Published in final edited form as:

Cancer Res. 2008 September 15; 68(18): 7332–7341. doi:10.1158/0008-5472.CAN-08-1087.

Functional Characterization of Filamin A Interacting Protein 1– Like, a Novel Candidate for Antivascular Cancer Therapy

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Abstract

Inhibiting angiogenesis has become a major therapeutic strategy for cancer treatment. To identify common intra-cellular mediators, we previously analyzed gene expression profiles of endothelial cells after treatment with angiogenesis inhibitors. *Filamin A interacting protein 1-like (FILIP1L*; previously known as *down-regulated in ovarian cancer 1*) was identified as one of the genes up-regulated in endothelial cells in response to these inhibitors. However, the expression and function of FILIP1L protein is uncharacterized. Here, we provide the first description of the expression and specific subcellular localization of FILIP1L protein in human tissue. Overexpression of FILIP1L resulted in inhibition of cell proliferation and migration and increased apoptosis. In addition, overexpression of FILIP1L truncation mutants showed differential antiproliferative activity. A COOH terminal truncation mutant (FILIP1LΔC103) was more potent than wild-type FILIP1L in mediating this activity. Targeted expression of FILIP1LΔC103 in tumor vasculature inhibited tumor growth *in vivo*. Overall, these findings suggest that the novel protein FILIP1L may be an important mediator of the effects of angiogenesis inhibitors and that FILIP1L has the potential to be an antivascular reagent for cancer therapy.

Introduction

Angiogenesis, the formation of new blood vessels from pre-existing ones, is required for the sustained growth, invasion, and spread of tumors. Thus, the inhibition of tumor angiogenesis has been considered to be an important component of anticancer therapy. Many angiogenesis inhibitors that prevent endothelial cells from proliferating and migrating or result in the induction of apoptosis have been identified (1,2). Endostatin, the 20-kDa COOH terminal fragment of collagen XVIII, inhibits endothelial cell proliferation, migration, invasion, and tube formation and induces apoptosis (3–6). Fumagillin, a natural metabolite from *Aspergillus fumigatus*, induces cell cycle arrest and apoptosis in endothelial cells (7–9). EMAP-II, a 20-kDa fragment of the aminoacyl-tRNA synthetase complex, induces endothelial cell apoptosis,

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

inhibits endothelial cell proliferation, and up-regulates tissue factor expression on the endothelial cell surface (10–14). Previously, our laboratory examined the effects of these different angiogenesis inhibitors, endostatin, fumagillin, and EMAP-II, on the gene expression profiles of human umbilical vascular endothelial cells (HUVEC) to elucidate commonly affected pathways (15,16). Interestingly, the majority of gene expression changes were observed as early as 1 and 2 hours after treatment. Among these, expression of genes, such as *filamin A interacting protein 1-like* [*FILIP1L*; synonym of *down-regulated in ovarian cancer 1* (*DOC1*), National Center for Biotechnology Information (NCBI) accession number NP_055705], *KLF4*, and *TC-1* was shown to be rapidly up-regulated. Using small interfering RNA, we showed that, when *FILIP1L* was silenced, *KLF4* and *TC-1* failed to show up-regulation in gene expression in response to endostatin treatment, suggesting that *FILIP1L* may be upstream of *KLF4* and *TC-1* in a pathway that mediates the antiangiogenic response to endostatin (15). Likewise, silencing of *FILIP1L* gene expression resulted in abrogation of the modulatory effect of EMAP II on *ADM*, *KLF4*, *SOCS3*, and *TNFAIP3* gene expression (16). Collectively, these data suggest that the *FILIP1L* gene may play a central role in mediating the common early response pathways seen in endothelial cells after exposure to angiogenesis inhibitors.

In the present study, we define expression and specific subcellular localization of *FILIP1L* protein in human tissue for the first time. Additionally, we show that overexpression of *FILIP1L* in endothelial cells results in a similar profile of antiangiogenic activity as the angiogenesis inhibitors. Finally, using a tumor vascular targeted gene therapy vector, we show that targeted expression of *FILIP1L* in the tumor vasculature results in inhibition of tumor growth *in vivo*, suggesting that *FILIP1L* could be developed as a potential cancer therapeutic.

Materials and Methods

Production of mouse monoclonal anti-FILIP1L antibody

The construct encoding a full-length *FILIP1L* cDNA was generated and expressed in baculovirus. The purified full-length *FILIP1L* protein (893 amino acids) was used as an antigen to immunize mice. Immunization of mice, production of hybridoma cells, screening by ELISA, and purification of monoclonal antibody were performed by Green Mountain Antibodies, Inc. Antibodies that recognize *FILIP1L* were further tested by Western blot, and a monoclonal antibody was selected.

Cell culture

HUVECs were cultured in complete EGM-2 medium as recommended by the manufacturer (Lonza). HEK293 cells were grown in DMEM containing 10% fetal bovine serum (FBS). DU145 human prostate carcinoma cells and M21 human melanoma cells were grown in RPMI 1640 containing 10% FBS.

Western blot

HUVECs were cultured, harvested, and fractionated with ProteoExtract Subcellular Proteome Extraction kit according to the manufacturer's protocol (Calbiochem). For endostatin experiment, HUVECs were starved in EGM-2 basal medium containing 1% FBS for 16 h, treated with 1 μ g/mL endostatin for 2, 4, and 8 h, and lysed with radio-immunoprecipitation assay (RIPA) buffer. HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen), with a series of NH₂ terminal and COOH terminal truncation mutants of *FILIP1L* containing a COOH terminal hemagglutinin (HA) tag, harvested at 24 h and lysed with RIPA buffer. Empty lentivirus or lentivirus expressing *FILIP1L* mutant 1–790 (hereafter called *FILIP1L* Δ C103)–transduced DU145 clones were cultured in the presence or absence of 1 μ g/mL doxycycline and lysed with RIPA buffer. Tumors from PBS-treated, null–adeno-associated

virus-phage (hereafter called AAVP-null)–treated, and AAVP expressing FILIP1L Δ C103 (hereafter called AAVP- Δ C103)–treated mice were removed 4 d after tail vein injection and snap frozen. Whole tumor lysates were prepared from RIPA buffer lysis of 60- μ m tumor section. Cellular fractionation (25–50 μ g), whole cell lysates, or whole tumor lysates prepared by above methods were separated on SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blotted with antibodies against FILIP1L, HA tag (Covance), and glycerol-dehyde-3-phosphate dehydrogenase (GAPDH; Chemicon) followed by incubation with antimouse antibody conjugated to horseradish peroxidase. The signal was detected using chemiluminescence (Millipore).

Immunohistochemistry

Frozen human colon tumors and their adjacent normal colon samples were obtained under an Institutional Review Board–approved protocol. Tissue sections (10 μ m) were fixed with 4% paraformaldehyde for 20 min and stained with mouse monoclonal antibodies against FILIP1L (7.5 μ g/mL) and CD31 (10 μ g/mL; DAKO). After visualization of staining by 3,3'-diaminobenzidine tetrahydrochloride, the slides were counterstained with hematoxylin. Images were acquired by Axioplan 2 microscope using a 20 $\times$ /0.75 objective with Axiovision 4.1 software (Zeiss).

Immunofluorescence and vessel density determination

HUVECs were starved in EGM-2 basal medium containing 1% FBS for 16 h and treated with 1 μ g/mL endostatin for 4 h. The cells were fixed with 4% paraformaldehyde for 10 min followed by permeabilization with 0.1% Triton X-100 for 5 min. The cells were washed with PBS, blocked with 5% bovine serum albumin (BSA) in PBS, and treated with mouse anti-FILIP1L antibody (4 μ g/mL) preincubated with 500-fold molar excess of either BSA or FILIP1L. The cells were then incubated with 2 μ g/mL Alexa Fluor 488 antimouse IgG (Invitrogen) and treated with 4',6-diamidino-2-phenylindole (DAPI) mounting media (Vector Laboratories). Images were acquired on an LSM-510 confocal microscope using a 25 $\times$ /0.8, 40 $\times$ /1.3, or 63 $\times$ /1.4 objective and analyzed by AxioVision LE software (Zeiss). For AAVP-targeting experiment, tumors from PBS-treated, AAVP- Δ C103–treated, and AAVP- Δ C243–treated mice were removed at 30 min and 6 d after tail vein injection (30 min only for PBS-treated tumor), snap frozen, and cut into 10- μ m tumor sections. These tumor sections were subjected to immuno-fluorescent staining as described above, except rabbit anti-AAVP antibody (Sigma), followed by Alexa Fluor 594 antirabbit IgG (Invitrogen) and rat anti-CD31 antibody (BD PharMingen), followed by Alexa Fluor 488 antirat IgG (Invitrogen) were used.

CD31-stained tumor sections from PBS-treated, AAVP-null–treated, and AAVP- Δ C103–treated mice were analyzed for vessel density as described previously (17). Three tumors from each treatment group were analyzed. Five random fields per tumor were imaged by Axiovert 200M microscope using a 10 $\times$ /0.3 objective (Zeiss). Axiovision 4.6 software (Zeiss) was used to quantify CD31-positive vessels. In addition, TUNEL staining was also performed on these AAVP-treated tumors as recommended by the manufacturer (Promega Corporation). Images were taken by Axiovert 200M microscope using a 5 $\times$ /0.15 objective (Zeiss).

Transfection of HUVECs with FILIP1L plasmids

Plasmids encoding wild-type, as well as truncation mutants of FILIP1L, were purified using Endo-free maxiprep kit (Qiagen). HUVECs were transfected with equimolar amount of each DNA using HUVEC nucleofector solution and Nucleofector II machine as provided by the manufacturer (Amaxa). Transfection efficiency was verified using a plasmid with an enhanced green fluorescent protein (eGFP) marker (2 μ g), as was calculated by the GFP expression. The percentage of transfection reached by this method was $50 \pm 10\%$. After transfection, the cells were subjected to proliferation, apoptosis, or migration assays.

Bromodeoxyuridine ELISA cell proliferation assay

The transfected HUVECs were plated with 2×10^4 per well in 96-well culture plates and incubated for 24 h. Cell proliferation was measured by Cell Proliferation Biotrak ELISA (GE Healthcare), as recommended by the manufacturer.

Apoptosis assay

The transfected HUVECs were plated with 2.5×10^4 per well in white-walled 96-well culture plates and incubated for 24 h. Early apoptosis was determined by the measurement of caspase-3/caspase-7 activity using the caspase-Glo 3/7 Assay (Promega Corporation) following manufacturer's instructions. The transfected HUVECs were plated with 2×10^6 /100-mm culture dishes and incubated for 48 h. Late-stage apoptosis was determined by the staining of Annexin V-FITC and 7-amino-actinomycin D (7-AAD) staining using the Annexin V-FITC Apoptosis Detection kit (BD PharMingen) following manufacturer's instructions. The stained cells were subjected to flow cytometric analysis using a FACSCalibur (BD PharMingen) and analyzed by the CELLQuest program.

Migration assay

The migratory potential of the transfected HUVECs was assessed by Electric Cell-Substrate Impedance Sensing (ECIS Model 9600, Applied Biophysics, Inc.; refs. 18,19). Cells (1.1×10^5) were inoculated in 8W1E plates in complete EGM-2 medium. The cells were allowed to completely adhere to the electrodes which produced maximum and nonvariable readings of impedance. The monolayers were then wounded (30 s, 4.0 V, 60 kHz) where impedance became a minimum. As cells migrated to heal the wound, the impedance was recorded at 15 kHz every 5 s for 10 h in real time. The differences in migration rate were evaluated by comparison of the slopes of the curves in linear range for early time points.

DU145 clones transduced with either empty lentivirus or lentivirus expressing FILIP1LAC103 (7.5×10^4 cells per chamber) were plated in the presence or absence of 1 μ g/mL doxycycline in upper chamber. Migration toward 10% FBS was measured at 15 h by QCM 24-well colorimetric cell migration assay kit (Chemicon), as recommended by the manufacturer.

Cloning of FILIP1L and its truncation mutants

Genes for FILIP1L and its truncation mutants were cloned into Gateway entry clones using multistep PCR. The subsequent entry clones were sequence verified throughout the entire cloned region. Entry clones were then subcloned by Gateway LR recombination using the manufacturer's protocols (Invitrogen) into different expression vectors.

Lentivirus generation and development of inducible clones over-expressing FILIP1LAC103

A lentiviral construct encoding *FILIP1LAC103* was used to generate lentivirus expressing FILIP1LAC103 by the ViraPower T-REx Lentiviral Expression System (Invitrogen) using the manufacturer's protocols. DU145 cells were transduced with the Tet repressor-lentivirus and screened for clones that expressed the Tet repressor by Western blot analysis. Tet repressor-expressing DU145 cells were then transduced with either empty lentivirus or lentivirus expressing FILIP1LAC103, and stable clones were screened by real-time reverse transcription-PCR (RT-PCR) analysis.

Quantitative real-time RT-PCR

DU145 clones transduced with lentivirus expressing FILIP1LAC103 were cultured in the presence or absence of 1 μ g/mL doxycycline for 48 h and harvested. Total RNA was prepared by RNeasy kit (Qiagen), and cDNA was prepared by Superscript II reverse transcriptase (Invitrogen). qPCR was performed using ABI 7500 SDS real-time PCR instrument following

manufacturer's instructions (Applied Biosystems). The expression of the FILIP1L gene was normalized to GAPDH expression. The primers used were 5'-AACGCTGGTATCATGGCTGAA-3' and 5'-ATCTCTGCACTGCTCCTCCATT-3' for FILIP1L; 5'-TCACCAGGGCTGCTTTTAACTC-3' and 5'-GGAATCATATTGGAACATGTAAACCA-3' for GAPDH.

Construction and generation of targeted AAVP particles

Cloning of both *FILIP1LAC103* (amino acid 1–790) and *FILIP1LAC243* (amino acid 1–650) mutant cDNA into the AAVP vector and the production of AAVP was performed as described previously (20,21).

Xenograft assay

M21 human melanoma cells were injected s.c. into female athymic nude mice and grown to an average size of 100 mm³. Mice were randomly sorted into four groups ($n = 11$ for each group), AAVP (1×10^{11} transducing units per dose) was injected i.v. at day 0 and day 7, and tumors were measured in a blinded manner. Tumor volume was calculated as the product of (length $\times$ width $\times$ height) $\times$ 0.52. All animal experiments were conducted according to protocols approved by the NIH Animal Care and Use Committee.

Statistical analysis

Statistical analyses were performed using a two-tailed Student's t test (GraphPad Prism 3.0), and differences were considered to be statistically significant at a value of $P < 0.05$. Xenograft and vessel density data were analyzed using one-way ANOVA with Newman-Keuls Multiple Comparison Test. A P value of <0.05 was considered significant.

Results

Expression of FILIP1L protein in HUVECs and human tissue

Although *FILIP1L* mRNA expression has been shown to be up-regulated in human endothelial cells in response to different angiogenesis inhibitors (15,16), the expression of FILIP1L protein has not been previously investigated. To determine whether FILIP1L protein is endogenously expressed in human tissue, we produced monoclonal antibodies that specifically recognize FILIP1L. To detect FILIP1L protein in endothelial cells and determine its subcellular localization, HUVECs were fractionized and a Western blot was performed using anti-FILIP1L antibody. A specific 110-kDa band, identical size to the purified FILIP1L protein, was detected by anti-FILIP1L antibody, suggesting that HUVECs express a full-length FILIP1L protein (Fig. 1A). In addition, FILIP1L was expressed predominantly in the cytoplasm with less expression in the membrane and nucleus. Having shown the expression of FILIP1L protein in cultured endothelial cells, we then examined the expression of FILIP1L in human tissue. Immunohistochemical analysis was performed on 15 frozen human colon cancers and matched normal colon tissues using anti-FILIP1L antibody. In normal colon, FILIP1L was expressed in the vasculature and muscularis mucosa (Fig. 1B, top). In colon cancer, FILIP1L was strongly expressed in tumor stroma and the vasculature (Fig. 1B, bottom). Thus, these data show that FILIP1L is expressed in vasculature and smooth muscle and in desmoplastic stroma in response to tumor invasion.

Up-regulation of FILIP1L protein by endostatin

Our previous studies have shown that *FILIP1L* mRNA expression is up-regulated in HUVECs within 1 hour after the treatment of endothelial cells with the angiogenesis inhibitors endostatin, fumagillin, and EMAP II (15,16). To further confirm that FILIP1L protein expression is up-regulated in endothelial cells in response to angiogenesis inhibitors, we treated HUVECs with

endostatin, harvested cells at 2, 4, and 8 h, and performed Western blot analysis using anti-FILIP1L antibody on whole cell lysates. Compared with vehicle-treated controls, endostatin-treated HUVECs expressed more FILIP1L protein at all the time points tested (densitometric quantitation values are also shown in Fig. 2A). To examine if endostatin treatment affects cellular distribution of FILIP1L protein in endothelial cells, we first serum starved HUVECs to synchronize them. We then treated those HUVECs with endostatin and immunofluorescently stained them with anti-FILIP1L antibody at 4 h. Serum-starved, vehicle-treated control cells showed weak cytoplasmic staining, whereas endo-statin-treated cells showed a stronger punctate distribution of staining in the cytoplasm (Fig. 2B; additional images are shown in Supplementary Fig. S1). FILIP1L expression measured by immuno-fluorescent staining in endostatin-treated HUVECs was significantly more than that in vehicle-treated control cells ($P = 0.0012$; Fig. 2C). In addition, this staining was FILIP1L-specific, as anti-FILIP1L antibody preincubated with FILIP1L protein, but not with BSA control, failed to show the staining (Fig. 2D). These results suggest that FILIP1L protein expression is increased after endostatin treatment and support our initial observations at the mRNA level.

Overexpression of FILIP1L in endothelial cells leads to inhibition of cell proliferation and an increase in apoptosis

Because FILIP1L protein expression was up-regulated by the angiogenesis inhibitor endostatin, we hypothesized that FILIP1L may mediate antiangiogenic activity in endothelial cells and that overexpression of FILIP1L in endothelial cells may result in antiproliferative and proapoptotic activity. To test this, we transfected HUVECs with a plasmid encoding *FILIP1L* cDNA and measured cell proliferation by bromodeoxyuridine (BrdUrd) ELISA 24 hours after transfection. Transfection efficiency was $50 \pm 10\%$, as verified by GFP expression after transfection of HUVECs with a control plasmid encoding an eGFP. Compared with control empty vector-transfected cells, *FILIP1L*-transfected cells showed a decrease in cell proliferation ($P < 0.0001$; Fig. 3A). To determine if overexpression of FILIP1L in endothelial cells results in an increase in apoptosis, we measured caspase-3/caspase-7 activity at 24 hours after transfection of HUVECs with *FILIP1L* cDNA. Although caspase-3/caspase-7 activity in control vector-transfected cells was present due to the cytotoxicity caused by the transfection procedure, *FILIP1L*-transfected cells showed significantly more activity ($P < 0.001$; Fig. 3B). To further detect apoptosis in these cells, we stained the transfected cells with Annexin V-FITC and 7-AAD at 48 hours after transfection and measured staining using flow cytometry. As shown in Fig. 3C, FILIP1L overexpression resulted in increased staining of both Annexin V-FITC and 7-AAD (44.4% versus 15.2%), suggesting that late-stage apoptosis is increased in *FILIP1L*-transfected cells compared with control vector-transfected cells.

FILIP1L truncation mutants have differential antiproliferative activity

A coiled-coil region (residues 3–542), two leucine zipper motifs (residues 83–111 and 218–253), and a prefoldin domain (residues 465–535) can be recognized in the NH₂ terminal half of the FILIP1L protein. In addition, an NCBI conserved domain search⁵ reveals that FILIP1L has an SbcC (COG0419; ATPase involved in DNA repair; residues 19–576) conserved domain in its NH₂ terminal half and a Herpes_BLLF1 (pfam05109; Herpes virus major outer envelope glycoprotein; residues 640–829) conserved domain in its COOH terminal half (Fig. 4A).

To examine which part of the FILIP1L protein mediates the antiproliferative activity in endothelial cells, we generated a series of NH₂ terminal and COOH terminal truncation mutants of FILIP1L as a fusion protein containing a COOH terminal HA tag (Fig. 4B). To determine if these mutant constructs produce proteins in cells, we transfected HEK293 cells with each construct and performed Western blot analysis using anti-HA tag antibody. All the constructs

⁵<http://www.ncbi.nlm.nih.gov/structure/cdd/cdd.shtml>

produced proteins of the predicted size, although NH₂ terminal truncation mutants 127–893 and 512–893 showed low levels of expression (Fig. 4C). To determine if these proteins are functional, we transfected HUVECs with the plasmid encoding each FILIP1L mutant and measured cell proliferation by BrdUrd ELISA 24 hours after transfection. FILIP1L truncation mutants 1–790, 1–650, 1–512, and 127–893 significantly inhibited cell proliferation compared with control. COOH terminal truncation mutant 1–790 was more potent in its ability to inhibit cell proliferation than wild type ($P = 0.001$; Fig. 4D). Thus, we chose to further evaluate the function of the COOH terminal truncation mutant 1–790 (called FILIP1LΔC103). To examine if overexpression of FILIP1LΔC103 in endothelial cells results in an increase in apoptosis, we measured caspase-3/caspase-7 activity at 24 hours after transfection of HUVECs with *FILIP1LΔC103* cDNA. As shown in Supplementary Fig. S2, *FILIP1LΔC103*-transfected cells showed significantly more apoptotic activity than control cells ($P < 0.0001$).

Overexpression of FILIP1LΔC103 in HUVECs and DU145 prostate cancer cells leads to inhibition of cell migration

Because inhibition of cell migration is one of the important characteristics of angiogenesis inhibitors, we tested whether overexpression of FILIP1LΔC103 results in inhibition of cell migration. To do this, we transfected HUVECs with a plasmid encoding *FILIP1LΔC103* cDNA and measured cell migration by Electric Cell-Substrate Impedance Sensing system (Applied Biophysics, Inc.; refs. 18,19). Compared with control empty vector-transfected cells, *FILIP1LΔC103*-transfected cells showed a significantly slower migration rate ($P < 0.0001$; Fig. 5A), suggesting that overexpression of FILIP1LΔC103 in HUVECs results in inhibition of cell migration.

We also tested the effects of overexpression of FILIP1LΔC103 on migration of neoplastic cell lines. We selected DU145 prostate cancer cells as a model system because *FILIP1L* mRNA expression was shown to be repressed in immortalized prostate epithelial cells (22,23), and *FILIP1L* mRNA expression is relatively low in this cell line compared with other cancer cell lines.⁶ Overexpression of FILIP1LΔC103 in DU145 cells also resulted in inhibition of cell proliferation (Supplementary Fig. S3). Thus, we chose to develop inducible FILIP1LΔC103-overexpressing clones. The system we used was the ViraPower T-REx Lentiviral Expression System (Invitrogen). In this system, the expression of a gene of interest is repressed by a Tet repressor in the absence of tetracycline (or doxycycline), whereas it is derepressed in the presence of tetracycline (or doxycycline). Clones were screened by real-time RT-PCR analysis. Several clones showed a 2.5-fold to 7-fold increase in *FILIP1LΔC103* mRNA expression after doxycycline induction compared with the uninduced condition (Fig. 5B). Unexpectedly, however, we observed that, at the uninduced basal level, most of these clones expressed 20-fold to 60-fold more *FILIP1LΔC103* mRNA than parental Tet repressor-expressing DU145 cells (Fig. 5B). FILIP1LΔC103 protein levels were shown to be increased considerably by doxycycline, but the basal level expression was also detectable in these clones (Fig. 5C). Control cells that were a mixed population from empty lentivirus-transduced Tet repressor-expressing DU145, however, did not produce any FILIP1LΔC103 protein (Fig. 5C). To measure cell migration for these cells, we used the Boyden chamber assay. Using this system, we measured cell migration for the FILIP1LΔC103 clones 2, 12, and 13, as well as control cells. All the FILIP1LΔC103 clones, but not control cells, showed a significantly slower migration in the presence of doxycycline ($P < 0.005$; Fig. 5D). Therefore, these data suggest that overexpression of FILIP1LΔC103 in DU145 cells also results in inhibition of cell migration.

⁶<http://dtp.nci.nih.gov/mtweb>

Targeted expression of FILIP1L Δ C103 in tumor vasculature results in inhibition of tumor growth *in vivo*

We have shown that overexpression of FILIP1L results in inhibition of cell proliferation and migration and increased apoptosis *in vitro*. We then set out to evaluate the effects of targeted FILIP1L expression *in vivo*. In particular, we chose to selectively deliver FILIP1L to tumor vasculature to determine if overexpression of FILIP1L in tumor vasculature leads to an antitumor effect. To achieve this, we used a hybrid AAVP vector, which has been shown to specifically target tumor vasculature in an RGD peptide-restricted manner (20,21,24). These hybrid vectors rely on the specific binding relationship between an RGD peptide and α_v integrin expressed on the surface of tumor vasculature. These vectors have been shown to specifically traffic to and specifically transfect tumor associated endothelial cells without evidence of transfection of normal endothelial cells. We tested two FILIP1L mutants for this purpose: FILIP1L Δ C103 (amino acid 1–790) and FILIP1L Δ C243 (amino acid 1–650). We cloned each FILIP1L mutant cDNA into the AAVP vector, produced RGD-targeted AAVP, and screened for the AAVP, which showed the highest expression of each mutant by real-time RT-PCR analysis.

To examine whether the AAVP specifically targets tumor vasculature in our M21 human melanoma model, the RGD-targeted AAVP was injected into the tail vein of female athymic nude mice harboring a 100 to 150 mm³ subcutaneous M21 tumor. After injection, tumors were harvested by time course and analyzed by immunofluorescent staining with anti-CD31 and anti-AAVP antibodies. Both RGD-4C-FILIP1L Δ C103 AAVP (AAVP expressing FILIP1L mutant 1–790; called AAVP- Δ C103) and RGD-4C-FILIP1L Δ C243 AAVP (AAVP expressing FILIP1L mutant 1–650; hereafter called AAVP- Δ C243) were shown to specifically target tumor vasculature (Fig. 6A; data for AAVP- Δ C243 shown in Supplementary Fig. S4), but not tumor cells (Fig. 6A) or the vasculature of normal organs (Supplementary Fig. S5). We also measured FILIP1L protein expression in these tumors by Western blot analysis using anti-FILIP1L antibody on whole tumor lysates. Tumors from AAVP- Δ C103-treated mice, but not PBS and RGD-4C AAVP (control null AAVP; called AAVP-null)-treated mice, showed FILIP1L protein expression (Fig. 6B). Therefore, these data support the observation that the FILIP1L mutant is expressed in the tumor vasculature of mice treated with AAVP- Δ C103.

To evaluate the efficacy of the FILIP1L mutant-AAVP treatment on tumor growth inhibition *in vivo*, M21 melanoma cells were injected s.c. into female athymic nude mice and grown to an average size of 100 mm³. Mice were randomly sorted into four groups ($n = 11$ for each group), AAVP (1×10^{11} transducing units per dose) was injected i.v. at day 0 and day 7, and tumors were measured in a blinded manner. The following groups were tested: PBS, AAVP-null, AAVP- Δ C243, and AAVP- Δ C103. PBS control tumors grew aggressively and started to show central necrosis by day 14. Thus, the experiments were terminated at day 14. Only tumors from AAVP- Δ C103-treated mice were significantly smaller than those from PBS-treated mice by day 14 ($P < 0.01$; Fig. 6C). Although tumors from AAVP-null-treated mice and AAVP- Δ C243-treated mice were smaller than those from PBS-treated mice, the differences were not statistically significant. In addition, tumors from AAVP- Δ C103-treated mice were significantly smaller than those from AAVP-null-treated mice ($P < 0.05$) and AAVP- Δ C243-treated mice ($P < 0.05$) by day 14 (Fig. 6C). These results suggest that targeted expression of FILIP1L Δ C103 in tumor vasculature results in inhibition of M21 melanoma growth *in vivo*.

To confirm that the inhibition of tumor growth is dependent on the antivascular effects of FILIP1L Δ C103, vessel density was analyzed for these AAVP-treated tumors. The percentage area of CD31-positive cells was used as a measure of vessel density (17). Vessel density from AAVP- Δ C103-treated tumors was significantly less than those from PBS-treated tumors ($P < 0.001$) and AAVP-null-treated tumors ($P < 0.01$) at day 4 (Fig. 6D), suggesting that the inhibition of tumor vasculature by AAVP- Δ C103 leads to the inhibition of M21 tumor growth.

In addition, AAVP- Δ C103-treated tumors showed extensive apoptosis as measured by TUNEL staining compared with PBS-treated or AAVP-null-treated tumors (Supplementary Fig. S6), further suggesting that the inhibition of tumor vasculature by AAVP- Δ C103 results in induction of apoptosis and necrosis in these M21 tumors.

Discussion

The development and maintenance of a blood supply is critical for the growth, invasion, and metastatic spread of tumors. Inhibiting the process of new blood vessel formation, as well as attacking established tumor vasculature, has been shown to be a viable strategy for treating cancer (25). A number of antiangiogenic agents have been identified, and several have entered clinical trials.⁷ To better understand the mechanisms of action of these agents on endothelial cells and determine if common pathways are shared between agents, we previously analyzed the gene expression responses of endothelial cells to a variety of angiogenesis inhibitors (15, 16). Based on these studies, we identified *FILIP1L* (previously termed *DOC1*) as a potentially important common regulator of the antiangiogenic activity on endothelial cells.

In the present study, we characterized the patterns of *FILIP1L* expression and elucidated important functions of *FILIP1L* in endothelial cells and cancer cells. We have shown that *FILIP1L* protein is expressed in the vasculature and muscularis mucosa of the colon. The same pattern of expression was observed in human prostate (data not shown). In endothelial cells, endogenous *FILIP1L* protein expression is up-regulated by the angiogenesis inhibitor endostatin, suggesting that *FILIP1L* may be a downstream mediator of some of endostatin's functions, including the inhibition of cell proliferation and migration and the induction of apoptosis. Overexpression of *FILIP1L* in endothelial cells results in inhibition of cell proliferation and increased apoptosis. The COOH terminal truncation mutant 1–790 (*FILIP1L* Δ C103) is more potent than wild-type *FILIP1L* in mediating this antiproliferative activity. In addition, overexpression of *FILIP1L* Δ C103 in HUVECs, as well as DU145 prostate cancer cells, leads to inhibition of cell migration. Finally, targeted expression of *FILIP1L* Δ C103 in tumor-associated vasculature results in the inhibition of M21 melanoma growth *in vivo*, demonstrating a similar effect as that seen by the delivery of angiogenesis inhibitors. Therefore, these data suggest that the novel protein *FILIP1L* could be a potential candidate for cancer therapy by targeted delivery.

Overexpression of *FILIP1L* *in vitro* resulted in inhibition of cell proliferation in other cell types, in addition to endothelial cells. Normal fibroblasts, as well as some cancer cells, including DU145 prostate carcinoma cells showed inhibition of cell proliferation after *FILIP1L* overexpression (Supplementary Fig. S3 and data not shown). To avoid possible toxicity, we chose to selectively deliver *FILIP1L* to tumor vasculature. To achieve this, we used a hybrid AAVP vector, which has been shown to specifically target tumor vasculature (21). Systemic delivery of our targeted *FILIP1L* Δ C103-expressing AAVP had the greatest effect on tumor growth inhibition (Fig. 6C).

Limited studies of *FILIP1L* from other laboratories have suggested that gene expression of *FILIP1L* is also implicated in neoplasia and senescence. *FILIP1L* mRNA was originally characterized as present in human ovarian epithelial cells, but consistently absent in ovarian carcinoma cells (26). Using cDNA microarray analysis, *FILIP1L* was identified as one of the genes whose transcription is induced in senescent human prostate epithelial cells, but significantly repressed in immortalized prostate epithelial cells (22,23). In addition, *FILIP1L* mRNA expression was shown to be down-regulated in microvascular endothelial cells infected with Kaposi's sarcoma-associated herpesvirus compared with uninfected microvascular

⁷<http://www.cancer.gov/clinicaltrials/developments/anti-angio-table>

endothelial cells (27). Furthermore, *FILIP1L* mRNA expression was also shown to be down-regulated in B cells transformed with an oncogene TaxBLV (bovine leukemia virus Tax) compared with untransformed B cells (28).

Although we have shown that overexpression of *FILIP1L* inhibits cell proliferation and migration, and induces apoptosis, the physiologic function of *FILIP1L* remains unclear. *FILIP1L* has a striking similarity to *FILIP1* (29). There is 46% amino acid identity between *FILIP1L* and *FILIP1*. Both have coiled-coil in NH₂ terminal halves, and *FILIP1L* has two leucine zipper motifs whereas *FILIP1* has four leucine zipper motifs in its NH₂ terminal halves. Both have two isoforms: isoform 2 of *FILIP1L* (893 amino acids) lacks the 240 amino-terminal residues of isoform 1 (1,135 amino acids) and S-*FILIP1* (965 amino acids) lacks the 247 amino-terminal residues of L-*FILIP1* (1,213 amino acids). Furthermore, *FILIP1L* is shown to be expressed in vessels and smooth muscle cells in human colon tissue (Fig. 1B), and *FILIP1* is shown to be expressed in cardiac, skeletal, and smooth muscle, as well as in the nervous system (29). In contrast, the *FILIP1L* gene is located on chromosome 3q12.1, whereas the *FILIP1* gene is located on chromosome 6q14.1. To date, the human *FILIP1L* gene is known to have 12 orthologues only in mammals, in contrast to the human *FILIP1* gene which has 30 orthologues throughout many species.

FILIP1 binds to filamin A and induces degradation of filamentous actin associated filamin A, thereby suppressing cell motility and formation of lamellipodia (29,30). In this study, we have shown that *FILIP1L* inhibits cell migration (Fig. 5A and D). In addition, immunofluorescence staining of both *FILIP1L* (Fig. 2B) and *FILIP1* (29) reveals a similar punctate distribution in the cytoplasm. Interestingly, LL5 β , a PIP3 and γ -filamin-binding protein, shows a punctate distribution in the cytoplasm with colocalization of γ -filamin (31). A novel α -helical coiled-coil domain-containing protein FIP, which is shown to bind *Dictyostelium* filamin also shows a punctate staining pattern in the cytosol (32). Therefore, we suspect that *FILIP1L* might be one of *FILIP1* family proteins that have a functional relationship to filamin A. Filamin A is an actin-binding protein which stabilizes three-dimensional actin filaments and links them to cellular membranes (reviewed in ref. 33). It is essential for cell motility and fetal development (34–36). Filamin A has been shown to bind a number of membrane receptors and signal transduction intermediates, including some regulatory cofactors of the Rho GTPases family (37–43). Angiogenesis inhibitors, such as endostatin, fumagillin, and EMAP II, have also been shown to regulate cytoskeletal organization to inhibit endothelial cell function (44–47). Therefore, the fact that *FILIP1L* expression is modulated by these inhibitors and that overexpression of *FILIP1L* in endothelial cells mimics the effects of these inhibitors further suggests that *FILIP1L* might exert its functions via the cytoskeleton. For instance, loss of filamin A was shown to result in not only inhibition of cell migration but also induction of apoptosis (48–50). Thus, it is possible that *FILIP1L* inhibits the function of filamin A after angiogenesis inhibitor treatment, thereby inhibiting endothelial cell function by the induction of apoptosis. Future studies to identify the physiologic binding partner(s) of *FILIP1L* and examine the potential relationship between *FILIP1L* and filamin A will be of interest.

In summary, we have characterized *FILIP1L* as a mediator of apoptosis and an inhibitor of cell migration and proliferation. Targeted delivery of the most potent *FILIP1L* truncation mutant to tumor vasculature results in tumor growth inhibition in a human xenograft mouse model. Further characterization of *FILIP1L* may lead to an improved understanding of the role played by agents that affect the cytoskeleton and may lead to the development of more effective anticancer agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support: Intramural Research Program of NIH, National Cancer Institute, Center for Cancer Research.

We thank Drs. Amin Hajitou, Renata Pasqualini, and Wadih Arap for providing us pfUSE5-MCS phage plasmid and pAAV-GFP plasmid and *E. coli* strains of MC1061 and K91Kan and Dr. Anita Tandle for helpful discussion.

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Figure 1.

Expression of FILIP1L protein in HUVECs and human tissue. *A*, a 110-kDa FILIP1L protein was detected in the cytoplasm, membrane, and nucleus of HUVECs by Western blot using anti-FILIP1L antibody. A purified FILIP1L protein was used as a standard. *B*, immunohistochemical staining of FILIP1L and CD31 in human colon cancers and matched normal tissues. H&E staining is also shown. FILIP1L was expressed in the vasculature (→) and muscularis mucosa (↓) of normal human colon tissues. FILIP1L was also expressed in tumor stroma and vasculature (→) of colon cancer tissues. Note that stroma was negative in FILIP1L staining in normal colon, whereas tumor stroma showed a strong positive staining.

Figure 2.

Up-regulation of FILIP1L protein by endostatin. *A*, increased expression of FILIP1L protein was detected in HUVECs treated with endostatin for 2, 4, and 8 h by Western blot using anti-FILIP1L antibody. GAPDH blot is shown as the loading control. The numbers underneath the blot are the densitometric values calculated as FILIP1L-GAPDH ratios using ImageQuant software. The result is representative of two independent experiments. *B*, the punctate distribution in the cytoplasm was detected in HUVECs treated with endostatin for 4 h by immunofluorescent staining using anti-FILIP1L antibody. Vehicle-treated control cells showed diffused cytoplasmic staining. Nuclear staining with DAPI is shown in blue. *Scale bar*, 10 μ m. The result is a representative image from two independent experiments. Additional

images are shown in Supplementary Fig. S1. *C*, FILIP1L expression in endostatin-treated HUVECs was significantly more than that in vehicle-treated control cells ($P = 0.0012$). Five images from each treatment group (*B*) were analyzed. Axiovision 4.6 software (Zeiss) was used to quantify the percentage area with FILIP1L-positive staining. Box and whiskers plot (GraphPad Prism 3.0) is shown. *D*, preincubation of anti-FILIP1L antibody with FILIP1L protein, but not with BSA, abrogated the punctuate staining seen in HUVECs treated with endostatin. Nuclear staining with DAPI is shown in blue. *Scale bar*, 20 μm .

Figure 3.

Overexpression of FILIP1L in endothelial cells leads to inhibition of cell proliferation and an increase in apoptosis. *A*, inhibition of cell proliferation by overexpression of FILIP1L in HUVECs was analyzed by BrdUrd ELISA 24 h after transfection. The amount of BrdUrd incorporated was measured by absorbance at 450 nm. Bars, SE ($n = 4$, $P < 0.0001$). The result is representative of three independent experiments. *B*, increased apoptosis by overexpression of FILIP1L in HUVECs was analyzed by caspase-3/caspase-7 assay 24 h after transfection. Caspase-3/caspase-7 activity was measured by luminescence. Bars, SE ($n = 4$, $P < 0.001$). The result is representative of three independent experiments. *C*, increased apoptosis by overexpression of FILIP1L in HUVECs was analyzed by Annexin V-FITC and 7-AAD staining followed by flow cytometry analysis 48 h after transfection. The numbers 15.2 for

control and 44.4 for FILIP1L indicate the percentage of cells in late apoptosis. The result is representative of two independent experiments.

Figure 4.

FILIP1L truncation mutants have differential antiproliferative activity. *A*, a schematic representation of FILIP1L protein (893 amino acids). Two leucine zipper motifs (*black rectangles*) and a prefoldin domain (*striped rectangles*) were recognized in the NH₂ terminal half of a coiled-coil region (*gray area*). A SbcC (COG0419; ATPase involved in DNA repair) conserved domain in its NH₂ terminal half and a Herpes_BLLF1 (pfam05109; Herpes virus major outer envelope glycoprotein) conserved domain in its COOH terminal half. *B*, a schematic diagram of FILIP1L truncation mutants. HA indicates COOH terminal HA tag. Amino acid residues are shown on top of each construct. *C*, expression of each mutant was confirmed in HEK293 cells transfected with each construct by Western blot analysis using

anti-HA tag antibody. GAPDH blot is shown as the loading control. The result is representative of two independent experiments. *D*, differential inhibition of HUVECs proliferation by FILIP1L truncation mutants was analyzed by BrdUrd ELISA 24 h after transfection. The amount of BrdUrd incorporated was measured by absorbance at 450 nm. Bars, SE ($n = 4$). FILIP1L truncation mutants 1–790 ($P = 0.0001$), 1–650 ($P = 0.004$), 1–512 ($P = 0.0114$), and 127–893 ($P = 0.0021$) significantly inhibited cell proliferation compared with control (*). COOH terminal mutant 1–790 was more potent than wild-type FILIP1L in mediating antiproliferative activity (**, $P = 0.001$). The result is representative of two independent experiments.

Figure 5.

Overexpression of FILIP1L Δ C103 in HUVECs, as well as DU145 prostate cancer cells, leads to inhibition of cell migration. *A*, FILIP1L Δ C103-transfected HUVECs showed a significantly slower migration rate than control vector-transfected HUVECs as measured by Electric Cell-Substrate Impedance Sensing System in real time ($P < 0.0001$). *Square box*, linear range in the curve that was used for analysis. The result is representative of three independent experiments. *B*, real-time RT-PCR analysis for FILIP1L on cDNA from DU145 clones transduced with FILIP1L Δ C103-lentivirus. Each clone was treated with either PBS (w/o Dox) or 1 μ g/mL doxycycline (Dox) for 48 h before harvest. The y axis represents a ratio between each clone and the parental Tet repressor-expressing DU145 cells where each value was standardized with

housekeeping gene GAPDH. *Columns*, average of three experiments. *C*, a 90-kDa FILIP1LΔC103 protein was detected in Tet repressor-expressing DU145 cells transduced with *FILIP1LΔC103*-lentivirus, but not control lentivirus, by Western blot using anti-FILIP1L antibody. The result shown is a representative (clone 12) from several clones. GAPDH blot is shown as the loading control. *D*, all three FILIP1LΔC103 clones, but not mixed population of control cells, showed a significantly slower migration in the presence of doxycycline. Error bars indicate SE ($n = 3$). *P* value comparisons between in the presence and in the absence of doxycycline were as follows: control cells, $P = 0.141$; clone 2, $P = 0.0014$; clone 12, $P = 0.0005$; and clone 13, $P < 0.0001$. The result is representative of two independent experiments.

Figure 6.

Targeted expression of FILIP1 Δ C103 in tumor vasculature results in inhibition of tumor growth *in vivo*. **A**, AAVP- Δ C103 injected i.v. specifically targeted tumor vasculature. Tumors from PBS-treated and AAVP- Δ C103-treated mice were immunofluorescently stained with anti-AAVP antibody followed by Alexa Fluor 594 antirabbit IgG (red, AAVP staining) and anti-CD31 antibody followed by Alexa Fluor 488 antirat IgG (green, blood vessel staining). Blue, nuclear staining with DAPI. Scale bar, 50 μ m. The result is a representative image from two independent experiments. Data for AAVP- Δ C243 are shown in Supplementary Fig. S4. **B**, a 90-kDa FILIP1 Δ C103 protein was detected in whole tumor lysates from AAVP- Δ C103-treated mice, but not PBS-treated and AAVP-null-treated mice, by Western blot using anti-FILIP1L antibody. Three different tumors per group were analyzed. GAPDH blot is shown as the loading control. **C**, tumors from AAVP- Δ C103-treated mice (empty circles) were significantly smaller than those from PBS-treated mice (empty triangles) in M21 xenograft model at day 14 ($P < 0.01$). Tumors from AAVP- Δ C103-treated mice were also significantly smaller than those from AAVP-null-treated mice (filled triangles; $P < 0.05$) and AAVP- Δ C243-treated mice (filled circles; $P < 0.05$) by day 14. AAVP was injected i.v. at day 0 and day 7 (arrow). Bars, SE ($n = 11$). The result is representative of two independent experiments. **D-a**, representative images of CD31-stained tumors from PBS-treated, AAVP-null-treated, and AAVP- Δ C103-treated mice. Tumors treated with each AAVP for 4 d were immunofluorescently stained with anti-CD31 antibody followed by Alexa Fluor 488 anti-rat IgG (green, vessel staining). Blue, nuclear staining with DAPI. Scale bar, 100 μ m. **D-b**, vessel density was significantly decreased in tumors from AAVP- Δ C103-treated mice compared with

those from AAVP-null-treated mice ($P < 0.01$) and PBS-treated mice ($P < 0.001$) at day 4. The percentage of cells in the tumors stained positive for CD31 was quantified using Axiovision 4.6 software (Zeiss). Box and whiskers plot (GraphPad Prism 3.0) of vessel density. Middle lines indicate median values. *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.05$; $n = 3$ mice per treatment group.