Fibulins 3 and 5 Antagonize Tumor Angiogenesis In vivo

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Abstract

Lethal tumor growth and progression cannot occur without angiogenesis, which facilitates cancer cell proliferation, survival, and dissemination. Fibulins (FBLN) 5 and 3 are widely expressed extracellular matrix proteins that regulate cell proliferation in a context-specific manner. Reduced FBLN-5 expression has been associated with cancer formation and progression in humans, whereas its constitutive expression antagonizes endothelial cell angiogenic sprouting in vitro. Thus, FBLN-5 may suppress tumorigenesis by preventing tumor angiogenesis. FBLN-3 is homologous to FBLN-5 and expressed in endothelial cells, yet its role in tumorigenesis and angiogenesis is unknown. We find FBLN-3 expression to be altered in some human tumors and that its constitutive expression in endothelial cells inhibited their proliferation, invasion, and angiogenic sprouting, as well as their response to vascular endothelial growth factor as measured by p38 mitogen-activated protein kinase activation. In endothelial cells, both FBLNs (a) reduced angiogenic sprouting stimulated by basic fibroblast growth factor (bFGF); (b) inhibited matrix metalloproteinase expression and activity; and (c) stimulated tissue inhibitor of metalloproteinase expression. More importantly, both FBLNs prevented angiogenesis and vessel infiltration into bFGF-supplemented Matrigel plugs implanted in genetically normal mice, as well as decreased the growth and blood vessel density in tumors produced by MCA102 fibrosarcoma cells implanted s.c. into syngeneic mice. Our findings establish FBLN-3 and FBLN-5 as novel angiostatic agents capable of reducing tumor angiogenesis and, consequently, tumor growth in vivo and suggest that these angiostatic activities may one day be exploited to combat tumor angiogenesis and metastasis in cancer patients. (Cancer Res 2006; 66(5): 2621-9)

Introduction

Fibulins (FBLN) comprise a small family of widely expressed extracellular matrix (ECM) proteins that localize to basement membranes, stroma, and ECM fibers where they mediate cell-to-cell and cell-to-matrix communication, as well as provide organization and stabilization to ECM structures during organogenesis and vasculogenesis (1, 2). A key component of physiologic tissue development and repair is angiogenesis (3), which when left unchecked can promote the pathogenicity of diabetic retinopathy, arthritis, macular degeneration, endometriosis, inflammation, and cancer (4–6). We independently identified FBLN-5 as a novel gene target of the tumor suppressor, transforming growth factor-β, and showed that (a) FBLN-5 regulates normal and cancer cell proliferation, migration, and invasion in a cell- and context-specific manner (7, 8); (b) tumorigenesis down-regulates FBLN-5 expression in metastatic human tumors (7); and (c) FBLN-5 antagonizes vascular endothelial growth factor (VEGF) signaling in and angiogenic sprouting by endothelial cells in vitro (8). Thus, FBLN-5 may suppress tumor formation and progression in part by antagonizing angiogenesis.

FBLN-3 is homologous to FBLN-5 and, like FBLN-5, regulates cell proliferation in a context-specific manner (9). FBLN-3 mutations are associated with the development of malattia leventinese (10–12), an inherited form of macular degeneration sometimes characterized by excessive angiogenesis. Despite these findings, a direct function for FBLN-3 in regulating tumor formation and angiogenesis remains to be established. We show herein that similar to FBLN-5, expression of FBLN-3 antagonized endothelial cell activities coupled to angiogenesis in vitro and that FBLN-3 and FBLN-5 both target endothelial cell expression of matrix metalloproteinases (MMP), tissue inhibitor of metalloproteinases (TIMP), and thrombospondin-1 (TSP-1), thereby potentially reducing ECM proteolysis and remodeling. Finally, we show for the first time that both FBLNs antagonize vessel development and angiogenesis stimulated by basic fibroblast growth factor (bFGF) both in vitro and in vivo, as well as the neovascularization and growth of fibrosarcomas implanted s.c. in mice. Collectively, our study establishes FBLN-3 and FBLN-5 as novel antagonists of endothelial cell activities coupled to angiogenesis both in vitro and in vivo, and as novel inhibitors of tumor angiogenesis and growth in mice. Our study further suggests that the angiostatic activities of these molecules may be exploited to prevent the growth and metastasis of human malignancies.

Materials and Methods

Plasmids. A retroviral vector encoding the short form of FBLN-3 (9) was constructed by PCR amplifying the full-length murine short FBLN-3 cDNA from EST BC031184. The resulting PCR fragment was ligated into pcDNA3.1/Myc-His B vector (Invitrogen, Carlsbad, CA) at EcoRI (COOH terminus) and restriction sites, which appended Myc and (His)6 tags to the COOH terminus of FBLN-3. Afterward, the tagged FBLN-3 cDNA was PCR amplified using oligonucleotides containing BglII (NH2 terminus) and XhoI (COOH terminus) restriction sites and ligated into identical sites in pMSCV-IRESS-GFP (7). All FBLN-3 cDNA constructs were sequenced on an Applied Biosystems 377A DNA sequencing machine.

Cell culture and retroviral infections. Murine brain microvascular MB114 endothelial cells were cultured as described previously (8). Human dermal microvascular HMEC-1 endothelial cells were kindly provided by Dr. Gerard C. Blobe (Duke University, Durham, NC) and were maintained in EBM-2 medium supplemented with 2% fetal bovine serum and growth factors according to the recommendations of the manufacturer (Cambrex, East Rutherford, NJ).

Control (i.e., pMSCV-IRESS-GFP) and FBLN-3 retroviral supernatants were produced and infected into MB114 endothelial cells, which subsequently were isolated by fluorescence-activated cell sorting for green fluorescent protein (GFP) expression (highest 10%) as described previously (8). MB114 cells stably expressing FBLN-5 were described previously (8).

Fusion protein construction and purification. Glutathione S-transferase (GST) fusion proteins containing full-length murine short...
matrices were removed and pelleted by microcentrifugation before Twenty-four hours later, the medium was discarded and the collagen in 0.5 mL collagen, which subsequently was solidified in 24-well plates. renaturing and developing were preformed using Novex Zymogram buffer mented with 0.1% gelatin (Sigma, St. Louis, MO). Afterward, zymogram were synthesized by iScript reverse transcription (Bio-Rad, Hercules, CA) and additional phenol/chloroform extraction and ethanol precipitation. cDNAs expressing MB114 cells was purified using the RNAqueous kit, followed by an

GGTGGCTT; reverse: 5′-TATCAGTCCAAACA; reverse: 5′-TGGCGTTGCTGATGCTCTT; (d) p38 mitogen-activated protein kinase (MAPK) activation using immunoblot analyses as described (7, 8). In addition, HMEC-1 endothelial cell adhesion was measured by coating 24-well plates overnight with 100,000 cells per well) were allowed to adhere to the various substrates for 15 minutes, at which point nonadherent cells removed by two PBS washes. The remaining adherent cells were fixed with 95% ethanol and stained with crystal violet and subsequently quantified by extracting the crystal violet dye with 10% acetic acid followed by spectrophotometry at 590 nm. Alterations in MB114 cell MMP activity were determined by mixing control-FBLN-3, or FBLN-5-expressing MB114 cells (1.2 x 10^6 cells per well) in 0.5 mL collagen, which subsequently was solidified in 24-well plates. Twenty-four hours later, the medium was discarded and the collagen matrices were removed and pelleted by microcentrifugation before fractionating supernatants (20-80 μL/lane) through 10% SDS-PAGE supplemented with 0.1% gelatin (Sigma, St. Louis, MO). Afterward, zymogram renaturing and developing were preformed using Novex Zymogram buffer system according to the instructions of the manufacturer (Invitrogen).

Gene expression assays. Total RNA from control-FBLN-3, and FBLN-5-expressing MB114 cells was purified using the RNAlater kit, followed by an additional phenol/chloroform extraction and ethanol precipitation. cDNAs were synthesized by iScript reverse transcription (Bio-Rad, Hercules, CA) and subjected to semiquantitative real-time PCR analyses on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) exactly as described previously (14). Murine oligonucleotide pairs used were as follows: (a) MMP-2, forward: 5′-TAACCTGGATGTGTGGCTGGA; reverse: 5′-GCCCA-GCCATGTGTATTTGAT; (b) MMP-3, forward: 5′-TGTTCTGAGTT-GGTGGCTT; reverse: 5′-TGTTCTGGCAATTCGCGT; (c) TIMP-1, forward: 5′-AACGGCTTGTTGATGCGCA; reverse: 5′-AACCAGAAACTGGG- CACT; (d) TIMP-2, forward: 5′-GTCCATGATCCTCAGCTCA; reverse: 5′-TGTGACGAGCGAAGAATTCT; (e) TIMP-3, forward: 5′-CCCTGGC- TATCGTCTCCAAAC; reverse: 5′-TGGCTTGCTGAGTCTCTT; (f) TSP-1, forward: 5′-GAATCTTACGTGACGCA; reverse: 5′-GGAATCTTGTTTGGCGA; and (g) FN-3, forward: 5′-GCCAATGTGCTGTTGGA; reverse: 5′-ACAGACTTGGTGGCGGTGTT. Relative gene expression levels were determined according to the recommendations and subsequently normalized to corresponding glyceraldehyde-3-phosphate dehydrogenase signals.

In vivo angiogenesis assay. The effect of FBLN-3 and FBLN-5 on angiogenesis in vivo was determined using the Matrigel implantation assay. Briefly, 6-week-old C57BL/6 female mice were injected twice s.c. in the ventral groin area with Matrigel (700 μL/insertion; BD Biosciences, Bedford, MA) supplemented either with diltuent (PBS) or with bFGF (300 ng/mL; R&D Systems, Minneapolis, MN) together with either recombinant GST (50 μg/mL), FBLN-3 (10 or 50 μg/mL), FBLN-5 (10 or 50 μg/mL), or RGE-FBLN-5 (10 or 50 μg/mL). Seven days postimplantation, the mice were sacrificed and the plugs were harvested. For each mouse, one plug was fixed overnight in 10% formalin before embedding in paraffin and sectioning in the National Jewish Histology Laboratory. Afterward, the sections were stained using the Masson's trichrome procedure to visualize infiltrating vessels, which were quantified under a light microscope by determining the average number of vessels present in ≥10 independent fields on two independent slides. Only fields containing at least one vessel in the area underlying the skin were quantified. The second plug was weighed and homogenized in PBS before determining plug hemoglobin content by Drabkin's assay according to the recommendations of the manufacturer (Sigma). Each sample was assayed in duplicate and resulting hemoglobin contents were normalized to corresponding plug protein concentrations. Two mice were used per experimental condition and this experiment was done thrice in its entirety. All animal studies were done according to protocol procedures approved by the Animal Care and Use Committee at National Jewish Medical and Research Center.

Tumor studies. Murine MCA102 fibrosarcoma cells (15, 16) were kindly provided by Dr. John M. Routes (National Jewish Medical and Research Center) and were engineered by retroviral transduction to stably express either GFP, FBLN-3, or FBLN-5. To maximize FBLN-3 secretion from MCA102 cells, we first shuttled the murine short FBLN-3 cDNA (less its signal sequence) through the pSecTag vector (Invitrogen) at HiiDIII (NH2 terminus) and NotI (COOH terminus) restriction sites. In addition to COOH-terminally tagging the FBLN-3 cDNA with the Myc and (His)6 tags, the pSecTag vector also appended the Igg leader sequence to its NH2 terminus, thus permitting more efficient FBLN-3 secretion when introduced into MCA102 cells (data not shown). Afterward, the resulting tagged FBLN-3 cDNA fragment was PCR amplified using oligonucleotides containing BgII (NH2 terminus) and EcoRI (COOH terminus) restriction sites, and subsequently ligated into identical sites in pMSCV-IRES-GFP to facilitate retroviral production.

The effect of FBLN-3 and FBLN-5 on tumor growth and angiogenesis was measured by s.c. injecting 10-week-old C57BL/6 female mice with GFP-, FBLN-3-, or FBLN-5-expressing MCA102 fibrosarcoma cells (500,000 cells per implant). Twenty-one days postimplantation, the mice were sacrificed and the tumors were harvested, weighed, and fixed overnight in 10% formalin before sectioning and H&E staining in the National Jewish Histology Laboratory. Afterward, tumor blood vessel densities were quantified under a light microscope by determining the average number of vessels present in five independent fields per slide. As for Matrigel implantation assays, only those fields containing at least one vessel were quantified. Six mice were used per experimental condition and this experiment was done thrice in its entirety.

Statistical analyses. Data were analyzed using Student’s t test and all comparisons in vitro studies were made to GFP-expressing MB114 cells, whereas those for in vivo studies were made to bFGF-supplemented Matrigel plugs. A value of P < 0.05 was considered significant.

Results

FBLN-3 is homologous to FBLN-5 and is expressed aberrantly in human tumors. Murine FBLN-3 is 48% identical to murine FBLN-5 (Fig. 1A) and, like FBLN-5 (7, 17, 18), is widely expressed throughout mammalian tissues (Fig. 1B; refs. 19, 20) and regulates cell proliferation in a context-specific manner (9). Although not as striking as FBLN-5 (7), we found FBLN-3

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expression to be altered in 47% (32 of 68) of sampled tumors, of which 37% (25 of 68) exhibited down-regulation and 10% (7 of 68) exhibited up-regulation of FBLN-3 mRNA (Fig. 1B). Reduced FBLN-3 expression was especially evident in cancers of the breast (67%; 6 of 9 cases), ovary (67%; 2 of 3 cases), lung (67%; 2 of 3 cases), kidney (47%; 7 of 15 cases), and colon (46%; 5 of 11 cases). Collectively, these findings suggest that altered FBLN-3 expression within tumor microenvironments may contribute to tumor formation and progression in humans.

FBLN-3 antagonizes angiogenic activities and sprouting in endothelial cells. Because tumors exhibit altered expression of FBLN-3 and FBLN-5 and because these ECM proteins have similar structures and expression profiles, we hypothesized that FBLN-3 may be functionally similar with FBLN-5 in mediating angiostatic activities. Interestingly, whereas tubulogenesis down-regulates FBLN-5 expression (8), FBLN-3 mRNA expression was up-regulated significantly in tubulating MB114 cells (Fig. 2A). However, analogous to FBLN-5 (8), constitutive FBLN-3 expression (Fig. 2B) inhibited MB114 cell proliferation and invasion (Fig. 2C), processes critical for angiogenesis activation and endothelial cell sprouting. Accordingly, FBLN-3 expression in (Fig. 2C) or recombinant FBLN-3 treatment of (Fig. 2D) quiescent layers of MB114 cells antagonized their formation of angiogenic sprouts. FBLN-3 expression in MB114 cells also prevented their activation of p38 MAPK, but not that of extracellular signal-regulated kinase 1/2 (ERK1/2), stimulated by VEGF (Fig. 2E). Collectively, these findings establish FBLN-3 as a novel antagonist of endothelial cell activities coupled to angiogenic sprouting.
Altered endothelial cell expression of MMPs, TIMPs, and TSP-1 by FBLN-3 and FBLN-5. The inhibition of endothelial cell invasion and angiogenic sprouting by FBLN-3 and FBLN-5 implicated these proteins as potential regulators of the expression and activity of ECM proteases, particularly MMPs and their inhibitors, the TIMPs. We tested this hypothesis by performing semiquantitative real-time PCR analyses on total RNA isolated from control-, FBLN-3-, and FBLN-5-expressing MB114 cells. Figure 3A shows that relative to control cells, FBLN-3 and FBLN-5 both decreased MB114 cell expression of MMP-2 and MMP-3 while simultaneously increasing that of the MMP antagonists, TIMP-1 and TIMP-3. Although MB114 cell expression of TIMP-2 was unaffected by either FBLN-3 or FBLN-5, both FBLNs induced MB114 cell expression of the angiostatic molecule, TSP-1 (Fig. 3A). More importantly, both FBLNs also inhibited MMP-2 and MMP-3 transcript synthesis in tubulating MB114 cells suspended in three-dimensional rat tail collagen matrices (Fig. 3B). Interestingly, although TIMP-3 expression was elevated basally in FBLN-5-expressing MB114 cells, only FBLN-3 stimulated TIMP-3 expression in tubulating MB114 cells (Fig. 3B). Gelatin zymography of MB114 cell conditioned medium confirmed that expression of either FBLN-3 or FBLN-5 significantly reduced MMP-2 protease activity in tubulating MB114 cells (Fig. 3C). Collectively, these findings suggest that FBLN-3 and FBLN-5 promote angiogenesis resolution by targeting MMP, TIMP, and TSP-1 expression in resting (i.e., steady-state) and activated endothelial cells, thereby potentially reducing ECM proteolysis and remodeling necessary for vessel development.

FBLN-3 and FBLN-5 antagonize angiogenesis in vivo. We next asked whether FBLN-3 and FBLN-5 could prevent angiogenesis in vivo by monitoring vessel development and infiltration into Matrigel plugs that were supplemented with proangiogenic factors and implanted s.c. into mice. Preliminary experiments established that bFGF was significantly better than VEGF in mediating vessel development in Matrigel plugs (data not shown). Interestingly, although the ability of bFGF to activate MAPKs (i.e., p38 MAPK and ERK1/2) in MB114 cells was unaffected by FBLN-3 or FBLN-5 (data not shown), both ECM proteins antagonized MB114 cell sprouting induced by bFGF in vitro (Fig. 4A). Moreover, recombinant FBLN-3 and FBLN-5 (Fig. 4B) both inhibited human HMEC-1 endothelial cell migration to fibronectin (Fig. 4C), whereas FBLN-5, but not FBLN-3, mediated their adhesion in an RGD-dependent manner (Fig. 4D; refs. 17, 21). Thus, the angiostatic activities of FBLN-3 and FBLN-5 are not restricted solely to murine endothelial cells nor solely to VEGF signaling in endothelial cells.

Figure 4A shows that bFGF stimulated significant vascularization of implanted Matrigel plugs, which was quantified by measuring plug hemoglobin contents (Fig. 5A) and microvessel

**Figure 2.** FBLN-3 antagonizes angiogenic activities and sprouting in MB114 cells. A, tubulating MB114 cells were incubated in collagen matrices for varying times as indicated. Total RNA was isolated and used to measure FBLN-3 expression by quantitative real-time PCR. Points, mean (n = 4); bars, SE. *, P < 0.05. B, resulting GFP expression profiles in MB114 cells infected with control (left) or FBLN-3 (right) retroviruses. Conditioned medium from control- and FBLN-3-expressing MB114 cells was tumbled with Ni²⁺-agarose and captured protein complexes were immunoblotted with anti-Myc antibodies to visualize recombinant FBLN-3 protein. C, the invasion (white column; n = 2), proliferation (black column; n = 3), and angiogenic sprouting (gray column; n = 5) of control- and FBLN-3-expressing MB114 cells were compared. Columns, mean; bars, SE. *, P < 0.05. D, MB114 cell tubulation proceeded on collagen gels for 5 days in the absence or presence of increasing concentrations of recombinant FBLN-3 (0-50 µg/ml). The quantity of invading angiogenic sprouts was determined by manual counting under a light microscope. Points, mean (n = 5); bars, SE. *, P < 0.05. FBLN-3 significantly inhibited p38 MAPK activation. #, P < 0.05.
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Figure 3. Regulation of ECM gene expression and activity in MB114 cells by FBLN-3 and FBLN-5. A, total RNA isolated from resting FBLN-5-expressing (filled columns) and FBLN-3-expressing (open columns) MB114 cells was reverse transcribed before analyzing the expression of MMP-2, MMP-3, TIMP-1, TIMP-2, TIMP-3, and TSP-1 by quantitative real-time PCR. Columns, mean (n = 3); bars, SE. B, control-expressing (filled squares), FBLN-3-expressing (open squares), and FBLN-5-expressing (open circles) MB114 cells were allowed to tubulate in collagen gels for 0 to 24 hours. Total RNA was isolated and subjected to semiquantitative PCR to monitor MMP-2, MMP-3, and TIMP-3 expression. Points, mean (n = 4); bars, SE. C, control-, FBLN-, and FBLN-3-expressing MB114 cells were allowed to tubulate in collagen gels for 24 hours and the resulting conditioned medium was fractionated through 10% SDS-PAGE supplemented with 0.1% gelatin. Afterward, the gel was processed for gelatin zymography. Columns, mean (n = 3); bars, SE. BG, background buffer control. *, P < 0.05.

Fibulins 3 and 5 antagonize the growth and vascularization of tumors in mice. We tested the aforementioned hypothesis by engineering MCA102 fibrosarcoma cells to stably express murine versions of either FBLN-3 or FBLN-5 (Fig. 6A). We chose to study MCA102 fibrosarcoma cells for two reasons. First, MCA102 fibrosarcoma cells were created by 3-methylcholanthrene treatment of C57BL/6 mice (16), thus enabling us to monitor the effects of FBLN-3 and FBLN-5 on tumor growth and angiogenesis in genetically normal mice. Second, we showed previously that FBLN-5 inhibits the proliferation of mink lung epithelial cells, while augmenting the proliferation, migration, and invasion of human HT1080 fibrosarcoma cells in vitro (7), indicating that FBLN-5 regulates various cellular responses in a context-specific manner.

Thus, MCA102 fibrosarcoma cells afforded a unique opportunity to study the context-specific effects of FBLN-3 and FBLN-5 on cancer cell activities in vitro and on cancer cell behavior in vivo.

As expected, MCA102 fibrosarcoma cells engineered to constitutively express FBLN-5 synthesized more DNA (15 ± 1.3%; n = 4; P = 0.0014) than did their control counterparts. Somewhat surprisingly, FBLN-3 expression had no effect on MCA102 fibrosarcoma proliferation (data not shown), whereas expression of both FBLNs significantly enhanced MCA102 fibrosarcoma invasion through synthetic basement membranes (Fig. 6C). Thus, both FBLNs increased the apparent tumorigenic potential of MCA102 fibrosarcoma cells when assayed in vitro, suggesting that the growth and angiogenesis of MCA102 tumors in mice might be enhanced by the expression of FBLN-3 and FBLN-5. In stark contrast, both FBLNs significantly reduced the growth and weights of MCA102 tumors produced in syngeneic C57BL/6 mice (Fig. 6D). More importantly, tumors derived from FBLN-expressing MCA102 fibrosarcoma cells also exhibited significantly reduced blood vessel densities compared with tumors derived from control cells (Fig. 6D). In addition, FBLN-3-expressing MCA102 tumors contained significantly enlarged regions of central and peripheral necrosis that were typically absent in their control counterparts. Indeed, quantifying these necrotic areas using NIH ImageJ (version 1.34s) showed FBLN-3-expressing MCA102 tumors to be 1.85 ± 0.18-fold (n = 3; P = 0.04) more necrotic than GFP-expressing MCA102 tumors. Collectively, these findings establish FBLN-3 and FBLN-5 as novel antagonists of tumor angiogenesis in vivo and, more importantly, suggest that these angiostatic activities may one day be developed to prevent the growth and progression of human malignancies.
Discussion

Tumor angiogenesis comprises a complex cascade of gene expression and repression that culminates in tumor neovascularization, which facilitates cancer cell proliferation, survival, and ultimately enhances cancer morbidity by establishing an escape route for metastatic cancer cells (4, 5, 22). Thus, identifying novel angiostatic molecules and deciphering their mechanisms of action holds the potential to limit and/or prevent tumor neovascularization, and, consequently, to thwart the proliferation, survival, and metastasis of malignant cells. To this end, we now present FBLN-3 as a novel angiogenesis antagonist that inhibits endothelial cell proliferation, invasion, and angiogenic sprouting (Fig. 2C), as well as endothelial cell activation of p38 MAPK stimulated by VEGF (Fig. 2F). We show for the first time that FBLN-3 and FBLN-5 both repress endothelial cell expression of MMP-2 and MMP-3, and induce that of TIMP-1, TIMP-3, and TSP-1 (Fig. 3). Finally, we provide the first definitive evidence that FBLN-3 and FBLN-5 antagonize vessel development in vivo (Fig. 5), leading to diminished tumor growth and neovascularization in mice (Fig. 6). These findings are especially important because FBLN-3 and FBLN-5 inhibited tumor angiogenesis in genetically normal mice, thereby excluding the possibility that diminished angiogenesis arose as a consequence of defective elastogenesis or other secondary effects associated with FBLN deficiency (21, 23).

Another especially important finding of our study concerns the nature of context-specific activities of FBLN-5 and, by extension, FBLN-3 (Fig. 6). For instance, we reported previously that FBLN-5 stimulates normal and malignant fibroblast proliferation, migration, and invasion (7) while simultaneously inhibiting these activities in endothelial cells (8). In addition, FBLN-5 expression inhibits epithelial cell proliferation and is down-regulated in the vast majority of epithelial-derived human tumors (7). Tumors are essentially miniature organs composed of malignant and normal cells, including fibroblasts, endothelial, and immune cells (24). Thus, given the context-specific activities of FBLN-5, it remained to be determined which cell type and biological activity is targeted predominantly by FBLN-5 (and FBLN-3) in developing tumor microenvironments. Our findings show that despite their ability to slightly, but significantly, enhance the apparent tumorigenicity of MCA102 fibrosarcoma cells in vitro (Fig. 6), FBLN-3 and FBLN-5 both significantly decreased the growth and angiogenesis of MCA102 fibrosarcoma tumors produced in mice. Thus, within the intricate context of the tumor microenvironments, the ability of FBLN-3 and FBLN-5 to promote angiostasis overrides their potential to enhance the growth and motility of fibrosarcoma cancer cells. Indeed, we suspect that within developing tumor microenvironments, these FBLNs suppress tumor growth and progression by targeting not only endothelial cells, but also their associated pericytes and vascular smooth muscle cells (25) and by normalizing tumor stroma in a manner that limits tumor growth and metastasis. Future studies need to determine which human tumors and associated microenvironments are sensitive to the tumor-suppressing activities of FBLN-3 and FBLN-5, as well as to...
assess whether these FBLNs or their peptide mimetics can induce stasis, or even regression, in established tumors.

FBLN-3 was isolated originally as an up-regulated gene product expressed in senescent cells (9) and again independently as a gene associated with malattia leventinese, an inherited form of macular degeneration characterized by the formation of drusen deposits that arise from the aberrant accumulation of misfolded mutant FBLN-3 proteins (i.e., Arg345Trp substitution; refs. 10–12). Likewise, missense FBLN-5 mutations have recently been identified and associated with the development of age-related macular degeneration (26). Interestingly, inappropriate ocular angiogenesis plays a prominent role in causing irreversible blindness, including that mediated by macular degeneration (27). A logical extension of our findings suggests that mutation-induced structural anomalies may inactivate the angiostatic function of FBLN-3 and FBLN-5, thereby contributing to macular degeneration development. Future studies need to test this hypothesis, as well as to determine whether patients housing FBLN-3 or FBLN-5 mutations are more susceptible to other angiogenic pathologies, particularly cancer.

A definitive function for FBLN-3 during tumorigenesis remains to be established. We find FBLN-3 expression to be altered in nearly half of all human malignancies surveyed (Fig. 1B), suggesting that FBLN-3 acts to suppress the formation and progression of human malignancies. Indeed, based on the overlapping expression and activity profiles attributed to FBLN family members, we suspect that additional FBLNs other than FBLN-3 and FBLN-5 will also regulate angiogenesis and vascular development. This notion is supported by the spatiotemporal manner in which FBLN-1 and FBLN-2 are expressed during cardiovascular development, which suggests involvement of these FBLNs in formation of the aortic arch and in endocardial and epicardial structures (1, 2, 28). Along these lines, neonatal and adult endothelial and vascular smooth muscle cells produce FBLNs, which incorporate into vascular basement membranes, elastic laminae, and vessel walls where they likely create an angiostatic environment (1, 2, 28). Indeed, FBLN-5-deficient mice exhibit exaggerated vascular remodeling following carotid artery ligation and injury, indicating that FBLN-5 functions to limit vascular remodeling and repair in injured vessels. Moreover, vascular smooth muscle cells isolated from these animals display elevated proliferative and migratory activities compared with those of their FBLN-5-expressing counterparts (25). In addition, FBLN-1 and FBLN-2 interact physically with the angiogenesis inhibitor, endostatin, presumably mediating its localization within vessel microenvironments (1, 29). Finally, FBLN-1 deficiency in mice elicits perinatal lethality due to hemorrhaging of irregularly shaped and aberrantly dilated neural and epidermal vessels formed from morphologically abnormal endothelial cells (30). Given these facts, future studies need to identify which FBLNs (and in which context) suppress and/or promote angiogenesis and to examine how manipulations of FBLN expression in mice mimics cancer formation and progression in humans.

Our study also indicates that FBLN-3 and FBLN-5 are functionally similar in their ability to antagonize angiogenesis. Indeed, both FBLNs inhibit endothelial cell proliferation and invasion and attenuate their response to VEGF, particularly its activation of stasis, or even regression, in established tumors.
MAPks (Fig. 2; ref. 8), which mediate endothelial cell actin cytoskeletal rearrangements (i.e., p38 MAPK) and MMP expression (i.e., ERK1/2; refs. 3, 31, 32). Thus, by inhibiting VEGF stimulation of MAPks, these FBLNs may reduce endothelial cell invasion and migration requisite for angiogenesis. This inhibitory state may be magnified by the ability of FBLN-3 and FBLN-5 to (a) repress MMP-2 and MMP-3 expression, while simultaneously inducing that of TIMP-1 and TIMP-3 in resting and tubulating endothelial cells (Fig. 3); and (b) induce TSP-1 expression, which antagonizes angiogenesis by stimulating endothelial cell apoptosis (33) and by inhibiting MMP-9 activation (34). Future studies need to examine the essentiality of these molecules in mediating angiostasis by FBLN-3 and FBLN-5 and to determine whether these FBLNs act extracellularly or intracellularly via direct or indirect mechanisms to inhibit VEGF and bFGF signaling in endothelial cells.

It is potentially noteworthy that tubulogenesis up-regulates FBLN-3 (Fig. 2) and down-regulates FBLN-5 expression (8) in activated endothelial cells. Why this is so remains unknown but may reflect distinct spatiotemporal requirements for each FBLN to negate vessel development. Recently, Bell et al. (35) found tubulating human umbilical vein endothelial cells to initially repress their expression of FBLN-3 (by 8 hours), which returned to basal levels by 24 hours. Although FBLN-3 function was not evaluated in this system, the discordant FBLN-3 expression profiles observed between our respective investigations may result from differences in the endothelial cells studied (i.e., tubulation kinetics) and matrices used. Alternatively, these discrepancies may reflect the detection of alternatively spliced FBLN-3 mRNAs, of which five unique transcripts having distinct 5’ sequences have been described (i.e., transcripts 1a, 1b, 2, 3, and 4; ref. 9). Our real-time PCR primers amplify a COOH-terminal fragment of the short form of FBLN-3, a region common to all FBLN-3 splice variants, whereas those used by Bell et al. (35) selectively amplify FBLN-3 transcript 2. Thus, quiescent and tubulating endothelial cells may differentially express FBLN-3 splice variants that mediate distinct angiogenic activities. Indeed, we find that the long form of FBLN-3 (i.e., transcript 1a), which contains an additional NH2-terminal 106 amino acids not found in its short counterpart studied herein, stimulates endothelial cell angiogenic sprouting in vitro.1 Thus, future studies need to determine (a) the function of individual FBLN-3 splice variants in endothelial cells, (b) whether cancer and other angiogenic diseases alter the expression of FBLN-3 variants, and (c) whether detection of FBLN-3 variants can be used clinically to detect and/or predict the clinical course of angiogenic diseases in humans.

**Figure 6.** FBLN-3 and FBLN-5 antagonize the growth and vascularization of tumors in mice. A, conditioned-medium from GFP-expressing (G), FBLN-5-expressing, or FBLN-3-expressing MCA102 fibrosarcoma cells was tumbled with Ni2+-agarose and captured protein complexes were immunoblotted with anti-Myc antibodies to visualize recombinant FBLN proteins. B, the invasion of control-expressing (GFP), FBLN-3-expressing, and FBLN-5-expressing MCA102 fibrosarcoma cells was compared. Columns, mean (n = 4); bars, SE. *, P < 0.05. C, C57BL/6 female mice were injected s.c. with control-expressing (GFP), FBLN-3-expressing, or FBLN-5-expressing MCA102 fibrosarcoma cells. Mice were sacrificed on day 21 and the resulting tumors were removed and weighed. Columns, mean (n = 3) tumor weights reported as the percentage GFP tumor weight; bars, SE. *, P < 0.05. D, MCA102 fibrosarcoma tumor sections were stained with H&E to determine blood vessel density (black arrows), which was quantified by manually counting five random high power fields (×400). Photomicrographs, representative H&E-stained tumor sections. Columns, mean (n = 3) blood vessel densities normalized to GFP tumors; bars, SE. *, P < 0.05.
Lastly, TIMP-3 mutations have been associated with the development of Sorsby fundus dystrophy (36–38), an early onset inherited form of macular degeneration that resembles age-related macular degeneration. Interestingly, VEGF-mediated angiogenesis is prevented by TIMP-3 binding to the VEGF KDR receptor, which antagonizes VEGF binding (39). We observed FBLN-3 to stimulate endothelial cell expression of TIMP-3 (Fig. 3B), which interacts physically with FBLN-3 via its COOH-terminal FBLN-type module (40). These findings raise the possibility that inhibition of VEGF signaling by TIMP-3 requires its association with FBLN-3, and, conversely, that FBLN-3-mediated inhibition of VEGF signaling in endothelial cells requires FBLN-3/TIMP-3 complex formation. Future studies need to test this hypothesis as well as the corresponding hypothesis that FBLN-5 interacts similarly with TIMP-3 or other TIMP family members when mediating antiangiogenic activities.

In summary, we have established FBLN-3 as a novel antagonist of angiogenesis, one that is biologically similar to FBLN-3 both in vitro and in vivo. Importantly, we show for the first time that both FBLNs prevent vessel development and tumor angiogenesis in genetically normal mice, suggesting that these ECM proteins mediate tumor suppression. Future studies using animal models of carcinogenesis are clearly warranted to determine the effectiveness of FBLN-based therapies to alleviate the growth, invasion, and angiogenesis of established tumors, as well as to identify minimal FBLN-3 and FBLN-5 determinants necessary in mediating their antiangiogenic activities. Indeed, such knowledge may enable the engineering of novel FBLN-3 and FBLN-5 proteins or peptide mimetics whose potential oncogenic activities are fully separated from their angiostatic activities, thereby improving their potential chemopreventive effectiveness.

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