Loss of Fibulin-2 Expression Is Associated with Breast Cancer Progression

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Fibulin-2, an extracellular matrix protein expressed by normal epithelia, was found to be down-regulated in several breast cancer cell lines. Fibulin-2 protein expression was also decreased in breast cancer tissue samples as evaluated by immunohistochemistry. Reintroduction of Fibulin-2 into breast cancer cell lines that do not express Fibulin-2 reduced cancer cell motility and invasion *in vitro* but had no effect on cell growth and adhesion properties. Together with evidence that Fibulin-2 contributes to wound healing and inhibits smooth muscle cell migration, our findings suggest that loss of Fibulin-2 expression may facilitate migration and invasion in breast cancer. (*Am J Pathol* 2007, 170:1535–1545; DOI: 10.2353/ajpath.2007.060478)

Fibulin-2, an extracellular matrix (ECM) protein first identified in 1990 by analysis of cDNA clones from a mouse fibroblast library,¹ belongs to a six member family of extracellular glycoproteins. Fibulins associate with a variety of extracellular ligands in diverse ECM structures, including basement membrane, elastic fibers, and microfibrils.^{2–7} Fibulin-2 contains multiple calcium-binding sites in a tandem array of 11 epidermal growth factor-like domains and forms an anti-parallel disulfide bonded homodimer.^{8–10} It has been shown that Fibulin-2 serves as a scaffold protein in the ECM by binding to a variety of ligands including type IV collagen, fibronectin, fibrinogen, fibrillin, laminins, aggrecan, and versican.^{6,7,11–13} The interactions with aggrecan and versican were directly mapped to epidermal growth factor-like domains.⁷ The association with fibronectin can be completely blocked by ethylenediaminetetraacetic acid, but binding to nidogen and type IV collagen is only partially abolished by ethylenediaminetetraacetic acid, suggesting that these

interactions are mediated by the epidermal growth factor-like domains that contain calcium-binding motifs.¹¹ This multifunctional binding capacity suggests that Fibulin-2 is involved in configuring, maintaining, and integrating basement membranes and other extracellular structures.

The components of ECM and their interactions are critical for the movement of cells during normal and disease processes. Fibulin proteins contribute to ECM remodeling during embryonic development,^{14–16} wound healing,^{17,18} and cancer cell invasion.^{19–22} Fibulin-2 is a prominent component of adult heart valves that is also transiently expressed in the embryonic endocardial cushion tissue^{15,23} and during development of central and peripheral nerve systems.^{16,24} Fibulin-2 mRNA levels are highly up-regulated during skin wound healing but return to normal levels once wound repair is completed,¹⁷ suggesting that it is important in regulating cell mobility during development and tissue repair. Fibulins are known to be involved in cancer, in which they have been reported to exhibit both tumor suppressive and oncogenic activities.²⁵ Fibulin-1 has been shown to be overexpressed in human ovarian epithelial tumors and breast cancers by immunohistochemical staining.^{20,26} Fibulin-4 mRNA is up-regulated in human colon cancer.²¹ Fibulin-5 overexpression increases migration and invasion in human HT1080 fibrosarcoma cells *in vitro* but is down-regulated in kidney, breast, ovary, and colon cancers.²²

We found that Fibulin-2 was expressed by normal mammary epithelial cells but was not expressed by several breast cancer cell lines or primary tumors. The role of Fibulin-2 in cancer progression and invasion has not previously been studied. In this report, we investigated potential contributions of Fibulin-2 to cell growth, adhesion, motility, and migration during breast cancer progression and invasion.

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Materials and Methods

Cell Lines and Cell Culture

Human breast cancer cell lines MDA-MB-231, T-47D, MCF-7, BT-20, BT-483, ZR-75-1, and SK-BR-3 were obtained from American Type Culture Collection (Manassas, VA) and maintained in cell culture medium (Invitrogen, Carlsbad, CA) according to American Type Culture Collection's instructions. All cells were grown in a humidified incubator at 37°C in 5% CO₂.

Reagents

Anti-Fibulin-2 rabbit serum and a pcDNA3 vector containing the full-length Fibulin-2 construct were obtained from Dr. Takako Sasaki, Max-Planck-Institut für Biochemie, Martinsried, Germany. Mouse monoclonal anti- β -actin was purchased from Sigma (St. Louis, MO). Alkaline phosphatase-conjugated goat anti-mouse and goat anti-rabbit immunoglobulin were purchased from Pierce (Rockford, IL).

cDNA Microarray Gene Expression of Breast Cancer Cell Lines

cDNA microarrays comprising 3157 genes were obtained from the I.M.A.G.E. Consortium cDNA clone set (ResGen; Invitrogen). Glass microarray slides were printed and postprocessed by the Microarray Core Facility at the Eppley Institute, University of Nebraska Medical Center. Briefly, polymerase chain reaction (PCR) products were purified and resuspended in 3 $\times$ standard saline citrate (SSC) at 30 μ mol/L and spotted onto poly-L-lysine-coated microscope slides using a MagnaSpotter robot (BioAutomation, Dallas, TX) with a 12-pin print head (Telechem, Sunnyvale, CA) in a humidified, HEPA-filtered hood. After spotting, the slides were rehydrated, and the DNAs were UV-cross-linked in a Stratalinker UV crosslinker (Stratagene, Inc., La Jolla, CA) and then blocked by succinic anhydride treatment and rinsed in ethanol. The printed slides were stored desiccated at room temperature until use.

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA extracted from human testis tissue was used as a universal reference standard, and total RNA obtained from the cell lines were hybridized against the control RNA. All hybridizations were repeated at least three times. For cDNA synthesis, 40 μ g of total RNA was incubated with 2 μ g of anchored oligodeoxythymidylate primers in a total volume of 20 μ l at 70°C for 10 minutes and chilled on ice. The labeling mixture added to the reaction contained 5 $\times$ Superscript II first-strand buffer, 0.1 mol/L dithiothreitol, deoxynucleotide triphosphate mixture (1 mmol/L dATP, dGTP, and dTTP; and 0.2 mmol/L dCTP), 1 mmol/L Cy3- or Cy5-conjugated dCTP, 30 U of RNasin, and 400 U of Superscript II (Invitrogen). The mixtures were incubated at 42°C for 2 hours, and an additional 400 U of Superscript II was added after 1 hour. The reaction

was stopped by 0.5 mol/L ethylenediaminetetraacetic acid, pH 8.0. Residual RNA was hydrolyzed by adding 10 μ l of 1 mol/L NaOH to the mixture followed by incubation at 65°C for 15 minutes and subsequent cooling to room temperature. Amplified DNA was purified by the Qiagen PCR purification kit (Qiagen, Valencia, CA). The cDNAs were conjugated with either Cy3 or Cy5 monofunctional *N*-hydroxysuccinimide-ester (Amersham Biosciences, Piscataway, NJ) and incubated for 90 minutes at room temperature in the dark. Cy3- and Cy5-labeled cDNAs were combined and purified again using the Qiagen PCR purification kit. The eluted mixtures were dried by speed vacuum (model RC 10.10; Jouan Inc., Winchester, VA) and resuspended in 60 μ l of hybridization solution consisting of 50% formamide, 4.1 $\times$ Denhardt's, and 4.4 $\times$ SSC, 0.15% sodium dodecyl sulfate, 10 μ g of polydeoxyadenylate (Amersham Biosciences), 2.5 μ g of yeast tRNA (Sigma), and 12.5 μ g of human Cot1 DNA (Invitrogen). The mixtures were boiled for 5 minutes and cooled to room temperature. After clarification by centrifugation at 13,000 rpm for 5 minutes, the hybridization solution was applied to cDNA microarrays and incubated at 65°C for 16 hours in a hybridization cassette (TeleChem International, Inc.). After hybridization, the arrays were washed with 1 $\times$ SSC/0.1% sodium dodecyl sulfate for 5 minutes, followed by a wash with 0.1 $\times$ SSC/0.01% sodium dodecyl sulfate, and 0.1 $\times$ SSC. The arrays were dried by centrifugation for 5 minutes at 1000 rpm and scanned with a ScanArray 4000 confocal laser system (Perkin-Elmer, Wellesley, MA).

Image analysis was performed by the QuantArray software package (Perkin-Elmer). In brief, a grid was placed on the image to identify individual spots. Fluorescence intensities of background and signal were determined by a histogram quantitation method, in which a histogram of intensities of all pixels in a spot was calculated, and the top 20% pixels that had the highest fluorescence intensities were set as real signal and the lowest 20% were set as background. Background fluorescence was subtracted from the signal and normalized using a global normalization method. A global normalization is based on the assumption that the total fluorescent intensities from the two channels should be equal. If they were not equal, one preselected channel was adjusted to equalize the intensities. The expression ratios were calculated after background subtraction and normalization. Differential expression was arbitrarily defined as a twofold increase or decrease of average ratios from three independent experiments.

Real-Time PCR Assay

Total RNA from cultured cells were prepared as described above. The reverse transcription reaction was performed using 2 μ g of total RNA with anchored oligodeoxythymidylate primers in a total volume of 20 μ l at 70°C for 10 minutes and chilled on ice and then adding reverse transcription mixture containing 5 $\times$ Superscript II first strand buffer, 0.1 mol/L dithiothreitol, 1 μ l deoxynucleotide triphosphate mixture (1 mmol/L dATP,

dGTP, dTPP, and dCTP), 30 U of RNasin, and 400 U of Superscript II (Invitrogen). The mixtures were incubated at 42°C for 2 hours, and an additional 400 U of Superscript II was added after 1 hour. The reaction products were diluted 1:5 with 1× TE buffer (10 mmol/L Tris-HCl and 1 mmol/L ethylenediamine tetraacetic acid), pH 8.0, and stored at -20°C until use.

TaqMan expression assays including fluorescent probes, forward and reverse primers for human Fibulin-2, and the internal control gene glucuronidase β (GUSB) were purchased from Applied Biosystems (Foster City, CA). All other reagents used in real-time PCR assays were purchased from the same manufacturer. Real-time quantitative PCR was performed in a 96-well microtiter plate with an ABI PRISM 7700 (Applied Biosystems). Each reaction contained 5 μ l of cDNA template, 2.5 μ l of 20× probe and primers mixture, 12.5 μ l of TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), in a total volume of 25 μ l. Reactions were amplified for 40 cycles (65°C for 5 minutes and 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute). The threshold was determined as 10 times the SD of the baseline fluorescence signal. The cycle number at the threshold was used as the threshold cycle (Ct). Standard curves for Fibulin-2 and GUSB were generated using serial dilutions of cDNA templates. The amount of mRNA of Fibulin-2 and GUSB in each test sample was calculated from the standard curve. The mRNA levels of Fibulin-2 were presented as the ratio of Fibulin-2 to GUSB in each sample.

Immunohistochemistry

Normal and malignant breast tissue samples were selected by a pathologist based on diagnosis and microscopic morphology. Tissue sections were deparaffinized with xylene and rehydrated in a series of ethanol washes (100, 90, 80, and 70%). Slides were washed with phosphate-buffered saline (PBS) and treated with 3% H₂O₂ for 30 minutes to block endogenous peroxidase activity. After washing with PBS three times, the sections were incubated with 10% horse serum for 1 hour to block non-specific binding. Next, sections were incubated with a 1:400 dilution of rabbit antiserum against human Fibulin-2 overnight at 4°C in a humidified chamber. The slides were rinsed with PBS and incubated with biotin-labeled goat anti-rabbit secondary antibody (1:200) (Vector Laboratories, Burlingame, CA) and for 1 hour at room temperature. After three washes with PBS, the slides were incubated for 30 minutes at room temperature with ABC reagent (Vector Laboratories). The slides were then rinsed with PBS and incubated for 3 to 5 minutes with 3,3'-diaminobenzidine substrate (Vector Laboratories) observing closely for color to develop, followed by counterstaining with Meyer's hematoxylin for 30 seconds. Coverslips were applied, and the slides were examined under a microscope (Nikon Eclipse 90i; Nikon, Tokyo, Japan). Images were captured using a digital camera (Nikon Digital Sight DS-5M; Nikon) and imaging software (Nikon ACT-2U).

Preparation of Cell Lysates

Cells were grown in 225-cm² flasks to 80% confluence and washed twice with ice-cold PBS. Lysates were prepared by scraping cells into 1.5 ml of lysis buffer (10 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L dithiothreitol, and 0.5% Nonidet P-40) with 15 μ l of phenylmethyl sulfonyl fluoride and complete mini protease inhibitors cocktail (Roche Applied Science, Indianapolis, IN). Lysates were incubated on ice for 30 minutes and centrifuged at 4°C for 15 minutes at 13,000 rpm to remove cell debris. Supernatants were transferred to fresh tubes, and protein content was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

Western Blotting

Lysate from each cell line (40 μ g) was resuspended in 4× lithium dodecyl sulfate sample buffer (Invitrogen) and incubated at 70°C for 10 minutes before resolution on 3 to 8% Tris-acetate gradient denaturing polyacrylamide gels (Invitrogen) at 125 V for 60 minutes. Proteins were electrophoretically transferred to a polyvinylpyrrolidone difluoride membrane (Bio-Rad) at 25 V for 2 hours. The blot was blocked in blotting buffer (5% dry milk in 1× Tris-buffered saline) overnight at 4°C and subsequently incubated with primary antibody in blotting buffer for 2 hours at room temperature with light shaking. The blot was washed three times with blotting buffer and incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated secondary antibody in blotting buffer. After three washes with blotting buffer and an additional wash with Tris-buffered saline, bands were visualized by chemiluminescence with the ECL kit (Amersham Biosciences) according to the manufacturer's instructions.

Stable Expression of Fibulin-2 in MDA-MB-231 and BT-20

Electroporation was performed using Nucleofector technology (Amaxa Biosystems, Gaithersburg, MD). Cells were passaged 3 days before transfection and grown to 80% confluence. Cells were released from the flasks by trypsinization and resuspended at 2×10^7 cells/ml. Supplement (0.5 ml) was added to Nucleofector solution V (2.25 ml; Amaxa Biosystems) before use. Cells (100 μ l) were mixed with 100 μ l of solution V, 2 μ g of pcDNA3.fibulin-2 (fib2), or 2 μ g of pcDNA3 vector alone. The transfection mixture was immediately transferred to an electroporation cuvette provided by the manufacturer and put into the Nucleofector machine. Transfection was performed using program A-23. The cuvette was removed from the holder, and 500 μ l of prewarmed culture medium was added before the cells were seeded into six-well plates. Recombinant selection was initiated 24 hours after transfection by the addition of G418. MDA-MB-231 and BT-20 cells were maintained in medium containing 1500 and 800 μ g/ml G418, respectively. Cell colonies were picked after 2 weeks and transferred to 96-well plates. Culture media containing 500 or 200 μ g/ml

G418 were used in the subsequent cell culture for MDA-MB-231 and BT-20 cell lines, respectively. Fibulin-2-expressing clones were initially screened by immunocytochemistry and confirmed by Western blotting. Two clones expressing high levels of Fibulin-2 were chosen from each cell line: MDA231.fib2 B6 and C6 and BT-20.fib2 C1 and C4 were used in the subsequent *in vitro* assays. The parental cell lines and cells transfected with vector control (MDA-MB-231.pcDNA3 and BT-20.pcDNA3) were used as controls.

Cell Growth Assay

Cells from each cell line were seeded in 48-well plates at a density of 1×10^4 cells/400 μ l of medium. Cells were harvested at 24, 48, 72, and 84 hours by trypsinization and counted using a Z2 cell and particle counter (Beckman Coulter, Fullerton, CA). Quadruple replicates for each cell line were counted at each time point. Cell growth curves were constructed using the cell numbers at each time point. One-way analysis of variance analysis was performed using the Prism program (GraphPad Software, San Diego, CA) to examine the difference of cell growth rate among the cell lines. A *P* value <0.05 was considered statistically significant.

Cell Adhesion Assay

Cell adhesion to type I collagen, type IV collagen, fibronectin, or laminins were determined using 96-well plates that were precoated with 5 μ g/cm² of the ECM proteins, or bovine serum albumin as a control, and incubated overnight at 4°C. All wells were blocked with 0.5% (w/v) bovine serum albumin for 4 hours at 4°C before use. Cells were serum-starved for 1 hour and harvested by trypsinization. Cells were resuspended in serum-free medium at 5×10^4 per ml. One hundred μ l of the cell suspension was added to each well. Experiments were performed using eight replicates and adhesion to bovine serum albumin controls and were repeated four times. The plates were incubated for 1 hour at 37°C. To remove nonadhesive cells, the plates were washed three times with serum-free medium. Adherent cells were fixed with 100 μ l of freshly diluted 1% glutaraldehyde for 10 minutes and stained with 100 μ l of 0.1% Crystal violet for 30 minutes and rinsed with distilled water. The crystal violet absorbed by the cells was solubilized with 0.1% Triton X-100 overnight at room temperature. Absorbance

was measured at 590 nm with a Titertek Multiskan Plus microtiter plate reader (Flow Laboratories, Mississauga, ON, Canada). Statistical significance of different groups was determined by one-way analysis of variance. An unpaired Student's *t*-test was used to assess differences between two groups. A *P* value <0.05 was considered statistically significant.

In Vitro Wound-Healing Assay

Cell motility was measured using an *in vitro* wound-healing assay. Cells were seeded on six-well tissue culture plates and grown to 100% confluence. Wounds were created by scraping monolayer cells with a sterile pipette tip. The wounded monolayers were washed twice with serum-free media to remove cell debris. Monolayers were incubated in cell culture medium and imaged through a microscope and photographed with a digital camera (CoolPix 950; Nikon) at 0, 18, and 36 hours.

Cell Invasion and Migration

Matrigel invasion assays were performed using 24-well BD BioCoat Matrigel Invasion Chambers (Becton Dickinson Labware, Bedford, MA) according to the manufacturer's instructions. Plates were warmed to room temperature and rehydrated with 1000 μ l of warm medium in a tissue culture incubator for 2 hours. Cells were harvested from 80% confluent tissue culture flasks by trypsinization, washed three times with PBS, and resuspended in serum-free medium at a concentration of 1×10^5 cells/ml. The lower chambers of the transwell cultures were filled with 600 μ l of medium with 10% fetal bovine serum as a chemoattractant. The cell suspension (500 μ l) was added to the upper chamber, and plates were incubated at 37°C for 36 hours. Noninvasive cells and Matrigel in the upper chamber were removed with a cotton swab, and cells that migrated through the membrane to the lower surface of the polycarbonate membrane were stained with a Diff-Quik kit (Allegiance Health, McGraw Park, IL). Membranes were removed with a scalpel blade and placed on glass slides. Cells that had migrated to the lower surface were quantified by counting 10 randomly selected microscope fields at $\times 400$ magnification. Cell migration assays were performed similarly using 24-well BD BioCoat Control cell culture inserts (Becton Dickinson Labware) lacking the Matrigel coating.

Table 1. Breast Cancer Cell Lines Used in This Study

Cell line	Histology	ER	Her2	EGFR	p53	PTEN
MDA-MB-231	Ductal adenocarcinoma	—	Low	Medium	Mut	High
T-47D	Ductal carcinoma	+	Medium	Low	Mut	High
BT-20	Ductal adenocarcinoma	—	Low	Medium	N/A	N/A
BT-483	Ductal carcinoma	+	N/A	N/A	N/A	N/A
SK-BR-3	Adenocarcinoma	—	High	Medium	Mut	High
ZR-75-1	Ductal carcinoma	+	High	Low	WT	Low
MCF-7	Adenocarcinoma	+	Medium	Low	WT	High

Mut, mutated; N/A, not available; EGFR, epidermal growth factor receptor.

Table 2. Highly Expressed Genes in the Seven Breast Cancer Cell Lines (>2 Compared with Reference RNA)

Keratin 8
S100 calcium-binding protein A11 (calgizzarin)
Nuclear transport factor (placental protein 15)
Protein phosphatase 1, catalytic subunit, α isoform
Homo sapiens cDNA DKFZp465E1616
Homo sapiens clone 23596 mRNA sequence
Keratin 19

Results

Gene Expression Profiling of Breast Cancer Cell Lines

We analyzed seven breast cancer cell lines for gene expression by cDNA microarrays: MDA-MB-231, T-47D, MCF-7, BT-20, BT-483, ZR-75-1, and SK-BR-3. These seven breast cancer cell lines presented a variety of known molecular characteristics of breast carcinomas, including differences in the expression or mutation status of estrogen receptor, Her2, epidermal growth factor receptor, p53, and PTEN (shown in Table 1). Total RNA extracted from human testis tissue was used as a universal reference RNA because of its abundant expression of most human mRNA species. Our intent in using a universal reference was to enable comparisons of multiple samples and ultimately to analyze samples from normal cells and malignant cells. As a starting point, we selected genes that showed a twofold alteration in expression. Among 3157 genes analyzed, we found seven genes with high expression levels in the seven breast cancer cell lines and 12 genes, including Fibulin-2, which exhibited lower expression in the breast cancer cell lines (Tables 2 and 3).

Quantitative Real-Time PCR Analysis of Fibulin-2 mRNA Expression in Breast Cancer Cell Lines

We used quantitative real-time PCR to determine the levels of Fibulin-2 mRNA and compared this to GUSB as an internal control. Glyceraldehyde-3-phosphate dehydrogenase was not used because its expression varies

Table 3. Poorly Expressed Genes in the Seven Breast Cancer Cell Lines (<0.5 Compared with Reference RNA)

Human mRNA for ankyrin motif, complete cds
Ribonuclease, RNase A family, 1 (pancreatic)
Heat shock 70 kd protein 2
Glutathione S-transferase A2
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 1
CAG repeat domain
Glycine amidinotransferase (L-arginine:glycine amidinotransferase)
FAT tumor suppressor (<i>Drosophila</i>) homolog
KIAA0077 protein
Lactate dehydrogenase C
Fibulin-2
Methionine adenosyltransferase II, α

Table 4. Relative Expression Levels of Fibulin-2 and GUSB mRNA

Cell lines/samples	Fibulin-2 mRNA expression	GUSB mRNA expression	Fibulin-2/GUSB
MDA-MB-231		3.4891	
T-47D		17.1316	
BT-20		2.6649	
BT-483	0.2901	4.3274	0.067
ZR-75-1		1.7991	
SK-BR-3	1.7831	11.6744	0.1527
MCF-7		4.9988	
Testis RNA	7.2665	6.4989	1.1181

among breast cancer cell lines. GUSB has been described to be the least differentially expressed in tumor cell lines and is therefore a more reliable control for real-time PCR experiments.²⁷ Standard curves were generated for both Fibulin-2 and GUSB to obtain mRNA expression levels for each sample, using a series of fivefold dilutions of BT-483 cDNA. Fibulin-2 mRNA expression levels were divided by the expression levels of GUSB. This ratio was used to present the relative expression levels of Fibulin-2 mRNA as shown in Table 4. Fibulin-2 mRNA was undetectable in most of the breast cancer cell lines except for BT-483 and SK-BR-3, in which the levels were 14- and 5.5-fold less than GUSB, respectively. Therefore, our real-time PCR analysis is consistent with the expression patterns observed in the microarray data.

Fibulin-2 Protein Expression Is Low in Breast Cancer Cell Lines

We used Western blotting methods using cell lysates extracted from the cultured breast cancer cells to determine Fibulin-2 protein expression in the breast cancer cell lines. Cell lysates were loaded onto 3 to 8% Tris-acetate gels that have a resolution of 10 to 400 kd under nonreducing conditions to detect Fibulin-2 dimers. Fibulin-2 forms anti-parallel disulfide-bonded homogeneous dimers with a molecular mass of ~375 kd, and monomer protein has a molecular mass of 195 kd when disulfide bonding is disrupted.¹⁰ In the breast cancer cell lines that express Fibulin-2, a band of ~195 kd was identified, but not the dimer. The results suggested that breast cancer cells did not form functional Fibulin-2 dimers or that the levels of dimer formation were too low to be detectable. Among all cell lines tested, BT-483 and SK-BR-3 showed low levels of Fibulin-2 expression, but the protein was not detected in any of the other cell lines (Figure 1).

Fibulin-2 Expression Is Reduced in Breast Cancer Tissues Compared with Normal Breast Tissues

Immunohistochemistry was performed on paraffin-embedded sections of normal breast tissues ($n = 15$) and breast carcinomas ($n = 17$). Two representative images of each group are shown in Figure 2. High expression of

Figure 1. Fibulin-2 protein expression in seven breast cancer cell lines, evaluated by Western blotting. Fibulin-2 was detected in breast cancer cell line BT-483 (lane 4) and SK-BR-3 (lane 7) but not in five other breast cancer cell lines.

Fibulin-2 protein was observed in the elastic tissue as previously reported^{28,29} (data not shown). There was moderate expression of Fibulin-2 in the 14 normal breast tissues. The protein was expressed in the cytosol and cell surface of normal ductal epithelial cells, especially on the apical side of the cells. However, Fibulin-2 protein expression was significantly reduced or not detectable in

14 of the 17 breast carcinoma tissue samples we examined. The results revealed that Fibulin-2 expression is reduced in invasive breast carcinomas compared with normal breast tissue. These results support the hypothesis that decreased Fibulin-2 in the ECM may facilitate the invasion of tumor cells. Lobular adenocarcinoma is rare, and the 17 breast cancer samples we used in this study included 16 cases of ductal adenocarcinoma and one case of lobular adenocarcinoma, which was positive for Fibulin-2 expression. The other three cases of Fibulin-2-positive tumors were ductal carcinomas. Therefore, there was no difference in Fibulin-2 expression in tumors of different histology. Fibulin-2 expression was not associated with lymph node metastasis, differentiation, stage, estrogen receptor status, or Her2 expression in this limited sample set; however, an expanded immunohistochemistry study with increased sample size should be performed to exclude conclusively any association between Fibulin-2 expression and these factors.

Expression of Fibulin-2 in Breast Cancer Cell Line MDA-MB-231 and BT-20

We expressed Fibulin-2 in breast cancer cell lines that do not express Fibulin-2 mRNA or protein to evaluate its functions. Two cell lines were used in this study. BT-20 was derived from a ductal adenocarcinoma, and MDA-MB-231 was established from a poorly differentiated ductal adenocarcinoma. Full-length Fibulin-2 cloned into the pcDNA3 vector was obtained from Dr. Takako Sasaki.¹¹ Fibulin-2

Figure 2. Fibulin-2 protein expression detected by immunoperoxidase in normal breast tissue and breast cancer tissue. **A** and **B**: Normal breast tissue. **C** and **D**: Breast carcinomas. In normal breast tissue, Fibulin-2 showed moderate expression in ductal epithelial cells but significantly decreased or nondetectable expression in breast carcinomas. Original magnifications, $\times 400$.

Figure 3. Fibulin-2 protein expression (Western blotting) in parental MDA-MB-231, BT-20 cell lines, and cell lines transfected with vector control and Fibulin 2. MDA-MB231.fib2.B6 has moderate Fibulin-2 expression compared with other cells transfected with Fibulin-2. In all cell lines transfected with Fibulin-2, there was a band of ~375 kd corresponding to the dimer form of Fibulin-2. In addition, there were other bands around the reported monomer and dimer protein, perhaps because of posttranslational modifications.

constructs or vector controls were transfected into these two cell lines by electroporation. After selection with G418 for 2 weeks, individual clones were selected and screened by immunocytochemistry. Expression of Fibulin-2 was confirmed by Western blotting. As shown in Figure 3, two individual clones that highly expressed Fibulin-2 were selected for each cell line and used for *in vitro* assays: MDA-MB-231.fib2.B6, MDA-MB-231.fib2.C6, BT-20.fib2.C1, and BT-20.fib2.C4. The latter three of these had similar expression levels of recombinant Fibulin-2, but MDA-MB-231.fib2.B6 had lower levels of Fibulin-2 protein.

Both the monomer (195 kd) and dimer (375 kd) forms of Fibulin-2 protein were detected in these four cell lines. The BT-20 and MDA-MB-231 transfectants showed other forms with mobilities above and below the predicted protein. The additional bands are likely to be products of posttranslational modifications such as O-glycosylation or N-glycosylation. The lower molecular mass bands less than 375 kd may be attributable to proteolysis. Analysis with the glycosylation prediction program NetOGlyc 3.1 (<http://www.cbs.dtu.dk/services/NetOGlyc/>) and NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>) suggested that Fibulin-2 bears five potential O-glycosylation and two potential N-glycosylation sites. It has been reported that Fibulin-2 has three N-glycosylation sites, but one is an Asn-Asp-Ser/Thr motif, which is unlikely to be an N-glycosylation site. To determine whether the additional bands were attributable to N-glycosylation, we incubated the protein lysates with glycosidase F at 37°C overnight. The treated and untreated lysates were evaluated by Western blotting with anti-Fibulin-2. There was no change in the mobility of these bands with or without glycosidase F treatment (data not shown), demonstrating that the

Figure 4. Adhesion assays to bovine serum albumin control, type I collagen, type IV collagen, fibronectin, and laminins ($n = 8$, mean, error bars represented ± 1 SD). **A:** Adhesion assay with MDA-MB-231 and MDA-MB-231.pcDNA3 transfectants. **B:** Adhesion assays with BT-20 and BT-20 transfectants. Cells showed adhesion to 96-well plates coated with type IV collagen, but not to other ECM proteins. There were no significant alterations in adhesion properties of cells overexpressing Fibulin-2.

different mobility of these bands is not attributable to N-glycosylation. It remains possible that the different mobility bands are attributable to differential O-linked glycosylation.

Fibulin-2 Expression Had No Effect on Cell Growth Rate and Adhesion to Other ECM Proteins

There were no significant changes in cell growth rate among parental cell line MDA-MB-231, vector control MDA-MB-231.pcDNA3, and Fibulin-2-expressing clones MDA-MB-231.fib2.B6 and C6. Likewise, neither the control BT-20 cell line transfected with vector alone nor those cells transfected with the recombinant Fibulin-2 construct demonstrated a difference in growth rate compared with the parental cell line (data not shown). Therefore, we conclude that Fibulin-2 expression does not affect cell growth rate *in vitro*.

Fibulin-2 is known to interact with several ECM ligands including fibronectin,⁶ laminins,^{6,12} and type IV collagen.^{11,30} Fibulin-2 binds to the latter with a weak affinity. To determine whether overexpression of Fibulin-2 increased cell adhesion to a solid surface coated with ECM proteins, we tested the adhesion properties of Fibulin-2-expressing MDA-MB-231 and BT-20 cells, parental cells, and pcDNA3

Figure 5. *In vitro* wound-healing assay. **A:** MDA-MB-231 cell line and Fibulin-2 transfectants. **B:** BT-20 cell line and Fibulin-2 transfectants. The images were taken at 0, 18, and 36 hours after wounding. At 36 hours, the wounds were nearly completely closed in parental cell lines and the vector control cell lines but not in cells expressing Fibulin-2.

vector control cells with four ECM proteins: type I collagen, type IV collagen, fibronectin, and laminins. Both parental cell lines demonstrated high levels of adhesion to type IV collagen and low levels of adhesion to the other three ECM proteins (Figure 4) compared with bovine serum albumin controls. Expression of Fibulin-2 or pcDNA3 vector alone did not alter cell adhesion to any of the ECM proteins.

In Vitro Wound-Healing Assay

We performed wound-healing assays to evaluate the effect of Fibulin-2 expression on cell motility. Wounds were introduced by scratching the cells with a 200- μ l pipette

tip, and images of cell migration into the wound were taken at 0, 18, and 36 hours. Wound-healing assays were repeated at least three times for each time point. The results (Figure 5) showed that expression of Fibulin-2 in both MDA-MB-231 and BT-20 cell lines reduced the speed of wound closure in the cell monolayer, suggesting that Fibulin-2 expression suppressed cell migration.

Expression of Fibulin-2 Reduces Breast Cancer Cell Invasion *In Vitro*

Matrigel chambers were used to assess the effect of Fibulin-2 on *in vitro* tumor cell invasion. MDA231.pcDNA3

Figure 6. Invasion through Matrigel. Number of cells invading through Matrigel-coated membranes/number of cells migrating through uncoated membranes. **A:** Invasion assays of MDA-MB-231 and MDA-MB-231 transfectants ($n = 3$, mean, error bars represented ± 1 SD). Both clones of MDA-MB-231.Fibulin-2 showed reduced invasion through Matrigel compared with parental cell lines and the vector control (* $P < 0.05$, ** $P < 0.01$ compared with MDA-MB-231). **B:** Invasion assays of BT-20 and BT-20 transfectants ($n = 3$, mean, error bars represent ± 1 SD). Both clones of BT-20.Fibulin-2 showed reduced invasion through Matrigel compared with the parental cell line and the vector control (*** $P < 0.001$ compared with BT-20).

and BT-20.pcDNA3 showed no differences in invasion assays compared with the parental cell lines MDA-MB-231 and BT-20. However, the MDA231.fib2.B6 and MDA231.fib2.C6 cell lines demonstrated a 50% reduction in cell invasion compared with parental MDA-MB-231 and MDA231.pcDNA3. BT-20.fib2.C1 and C4 also demonstrated a 50% reduction in the level of cell invasion compared with BT-20 and BT-20.pcDNA3 (Figure 6).

Discussion

The Fibulins are a recently discovered family of ECM proteins.²⁵ Previously described functions of Fibulins include organogenesis, tissue remodeling, and tumorigenesis.^{17–22,31,32} We provide evidence that loss of an ECM protein, Fibulin-2, is associated with breast cancer progression. We initially determined that Fibulin-2 mRNA was not expressed in several breast cancer cell lines and confirmed that Fibulin-2 protein expression was de-

creased in breast cancer cell lines and in invasive breast carcinomas compared with normal mammary epithelial cells.

Fibulin-2 is known to bind many ECM ligands and therefore may serve as a scaffold for extracellular matrices. Based on its hypothesized function and our observation that Fibulin-2 expression is decreased in breast carcinomas, we hypothesized that decreased expression of Fibulin-2 leads to disruptions in the molecular architecture of the basement membrane, which would enhance the ability of tumor cells to migrate and invade through the ECM. Fibulin-2 is believed to be involved in maintaining the normal rigid structure of the basement membrane by binding and cross-linking other ECM proteins, given that it is a ligand for many ECM components. A decrease in Fibulin-2 expression in breast cancer may facilitate tumor cell migration and invasion by physically weakening the basement membrane.

To examine this possibility, we expressed Fibulin-2 in breast cancer cell lines BT-20 and MDA-MB-231, which do not express Fibulin-2 mRNA or protein. Several *in vitro* assays were used to examine the effect of Fibulin-2 expression on properties of cell growth, adhesion of ECM proteins, motility, migration, and invasion. Expression of Fibulin-2 in MDA-MB-231 and BT-20 cell lines did not affect growth rates. We predicted that expression of Fibulin-2 would increase cellular adhesion to ECM proteins such as fibronectin and laminins, which are known to bind Fibulin-2 *in vitro*¹²; however, the MDA-MB-231 and BT-20 cell lines (parental or those expressing recombinant Fibulin-2) showed very little adhesion to these two ECM proteins. Type IV collagen is a weak ligand for Fibulin-2.¹¹ Although the parental cells showed adhesion to type IV collagen, there were no significant changes in adhesion to type IV collagen for cells expressing Fibulin-2, suggesting that Fibulin-2 is not involved in cellular adhesion to the four ECM proteins tested.

The results of *in vitro* wound-healing assays revealed that expression of Fibulin-2 reduced cellular migration for both cell lines tested, MDA-MB-231 and BT-20. Likewise, the results of Matrigel invasion assays demonstrated that Fibulin-2 expression reduced cellular invasion by 50% in two clones of each of these two breast cancer cell lines. Two of the clones for MDA-MB-231, MDA-MB-231.fib2.B6 and C6, express medium and high levels of Fibulin-2, respectively. However, there were no differences in invasion among these two cell lines. These results indicate that a modest level of Fibulin-2 is sufficient to reduce invasion and that higher levels of expression do not further reduce invasion activity.

To determine whether knockdown of Fibulin-2 in BT-483 and SK-BR-3 cell lines enhanced cellular invasion, we performed a transient knockdown of Fibulin-2 expression by small RNA interference, using Fibulin-2 siRNA oligonucleotides from Dharmacon Inc. (Chicago, IL). Invasion assays and migration assays were conducted using both the parental and Fibulin-2 knockdown SK-BR-3 and BT-483 cells. There were no significant changes in invasive properties of SK-BR-3 and BT-483 cell lines on Fibulin-2 knockdown (data not shown). We offer two explanations for these results. First, these two

cell lines are not inherently invasive.³³ Consistent with this possibility, we observed that only a few cells migrated through the membrane with or without Matrigel. Secondly, we were unable to knock down completely Fibulin-2 in these cell lines. For both SK-BR-3 and BT-483, the knockdown was performed twice and Fibulin-2 expression was determined by Western blotting. Fibulin-2 expression was significantly reduced after 3 days of knockdown but was not completely silenced, even after increasing the dose of siRNA oligonucleotides and numbers of days of incubation.

Overexpressing Fibulin-2 in MDA-MB-231 and BT-20 cell lines resulted in additional forms of Fibulin-2 protein (Figure 3) that may be attributable to posttranslational modification, potentially glycosylation. The posttranslational modification of Fibulin-2 has not been reported previously. We do not know if posttranslational modification may affect the function of Fibulin-2. For example, glycosylation may affect binding to other ECM proteins because Fibulin-2 functions by binding to ligands on ECM proteins. Future studies should evaluate the affinity of purified Fibulin-2 protein with or without this posttranslational modification for known Fibulin-2 ligands including type IV collagen, fibronectin, and fibrinogen.

The only known Fibulin protein in *Caenorhabditis elegans* has been reported to be involved in directing cellular migration *in vivo*. Two independent studies have shown that during gonad organogenesis, Fibulin was expressed in the surrounding nongonadal tissues and was involved in controlling organ shape by antagonizing the GON-1 and ADAM metalloproteases.^{31,32} The authors initially hypothesized that Fibulin is a direct substrate of GON-1 and ADAM, but cleavage of Fibulin *in vitro* was not detected. Therefore, they proposed that Fibulin might counteract metalloprotease action by interacting with other ECM proteins that were the substrates of the metalloproteases. The functions of Fibulin in *C. elegans* may provide insight into the function of its mammalian orthologs. Lee and colleagues³⁴ reported that Fibulin-1 is not a substrate for another metalloprotease, ADAMTS-1, but it can assist ADAMTS-1 in cleavage of aggrecan, which binds Fibulin-1. Recently, it was shown that Fibulin-2 is coexpressed and is in complex with the ECM network components versican and hyaluronan in murine vascular lesions. Blocking interactions between versican and Fibulin-2 by recombinant peptides (FBLN2 III 3 to 5 and aggrecan C-type lectin-like domain) inhibited the *in vitro* migration of smooth muscle cells.³⁵ Taken together with our results, these data support the hypothesis that Fibulin-2 can interact with ECM components, influence the activity of ECM remodeling metalloproteases, and thereby regulate cell migration and invasion. The underlying mechanism for this activity is not clear. However, direct cell migration and invasion is a likely mechanism by which Fibulin-2 affects tumor cell invasion. In the current study, there were no differences in Fibulin-2 expression with respect to incidence of lymph node metastasis. The loss of Fibulin-2 may contribute to the progression of noninvasive lesions to invasive breast carcinomas, because cell migration and stromal invasion is a hallmark of invasive breast cancer and is not seen *in*

situ breast cancer lesions. Future prospective studies should evaluate cases of breast carcinoma *in situ* for Fibulin-2 expression and correlate expression with incidence of recurrence to test this hypothesis and determine the potential of Fibulin-2 as a prognostic marker.

Fibulin family members have multiple and diverse effects in human malignancies. Fibulin-1 and Fibulin-4 are overexpressed in breast, ovarian, and colon cancers. We have shown here that Fibulin-2 is down-regulated in breast cancer, and others have shown that Fibulin-5 is down-regulated in many cancers. There is also evidence that alternate forms of these proteins can have divergent roles in tumors. Fibulin-1 has four splice variants (A, B, C, and D), with variations at the C terminus.²⁵ Fibulin-1C and -1D are predominant isoforms,³⁶ and they seem to have distinct functions.³⁷ Fibulin-1D decreases tumor growth *in vivo*³⁸ and delays cell invasion and migration *in vitro*.^{38,39} Fibulin-1C is elevated in ovarian cancer, suggesting it may be involved in ovarian carcinogenesis.²⁶ Although Fibulin-5 is down-regulated in many human malignancies, overexpression of Fibulin-5 increases human fibrosarcoma cell migration and invasion *in vitro*. Very little is known about how Fibulin proteins are regulated. Fibulin-6 homologue is transcriptionally regulated by the fos-1 transcription factor,⁴⁰ and Fibulin-1 is regulated by estrogens.^{19,41} The divergent activities of Fibulin proteins may be attributable to diversity in regulation or interactions with other proteins in different tissues.

This report is the first to propose that Fibulin-2 may be involved in suppression of human malignancy. Our results suggest that expressing recombinant Fibulin-2 reduces breast cancer cell migration and invasion and that loss of Fibulin-2 expression in breast cancer cells may facilitate cell migration and invasion. Deciphering the exact mechanism by which Fibulin-2 affects cell invasion and migration will require further investigation.

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