Context-specific Effects of Fibulin-5 (DANCE/EVEC) on Cell Proliferation, Motility, and Invasion

FIBULIN-5 IS INDUCED BY TRANSFORMING GROWTH FACTOR-β AND AFFECTS PROTEIN KINASE CASCADES*

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† The abbreviations used are: FBLN, Fibulin; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; TGF, transforming growth factor; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; CDM, conditioned medium; GFP, green fluorescent protein; β-gal, β-galactosidase; JNK, c-Jun N-terminal kinase; HMEC, human dermal microvascular endothelial cell.

Fibulin-5 (FBLN-5; also known as DANCE or EVEC) is an integrin-binding extracellular matrix protein that mediates endothelial cell adhesion; it is also a calcium-dependent elastin-binding protein that scaffolds cells to elastic fibers, thereby preventing elastinopathy in the skin, lung, and vasculature. Transforming growth factor-β (TGF-β) regulates the production of cytokines, growth factors, and extracellular matrix proteins by a variety of cell types and tissues. We show here that TGF-β stimulates murine 3T3-L1 fibroblasts to synthesize FBLN-5 transcript and protein through a Smad3-independent pathway. Overexpression of FBLN-5 in 3T3-L1 cells increased DNA synthesis and enhanced basal and TGF-β-stimulated activation of ERK1/ERK2 and p38 mitogen-activated protein kinase (MAPK). FBLN-5 overexpression also augmented the tumorigenicity of human HT1080 fibrosarcoma cells by increasing their DNA synthesis, migration toward fibronectin, and invasion through synthetic basement membranes. In stark contrast, FBLN-5 expression was down-regulated in the majority of metastatic human malignancies, particularly in cancers of the kidney, breast, ovary, and colon. Unlike its proliferative response in fibroblasts, FBLN-5 overexpression in mink lung Mv1Lu epithelial cells resulted in an antiproliferative response, reducing their DNA synthesis and cyclin A expression. Moreover, FBLN-5 synergizes with TGF-β in stimulating AP-1 activity in Mv1Lu cells, an effect that was abrogated by overexpression of dominant-negative versions of either MKK1 or p38 MAPKα. Accordingly, both the stimulation and duration of ERK1/ERK2 and p38 MAPK by TGF-β was enhanced in Mv1Lu cells expressing FBLN-5. Our findings identify FBLN-5 as a novel TGF-β-inducible target gene that regulates cell growth and motility in a context-specific manner and affects protein kinase activation by TGF-β. Our findings also indicate that aberrant FBLN-5 expression likely contributes to tumor development in humans.

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regulated proliferation in a context-specific manner. Moreover, FBLN-5 expression enhanced the tumorigenicity of human fibrosarcoma cells and is altered dramatically during carcinogenesis, particularly in cancers of the kidney, breast, ovary, and colon. Finally, by enhancing activation of ERK1/2 and p38 MAPK, FBLN-5 expression synergizes with TGF-β in stimulating AP-1 activity in mink lung epithelial cells. Taken together, our findings have identified FBLN-5 as a novel gene target of TGF-β and provided new insights into the molecular mechanisms underlying the consequences of FBLN-5 expression on normal and abnormal cells.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human TGF-β1 was kindly provided by R & D Systems. The cDNA constructs encoding dominant-negative versions of FLAG-tagged Smad3/3A (pMX-Smad3/3A-IRE5-GFP) and he-maggutinin-tagged MKK1 (pMCL-MKK1-R & D Systems. The cDNA constructs encoding dominant-negative ver-

MATERIALS

Human dermal microvascular HMEC-1 endothelial cells were kindly provided by Dr. Rik Derynck (University of California at San Francisco). Human dermal microvascular HMEC-1 endothelial cells were kindly provided by Drs. Edwin Ames, Thomas Lawley, and Francisco Candall (Centers for Disease Control). All additional supplies or reagents were routinely available.

Fibulin-5 Plasmids—Full-length human and murine FBLN-5 cDNAs were PCR-amplified from expressed sequence tags H172726 and AW106432, respectively. The resulting PCR fragments were engineered to contain unique HindIII (N terminus) and SacII (C terminus) restriction sites for subcloning into the corresponding sites in pcDNA3.1Myc-His B vector (Invitrogen) to C-terminally tag FBLN-5 with Myc and His tags.

A retroviral FBLN-5 vector was synthesized by PCR amplifying the full-length Myc-His-tagged murine FBLN-5 cDNA using oligonucleotides containing XhoI (N terminus) and EcoRI (C terminus) restriction sites. The resulting PCR product was ligated into identical sites located immediately upstream of the IRES in the bicistronic retroviral vector, pMSCV-IRE5-GFP (11). All of the FBLN-5 cDNA inserts were sequenced on an Applied Biosystems 377A DNA sequencing machine.

Mass Spectrometry—Murine 3T3-L1 fibroblasts were plated onto 10-cm dishes and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. Upon reaching ~90% confluency, the cells were washed extensively in PBS and serum-starved in DMEM for 12 h at 37 °C. The quiescent 3T3-L1 cells were then metabolically labeled with [35]S)methionine in the absence or presence of TGF-β1 12 h at 37 °C. Afterward, naive and TGF-β1-CDM were collected, clarified by centrifugation, and concentrated by acetone precipitation prior to fractionation through 15% SDS-PAGE. The secreted proteins were visualized by silver staining and autoradiography of the dried gel. A differentially expressed ~60-kDa protein that was evident in TGF-β1-CDM was excised from the gel and subjected to trypsin digestion as described previously (12). The resulting peptides were sprayed from nanoelectrospray needles supplied by Proxana A/S (Odense, Denmark) and analyzed on a Micromass q-Tof mass spectrometer (Manchester, UK) essentially as described earlier (13). Data acquisition and analysis were performed on a Mass Lynx Windows NT PC data system.

Northern Blotting—The quiescent 3T3-L1 cells and human dermal microvascular HMEC-1 endothelial cells were stimulated with TGF-β1 as above. Afterward, total RNA was isolated using the RNazol B reagent (Tel-Test), and 10 µg was subsequently fractionated through 1.2% agarose, formaldehyde gels. After immobilizing to nylon membrane, the RNA was probed with a [32]P-labeled human FBLN-5 cDNA probe (nucleotides 887–1437) for 60 min at 68 °C. After hybridization, the membrane was washed for 45 min at room temperature in 2× SSC, 0.05% SDS, followed by 45 min of washing at 50 °C in 0.1× SSC, 0.1% SDS prior to visualization of the FBLN-5 mRNA by autoradiography.

Tissue-specific expression of FBLN-5 mRNA in humans and mice was monitored by hybrizing human and murine multiple tissue Northern blots (CLONTECH) and a murine embryonic Northern blot (CLONTECH) to [32]P-labeled species-specific FBLN-5 cDNA probes (human, nucleotides 857–1437; murine, 1056–1579). The procedures for hybridizing, washing, and visualizing FBLN-5 mRNA were identical to those above.

Retroviral Infections—Phoenix retroviral packaging cells (Dr. Gary Nolan, Stanford University) were cultured on 10-cm plates and transiently transfected at 70% confluency by overnight exposure to calcium phosphate precipitate containing 1 µg of pcDNA3.1/Myc-His B vector (Invitrogen) to C-terminally tag FBLN-5 with Myc and His tags. The retroviral supernatants were collected 48 h later. Murine 3T3-L1, human HT1080 fibrosarcoma (15), and mink lung MvLu cells epithelial cells (16) were infected overnight with 5 ml of either control (i.e. pMSCV-IRE5-GFP) or FBLN-5 retroviral supernatants in the presence of 4 µg/ml of Polybrene. The cells were analyzed at 48 h for GFP expression on a FACSVantage cell sorter (Becton Dickinson), and the highest 10% of GFP-expressing cells were expanded to generate stable populations that were ~70% GFP-positive. These initial populations were subjected to a second round of GFP sorting on a MoFlo cell sorter (Cytomation), which yielded stable populations of control or FBLN-5-expressing cells with equivalent GFP levels at a positivity rate of ~90%. These stable populations of 3T3-L1, HT1080, and MvLu cells were used to analyze the effects of FBLN-5 on cell growth, motility, and invasion, as well as reporter gene expression and protein kinase activation.

[3H]Thymidine Incorporation Assays—To detect inhibitory effects on DNA synthesis, GFP- or FBLN-5-expressing cells were cultured onto 96-well plates at a density of 5,000 cells/well in complete DMEM supplemented with various concentrations of TGF-β1 (0.5–5 ng/ml) for 48 h at 37 °C. During the final 4 h of stimulation, newly synthesized DNA was radiolabeled by adding [3]H]thymidine to the culture medium. Afterward, the cells were washed twice in ice-cold PBS, precipitated with ice-cold 5% trichloroacetic acid, and subsequently solubilized in 0.5 N NaOH prior to scintillation counting to determine radiolabeled incorporation into DNA.

To detect stimulatory effects on DNA synthesis, GFP- or FBLN-5-expressing cells were also cultured onto 96-well plates at a density of 5,000–10,000 cells/well and were then incubated overnight in complete DMEM. The next day, the cells were washed twice in PBS and plated in serum-free DMEM with or without TGF-β1 as above. Cellular DNA was radiolabeled by the addition of [3]H]thymidine during the final 12 h of stimulation and subsequently prepared for scintillation counting as above. The data are the means ± S.E. of five independent experiments presented as the percentages of [3]H]thymidine incorporation normalized to untreated GFP-expressing cells.

Tumor Array—The effects of tumorigenesis on FBLN-5 expression were examined by hybridizing a 35,000-celled human normal or cancer-related microarray according to the manufacturer’s instructions (CLONTECH). FBLN-5 expression in normal and malignant human tissues was subsequently visualized by autoradiography. cDNA arrays were subsequently hybridized with a [32]P-labeled ubiquitin cDNA probe provided by the manufacturer. The expression of FBLN-5 was normalized to that of ubiquitin, and the ratio of FBLN-5 expression between individual pairs of normal and cancerous tissue was determined. A ratio of ≥2 or ≤0.5 was considered significant.

In Vitro Migration and Invasion Assays—The effects of FBLN-5 on the motility and invasiveness of HT1080 cells were determined using a modified Boyden chamber assay essentially as described (17, 18). Briefly, the underside of a porous membrane (8-µm pore, 24-well format, Becton Dickinson) was coated overnight at 4 °C in PBS containing 50 µg/ml fibronectin (Invitrogen). Afterward, the fibronectin mixtures were removed and replaced with serum-free DMEM containing 0.1% bovine serum albumin (SFM, 0.1% bovine serum albumin). Dissociated GFP-expressing (i.e. control) or FBLN-5-expressing HT1080 cells were washed twice in serum-free media, 0.1% bovine serum albumin and subsequently cultured for 18–24 h at 37 °C in the upper chambers at a density of 100,000 cells/well. Afterward, the cells were washed twice in ice-cold PBS and immediately fixed for 10 min in 95% ethanol. After fixation, the cells remaining in the upper chamber were removed with a cotton swab, whereas those remaining in the lower chamber were stained with crystal violet.

The effects of FBLN-5 on the invasiveness of HT1080 cells were determined similarly, except that GFP- or FBLN-5-expressing HT1080 cells were cultured onto 96-well plates at a density of 100,000 cells/well. Afterward, the cells were washed twice in ice-cold PBS and immediately fixed for 10 min in 95% ethanol. After fixation, the cells remaining in the upper chamber were removed with a cotton swab, whereas those remaining in the lower chamber were stained with crystal violet.
using the IPLab Spectrum Software package (Scanalytics Inc.), and (iii) extraction of crystal violet dye by incubating the membranes in 10% acetic acid, followed by spectrophotometry at 590 nm. The data are the means ± S.E. of nine independent experiments presented as the percentage of migration or invasion relative to GFP-expressing HT1080 cells.

**Fig. 1. Identification of FBLN-5 as a novel TGF-β-inducible secretory protein.** Metabolically labeled naive or TGF-β-CDM were collected and concentrated by acetone precipitation prior to their fractionation through 15% SDS-PAGE. Silver staining and autoradiography (data not shown) of the gel revealed the induction of a ~60-kDa protein by TGF-β. This protein was excised from the gel and subjected to trypsin digestion prior to sequencing by mass spectrometry as described under “Experimental Procedures.” The resulting mass spectra and its corresponding amino acid sequence are shown.

**RESULTS**

**FBLN-5 Is a Novel TGF-β-inducible Fibroblast Secretory Protein**—To identify secretory proteins whose expression are regulated by TGF-β, the proteins present in naive and TGF-β-CDM of murine 3T3-L1 fibroblasts were concentrated and fractionated through SDS-PAGE. Differentially expressed proteins regulated by TGF-β were excised and subjected to in situ trypsinization prior to identification by mass spectrometry. As shown in Fig. 1, a ~60-kDa protein was prominent in TGF-β-CDM but not in naive CDM. Sequencing of a single peptide by nanoelectrospray mass spectrometry/mass spectrometry analysis returned an amino acid sequence of NH$_2$-YPGAYYIFQIK-NH$_2$ (data not shown) of the gel revealed the 60-kDa protein by TGF-β-CDM but not in naive CDM. Sequencing of a single peptide by nanoelectrospray mass spectrometry/mass spectrometry analysis returned an amino acid sequence of NH$_2$-YPGAYYIFQIK-NH$_2$, corresponding to residues 374–384 of murine FBLN-5.

FBLN-5 is a recently identified 448-amino acid member of the Fibulin family (9, 10). Like other Fibulins, FBLN-5 contains multiple calcium-binding epidermal growth factor-like repeats (i.e. six) and a globular C-terminal domain; it also contains an integrin-binding RGD motif that binds $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_\text{IIa}\beta_3$ integrins (4) and mediates endothelial cell adhesion (10). Mice deficient in FBLN-5 expression are viable but exhibit pronounced abnormalities of the skin, lung, and vasculature resulting from profound elastic fiber disorganization (i.e. elastinopathy). Thus, FBLN-5 regulates organogenesis and, like other Fibulins, is widely expressed in throughout human and murine tissues (Fig. 2, A and B, and Refs. 9 and 10). Not surprisingly, we found that FBLN-5 expression is induced significantly during embryogenesis (Fig. 2C).

To confirm that FBLN-5 expression was induced by TGF-β and to establish the mechanism for this effect, we performed Northern blot analysis on total RNA prepared from TGF-β-treated 3T3-L1 cells. As shown in Fig. 3A, treatment of 3T3-L1 cells with TGF-β stimulated the synthesis of FBLN-5 transcript in a time- and dose-dependent manner. Thus, FBLN-5 is indeed a novel gene target for TGF-β in 3T3-L1 cells.

The biological actions of TGF-β are mediated primarily through its stimulation of a relatively simple signaling system that at its core is comprised of three TGF-β receptors, types I, II, and III, and three latent transcription factors, Smad2, Smad3, and Smad4 (20, 21). To determine whether TGF-β-mediated induction of FBLN-5 expression was Smad2/3-dependent, we infected 3T3-L1 cells with control (i.e. GFP) or dominant-negative Smad3/3A retrovirus (pMX-Smad3/3A-IRES-GFP) (16), and cells expressing GFP were isolated by flow cytometry to establish stable polyclonal populations of control and Smad3/3A expressing 3T3-L1 cells (data not shown). As shown in Fig. 3B, stable expression of dominant-negative Smad3/3A in 3T3-L1 cells significantly inhibited their
TGF-β signaling as a mediator of 3T3-L1 cell growth. Accordingly, treatment of 3T3-L1 cells with TGF-β significantly stimulated their synthesis of DNA in a dose-dependent manner; this response to TGF-β was completely abrogated by overexpression of dominant-negative Smad3/3A in 3T3-L1 cells (Fig. 3B). In contrast, overexpression of dominant-negative Smad3/3A failed to inhibit the ability of TGF-β to stimulate FBLN-5 expression in 3T3-L1 cells (Fig. 3C). Taken together, these findings establish FBLN-5 as a novel TGF-β-inducible gene target whose expression when stimulated by TGF-β proceeds through a Smad2/3-independent pathway.

In addition to mediating endothelial cell adhesion (10), FBLN-5 expression is induced dramatically in mechanically injured vascular endothelial and smooth muscle cells (9, 10). Because TGF-β induces FBLN-5 expression and because TGF-β regulates endothelial cell activity, we asked whether TGF-β induces FBLN-5 expression in human dermal microvascular HMEC-1 endothelial cells. As shown in Fig. 3D, TGF-β treatment of HMEC-1 cells stimulated their transcription of FBLN-5 mRNA. This finding indicates that the ability of TGF-β to stimulate FBLN-5 expression is not restricted to fibroblasts but may instead represent a generalized cellular response to TGF-β. Moreover, this finding establishes FBLN-5 as a potential mediator of the effects of TGF-β on endothelial cell activity.

**FBLN-5 Stimulates 3T3-L1 Cell Proliferation and MAPKs—** Fibulins stimulate (Fibulin-4 (22); Fibulin-3 (23)) or inhibit (Fibulin-3 (23); Fibulin-1 (18, 24)) proliferation in a context- and cell type-specific manner. However, the role of FBLN-5 in regulating cell growth remains to be established. Our finding that FBLN-5 is secreted by 3T3-L1 cells suggested that FBLN-5 expression may function in governing cell growth. To test this hypothesis, 3T3-L1 cells were infected with control (i.e. pMSCV-IRE-38-GFP) or FBLN-5 retrovirus, and cells expressing GFP were isolated by flow cytometry to establish stable polyclonal populations of control and FBLN-5 expressing 3T3-L1 cells (data not shown). As shown in Fig. 4A, expression of FBLN-5 in 3T3-L1 cells did not affect their sensitivity to TGF-β (inset). However, in the absence of added TGF-β or growth factors, overexpression of FBLN-5 in 3T3-L1 cells significantly enhanced their synthesis of DNA as compared with control cells. Similarly, treatment of 3T3-L1 cells with recombinant FBLN-5 also stimulated their synthesis of DNA (data not shown). Thus, these data indicate that FBLN-5 is a positive regulator of fibroblast growth.

Mammalian MAPKs (e.g. ERKs, JNKs, and p38 MAPKs) are important mediators in virtually all physiological processes, including the control of gene expression, programmed cell death, and cell proliferation (25, 26). We therefore hypothesized that FBLN-5 expression would lead to stimulation of MAPKs. This hypothesis seemed especially attractive given that FBLN-5 binds integrins (4, 10) and integrins stimulate MAPKs (27). To test this hypothesis, GFP- or FBLN-5-expressing 3T3-L1 cells were serum-starved for 60 min prior to stimulation with TGF-β for various lengths of time. Afterward, the activation status of MAPKs was determined by immunoblot analysis using phospho-specific anti-MAPK antibodies. When constitutively overexpressed in 3T3-L1 cells, FBLN-5 failed to affect the rate and extent of activation of p38 MAPK (Fig. 4B) or ERK1/2 (Fig. 4C) by TGF-β. However, in the absence of added TGF-β or growth factors, serum-starved FBLN-5-expressing 3T3-L1 cells exhibited significantly higher activities of p38 MAPK (Fig. 4B) and ERK1/2 (Fig. 4C) as compared with control cells. Likewise, ERK1/2 activities were greater in 3T3-L1 cells stimulated with CDM from FBLN-5-expressing 293T cells as compared with CDM from control cells (Fig. 4D).

Lastly, it is worth noting that although TGF-β treatment of 3T3-L1 cells did indeed stimulate their activation of JNK, FBLN-5 expression did not promote JNK activation in response to serum starvation (data not shown). Taken together, these findings clearly show that expression of FBLN-5 in 3T3-L1 cells selectively stimulates the activation of p38 MAPK and ERK1/2 but not JNK. Moreover, these findings implicate p38 MAPK and ERK1/2 as potential mediators of the effects of TGF-β on 3T3-L1 cell proliferation.  

**FBLN-5 Expression Enhances Human Fibrosarcoma Malignancy—** The ability of FBLN-5 to promote DNA synthesis and MAPK activation in 3T3-L1 fibroblasts led us to speculate that FBLN-5 may also function in regulating tumorigenesis. In support of this hypothesis, recent studies have established that Fibulins can either augment (Fibulin-4 (22, 28)) or attenuate (Fibulin-1 (18, 24)) the malignancy of cancer cells. We therefore sought to examine the role of FBLN-5 in regulating the malignancy of human HT1080 fibrosarcoma cells. These cells were chosen because they lack expression of FBLN-5 (data not shown) and its relative, Fibulin-1 (18). Importantly, re-expression of Fibulin-1 in Fibulin-1-deficient fibrosarcomas negates their malignancy by inhibiting their invasion and by preventing their growth in soft agar or mice after implantation (18).

To determine the effects of FBLN-5 on the malignancy of cancer cells, we infected HT1080 cells that stably express the murine ectopic receptor (15) with control (i.e. GFP) or FBLN-5 retrovirus. Afterward, the cells that expressed GFP were isolated by flow cytometry to establish stable polyclonal populations of GFP control and FBLN-5-expressing HT1080 cells. As shown in Fig. 5A, the resulting HT1080 cell lines had purities >90% and expressed GFP indistinguishably. As expected, HT1080 cells infected with FBLN-5 retrovirus expressed and secreted high levels of FBLN-5 protein into the medium, whereas those infected with GFP control retrovirus were negative for expression of recombinant FBLN-5 protein (i.e. Myc immunoreactivity; Fig. 5A). These stable populations of HT1080 cells were used to examine the effects of FBLN-5 expression on fibrosarcoma malignancy.
As shown in Fig. 5B, FBLN-5-expressing HT1080 cells exhibited a trend toward enhanced DNA synthesis as compared with control cells (p = 0.11); however, TGF-β treatment in combination with FBLN-5 expression significantly stimulated DNA synthesis in HT1080 cells (Fig. 5B). Thus, similar to its effects on 3T3-L1 cell proliferation, FBLN-5 also enhances the growth of human fibrosarcoma cells.

We also investigated whether FBLN-5 expression regulated HT1080 cell migration toward fibronectin. As shown in Fig. 5C, FBLN-5-expressing cells migrated more readily to fibronectin as compared with control HT1080 cells. Moreover, when confronted with synthetic basement membranes, FBLN-5 expressing HT1080 cells invaded significantly better than control HT1080 cells (Fig. 5D). Taken together, these findings demonstrate that FBLN-5 expression enhances the malignancy of human HT1080 fibrosarcoma cells by increasing their migration, their invasion, and their production of DNA in response to TGF-β.

**Tumorigenesis Alters FBLN-5 Expression in Human Tissues**—Our finding that FBLN-5 expression enhanced the malignancy of human fibrosarcoma cells prompted us to examine the effects of tumorigenesis on FBLN-5 expression in human tissues. To address this question, we hybridized a radiolabeled human FBLN-5 cDNA probe to a membrane arrayed with matched normal/tumor cDNAs generated from cancer patients. Of the 68 patients surveyed, FBLN-5 expression was altered in 65% (44 of 68 cases) of the tumors, of which 95% (42 of 44 cases) showed down-regulation and 5% (2 of 44 cases) up-regulation (Fig. 6). The reduction or loss of FBLN-5 expression was especially evident in cancers of the kidney (93%; 14 of 15 cases), breast (100%; 9 of 9 cases), ovary (100%; 3 of 3 cases), and colon (55%; 6 of 11 cases) (Fig. 6). Importantly, FBLN-5 was expressed aberrantly in 68% of metastatic human malignancies (17 of 25 cases), of which 100% showed down-regulated expression of FBLN-5 (Fig. 6). Taken together, these findings provide the first evidence that FBLN-5 expression is altered dramatically during tumorigenesis, suggesting that aberrant FBLN-5 expression may affect cancer cell growth and metastasis in a context- and/or tumor-specific manner. Moreover, despite the ability of FBLN-5 to enhance the malignancy of human fibrosarcoma cells (Fig. 5), the overwhelming trend for its down-regulated expression in human tumors, particularly in cancers of the kidney, breast, ovary, and colon, suggests that FBLN-5 expression normally functions as a tumor suppressor.

**FBLN-5 Inhibits Mv1Lu Cell Proliferation and Cyclin A Expression**—Fibulin family members, including FBLN-5, are produced predominantly by fibroblasts localized to boundaries between epithelial and mesenchymal tissues (9, 10, 29). Fibulin family members, including FBLN-5, are pro-

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**Fig. 3.** TGF-β stimulates FBLN-5 mRNA expression through a Smad3-dependent pathway. *A*, total RNA prepared from TGF-β1-treated 3T3-L1 cells (10 μg/ lane) was hybridized with a radiolabeled human FBLN-5 cDNA probe as described under “Experimental Procedures.” Differences in mRNA loading were monitored by ethidium bromide staining to visualize the 28S rRNA. Shown is a representative autoradiograph of an experiment that was repeated three times. *B*, GFP- or Smad3/3A-expressing 3T3-L1 cells were incubated in the absence or presence of increasing concentrations of TGF-β1 (0–0.5 ng/ml) for 48 h, and cellular DNA was radiolabeled with [3H]thymidine and quantitated by scintillation counting as described under “Experimental Procedures.” The data are the means ± S.E. of two independent experiments presented as the percentages [3H]thymidine incorporation normalized to untreated GFP-expressing cells. *C*, GFP- or Smad3/3A-expressing cells were stimulated with TGF-β1 (0.5 ng/ml) as indicated. Afterward, the total RNA was prepared, fractionated, and immobilized to nylon prior to hybridization with a radiolabeled human FBLN-5 cDNA probe as described under “Experimental Procedures.” The differences in mRNA loading were monitored by ethidium bromide staining of the 28S rRNA. Shown is a representative autoradiograph of an experiment that was repeated two times. *D*, total RNA prepared from TGF-β1-treated (5 ng/ml) human Mv1Lu epithelial cells (16) expressing either GFP or FBLN-5 were generated by retroviral infection and compared for their ability to synthesize DNA by [3H]thymidine incorporation assay. Similar to 3T3-L1 fibroblasts, expression of FBLN-5 in Mv1Lu epithelial cells failed to affect their sensitivity to TGF-β (Fig. 7A). However, in stark contrast to its stimulation of
3T3-L1 cell proliferation, FBLN-5 expression significantly inhibited Mv1Lu cell DNA synthesis (Fig. 7A). Accordingly, Mv1Lu cell proliferation was also inhibited by addition of recombinant FBLN-5 (data not shown). Thus, these findings demonstrate that FBLN-5 is a negative regulator of epithelial cell growth. As an additional test for FBLN-5-mediated growth arrest and to establish a mechanism for this effect, we performed a reporter gene assay that measured changes in luciferase expression driven by the cyclin A promoter. Expression of this reporter gene is repressed by TGF-β (39), and as expected, TGF-β treatment of Mv1Lu cells repressed their expression of luciferase driven by the cyclin A promoter (Fig. 7B). This response of Mv1Lu cells to TGF-β was not affected by FBLN-5 expression; however, FBLN-5 was able to significantly reduce cyclin A-luciferase expression in unstimulated Mv1Lu cells (Fig. 7B). Taken together, these findings demonstrate that
FBLN-5 suppresses cyclin A expression in Mv1Lu cells and thereby reduces DNA synthesis. Moreover, comparing the effects of FBLN-5 expression on DNA synthesis by 3T3-L1 and Mv1Lu cells (i) denotes FBLN-5 as a multifunctional signaling molecule that regulates proliferation in a cell type-specific manner and (ii) supports the hypothesis that FBLN-5 expression mediates a variety of distinct biological activities in a context-specific manner.

**FBLN-5 Stimulates Mv1Lu Cell AP-1 Activity and MAPKs**—Although FBLN-5 regulates proliferation in a context-specific manner, we suspected that this ability was likely mediated through the stimulation of a signaling pathway whose effect is modified by the particular genetic makeup of an individual cell. Indeed, our finding that FBLN-5 stimulated MAPKs in 3T3-L1 cells (Fig. 4) prompted us to speculate that FBLN-5 would similarly stimulate MAPKs in Mv1Lu cells, leading to enhanced AP-1 activity in them. This hypothesis seemed especially attractive given the fact that (i) MAPKs phosphorylate and regulate the activity of AP-1 transcription factors (25, 26) and (ii) AP-1 activity both enhances and inhibits cell cycle progression (32). In testing this hypothesis, we measured changes in luciferase expression driven by a synthetic AP-1 promoter (\(\text{TGAC-TAA}^-\)). Although FBLN-5 expression failed to affect basal AP-1 activity in Mv1Lu cells, its expression in combination with TGF-\(\beta\) treatment did synergistically stimulate AP-1 activity as compared with control cells stimulated solely with TGF-\(\beta\) (Fig. 8A). The synergistic stimulation of AP-1 activity by FBLN-5 and TGF-\(\beta\) was readily inhibited by overexpression of dominant-negative versions of either MKK1 or p38 MAPKs (Fig. 8B). Thus, this result indicates that ERK1/2 and p38 MAPK activities converge in stimulating synergistic AP-1 activity in Mv1Lu cells of dominant-negative versions of either MKK1 or p38 MAPKs.

Finally, we were unable to detect any changes in JNK activity in serum-starved FBLN-5-expressing Mv1Lu cells (data not shown). Taken together, these findings show that in Mv1Lu cells FBLN-5 and TGF-\(\beta\) collaborate in stimulating AP-1 activation through ERK1/2- and p38 MAPK-dependent pathways.
DISCUSSION

The biology of TGF-β can largely be divided into two broad categories: regulation of cell cycling versus regulation of cell microenvironments. Although the ability of TGF-β to inhibit cell cycling and therefore to suppress tumor formation has been thoroughly established (20, 21), less is known concerning its role in regulating cell microenvironments. Indeed, although TGF-β is clearly an important player governing the production of cytokines, growth factors, and extracellular matrix proteins by a variety of cell types and tissues, the identities of these proteins and their role in mammalian biology remain to be fully elucidated. We therefore have sought to identify these secretory proteins, believing that in doing so and in determining their function we will significantly strengthen our understanding of the molecular mechanisms that underlie both the biology and pathology of these secretory proteins and perhaps that of TGF-β as well.

To this end, we now present FBLN-5 as a novel gene target for TGF-β in fibroblasts and endothelial cells. Moreover, our findings have also identified the TGF-β receptor system as being the first signaling system coupled to FBLN-5 expression. We also show for the first time that FBLN-5 expression: (i) regulates proliferation in a context-specific manner (Figs. 4, 5, and 7), (ii) enhances the growth, motility, and invasion of human fibrosarcoma cells (Fig. 5), (iii) is aberrant in the majority of metastatic human malignancies, and (iv) stimulates MAPKs that enhance AP-1 activity stimulated by TGF-β (Figs. 4, 7, and 8). Whereas previous studies implicated FBLN-5 in mediating the assembly and stabilization of extracellular matrix structures (4, 5), our findings demonstrate that FBLN-5 also functions as a multifunctional signaling molecule capable of propagating messages between cells or between matrix and cells.

The biological significance of Fibulin family members and the molecular mechanisms whereby they mediate these activities have yet to be fully appreciated. Based on their expression patterns in fetal and adult tissues, Fibulins have been suggested to regulate a variety of normal and abnormal biological processes, including organogenesis, thrombosis, fibrogenesis, and tumorigenesis (6–8). Gene targeting experiments in mice support this assertion. For instance, Fibulin-1-deficient mice suffer perinatal lethality because of impaired endothelial function, resulting in fatal hemorrhaging in neural and epidermis tissues; they also exhibit malformations of the kidney and lung (3). Thus, Fibulin-1 is essential for proper development of the
kidneys, lungs, and the vascular system. Interestingly, FBLN-5-null mice also exhibit lung and vasculature malformations (4, 5); however, these developmental deficiencies are distinct from those resulting from Fibulin-1 inactivation, suggesting that expression of Fibulins 1 and 5 fulfill similar, yet nonoverlapping functions during development. FBLN-5-deficient mice are also distinguished from their Fibulin-1-null counterparts by their profound elastinopathy of the skin, which is reminiscent of cutis laxa syndrome in humans (4, 5).

Additional insights into the function of FBLN-5 are suggested by its expression patterns. For instance, we and others (9, 10) find that FBLN-5 is widely expressed throughout human and murine tissues. FBLN-5 expression is induced significantly during embryogenesis (Fig. 2 C), predominantly in developing arteries and mesenchymal tissues and in migrating neural crest cells (9, 10). These findings suggest that FBLN-5 may be an important regulator of epithelial-to-mesenchymal transdifferentiation. Consistent with this idea, FBLN-5, like other Fibulins (6, 29–31), localizes to boundaries between epithelium and mesenchyme (9, 10). Thus, FBLN-5 likely plays a role during tissue development, remodeling, and repair. Accordingly, FBLN-5 expression is induced

**FIG. 8.** FBLN-5 synergizes with TGF-β to stimulate AP-1 activity through MAPKs in Mv1Lu cells. A, GFP- or FBLN-5-expressing Mv1Lu cells were transiently transfected with pAP-1-luciferase and pCMV-β-gal as described under “Experimental Procedures.” The transfectants were stimulated with TGF-β1 (5 ng/ml) for 18 h at 37°C and subsequently processed to measure luciferase and β-gal activities as described under “Experimental Procedures.” The data are the mean luciferase activities ± S.E. of five independent experiments normalized to untreated GFP-expressing cells. B, GFP- or FBLN-5-expressing Mv1Lu cells were transiently transfected with pAP-1-luciferase and pCMV-β-gal, together with or without the indicated amounts (0–100 ng/well) of dominant-negative versions of either MKK1 and p38 MAPKα. Afterward, the transfectants were stimulated with TGF-β1 and subsequently processed for luciferase and β-gal activities as above. Shown is a representative of three experiments. The data are the mean luciferase activities ± S.E. normalized to untreated GFP-expressing cells. C and D, GFP- or FBLN-5-expressing Mv1Lu cells were serum-starved for 1 h prior to stimulation with TGF-β1 (5 ng/ml) as indicated. Afterward, whole cell extracts were fractionated through 10% SDS-PAGE and immobilized to nitrocellulose as described under “Experimental Procedures.” The activation status of ERK1/2 (C) or p38 MAPK (D) was determined by immunoblot analysis using phospho-specific antibodies as described under “Experimental Procedures.” The differences in protein loading were monitored by reprobing stripped membranes with either anti-ERK1 or anti-p38 MAPK polyclonal antibodies. The accompanying graphs show the densitometric analysis of MAPK activation throughout the entire time course (left panel) or the first hour (right panel). Shown is a representative experiment that was repeated at least once with similar results.
dramatically in vascular endothelial and smooth muscle cells in response to mechanical injury (9, 10) and in uterine myometrial arteries undergoing cyclic angiogenesis (9). Moreover, we have recently found that retroviral delivery of FBLN-5 to animal wounds significantly increased granulation tissue volume, thereby enhancing wound closure and healing.2 This observation is reminiscent of the effects of wounding on Fibulin-2 expression, which is significantly up-regulated throughout the granulation layer (33). Although we have yet to define the mechanism(s) whereby FBLN-5 promotes wound healing, our findings demonstrating that FBLN-5 regulates proliferation, migration, and invasion are entirely consistent with its designation as a positive mediator of tissue remodeling and wound healing. Future studies clearly need to address which cell types are targeted by FBLN-5 during wound healing and, more importantly, what signaling systems are activated in them by FBLN-5. Because TGF-β stimulates wound healing and FBLN-5 expression, it will also be interesting to determine the role of FBLN-5 in the context of TGF-β-mediated tissue remodeling and wound healing.

Endothelial cells are clearly targets of FBLN-5. For instance, endothelial cells express and secrete FBLN-5, especially in response to mechanical injury (9, 10). Moreover, FBLN-5 mediates endothelial cell adhesion by binding α₁β₁, α₁β₃, and α₁β₇ integrins via its RGD motif (4, 10). These findings indicate that FBLN-5 functions in an autocrine manner to regulate endothelial cell activities and vasculogenesis. TGF-β is a potent regulator of endothelial activities (e.g., proliferation, migration, invasion, and tubule formation) (34) and, as shown herein, a stimulator of FBLN-5 expression in endothelial cells (Fig. 3). As such, it is tempting to speculate that FBLN-5 expression participates in mediating some of the effects of TGF-β on endothelial cells. It therefore will be interesting to determine precisely how FBLN-5 signaling affects endothelial cell activities and ultimately to examine the relative contribution of these events during endothelial cell activation by TGF-β.

The finding that Fibulin family members, including FBLN-5, are highly expressed at the boundaries between epithelial and mesenchymal cells (9, 10, 29–31) prompted us to speculate that FBLN-5 expression serves to mediate one set of biological activities in fibroblasts, whereas mediating a distinctly different set of activities in neighboring epithelial cells. In support of this hypothesis, we show that FBLN-5 expression regulates proliferation in a cell type-specific manner, stimulating DNA synthesis in fibroblasts while inhibiting that in epithelial cells (Figs. 4 and 7). Interestingly, in both cell types FBLN-5 expression led to activation of MAPKs (i.e. ERK1/2 and p38 MAPK), whose activities figure prominently in a variety of physiological processes including the stimulation or inhibition of cell proliferation (25, 26). With respect to epithelial cells, we found that FBLN-5 expression synergized with TGF-β in stimulating AP-1 activity, a response that required the activities of ERK1/2 and p38 MAPK. Moreover, FBLN-5 also repressed cyclin A expression in Mv1Lu cells, thereby contributing to the reduced synthesis of DNA. Although not yet determined, we suspect that FBLN-5 expression and its consequential stimulation of ERK1/2 and p38 MAPK activities will also induce AP-1 activity in 3T3-L1 cells. Along these lines, our preliminary experiments have shown that FBLN-5 expression elevates cyclin A expression in 3T3-L1 cells (data not shown), a finding consistent with the ability of FBLN-5 to promote DNA synthesis in these cells. Recently, Bhowmick et al. (35) determined that TGF-β-mediated epithelial-to-mesenchymal transdifferentiation and p38 MAPK activation require β₁ integrin expression. Although direct binding of FBLN-5 to β₁ integrins has yet to be established definitively, it is nonetheless tempting to speculate that engagement of α₁β₁, α₁β₃, or α₁β₇ integrins by FBLN-5 similarly serves in mediating MAPK activation by TGF-β. Thus, based on our findings, we propose that engagement of integrins by FBLN-5 results in the activation of a conserved signaling system(s) whose ultimate biological outcome depends upon the genetic makeup of the individual cell in question. In other words, FBLN-5-mediated stimulation of MAPKs leads to the activation of distinct forms of AP-1 dimers that arise from cell type-specific expression of Jun, Fos, and ATF members, which ultimately results in context-specific effects on gene expression and cellular activities.

As a Fibulin family member, FBLN-5 is not unique in its ability to regulate proliferation in a context-specific manner. For instance, Fibulin-3 expression is elevated in fibroblasts undergoing growth arrest or senescence (23), indicating its involvement in inhibiting cell cycle progression. However, microinjection of Fibulin-3 mRNA into fibroblasts stimulates DNA synthesis in the injected cells and in their noninjected neighbors. Thus, Fibulin-3 regulates cell growth in a context-specific manner via autocrine and paracrine signaling mechanisms. Likewise, Fibulin-4 expression promotes the growth of normal and abnormal cells through p53-independent and -dependent mechanisms, respectively. With respect to the latter, signal sequence polymorphisms in the Fibulin-4 gene prevent its secretion from human colon cancer cells (28). This intracellular variant of Fibulin-4 then interacts with and inhibits the activity of p53, leading to enhanced proliferation of tumor cells (22). Thus, expression of Fibulins 3 and 4, like that of FBLN-5, governs proliferation in a cell type-specific manner.

Along these lines, our findings identify FBLN-5 as a candidate regulator of tumorigenesis. Indeed, we have shown that FBLN-5 enhances the malignancy of human HT1080 fibrosarcoma cells by (i) increasing their DNA synthesis in response to TGF-β, (ii) enhancing their migration toward fibronectin (Fig. 5C) and laminin (data not shown), and (iii) augmenting their invasion through synthetic basement membranes (Fig. 5C). Accordingly, tumors of the majority of patients surveyed expressed FBLN-5 aberrantly (44 of 68 cases); however, contrary to our expectations, FBLN-5 expression was down-regulated in 95% of these cases, particularly in cancers of the kidney, breast, ovary, and colon (Fig. 6). More importantly, FBLN-5 expression was inversely related to tumor metastasis, being expressed aberrantly in ~65% (17 of 25 cases) of metastatic malignancies and being down-regulated in 100% of these cases (Fig. 6). Taken together, our findings highlight the context-specific nature of FBLN-5 in regulating the activities of normal (i.e. noncancerous) and abnormal (i.e. cancerous) cells. Moreover, the striking down-regulation of FBLN-5 expression in human tumors suggests that FBLN-5 functions predominantly to suppress, not promote, tumor formation. It is interesting to note that TGF-β both suppresses and promotes tumor formation in a context-specific manner (20, 36). Therefore, future studies clearly need to determine precisely how FBLN-5 signaling suppresses and promotes tumor formation and ultimately to examine how alterations in these processes contribute to tumor suppression and promotion mediated by TGF-β.

It will be equally important to ascertain the identity of the cells whose expression of FBLN-5 becomes aberrant during tumorigenesis. Whether down-regulation of FBLN-5 in these cells results from altered TGF-β signaling or additional compensatory mechanisms (i.e. elevated FBLN-5 protein expression diminishes FBLN-5 mRNA expression via a negative feedback loop) is currently unknown. Regardless, this study demonstrates that alterations in tumor microenvironments

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2 W. P. Schiemann and T. Mustoe, unpublished observations.
negatively impacts FBLN-5 expression, an event capable of stimulating (e.g. fibrosarcoma cells) or inhibiting (e.g. potentially cancers of the kidney, breast, ovary, and colon) tumorigenesis in a context-specific manner. A similar paradox has been described for Fibulin-3, whose expression positively and negatively regulates cell growth and is up-regulated in transformed cell lines (23). Likewise, Fibulin-1 expression has been shown to inhibit the tumorigenicity of fibrosarcoma cells (18, 23). FBLN-5 expression was down-regulated dramatically during carcinogenesis, pointing toward a prominent role in mediating tumor suppression. Indeed, in terms of disease development, we expect the inappropriate absence or presence of FBLN-5 in cell microenvironments to elicit profound effects on a variety of cellular activities and processes, particularly those involved in tissue development, remodeling, and repair. This hypothesis is currently under investigation.

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Context-specific Effects of Fibulin-5 (DANCE/EVEC) on Cell Proliferation, Motility, and Invasion: FIBULIN-5 IS INDUCED BY TRANSFORMING GROWTH FACTOR-β AND AFFECTS PROTEIN KINASE CASCADES
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