

Basement Membrane Derived Fibulin-1 and Fibulin-5 Function as Angiogenesis Inhibitors and Suppress Tumor Growth

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The fibulins are a family of secreted glycoproteins that are characterized by repeated epidermal-growth-factor-like domains and a unique C-terminus structure. Fibulins modulate cell morphology, growth, adhesion, and motility. Our initial basement membrane degradome screen using Cathepsin D, a tumor microenvironment-associated protease, contained fragments of fibulin-1 and full length fibulin-5. In this report, we evaluate the anti-angiogenic activity of fibulin-1 and fibulin-5. Tumor studies demonstrate that both fibulin-1 and fibulin-5 suppress HT1080 tumor growth. CD31 labeling and TUNEL assay further reveal that fibulin-1 suppression of HT1080 tumor growth is associated with diminished angiogenesis and also enhanced apoptosis of endothelial cells and tumor cells. In contrast, fibulin-5 inhibits tumor angiogenesis with a minimal anti-apoptotic affect. Cathepsin D digestion of fibulin-1 produces a fragment with nearly the same molecular weight as fibulin-5, and this fragment (named Neostatin) inhibits endothelial cell proliferation. Additionally, degradation of basement membrane by cathepsin D liberates both fibulin-1 fragments and fibulin-5, which function to inhibit angiogenesis. *Exp Biol Med* 233:155–162, 2008

Key words: fibulin; angiogenesis; tumor; neostatin; basement membrane; cancer

Introduction

The fibulins are a family of secreted glycoproteins, which are characterized by epidermal-growth-factor-like domains repeats and a unique C-terminus structure (1). Five distinct fibulin genes, encoding at least nine protein products generated by alternative splicing, have been identified. Fibulins modulate cell morphology, growth, adhesion, and motility [see reviews (1–3)]. Fibulin-1, the first member identified, was discovered through affinity chromatography experiments utilizing the short cytoplasmic tail of $\beta 1$ integrin receptors (4).

A correlation is reported between the expression of fibulins and certain types of malignancies. Upregulation of fibulin-4 is reported in colon carcinomas (5), and fibulin-1 is overexpressed in estrogen receptor (ER)-positive ovarian carcinomas (6–8). It is likely that such an upregulation inhibits the mobility of cancer cells by suppressing their invasive properties and also inhibits angiogenesis. High concentration of the fibulin-1D splice variant selectively delays tumor transformation, although the mechanism of this delay is still unclear.

The lysosomal aspartic protease cathepsin D plays a role in cancer cell progression and metastasis, with dual apoptosis regulatory functions [for reviews, see (9) (10)]. It serves as a prognostic molecular marker in early breast cancer (11). In addition, an inverse relationship between fibulin-1 and cathepsin D expression patterns are observed in human breast carcinomas (12).

A few years ago, we developed a basement membrane degradation screen to evaluate the potential contribution of the basement membrane degradome in the regulation of angiogenesis (13). We employed many tumor microenvironment-associated proteases to degrade basement membrane preparations from amnion and placental tissue. When cathepsin D was used in this degradation screen, fibulin-1 fragments and full-length fibulin-5 were among the

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degradation products. This prompted us to further analyze the role of these fibulin proteins in the regulation of angiogenesis.

Angiogenesis, the growth of new blood vessels from preexisting vessels, is essential for the progression of cancer. Upregulation of fibulin-1 is reported during cutaneous wound healing (14, 15). Fibulin-5 is essential for elastogenesis *in vivo* (16, 17), antagonizes VEGF signaling in endothelial cells, and induces the expression of TSP-1 (18). Fibulin-5 suppresses tumor formation by controlling cancer cell proliferation, motility, and angiogenic sprouting (18–20).

To further investigate the potential mechanism of fibulins in the regulation of cancers, we generated fibrosarcoma HT1080 cell lines with stable expression of human fibulin-1 and fibulin-5, respectively. We establish that fibulin-1 and fibulin-5 possess anti-angiogenic activity and inhibit tumor growth. Additionally, we demonstrate that cathepsin D can degrade fibulin-1 to generate a fragment similar both in molecular weight to fibulin-5 and in anti-angiogenic activity. Collectively our data demonstrate that fibulins can play a key role in the regulation of angiogenesis.

Materials and Methods

Cell Lines and Reagents. CPAE cells (cow pulmonary artery endothelial cells), HT1080 cells (derived from human fibrosarcoma), and HEK 293 cells (epithelial cells derived from human embryonic kidney cells) were purchased from American Type Culture Collection (Manassas, VA). HUVEC cells (human umbilical vein endothelial cells), EGM-2, and EBM media were purchased from Lonza (Walkersville, MD). DMEM medium was purchased from Mediatech, Inc. (Herndon, VA). BALB/c SCID mice were purchased from Charles River (Wilmington, MA). Lipofectamine2000, pfx DNA polymerase, zeocin, and pSecTag-2A vector were bought from Invitrogen (Carlsbad, CA). pQE-Tri system vector was obtained from Qiagen (Valencia, CA). Human placental cDNA library was bought from Clontech (Mountain View, CA). *In situ* cell death detection kit (TUNEL) and WST-1 kit were obtained from Roche (Basel, Switzerland). Rat anti-mouse CD31 antibody was purchased from BD Pharmingen (San Jose, CA). MTT assay kit was purchased from Chemicon (Temecula, CA). Talon metal affinity resins and Matrigel were obtained from BD Biosciences (Bedford, MA). Cathepsin D, cathepsin B, Polymyxin-B, and anti-FLAG M2 Affinity Gel were purchased from Sigma-Aldrich (St. Louis, MO). The Boyden chamber and the 8- μ m polycarbonate membrane were purchased from Neuro Probe Inc. (Gaithersburg, MD). The PROTOCOL HEMA3 Stain Set was purchased from Fisher Scientific (Kalamazoo, MI). Recombinant human VEGF was obtained from R&D Systems (Minneapolis, MN).

Molecular Cloning of Human Fibulin-1 and Fibulin-5. DNA fragments containing the human fibulin-

1D gene (a splice variant of fibulin-1) and human fibulin-5 gene were generated by PCR using human placental cDNA library as the template. For fibulin-1, the primers were 5'-ATCCGCCCATGGAGCGCGCCGCGCCGTC-3' and 5'-GACCAGCTCGAGGAACCAGTACTCAGAGACGTCC-3'. For fibulin-5, the primers were 5'-ATCTGACCATGGCAGGAATAAAAAGGATACTCAC-3' and 5'-GACTGACTCGAGGAATGGGTACTGCGACAC-3'. The PCR conditions were: 95°C for 2 minutes followed by 30 cycles of 95°C for 1 minute, 65°C for 1 minute, and 68°C for 3 minutes. The resulting PCR products were gel-purified and ligated into the pQE-tri plasmid for expression.

Protein Expression in *E. coli*. pQE-Fibulin-1 and pQE-Fibulin-5 plasmids were transformed into XL1-Blue cells for protein expression in the presence of 100 μ g/ml of ampicillin and 12.5 μ g/ml of tetracycline. Human fibulin-1 and fibulin-5 were expressed in *E. coli* under the induction of 1mM IPTG at 37°C for 3 hours. The proteins were purified using talon metal affinity resins according to the instructions from BD Biosciences. The cell pellet was resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 25 mM MgCl₂) at about 2–5 ml per gram wet weight. Lysozyme was added to 1 mg/ml and incubated on ice for 30 minutes followed by sonication on ice for six 10 second bursts at 300W with a 10 second cooling period between each burst. The lysates were centrifuged at 10,000 $\times$ g for 30 minutes at 4°C. The supernatant was applied to talon metal affinity column, which was equilibrated with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 25 mM MgCl₂). After the column was washed with washing buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 25 mM MgCl₂, 10 mM imidazole), the proteins were eluted with elution buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 25 mM MgCl₂, 100 mM imidazole). The protein was analyzed by SDS-PAGE and visualized by Coomassie Blue.

***In Vitro* Proliferation Assay of Human Fibulin-1 and Fibulin-5.** Polymyxin B at a final concentration of 5 μ g/ml was used to inactivate endotoxin from the purified proteins. CPAE cells were seeded at a concentration of 4×10^3 cells/well in 100 μ l DMEM medium containing different concentrations of human fibulin-1 or fibulin-5 using 96-well plates. After incubation for 48 hours at 37°C and 5% CO₂, 10 μ l of MTT solutions A and B were added to each well and incubated for 4 hours at 37°C and 5% CO₂. The MTT solution C was added and the absorbance at 595 nm was measured against a background control as a blank using a microplate reader.

Establishment of Tumor Cell Lines with Stable Expression of Human Fibulin-1 and Fibulin-5. The fibulin-1 or fibulin-5 gene, with a FLAG sequence tag at the N-terminus, was generated by PCR and gel purified. The genes were then inserted into pSecTag-2A plasmid to obtain the pSecTag-Fibulin-1 and pSecTag-Fibulin-5 plasmids. To produce cell lines with stable transfections, cells in exponential growth were plated in 100 mm dishes. After

18–24 hours, 2 µg plasmid DNA was transfected into HT1080 fibrosarcoma cells using Lipofectamine 2000 according to the manufacturer's instructions. Transfected HT1080 cells were selectively grown in culture containing 400 µg/ml zeocin for 3 weeks. Drug-resistant clones were randomly selected and subcloned. The expression levels of fibulin-1 or fibulin-5 were examined by western blot using anti-FLAG-tag antibody.

In Vivo Tumor Studies. One million HT1080 cells were injected subcutaneously onto the backs of male 8-week-old BALB/c SCID mice. Tumor dimensions were measured every three days using a digital caliper, and the volume of the tumors was calculated using the formula: $\text{Volume} = 0.52 \times \text{length} \times \text{width} \times \text{width}$. Six mice were used in each experimental group. All mouse studies were reviewed and approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

Immunohistochemistry and TUNEL Assay. Immunohistochemistry was performed as previously described (21). In each group, the number of blood vessels comprised of CD31-positive endothelial cells was counted at $\times 200$ magnification in a blinded fashion for 10 separate fields per group and averaged. The detection and quantification of apoptosis at the single cell level was carried out according to the manual using the *in situ* cell death detection kit (TUNEL). In each group, the number of apoptotic cells was counted at $\times 400$ magnification in a blinded fashion for 10 separate fields per group and averaged. In double immunofluorescence experiments, TUNEL staining was performed after CD31 staining. The number of cells which stained for both TUNEL and CD31 were evaluated at $\times 400$ magnification.

Protein Expression in HEK 293 Cells. pSecTag-Fibulin-1 and pSecTag-Fibulin-5 plasmids were transfected into HEK 293 cells using Lipofectamine2000. The protein was expressed in DMEM medium containing 400 µg/ml of zeocin and purified using anti-FLAG M2 affinity gel.

Cathepsin Digestion. Twenty µg of proteins were digested with 10 units of cathepsin D in buffer containing 50 mM NaAc, pH 4.0, 50 mM NaCl at 37°C overnight. For cathepsin B digestion, 20 µg of proteins were digested with 10 units of enzyme in the buffer containing 50 mM NaOAc, pH 6.0, 50 mM NaCl at 40°C overnight. The digested product/s was analyzed by SDS-PAGE.

Molecular Cloning and Protein Expression of Neostatin. The Neostatin DNA fragment was produced by PCR using human placenta cDNA library as the template with the forward primer (5'-ATGCTAGGCC-CAGCCGGCCGACTACAAGGACGACGATGACAA-GAGCTGCCGGCTTGGAATCCTGCATC-3') and the reverse primer (5'-GCTAAGCTCGAGTCAGAACCTAGTACTCAGAGACGAAGATGC-3'). The PCR protocol was: 95°C for 2 minutes, followed by 35 cycles of 95°C for 1 minute, 65°C for 1 minute, and 68°C for 2 minutes. The resulting PCR products were gel-purified and ligated into pSecTag-2A plasmid for protein expression.

The pSecTag-Neostatin plasmid was transfected into HEK 293 cells using Lipofectamine2000. The protein was expressed in DMEM medium containing 400 µg/ml of zeocin and purified using anti-FLAG M2 affinity gel.

In Vitro Proliferation Assay with Neostatin. Four thousand CPAE cells/well were seeded in 96-well plates in 100 µl DMEM medium containing different concentrations of neostatin. After incubation for 48 hours, 10 µl of WST-1 was added to each well and incubated for 4 hours. The absorbance at 440 nm was measured against a background control as a blank using a microplate reader.

In Vitro Tube Formation Assay with Neostatin. Matrigel (BD Biosciences, San Jose, CA) was added (100 µL) to each well of a 24-well plate and allowed to polymerize. A suspension of 50,000 HUVEC cells in 5% EGM-2 medium in EBM medium (Lonza, Walkersville, MD) was seeded into each well. Cells were treated with either PBS or increasing concentrations of neostatin (1–10 µg/mL). All assays were performed in triplicate. Cells were incubated for 24–48 h at 37°C and viewed using an inverted microscope (Zeis Axiovert 200m, Serial number 240013486). Representative images were taken from each well using a digital camera (Zeis AxioCam Hrc, Serial number 240013486).

Statistical Analysis

All results are shown as mean $\pm$ SEM. Statistical differences between two groups were calculated by using student's *t* test or Welch's *t* test. ANOVA was used to determine statistical differences between three or more groups. As needed, further analysis was carried out by using *t* test with Bonferroni correction to identify significant differences. *P* values less than 0.05 were considered statistically significant. *, 0.01 < *P* < 0.05, **, 0.001 < *P* < 0.01, ***, 0.0001 < *P* < 0.001.

Results

Molecular Cloning and Protein Purification of Human Recombinant Fibulin-1 and Fibulin-5. The genes for human fibulin-1 (fibulin-1D splice variant) and fibulin-5 were cloned by PCR using human placental cDNA library as the template. A 2.1-kb fragment of human fibulin-1 and a 1.3-kb fragment of human fibulin-5 were generated (Fig. 1A–B). DNA sequencing confirmed that the sequences of both genes were correct.

In order to express human fibulin-1 and fibulin-5 in *E. coli*, the gel-purified DNA fragment was ligated into pQE-Tri vector and transformed into XL1-Blue cells. As shown in Figure 1C–D, both fibulin-1 and fibulin-5 were successfully expressed in *E. coli* under an induction by 1 mM of IPTG. The molecular weight of recombinant fibulin-1 protein was around 72 kDa, and that of fibulin-5 was around 50 kDa. After the protein was purified by talon metal affinity chromatography, the fraction containing greater than 95% of pure protein was visualized using Coomassie Blue.

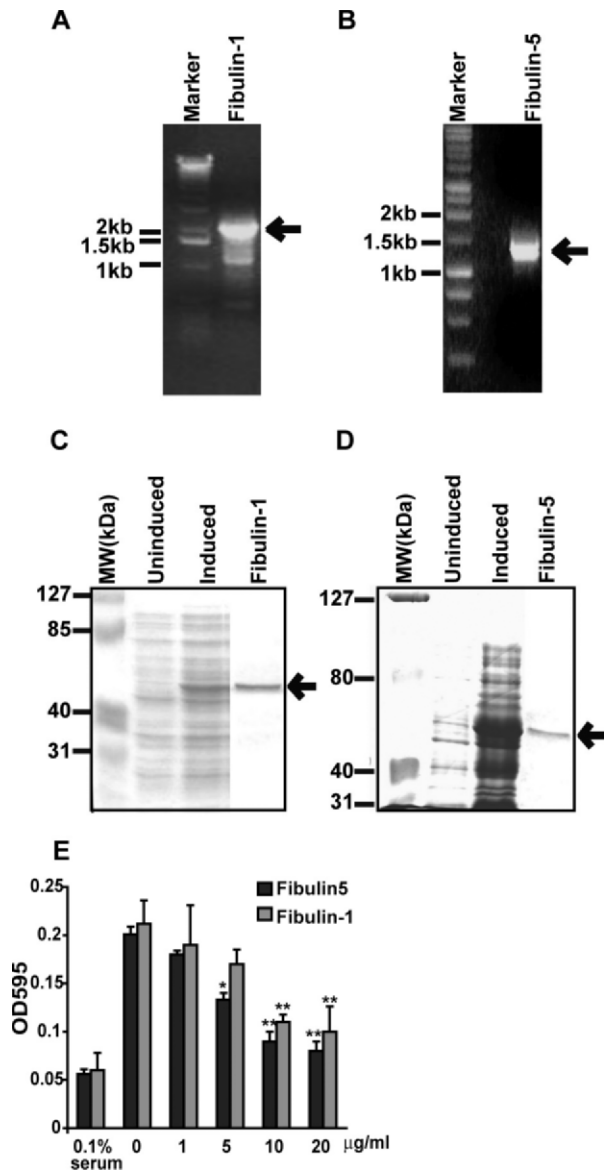


Figure 1. Molecular cloning, protein expression and purification of human fibulin-1 and fibulin-5. (A–B) PCR products of human fibulin-1 and fibulin-5 were analyzed by 1% agarose gel. The arrow indicates fibulin-1 in panel A and fibulin-5 DNA in panel B. (C–D) Expression and purification of fibulin-1 and fibulin-5 in *E. coli*. The total cell extracts with or without 1 mM IPTG induction and the purified protein were applied to 12% or 10% SDS-PAGE and visualized by Coomassie blue. The arrow indicates fibulin-1 protein in panel C and fibulin-5 in panel D, respectively. (E) *In vitro* proliferation assay of fibulin-1 and fibulin-5. 0, 1, 5, 10, 20 μg/ml of proteins were applied to CPAE cells individually. The cell density was measured at 595 nm. Cells growing in 0.1% serum were used as a negative control, and cells growing in 10% serum without fibulin-1 or fibulin-5 were used as a positive control. Ten μg/ml of both human fibulin-1 and fibulin-5 significantly inhibits endothelial cell proliferation.

Western blot using anti-HIS tag antibody further demonstrated that the expressed proteins were indeed human recombinant fibulin-1 and fibulin-5 (data not shown).

Fibulin-1 and Fibulin-5 Inhibits Endothelial Cell Proliferation *in Vitro*. In order to evaluate whether fibulin-1 and fibulin-5 inhibit endothelial cell proliferation,

CPAE cells cultured with 10% FCS were exposed to different amounts of purified proteins *in vitro*. CPAE cells cultured in 0.1% FCS were used as a control to demonstrate low cell proliferation levels. As shown in Figure 1E, fibulin-5 inhibits endothelial cell proliferation starting at a concentration of 5 μg/ml. 10 μg/ml of fibulin-5 maximally inhibits endothelial cell proliferation. Cells treated with 10 μg/ml or 20 μg/ml of fibulin-5 had a 44.8% or 39.8% proliferation rate, respectively, relative to the positive control. Cells treated with 10 μg/ml or 20 μg/ml fibulin-1 had a 51.8% or a 47.2% proliferation rate, respectively.

***In Vivo* Tumor Studies Human Fibulin-1 and Fibulin-5.** To investigate the function of fibulin-1 and fibulin-5 in the regulation of tumor growth, we constructed human fibulin-1 and fibulin-5 cDNA expression vectors. The vectors were used to generate HT1080 cancer cell lines with stable expression of fibulin-1 and fibulin-5. In order to evaluate the overexpression of fibulins in cancer cells, a FLAG-tag was added at the N-terminus of both fibulin-1 and fibulin-5 by PCR. Zeocin resistant clones were isolated and immunoblot analysis of whole cell extracts was used to identify clones that expressed high levels of fibulin-1 and fibulin-5. The amount of protein secreted into the conditioned culture medium by HT1080 cell lines expressing fibulin-1 or fibulin-5 was evaluated by western blot using the FLAG-tag antibody (Supplemental Figure, SFig. 1, available online). One clonal cell line expressing fibulin-5 (lane 2, SFig. 1) and one clonal cell line expressing fibulin-1 (lane 3, SFig. 1), both of which expressed high but equal amounts of protein, were used for tumor experiments.

To investigate whether the expression of fibulin-1 and fibulin-5 can suppress tumor growth, the HT1080 cells with expression of fibulin-1 or fibulin-5 were injected subcutaneously onto the backs of BALB/c SCID mice, and the tumor growth was monitored. As shown in Figure 2A, the latency of tumor growth in mice with either fibulin-1 or fibulin-5 expressing HT1080 cancer lines was significantly longer when compared to the control vector-transfected line. In the control experiment the tumor reached 500 mm³ around 18 days after injection, while for the fibulin-5-expressing cells the average time was 25 days. For fibulin-1-expressing cells, the tumors were smaller than 500 mm³ even after 28 days.

Fibulin-1 and Fibulin-5 Inhibit Tumor Angiogenesis. HT1080 tumors were evaluated for blood vessel density. The mice were sacrificed 28 days after tumor cell injections and tumors in each group were harvested. The tumors were sectioned and labeled with CD31, an endothelial cell-specific marker. Immunohistochemistry results revealed that both fibulin-1 and fibulin-5 significantly inhibit tumor angiogenesis (Fig. 2D–F). Blood vessel quantification reveals a 45% reduction in the HT1080 tumors overexpressing either fibulin-1 or fibulin-5 (Fig. 2B).

Fibulin-1, but Not Fibulin-5, Induces Apoptosis in Tumors. Next, apoptosis in tumor samples was detected and quantified by TUNEL assay. Fibulin-1 induces

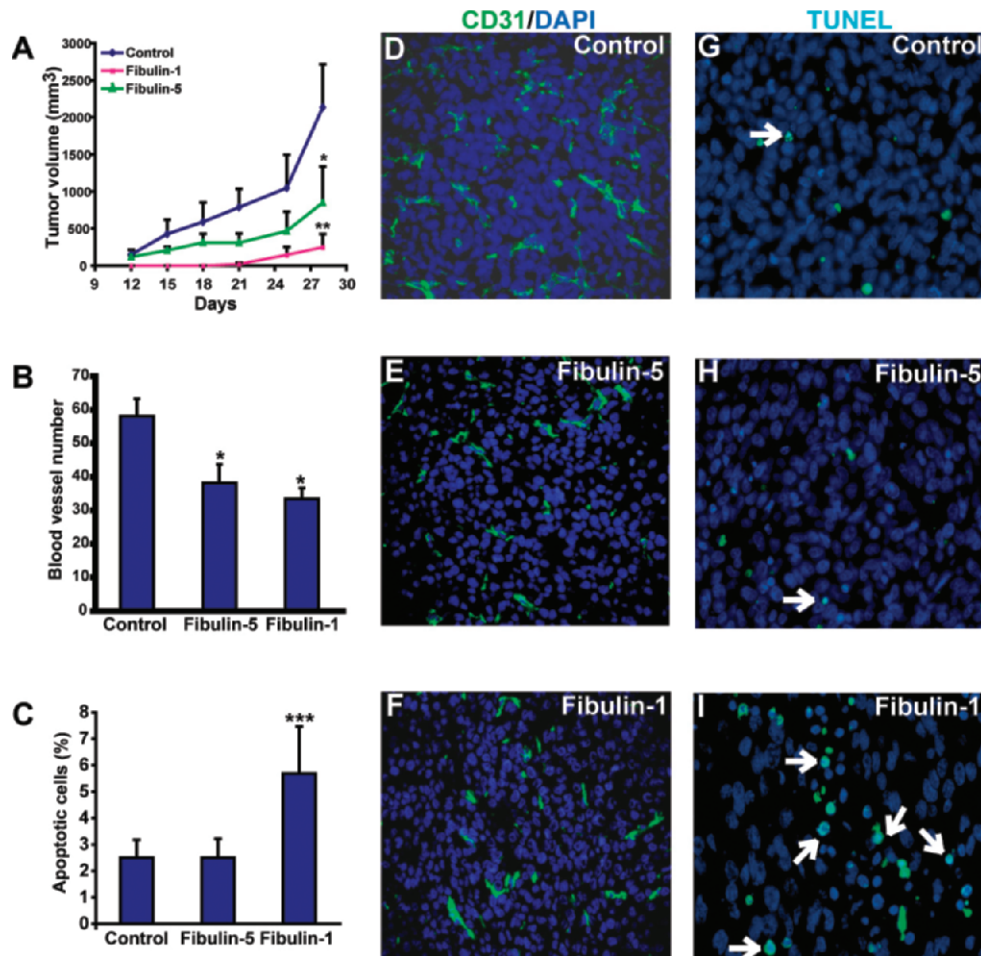


Figure 2. *In vivo* tumor studies with fibulin-1 and fibulin-5. (A) Tumor growth curve of HT1080 cells with stable expression of fibulin-1 or fibulin-5. One million cells were injected into BALB/c SCID mice subcutaneously and the tumor volume was measured every three days. Six mice were used in each group. ◆, vector-transfected control cells; ■, pSecTag-fibulin-1D-transfected cells; ▲, pSecTag-fibulin-5-transfected cells. *, $0.01 < P < 0.05$, **, $0.001 < P < 0.01$. (B) Quantification of CD31 staining in HT1080 tumors. The mice were sacrificed 28 days after injection, and the tumors were sectioned and stained with CD31. Ten sections for each sample were analyzed at $\times 200$ magnification in a blinded fashion. The number of blood vessels was significantly decreased in HT1080 fibrosarcoma overexpressing either fibulin-1 or fibulin-5, compared to the vector-transfected control cells. *, $P < 0.05$. (D–F) Representative pictures of CD31 staining on HT1080 fibrosarcoma control (Panel D) or HT1080 fibrosarcoma with fibulin-5 overexpression (Panel E) or with fibulin-1 overexpression (Panel F). (C) Quantification of apoptotic cells in HT1080 tumors with the overexpression of human fibulin-1 or fibulin-5 by TUNEL assay. Overexpression of fibulin-1 significantly induced apoptosis of HT1080 cancer cells. ***, $P < 0.001$. (G–I) Representative pictures of TUNEL assay of apoptotic cells in HT1080 tumors with overexpression of control (Panel G), fibulin-5 (Panel H) or fibulin-1 (Panel I). Ten sections for each sample were analyzed at $\times 400$ magnification in a blinded fashion. The apoptotic cells, co-localization of TUNEL (green) and the nuclei (blue), are indicated with arrows. All results were shown as mean $\pm$ SEM.

apoptosis much more robustly when compared to fibulin-5 (Fig. 2G–I). Apoptosis was observed in 2.5% of all cells in the fibulin-5 and control tumor sections, while fibulin-1 induced apoptosis in 5.7% of all cells (Fig. 2C). Next, tumor sections were stained with both CD31 and TUNEL, and co-localization was detected in the endothelial cells and also tumor cells, with the majority of them being cancer cells (Supplemental Figure, SFig. 2A–C, available online).

Cathepsin D Degrades Fibulin-1 to Generate A Fragment Similar in Molecular Weight to Full Length Fibulin-5. An alignment of human fibulin-1 and fibulin-5 protein sequences with Cluster W software reveals a high degree of homology (Fig. 3A). Sequence analysis

reveals three potential cathepsin D cleavage sites (red highlights in Fig. 3A) (22, 23).

Human recombinant fibulin-1 and fibulin-5 proteins were produced in HEK293 cells and purified. It should be noted that fibulin-1 and fibulin-5 produced in mammalian cells undergo glycosylation and therefore have higher molecular weights than those produced in *E. coli* (24, 25). Fibulin-1 was digested using active cathepsin D. Two fragments were generated upon degradation. One fragment, later named neostatin, was similar to fibulin-5 in size, and the other fragment was about 35 kDa (Fig. 3B). However, cathepsin B digestion of fibulin-1 did not generate this fragment, suggesting that the digestion of fibulin-1 by cathepsin D specifically yielded the neostatin fragment.

with anti-FLAG antibody demonstrates that the protein fragment derived from human fibulin-1 was successfully expressed in 293 cells (Fig. 4B). This fragment was not generated when cathepsin B was used in the degradation assay (Fig. 3B–C). Next, the fibulin-1 fragment was purified from 293 cells for further *in vitro* analysis. To evaluate the effect of this novel fragment on endothelial cell proliferation, CPAE cells cultured with 10% FCS were exposed to different amounts of fragment *in vitro*. CPAE cells cultured in 0.1% FCS were used as a control to demonstrate low cell proliferation levels. The *in vitro* proliferation assay demonstrates that 10 $\mu\text{g/ml}$ of this novel fragment significantly inhibits endothelial cell proliferation (Fig. 4C).

To further characterize the *in vitro* effects of neostatin, an endothelial tube assay was performed. HUVEC cells were cultured on Matrigel with increasing concentrations of neostatin (1–10 $\mu\text{g/ml}$). The results of this assay demonstrate that 10 $\mu\text{g/ml}$ inhibited tube formation when compared to PBS control (Fig. 4D). This supports the notion that neostatin may also inhibit angiogenesis *in vivo*. Based on this activity, the novel fibulin-1 fragment was named “Neostatin” for its potential capacity to inhibit neo-vascularization.

Discussion

Fibulin-1 and fibulin-5 belong to a family of extracellular matrix proteins, which can adhere to endothelial cells and regulate their motility and proliferation. Some studies have suggested a possible role for fibulin-1 as a tumor suppressor, but a mechanism behind such activity was not established. In this study, using HT1080 cell lines with stable expression of fibulin-1 and fibulin-5, we demonstrate that fibulin-1 inhibits tumor growth by suppressing tumor angiogenesis and inducing apoptosis of cells in the tumor. Fibulin-5 also inhibits tumor angiogenesis.

We provide compelling proof that fibulins associated with basement membrane can function as endogenous angiogenesis inhibitors and control the rate of tumor growth. Our studies, in conjunction with reports from other laboratories, suggest that among the five members of the fibulin family, fibulin-1, fibulin-3 and fibulin-5 exhibit anti-angiogenesis activity and demonstrate an ability to suppress tumor growth (18). It is likely that these inhibitors have a restricted distribution in select tissue-specific basement membranes and are liberated to function as angiogenesis inhibitors upon the degradation of pre-existing vasculature.

There is an inverse relationship between the expression of fibulin-1 and cathepsin D in human breast cancers (12). This notion is further favored by the fact that cathepsin D degrades fibulin-1 to generate a fragment with the ability to inhibit the proliferation of endothelial cells. Our results suggest that an increase of cathepsin D in the tumor microenvironment can induce the degradation of fibulin-1, leading to a potential decrease of its protein level in the

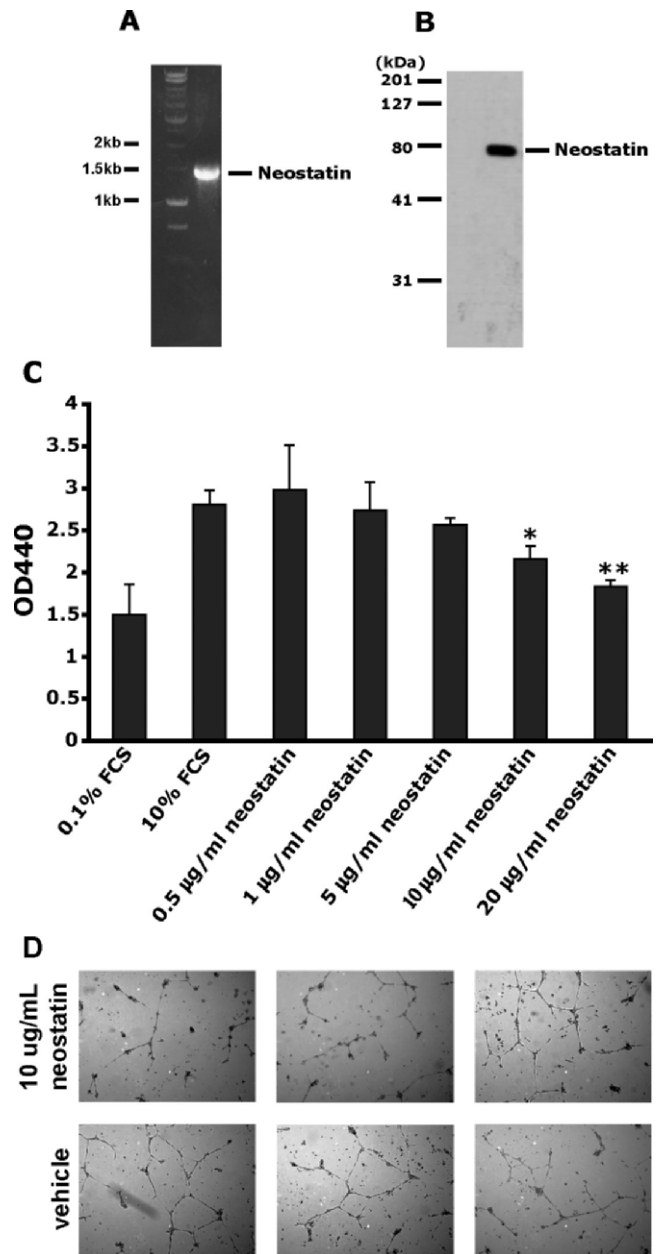


Figure 4. Neostatin, a fragment from human fibulin-1, inhibits endothelial cell proliferation. (A) Analysis of neostatin PCR products using a 1% agarose gel. (B) Western blot of neostatin expression in 293 cells using anti-FLAG antibody. Neostatin was successfully expressed by transfecting pSecTag-neostatin into 293 cells. (C) *In vitro* proliferation assay of neostatin. Cells growing in 0.1% serum were used as a negative control, and cells growing in 10% serum without fibulin-1 or fibulin-5 were used as a positive control. Ten $\mu\text{g/ml}$ of neostatin significantly inhibits endothelial cell proliferation *in vitro*. (D) Representative images from endothelial tube assay. Top panels were taken from replicate wells, and show disrupted tube formation in the presence of 10 $\mu\text{g/ml}$ neostatin (in PBS). Bottom panels were taken from replicate wells.

tumors (12). This novel anti-angiogenic fragment from fibulin-1 exhibits the same molecular size as fibulin-5 and shares significant amino acid homology with it. In fact, one of the putative cathepsin D cleavage sites in the fibulin-1 sequence aligns with the N-terminal start of fibulin-5

sequence. Therefore, it is likely that the anti-angiogenic sites of fibulin-5 and the fibulin-1 fragment are similar.

The novel fibulin-1 fragment generated by cathepsin D was named "Neostatin" for its ability to inhibit endothelial cell proliferation and tube formation *in vitro*. This novel basement membrane derived fibulin-1 fragment adds to growing list of endogenous inhibitors of angiogenesis derived from basement membrane proteins, such as endostatin, tumstatin, canstatin, and arresten (26). Future studies will shed light on the mechanism of how neostatin functions as an endogenous inhibitor of angiogenesis.

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