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Serpin-Derived Peptides Are Antiangiogenic and Suppress Breast Tumor Xenograft Growth[1]

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Abstract

Angiogenesis is the formation of neovasculature from preexisting microvessels. Several endogenous proteins regulate the balance of vessel formation and regression in the body including pigment epithelium–derived factor (PEDF), which has been shown to be antiangiogenic and to suppress tumor growth. Using sequence homology and bioinformatics, we previously identified several peptide sequences homologous to an active region of PEDF existing in multiple proteins in the human proteome. These short 11-mer peptides are found in a DEAH box helicase protein, CKIP-1 and caspase 10, and show similar activity in altering endothelial cell adhesion, migration and inducing apoptosis. We tested the peptide derived from DEAH box helicase protein in a triple-negative MDA-MB-231 breast orthotopic xenograft model in severe combined immunodeficient mice and show significant tumor suppression.

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Introduction

Angiogenesis has received much attention as a therapeutic target to treat various pathologic diseases such as solid tumors. Tuning vascular growth by endogenous proangiogenic and antiangiogenic factors provides a plethora of information useful for the development of therapeutic antiangiogenic agents. A review by Judah Folkman [1], the pioneer in the studies of tumor angiogenesis, discussed multiple experimentally identified proteins with antiangiogenic properties, one being pigment epithelium–derived factor (PEDF). First identified as a retinal factor, PEDF is now shown to be expressed across multiple tissues and organs in the body including the brain, eye, heart, and lung [2–4]. PEDF was shown to inhibit the migration and proliferation of endothelial cells, blocking the angiogenic effects of vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF-2) [5]. In addition to being a potent antiangiogenic agent, PEDF possesses other physiological properties including antitumor and neurotrophic activities [6,7]. Its anticancer effects, for example, in lung, ovarian, and breast cancer, have been further characterized [8].

The crystal structure of PEDF was resolved, showing a distinct asymmetric charge distribution and reactive center loop [9]. Further analysis revealed distinct regions with antiangiogenic activity [10], including a 34-mer region and upstream TGA fragment and a 44-mer neurotrophic region [11]. The 34-mer and TGA sequences induced *c-jun*–NH2 kinase (JNK) phosphorylation and induced apoptosis of endothelial cells. Additional studies characterized the 34-mer region and identified a potent 18-mer active segment within the 34-mer region that inhibited angiogenesis by blocking endothelial cell chemotaxis and suppressed prostate (PC-3) and renal cell carcinoma (RENCA) tumor growth *in vivo* [12].

Bioinformatics methodology revealed three sequences in the human proteome similar to the TGA epitope [13]. These fragments are contained in several proteins including the RNA helicase DEAH box helicase protein, the pleckstrin homology domain-containing protein casein kinase 2–interacting protein 1 (CKIP-1), and apoptotic factor

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caspase 10. Physiologically distinct, these parent proteins have many diverse roles including releasing RNA from ribosomes (DEAH box helicase protein) [14], regulating cell differentiation and apoptosis (CKIP-1) [15], and initiating apoptosis (caspase 10) [16]. Although these proteins have been previously characterized in part, the bioinformatics methodology used in our laboratory and experimental screening described in the present work show antiangiogenic activity resides within these proteins previously having been unrecognized as containing antiangiogenic potential.

Breast cancer has been shown to be angiogenesis dependent [17–19]. Worldwide, breast cancer remains a major killer, contributing to more than 458,000 deaths annually and 1.4 million newly diagnosed cases [20]. Triple-negative breast cancer represents a heterogeneous subpopulation of breast tumors, accounting for ~15% of breast tumors and is not amenable to standard hormone therapy, contributing to poor prognosis and survival [21]. In this study, we focus on the aggressive MDA-MB-231 triple-negative breast cancer cell line owing to the unmet medical need in treating triple-negative breast cancer.

We show that the bioinformatically identified serpin peptides, derived from a DEAH box helicase protein (seq: EIELVEEEPPF), which we name SP6001, SP6023 from CKIP-1 (seq: TLDLIQEEDPS), and SP6024 from caspase 10 (seq: AEDLLSEEDPF), are potent inducers of endothelial cell apoptosis as measured by caspase 3/7 release. Interestingly, all three peptides also increase endothelial cell adhesion, and SP6001 sustains phosphorylation of focal adhesion kinase (FAK), consequently, diminishing cell migration. Most significantly, we show that SP6001 potently suppresses growth of MDA-MB-231 breast tumors through intraperitoneal or subcutaneous administration; therefore, there is potential for this and other serpin-derived peptides to be developed as therapeutic antiangiogenic agents against breast cancer.

Materials and Methods

Peptide Synthesis and Handling

Peptides were produced by a commercial provider (American Peptide, Sunnyvale, CA) using solid-phase synthesis. Purity was guaranteed to be more than 95% as determined by HPLC and MS data provided by the manufacturer. A scrambled sequence was generated for SP6001 identified herein as SP6001SCR (PEFEPEIEVEL) as a separate control. For *in vitro* experiments, peptides were solubilized in 1% dimethyl sulfoxide (DMSO) and phosphate-buffered saline (PBS) and stored at −20°C. Total DMSO content in all experiments in all wells was held at parity. For *in vivo* experiments, the peptide was solubilized in 5% DMSO and PBS, and a negative control of 5% DMSO and PBS was injected for tumor studies. Peptide was injected daily at 1 or 5 mg/kg intraperitoneally (i.p.) or subcutaneously (s.c.).

Cell Culture

For *in vitro* experiments, human umbilical vein endothelial cells (HUVECs) were purchased from a commercial provider (Lonza, Walkersville, MD). Cells were passaged at constant ratios, and passages 3 to 6 were used in all experiments according to manufacturer's recommendations. We use the endothelial basal medium-2 and endothelial growth medium-2 bullet kit for cell passage and treatments, purchased from Lonza.

For breast xenograft assays, MDA-MB-231 breast cancer cells were provided by Dr Zaver Bhujwalla's group (JHMI Radiology and Oncology), providing the following details about the cell line: MDA-MB-231 breast cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and used within 6 months of obtaining them from ATCC. The cell line was tested and authenticated by ATCC by two independent methods, the ATCC cytochrome *C* oxidase I polymerase chain reaction assay and short tandem repeat profiling using multiplex polymerase chain reaction. Cells were propagated in RPMI-1640 medium (Gibco, Carlsbad, CA), containing 10% FBS and 1% penicillin/streptomycin, and grown at 37°C and 5% CO_2 under standard conditions.

Cell Adhesion Assay

A real-time cell adhesion assay was completed using a RT-CIM system (ACEA Biosciences, Inc, San Diego, CA) and E-plates (Roche, IN), which are 16-well plates suitable for cell culture and contain sensor electrodes. The change in impedance is measured by electrodes and expressed as a cell index, which is proportional to the degree of cell adhesion to the wells. HUVECs were gently trypsinized and plated at a density of 25,000 cells/well in the presence or absence of serpin peptides at 10 and 100 μM. Cells were allowed to settle at room temperature for 30 minutes and then loaded into the RT-CIM system. Values are scaled to percent increase above negative control (complete endothelial cell media) and taken at 1.5 hours. Statistical significance was determined at $^*P < .05$ by Student's *t* test and compared with the negative control.

Western Blot Analysis

HUVECs were grown in complete endothelial cell media, plated in six-well tissue culture-treated plates at high density (360,000 cells/well), and allowed to adhere for 2 hours. At this point, the medium was removed; fresh endothelial cell media, SP6001 or SP6001SCR (10 μM) with VEGF, or VEGF alone at 20 ng/ml were added, and the cells were incubated at 37°C for 24 hours. The reactions were stopped by removing the medium containing VEGF and adding PBS at 4°C and cell lysis buffer (150 mM NaCl, 1 mM EDTA, 100 μl/ml protease inhibitors [Sigma, St. Louis, MO], 10 μl/ml phosphatase inhibitors [Sigma], and 1% Triton) was added after removing the cold PBS. The cell lysates were spun for 15 minutes at 14,000*g* to remove cell membranes and debris and were separated by SDS-PAGE; the transferred blots were blocked for 1 hour with 5% milk and 1% bovine serum albumin in Tris-buffered saline and Tween. The 4G10 pan-tyrosine phosphorylation antibody at 1:2000 dilution (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) was added, and the blot was incubated overnight. Secondary antibodies were added the next day at 1:2000 dilution and the phosphorylated protein bands were detected by chemiluminescence detection reagent (GE Healthcare, Little Chalfont, United Kingdom). Blots were stripped and probed with FAK primary antibody (Cell Signaling Technology, Inc, Danvers, MA).

We followed a similar protocol for analysis of pJNK-1. Briefly, SP6001 or SP6001SCR were applied at 1, 10, and 30 μM for 4 hours, and cells were lysed with cell lysis buffer, separated by SDS-PAGE, and probed with pJNK-1 antibody (Cell Signaling Technology, Inc). Blots were then stripped and probed for JNK-1 (Cell Signaling Technology, Inc). Experiments were repeated at least once. The ratio of phospho-protein to total protein was quantified with ImageJ software (National Institutes of Health, Bethesda, MD).

Cell Migration Assay

We used the Oris Pro migration assay (Platypus Technologies, Madison, WI), a modified wound healing assay to assess SP6001 effects on cell migration. Plates are 96-well tissue culture plates that contain stoppers that block the center of the wells. HUVECs were plated at 20,000 cells/well in the presence or absence of peptide SP6001 at 25, 50, or 100 μM and SP6001SCR at 10, 50, or 100 μM

in complete endothelial cell media and were allowed to adhere. Two hours later, the stoppers were removed, and the cells were allowed to migrate into the center of the well for 20 hours, at which point cells were stained with calcein AM (Invitrogen, Carlsbad, CA), and fluorescence was measured with a fluorescent Victor V plate reader (Perkin Elmer, Waltham, MA). In some wells, stoppers were removed only after 20 hours to determine background migration, which was negligible.

Apoptosis Assay

A Caspase-Glo 3/7 Assay apoptosis detection assay was used to determine whether the peptides were causing endothelial cells to undergo apoptosis (Promega, Madison, WI). Briefly, cells were plated at 5000 cells/well in opaque 96-well plates to minimize well-to-well crosstalk. The next morning, the cells were serum starved for 24 hours by replacing complete medium with serum-free media. A combination of basic fibroblast growth factor (bFGF)/VEGF (30/10 ng/ml) with or without peptide (0.1, 1 or 10 μM) was added at this point. Forty-eight hours later, the Caspase-Glo chemiluminescent substrate (100 μl/well) was added, and luminescence was measured with a Victor V plate reader (Perkin Elmer). A serum-free medium control was used as an apoptosis inducer. Each concentration of peptide was tested in triplicate, and the experiments were repeated three times. The degree of apoptosis in wells containing peptide is compared to only bFGF/VEGF–treated wells. *P* values for the Student's *t* test were calculated and considered to be significant if they are $P < .01$. Experimental values shown are a composite graph of $n = 3$ independent experiments.

MDA-MB-231 Breast Xenograft Assay

Orthotopic breast xenografts were established in severe combined immunodeficient (SCID) mice using previously established techniques [22]. Briefly, 2×10^6 cells were injected into the mammary fat pad of animals, and tumors were allowed to grow to a palpable starting size (~100 mm^3), which usually took 2 to 3 weeks. The mice were randomized and grouped into sets of eight for control or 1- or 5-mg/kg peptide treatments. There was no statistical difference in starting values among sets as determined by *P* values from Student's *t* test. Tumor sizes were determined every fourth day using calipers and applying the formula $V = ab^2/2$, where *a* is the long axis and *b* is the short axis of the tumor. The peptides were administered daily i.p. or s.c. into the flank of the animal for a total of 25 days. Statistical significance was determined on

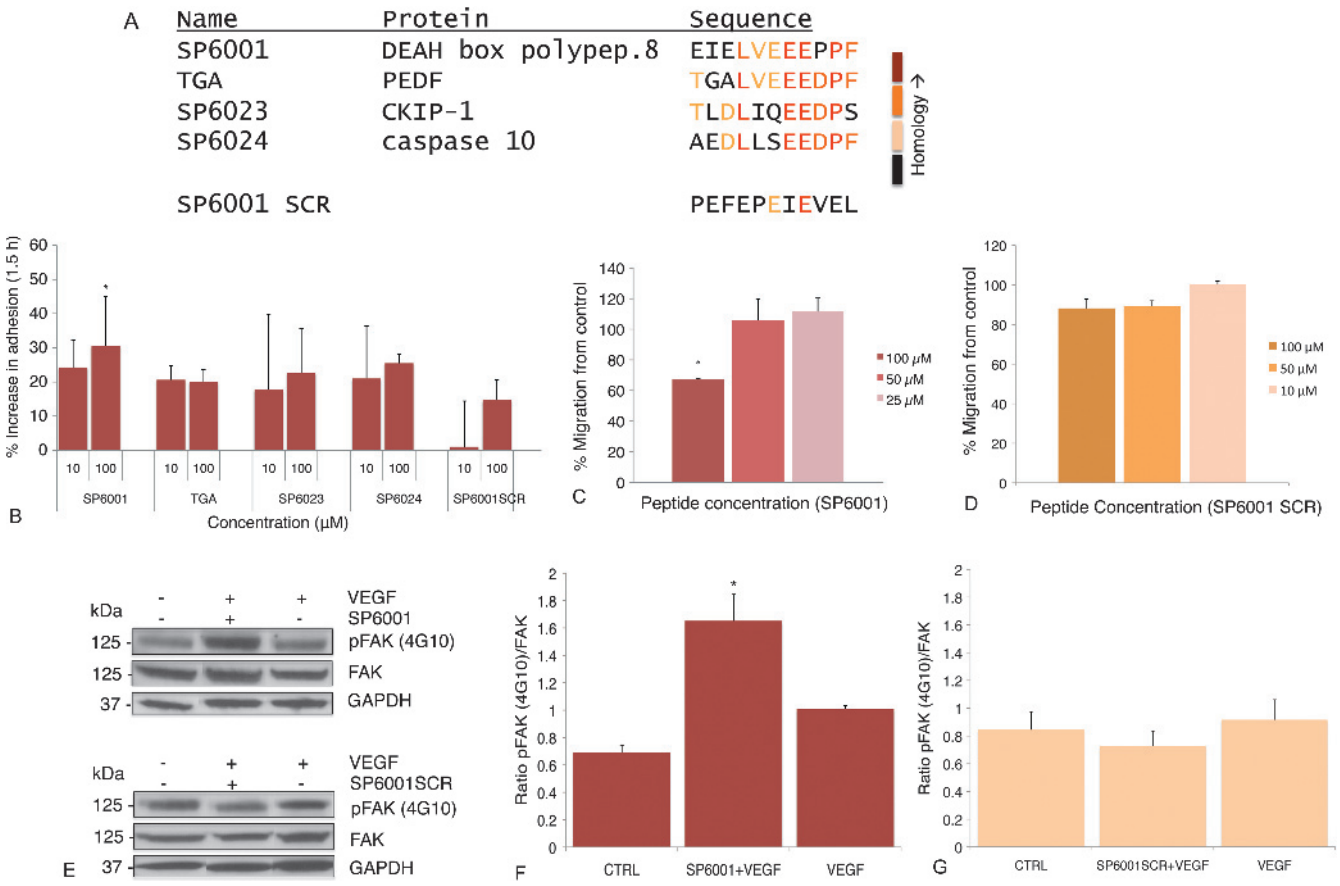

Figure 1. Serpin sequences and their activities in cell adhesion and migration assays. (A) Bioinformatics methodology showing novel sequences in the human proteome termed SP6001, SP6023, and SP6024. We generated a scrambled sequence based on the original SP6001 peptide for test in adhesion assays. (B) SP6001, SP6023, and SP6024 promote endothelial cell adhesion. Adhesion in the presence of SP6001 is significant over the complete medium control at 100 μM. (C) Oris Pro endothelial cell migration assay for HUVECs. Cells were plated for 2 hours in the presence of SP6001 or complete medium around stoppers, which were then removed and cells were then allowed to migrate. After 20 hours, cells were stained with calcein AM and fluorescence intensity measured with a Victor V plate reader. (D) Oris Pro endothelial migration assay testing SP6001SCR. (E) Western blot analysis showing that SP6001 promotes endothelial cell adhesion by sustaining phosphorylation of FAK over time in the presence of VEGF for 24 hours *versus* untreated cells and VEGF-treated cells. The scrambled peptide did not show this same increase. (F) pFAK quantification for SP6001 (*$P < .05$) and (G) SP6001SCR.

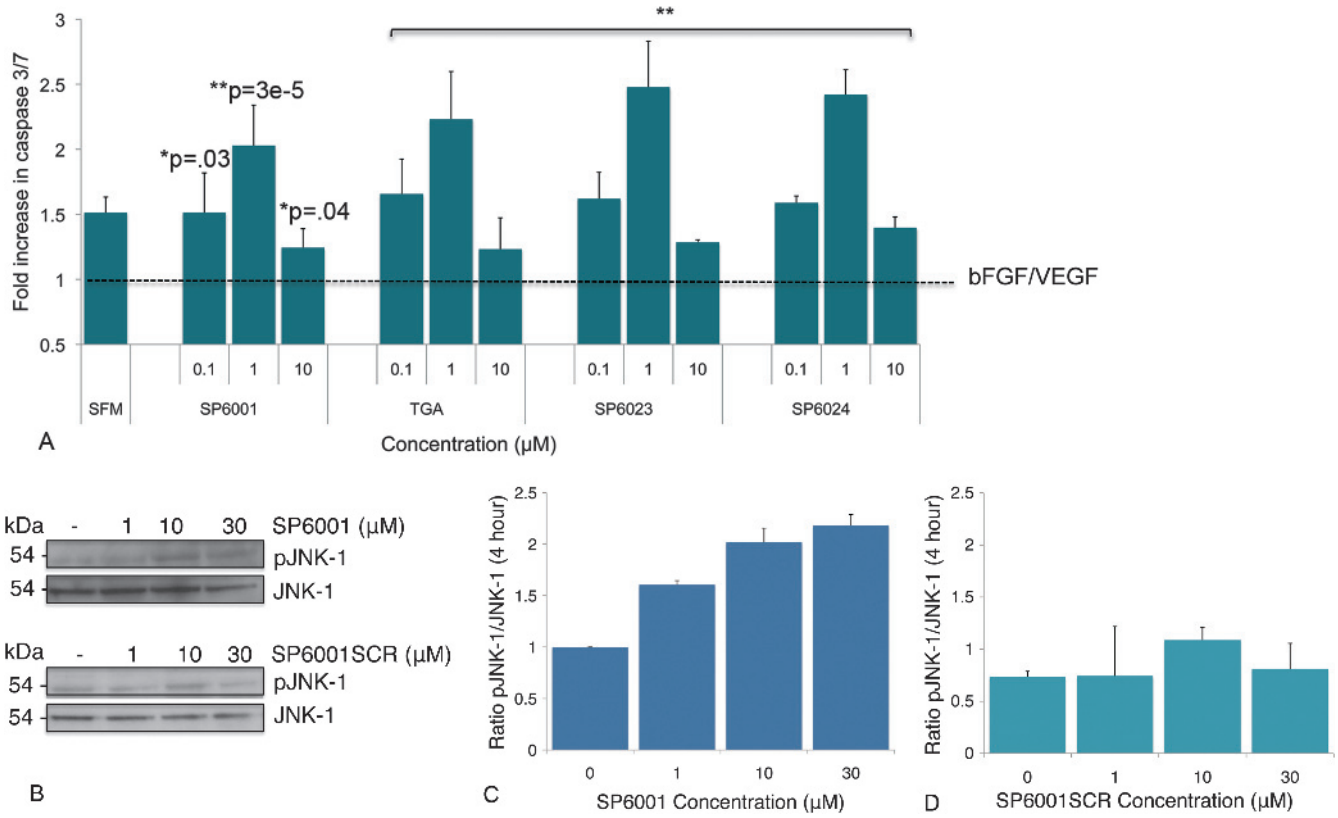

Figure 2. Serpin-derived peptides induce apoptosis in HUVECs. (A) Caspase 3/7 assay showing an increase in caspase 3/7 for peptide-treated cells in the presence of bFGF and VEGF (30/10 ng/ml) after 48 hours. Values are normalized to 1 (bFGF/VEGF control). **Significant compared to bFGF/VEGF controls for $P < .01$. (B) Western blot analysis showing JNK-1 stress kinase phosphorylation increases on SP6001 administration over SP6001SCR at 4 hours (1, 10, and 30 μM SP6001 or SP6001SCR treatment). Quantification of pJNK-1 increase for SP6001 (C) and SP6001SCR (D).

termination of the experiment by Student's *t* test, and *P* values shown compare PBS-treated controls and peptide-treated groups.

Immunohistochemical Staining and Quantification

After the sacrifice of animals on day 25 of the experiment, the tumors were excised and stored in a zinc-based fixative (BD Biosciences, San Jose, CA) for 2 weeks and sent to the JHMI Immunohistochemistry Core Facility where they were centrally cross-sectioned and embedded in paraffin wax. Samples were then sent to Covance, Inc (Princeton, NJ), for immunohistochemical staining for the CD34 antigen. After receipt of images, they were quantified for CD34 staining by FRiDA (free software available from Johns Hopkins University, Baltimore, MD) and measured pixel intensity at 20× magnification/frame. Statistical significance was assessed at $^{**}P < .01$ by Student's *t* test. Images shown are magnified at 4× to visually show greater surface area.

Results

The three bioinformatically identified serpin domain–derived sequences enhanced adhesion of endothelial cells by similar amounts, although SP6001 was the only statistically significant result above the non–peptide-treated negative control (Figure 1*B*). The scrambled peptide, with the same amino acid content as SP6001, showed markedly reduced activity from the native sequence and was not statistically different from the negative complete medium control. SP6001 but not SP6001SCR also sustained FAK phosphorylation in the presence of VEGF as determined by Western blot analysis (Figure 1*E*). VEGF is known to induce FAK phosphorylation, and in the VEGF-treated controls, this phosphorylation decreased during 24 hours. SP6001 constitutively activates FAK for 24 hours in endothelial cells as determined by the ratio of pFAK (4G10) to FAK. Figure 1*F* and Figure 1*G* quantify this effect and show SP6001 but not SP6001SCR increase pFAK. In the endothelial cell migration assay, SP6001 decreased HUVEC migration at 100 μM compared with the complete medium control (Figure 1*C*). A serum-free medium control was also taken (containing no chemoattractant; data not shown) and was negligible compared to the induction of migration by complete media. The scrambled peptide, SP6001SCR, shows markedly reduced activity from SP6001 in migration and is not statistically different from the complete medium control at 10, 50, or 100 μM (Figure 1*D*).

These serpin peptides were further characterized by showing that they induce apoptosis of HUVECs in the presence of bFGF and VEGF as measured by caspase 3/7 release (Figure 2*A*). In this case, all peptides behaved similarly by increasing apoptosis significantly compared to bFGF/VEGF treatment. Some peptide concentrations were significantly more apoptotic than the serum-free medium control ($^{**}P < .01$). All peptides tested have a similar biphasic profile with activity peaking at 1 μM, consistent with previously published results [11]. This effect was most prominent during 48 hours of peptide treatment. SP6001 but not the scrambled control also begins inducing JNK-1 stress kinase phosphorylation at 4 hours in comparison to untreated controls (Figure 2*B*). These results are quantified in Figure 2, *C* and *D*.

SP6001 was then tested *in vivo* against a triple-negative MDA-MB-231 orthotopic breast xenograft model. Compared to the PBS-

treated controls, SP6001 significantly suppressed breast tumor growth at 5 mg/kg (i.p. injection; Figure 3*A*) and 5 mg/kg (s.c. injection; Figure 3*B*). The subcutaneous peptide administration had a more pronounced effect than i.p. administration when comparing mean tumor volumes on day 25 (687 *vs* 453 mm^3, respectively). Both values were statistically lower than PBS-treated control, which had a final value of 1274 mm^3 on day 25. At 1 mg/kg, the i.p. injection was very similar to control with virtually no tumor-suppressing activity, although s.c. injection again showed improved activity *versus* PBS-treated control but not quite statistically significant ($P = .125$). Immunohistochemistry showed that at 5-mg/kg subcutaneous injection, there were significantly fewer vessels as marked by CD34 staining *versus* the control (Figure 3*C*). Microvessels were quantified and confirmed a 43% decrease in CD34 from the PBS-treated control for SP6001 5 mg/kg s.c., significant at $^{**}P < .01$ (Figure 3*D*).

Discussion

Triple-negative breast cancer is a virulent disease with poor prognosis and few treatment options. Bevacizumab, a VEGF-neutralizing antibody, initially showed promise and was approved by the Food and Drug Administration as breast cancer therapy in combination with chemotherapy [23]; however, in 2011, the FDA recommended removing the breast cancer indication from bevacizumab because it did not lead to an increase in overall survival of patients [24]. Breast cancer is an angiogenesis-dependent disease and may be amenable to peptide-based therapies, which do not target standard hormone receptors. In this study, we experimentally show that bioinformatically identified serpin peptides suppress tumor growth by altering adhesion/migration of endothelial cells and inducing endothelial cell apoptosis.

The SP6001, SP6023, and SP6024 peptides have similar sequences and are found in the human proteome. Their parent proteins are not

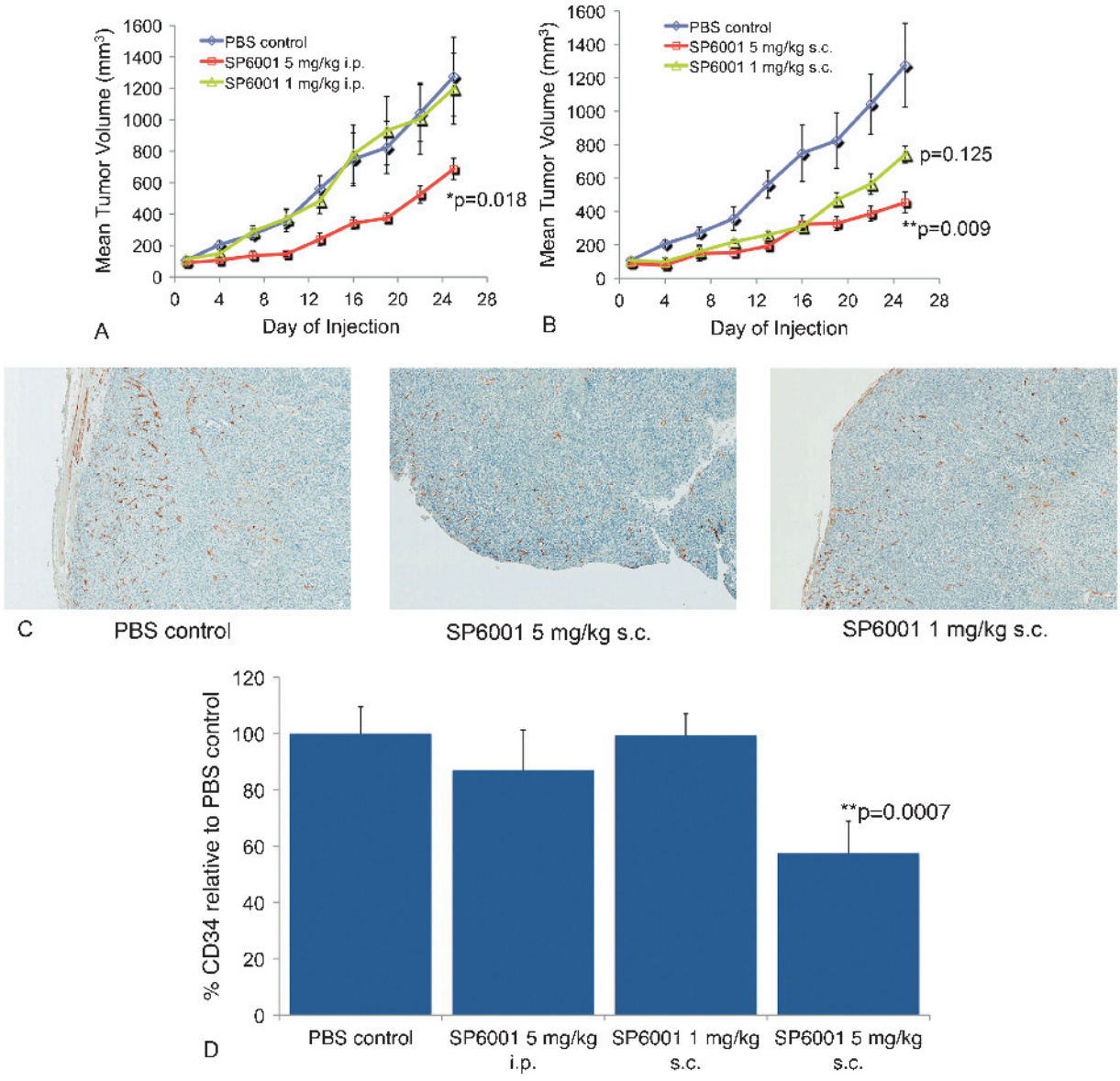

Figure 3. MDA-MB-231 breast xenograft assay. SP6001 significantly suppresses breast tumor growth by two methods of administration—intraperitoneal (i.p.) (A) and subcutaneous (s.c.) (B) peptide delivery. Tumors were measured every fourth day. Peptides were injected daily at 1 or 5 mg/kg. (C) CD34 immunohistochemistry for endothelial cells in tumor cross sections. (D) SP6001 at 5 mg/kg subcutaneous administration significantly decreases CD34 below PBS-treated controls ($^{**}P < .01$).

known to have antiangiogenic properties. The similarity in sequence and activity suggest a common mechanism of action for this peptide family. In this study, we show that these serpin-derived peptides increase cell adhesion above a negative control, and SP6001, but not the scrambled control, sustains phosphorylation of FAK. FAK is responsible for tyrosine phosphorylation in many focal adhesion-associated proteins to sustain integrin-mediated adhesion [25]. In a previous study, Ek et al. [26] generated four 25-mer PEDF-derived peptides termed *StVOrth-1*, *-2*, *-3*, and *-4* and showed that three sequences increased adhesion of osteosarcoma cells, resulting in a reduction of their invasiveness. The serpin-derived peptides may also primarily exhibit their cellular effects through this mechanism whereby adhesion is increased resulting in a decrease in migration. In contrast, several nonserpin peptides are antiangiogenic by blocking endothelial cell adhesion and migration, including the proapoptotic peptide CIGB-300 [27], the human decorin leucine-rich peptide LRR5 [28], and the galectin 1–binding peptide Anginex [29]. The relative importance of endothelial cell adhesion and how adhesion may determine efficacy of targeted therapies in tumor angiogenesis by inhibiting endothelial cell migration should be further characterized by more comprehensive peptide-peptide comparisons.

We show that serpin-derived peptides induce endothelial cell apoptosis by caspase 3/7 release and induction of JNK-1. These results are consistent with previously published PEDF literature [8,11]. The proapoptotic activity is biphasic for each peptide and peaks at 1 μM, suggesting the presence of multiple receptors. JNK-1 may be activated by numerous stresses on the cell including UV light, reactive oxygen species, and other stress factors contributing to apoptosis [30]. SP6001 but not the scrambled control increases pJNK-1.

SP6001 is potent when administered i.p. or s.c. in tumor-bearing mice. Mean tumor volumes for 1- and 5-mg/kg dosing were lower in the s.c. treatment compared to i.p. CD34 immunohistochemical staining of the tumor, which was significantly lower in the case of 5 mg/kg s.c. administration, confirming our hypothesis that the peptide is antiangiogenic and affects endothelial cells in the tumor microenvironment. The improved activity after subcutaneous administration, namely, lower tumor volume and decreased CD34 expression, suggests better bioavailability of the peptide to the tumor microenvironment, which we plan to more comprehensively study in future work. PEDF and phosphomimetic variants of the full-length protein have been proposed as treatment for breast cancer [31]; however, to our knowledge, this is the first study of short serpin-derived peptides showing efficacy in a breast cancer model. Indeed, the efficacy of SP6001 suggests that these peptides should be further optimized to become clinical candidates for breast cancer treatment.

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