70/15/6247.full.html#ref-list-1</a>
-----------------------	--

<b>Citing articles</b>	This article has been cited by 6 HighWire-hosted articles. Access the articles at: <a href="/content/70/15/6247.full.html#related-urls">/content/70/15/6247.full.html#related-urls</a>
------------------------	---

<b>E-mail alerts</b>	<a href="#">Sign up to receive free email-alerts</a> related to this article or journal.
----------------------	--

<b>Reprints and Subscriptions</b>	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a> .
-----------------------------------	--

<b>Permissions</b>	To request permission to re-use all or part of this article, contact the AACR Publications Department at <a href="mailto:permissions@aacr.org">permissions@aacr.org</a> .
--------------------	---