Integrin (α6β4) Signals Through Src to Increase Expression of S100A4, a Metastasis-Promoting Factor: Implications for Cancer Cell Invasion

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Abstract

Integrin α6β4 is linked to cancer cell motility and invasion in aggressive and metastatic cancer cells. In this study, we showed that expression of the β4 integrin in MDA-MB-435 cancer cells (MDA-MB-435/β4) leads to a dramatic increase in expression of a metastasis-promoting factor, S100A4, as determined by affymetrix gene chip microarray, quantitative real-time PCR, and Western blot analysis. Alternatively, knocking down β4 integrin expression in MDA-MB-231 breast carcinoma cells by shRNA reduced the level of S100A4 expression. The mechanism by which α6β4 enhances S100A4 expression involves Src, Akt, and NFAT. We have further shown that Y1494, a tyrosine residue of the ITIM motif in the cytoplasmic domain of the β4 integrin subunit, is essential for α6β4-dependent S100A4 expression. Reduction of S100A4 expression by shRNA blocked migration, invasion, and anchorage-independent growth of MDA-MB-435/β4, SUM-159, and MDA-MB-231 cells. These studies define a novel mechanism by which integrin α6β4 promotes cancer cell motility and invasion, and provides insight into how S100A4 expression is regulated in cancer cells. (Mol Cancer Res 2009;7(10):1605–12)

Introduction

The α6β4 integrin, an epithelial-specific integrin, functions as a receptor for members of the laminin family of extracellular matrix proteins (1-3). Although the primary known function of α6β4 is to contribute to tissue integrity through its ability to mediate the formation of hemidesmosomes (4, 5), there is growing evidence suggesting that this integrin also plays a pivotal role in functions associated with carcinoma progression (4, 6, 7). In aggressive and metastatic carcinoma cells, the host-tumor microenvironment induces the relocalization of α6β4 from hemidesmosome into filamentous actin in lamellipodia and filopodia, where it becomes signaling competent by functionally interacting with other growth factor receptors and G protein-coupled receptors (8-11).

The enhanced signaling function of α6β4 contributes to a motile and invasive phenotype of cancer cells (12-16). Although an α6β4-dependent invasive phenotype has been linked to the activation of transcription factors such as nuclear factor of activated T cells (NFAT) and NF-κB (17, 18), α6β4-dependent transcriptional target genes important for cancer cell motility need to be identified. To address this issue, we performed microarray analysis using MDA-MB-435/mock (α6β4 negative) and MDA-MB-435/β4 transfectant (α6β4 positive) cell lines and found that an invasion and metastasis promoting factor, S100A4, is upregulated >7-fold as a function of α6β4 expression.

S100A4, a member of the S100 family of calcium binding proteins, has an established role in tumor invasion and metastasis (19-21). Elevated S100A4 expression levels are associated with tumor progression and poor prognoses in many cancers, including breast, non–small cell lung, primary gastric, colon carcinoma, invasive pancreatic carcinoma, bladder, malignant melanoma, and prostate (22-29). S100A4 itself is not a tumor-inducing factor, because transgenic mice overexpressing S100A4 do not develop tumors (30). However, under tumorigenic conditions, S100A4 expression in tumor cells facilitates development of highly aggressive tumors and formation of metastases (31, 32).

In this study, we assessed the relationship between α6β4 and S100A4 based on our microarray data. Specifically, we focused on the signaling mechanism by which α6β4 increases S100A4 expression. We found that integrin α6β4 regulates S100A4 expression through Src, Akt, and NFAT pathways, and that α6β4-dependent cancer cell motility, invasion, and anchorage-independent growth require S100A4. These results suggest a novel mechanism whereby α6β4 promotes cancer cell motility and invasion through upregulation of S100A4 expression.

Results

α6β4 Integrin Upregulates S100A4 Expression in Human Cancer Cells

α6β4 has been shown to signal through the transcription factor, NFAT, to enhance cell migration and invasion (17, 33, 34), but the target genes that mediate α6β4-dependent cell motility have not been well characterized. Therefore, we studied the contribution of integrin α6β4 to the expression of genes involved in cancer cell motility at the transcriptional level.
S100A4 Mediates α6β4-Dependent Cancer Cell Motility, Invasion, and Anchorage-Independent Growth

Given that S100A4 enhances cancer metastasis (21, 32), we investigated whether S100A4 mediates α6β4-dependent cancer cell motility, invasion, and anchorage-independent growth, which are essential components of metastasis. As reported previously by us and others, the ability of MDA-MB-435 β4 transfectants, MDA-MB-231, and SUM-159 human cancer cells to migrate and invade toward chemoattractants such as lysophosphatidic acid (LPA) depends on integrin α6β4 (12, 16, 35). To test the role of S100A4 in α6β4-dependent functions important for metastasis, we stably knocked down S100A4 expression in these three cell lines using lentivirus-delivered shRNA. The knockdown was confirmed by Western analysis as well as quantitative real-time PCR (Fig. 2A). Knockdown of S100A4 expression by shRNA did not influence the expression of β4 integrin expression, as shown in Fig. 2A. Knockdown of S100A4 expression in these cell lines effectively blocks both α6β4-dependent cell motility and invasion (Fig. 2B), suggesting that S100A4 is an essential component of α6β4-dependent functions related to metastasis. We then measured the impact of S100A4 knockdown on colony formation in soft agar, because in addition to migration and invasion, cancer cell growth via stimulation of anchorage-independent growth is another important aspect of successful metastasis. MDA-MB-435 β4 cells that stably express S100A4 shRNA formed fewer and smaller colonies when compared with colonies formed by cells that express control green fluorescent protein (GFP) shRNA (Fig. 2C).

β4 integrin regulates S100A4 expression in human cancer cells. A, RNAs were isolated from MDA-MB-435 parental, mock-1, mock-2 (clone: 6D2, 6D7), and β4 integrin transfectant-1 and β4 integrin transfectant-2 (clone: 3A7, 5B3) cells and used for quantitative real-time PCR. The statistical analysis was done using the two-tailed Student's t test. P < 0.01 (**), compared with results of the parental MDA-MB-435 cells. B, MDA-MB-435 human cancer cells (parental, mock-1, mock-2, and β4 transfectant-1 and β4 transfectant-2) and (C) MDA-MB-231 breast carcinoma cell lines (parental, GFP, and β4 shRNA infectants) were maintained as described in the Materials and Methods section and lysed using radioimmunoprecipitation assay (RIPA) buffer. Equal amounts of protein were isolated from extracts of these cell lines for Western blot analysis using antibodies against β4 integrin, S100A4 and actin.

Y1494 of the Integrin β4 Subunit Is Essential for Mediating α6β4-Dependent Migration and S100A4 Expression

Our studies have shown that a critical tyrosine residue (Y1494) in the third fibronectin type III domain of the β4 cytoplasmic tail is essential for initiating α6β4-dependent signaling cascades required for cancer cell invasion and survival (14, 36). To determine the importance of this residue in mediating S100A4 expression, we used stable sublines of MDA-MB-435 cells that expressed the β4 mutant that substitutes tyrosine 1494 into phenylalanine, as well as a mutant in which the β4 subunit cytoplasmic domain is deleted. Y1494 of β4 integrin is constitutively phosphorylated upon expression of this integrin subunit in the MDA-MB-435 cell line (data not shown). Compared with results from wild-type β4 integrin transfectants, we were unable to detect S100A4 expression in

We used MDA-MB-435 cells, which endogenously lack β4 integrin expression. Analysis of gene expression patterns by affymetrix HG-U133A_2 microarray, using MDA-MB-435 clones that were either mock (MDA-MB-435/mock) or β4 integrin transfected (MDA-MB-435/β4), led to the discovery that S100A4, a metastasis- and motility-promoting factor, is one of the most upregulated genes as a function of β4 integrin expression in MDA-MB-435 cells (data not shown). To validate the result from the gene chip microarray analysis, the relationship between α6β4 and S100A4 expression was further investigated using quantitative real-time PCR and Western blot analysis (Fig. 1A and B). In agreement with the results of the microarray gene expression profiling, the level of S100A4 mRNA expression was over 100-fold higher in MDA-MB-435/β4 cells compared with MDA-MB-435 parental or mock control cells as determined by quantitative real-time PCR (Fig. 1A). Western blot analysis showed that the level of S100A4 protein is also dramatically upregulated in MDA-MB-435/β4 cells, whereas we were not even able to detect S100A4 protein signal in either MDA-MB-435 parental or mock control cells (Fig. 1B). To address the relationship between α6β4 and S100A4 expression in another cancer cell line, we used the MDA-MB-231 breast carcinoma cell line, which endogenously expresses α6β4. We knocked down β4 integrin expression in this cell line using lentivirus-delivered shRNA (Fig. 1C). Reduction of β4 integrin correlates with decreased S100A4 expression, which further supports the idea that α6β4 is a key upstream regulator of S100A4 expression in human cancer cells (Fig. 1C).
FIGURE 2. S100A4 contributes to integrin α6β4-dependent cancer cell motility and invasion. A. Equal extracts from the lysates of either GFP or S100A4 shRNA infectants of MDA-MB-435 β4, MDA-MB-231, and SUM-159 cells were used for Western analysis and quantitative real-time PCR to check S100A4 expression. B. The ability of the cell lines mentioned in Fig. 2A to migrate or invade toward 100 mmol/L LPA was measured using a transwell cell motility assay as described in the Materials and Methods section. The statistical analysis was done using the two-tailed Student’s t test. \( P < 0.01 \) (**), or \( P < 0.05 \) (*) compared with the results of the GFP shRNA–infected cells (G, GFP; S1, S100A4 #1; S2, S100A4 #2). C. Representative bright-field images of GFP and S100A4 shRNA–infected MDA-MB-435 β4 cells (captured at x10 magnification) grown in 0.7% agar with growth medium containing 2.5% fetal bovine serum for 2 wk. Columns, mean of three representative experiments done in triplicate; bars, SEM. Fifty fields per well were counted for each assay. **, \( P < 0.01 \).
of Src in regulating integrin α6β4 expression, we knocked down expression of c-Src using siRNA in MDA-MB-435/α6β4 transfectants (Fig. 4B). As shown in Fig. 5B, stable expression of c-Src siRNA effectively reduced the level of c-Src as well as S100A4 expression, confirming that α6β4 signals through c-Src to increase S100A4 expression. Considering evidence from previous studies showing that phosphoinositide 3-kinase/Akt and Ras/Erk are two major downstream signaling pathways activated by α6β4 (12, 39-42), Src is likely to be involved in regulation of phosphoinositide 3-kinase/Akt, but not Ras/Erk, pathways (results shown in Fig. 4A). To further test this hypothesis, we assessed the impact of pharmacologic inhibitors that target Akt and Erk pathways on α6β4-dependent S100A4 expression. As shown in Fig. 4C, inhibition of Akt by Akti, as judged by reduction of Akt phosphorylation at Ser 473, effectively blocks α6β4-dependent S100A4 expression. Meanwhile, treatment with Mek (immediately upstream of Erk) inhibitor, which effectively reduced the phosphorylation of p42/44 Erk1/2, did not block α6β4-dependent S100A4 expression (Fig. 4D). Instead, inhibition of the Erk pathway slightly enhanced Akt phosphorylation and S100A4 expression, suggesting that the Akt, but not Erk pathway plays a major role in mediating S100A4 expression.

Src Regulates NFAT Activity to Mediate Integrin α6β4-Dependent S100A4 Expression

The transcription factor NFAT, whose activity is enhanced by α6β4, is involved in α6β4-dependent human cancer cell migration and invasion (17, 34). Therefore, we tested the possibility that NFAT mediates α6β4-dependent S100A4 expression. Pretreatment with an NFAT inhibitor in MDA-MB-435/α6β4 transfectants effectively blocks α6β4-dependent S100A4 expression (Fig. 5A) and human cancer cell motility as reported previously (34). These results suggest that NFAT is indeed essential in mediating α6β4-dependent cell motility-promoting factor expression such as ENPP2 (34), Cox-2 (43), and S100A4. We next investigated whether Src and Akt act upstream of NFAT activation, because Src and Akt also mediate α6β4-dependent S100A4 expression (Fig. 4). Consistent with previous reports (17, 34), we confirmed that expression of β4 integrin dramatically upregulates the activity of NFAT in MDA-MB-435 cells (Fig. 5B). Pretreatment with PP2 (a Src inhibitor) and Akti (Akt 1/2 inhibitor) in MDA-MB-435/α6β4 transfectants, effectively blocks NFAT activation, whereas PD98059 (Mek inhibitor) has no major effect on inhibition of NFAT activity (Fig. 5B). These data suggest a novel signaling mechanism in which Src and Akt mediate α6β4-dependent NFAT activation (Fig. 5C).

α6β4 Signals through Src and Akt to Increase S100A4 Expression

We next investigated the signaling mechanism by which α6β4 enhances S100A4 expression. Although we have shown the importance of Y1494 of the β4 cytoplasmic tail in mediating S100A4 expression, the mechanism by which phosphorylation of this residue initiates the signaling cascade is poorly understood. Recent reports showed that Y1494 is an essential factor for Shp2-dependent Src activation (37, 38). Although the role of Src in α6β4 signaling still needs to be determined, it seems to be an important downstream effector of α6β4. Pharmacologic inhibition of Src activity by PP2 in MDA-MB-435/α6β4 transfectants effectively blocks α6β4-dependent S100A4 expression in a concentration-dependent manner (Fig. 4A), suggesting that Src is indeed an important mediator of α6β4 signaling leading to S100A4 expression. It is interesting to note that inhibition of Src activity also blocks Akt phosphorylation, but not Erk phosphorylation, in MDA-MB-435 cells that express β4 integrin (Fig. 4A). To further confirm the role of Src in α6β4-dependent signaling important for S100A4 expression, we knocked down expression of c-Src using siRNA in MDA-MB-435/α6β4 transfectants (Fig. 4B). As shown in Fig. 5B, stable expression of c-Src siRNA effectively reduced the level of c-Src as well as S100A4 expression, confirming that α6β4 signals through c-Src to increase S100A4 expression.

FIGURE 3. Y1494 of the integrin β4 subunit is essential to S100A4 expression and cancer cell migration. A, MDA-MB-435 parental, MDA-MB-435/α6β4 wild-type, MDA-MB-435/β4Δcyt (cytoplasmic domain–deleted mutant), and MDA-MB-435/α6β4 Y1494F mutant–expressing cells were lysed using RIPA buffer and their extracts used for Western blot analysis with antibodies against β4 integrin, S100A4, and actin. B, The ability of subclones of MDA-MB-435 cells (mock, β4, β4Δcyt, β4 Y1494F) to migrate toward 100 nmoi/L LPA was measured using a transwell cell motility assay as described previously. Columns, mean; bars, SD. The statistical analysis was done with the two-tailed Student’s t test. **P < 0.01 (*) compared with the results of the MDA-MB-435/mock cells.

cells transfected with the β4 mutants (Fig. 3A). Expression of these mutants also did not enhance the ability of MDA-MB-435 cells to migrate toward LPA (Fig. 3B). These data suggest the importance of the β4 cytoplasmic domain and a critical tyrosine residue, Y1494, in regulation of S100A4 expression and cancer cell motility.
Discussion

Although the role of α6β4 in tumorigenesis has recently been highlighted in studies using animal models (6, 44), the mechanism by which α6β4 mediates metastasis is not well understood. This study revealed a novel mechanism by which integrin α6β4 regulates expression of a metastasis promoting factor, S100A4, which is essential for α6β4-dependent cell motility and invasion. We further define a signaling pathway by which α6β4 signals through Src and Akt to mediate NFAT-dependent S100A4 expression. Our data support earlier reports of the involvement of Akt, NFAT, and α6β4 in cancer cell motility, and suggest a potential role for α6β4 in later stages of progression such as invasion and metastasis, resulting from regulation of S100A4 expression.

Consistent with previous reports that NFAT is activated by α6β4 and mediates α6β4-dependent cell motility (17, 34), our data also suggest that NFAT is an essential mediator of α6β4-dependent expression of S100A4 and cancer cell motility. The novel aspect of our study is the finding that α6β4 signals through Src to activate NFAT and NFAT-dependent S100A4 expression. Indeed, recent reports suggest that Src is a key downstream effector of α6β4 (37, 38) and is involved in α6β4-dependent anchorage-independent growth (7, 38). This study also showed that Y1494 of β4 integrin is essential in mediating Shp2 binding to β4 integrin and that Shp2/β4 integrin interaction leads to activation of Src. Although the exact mechanism by which Src mediates α6β4-dependent signaling remains to be determined, and Src can act upstream of many other signaling pathways depending on the cell lines tested, our study suggests that Src mediates α6β4-dependent Akt and NFAT activation to increase S100A4 expression in MDA-MB-435 β4 cells. As shown by our study involving shRNA (Fig. 5), c-Src is likely to play a major role in regulating α6β4-dependent S100A4 expression, but other Src family kinase isoform(s) could be involved as well. The identity of the Src kinase isoform(s) that mediates α6β4 signaling is currently under investigation using shRNA-dependent selective knockdown. The role of Akt in cell migration and invasion remains controversial. Recent reports show that Akt isoforms have opposite effects on cell motility (33, 45), suggesting that Akt1 is inhibitory, whereas Akt2 is stimulatory, to cancer cell motility. Akti, the pharmacologic inhibitor that we used, blocks activity of both Akt1 and Akt2. Therefore, it seems likely that Akt2 is the Akt isoform that mediates Src-dependent S100A4 expression and motility. However, the selective knockdown of each Akt isoform by shRNA needs to be done to address this issue. The relative role of Akt isoforms in mediating α6β4-dependent S100A4 expression is currently under investigation.

As previously reported, the level of S100A4 expression correlates with metastatic potential (46-49). However, the mechanism that regulates expression of S100A4 in aggressive cancer cells has not been elucidated. These studies, taken together with our current data, strongly suggest that increased S100A4 expression by α6β4 integrin could be associated with the

![FIGURE 4. α6β4 promotes S100A4 expression via the Src/Akt pathway. MDA-MB-435/β4 cells were incubated with or without various concentrations of PP2 (Src kinase inhibitor; A), Akti (Akt inhibitor; C), or PD98059 (Mek inhibitor; D) for 24 h before lysis by RIPA buffer. Equal amounts of protein from these lysates were prepared for Western blot analysis using antibodies against phosphor-Src (Y416), Src, phospho-Akt (Ser 473), Akt, phospho-Erk (T202/Y204), Erk, S100A4, and actin. B. MDA-MB-435/β4 cells were infected with lentivirus that expresses shRNA against c-Src or GFP and selected under 2 μg/mL puromycin. After the selection, these cell lysates were prepared and equal amounts of protein from these lysates were analyzed by Western blot analysis as mentioned above.](https://mcr.aacrjournals.org/doi/10.1158/1541-7786.MCR-09-0102)
development of metastasis in malignant cancer cells. Therefore, future studies will involve experimental as well as spontaneous metastasis assays to determine the contribution of S100A4 to α6β4-dependent carcinoma progression. We will also attempt to obtain in vivo evidence that the level of α6β4 expression correlates with that of S100A4 expression in tumors by performing tissue microarray.

In summary, our data provide evidence that α6β4 integrin regulates expression of S100A4 and further substantiate the role of the α6β4-NFAT pathway in human cancer cell motility and invasion. These data also suggest the possibility that α6β4 may enhance later stages of progression, such as invasion and metastasis, through upregulation of S100A4.

Materials and Methods

Cell Lines and Cultures

The MDA-MB-435 human cancer cells and MDA-MB-231 human breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository at Georgetown University, SUM-159 cells were obtained from Dr. Steve Ethier (University of Michigan, Ann Arbor, MI). The generation of MDA-MB-435 subclones [MDA-MB-435/mock (vector only), MDA-MB-435/β4 (β4 overexpression), MDA-MB-435/β4Δcyt (deletion of β4 cytoplasmic domain), and MDA-MB-435/β4 Y1494F (mutation of Y1494 residue to F in β4 integrin)] was done as previously described (12, 14, 36). MDA-MB-231 cells were stably infected with lentivirus-expressing shRNA targeted against either GFP or the β4 integrin subunit as previously described (37). For the generation of cell lines with stable knockdown of c-Src and S100A4, lentiviral particles with the shRNA for c-Src and S100A4 were purchased from Sigma and infection carried out according to the manufacturer’s procedure. Puromycin (2 μg/mL) was used for lentivirus-infected cell selection. Cells were maintained in DMEM/low glucose (Hyclone) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies). Lentivirus-expressing shRNA against β4 integrin and S100A4 were purchased from Sigma and infection was done per manual’s protocol.

Antibodies and Reagents

The integrin β4 (clone H-101) and actin (clone C-11) antibodies were purchased from Santa Cruz Biotechnology. The p-Src (Y416), p-Akt (S473), p-p44/42 Erk1/2 (T202/Y204), Src (clone 36D10), Akt, and p44/42 Erk1/2 antibodies were obtained from Cell Signaling Technology. The S100A4 antibody was obtained from DakoCytomation, and PP2 (Src kinase inhibitor), Akti (Akt inhibitor), and NFAT inhibitors were purchased from EMD Chemicals, Inc. For the pharmacologic inhibition, cells were incubated with or without 10 to 50 μmol/L PP2, Akti, PD98059, and 5 to 100 μmol/L NFAT inhibitor for 24 h before lysis for Western blot analysis.

Taqman Based Quantitative Real-time PCR

Total RNA was prepared from MDA-MB-435, MDA-MB-435/mock-1 (clone: 6D2), MDA-MB-435/mock-2 (clone: 6D7), MDA-MB-435/β4-1 (clone: 3A7), and MDA-MB-435/β4-2 (clone: 5B3) cells using the RNeasy mini kit (Qiagen), and reverse transcription-PCR for analysis of S100A4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was carried out with the Qiagen one-step RT-PCR kit (Qiagen) according to the manufacturer’s procedure. S100A4 and GAPDH primers were synthesized from Invitrogen.

S100A4-forward, 5′-CAGATCTTCACTGCTGC-CATGCGG-3′
S100A4-reverse, 5′-ACGTGCTGAAGGACGCATGGTG-3′
GAPDH-forward, 5′-CCACCAGGGCAATTTCCATC-CA-3′
GAPDH-reverse, 5′-TCAGACGGAGCTGAGGT-CACC-3′
Amplified PCR products were electrophoresed on an agarose gel containing ethidium bromide and visualized under UV light.

**Western Blot Analysis**

Cells were lysed with 50 mmol/L Tris buffer (pH 7.4), containing 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L sodium orthovanadate, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and 1% protease inhibitor (Pierce), scraped with a rubber policeman, and collected into 1.5-ml tubes. The protein concentration was determined using a BCA protein assay kit (Pierce). Total cellular protein was resolved on 4% to 20% gradient SDS-PAGE, transferred to polyvinylidene fluoride membranes, and incubated with a primary antibody. After three 10-min washes in 50 mmol/L Tris buffer (pH 7.5), containing 0.15 mol/L NaCl and 0.1% Tween 20, protein was detected, in turn, by means of a peroxidase-conjugated secondary antibody and visualized using the Luminol and Oxidizing solutions (Boston Bioproducts).

**Cell Motility and Invasion Assay**

For the cell motility assay, the upper chambers (8 μm pore size) of transwells (Costar) were coated with collagen at 4°C. Serum-free DMEM/bovine serum albumin, and LPA was added to the lower chamber as a chemottractant. The chamber was incubated for 2 h at 37°C with 10% CO2. The cells that attached to the bottom side of the membrane were stained and counted using crystal violet. Assays were done in triplicate and repeated several times.

**Soft Agar Assay**

MDA-MB-435 β4 cells (1 × 10³) that express either GFP or S100A4 shRNA were suspended in serum-containing (2.5% fetal bovine serum) DMEM (2 mL) containing 0.35% low-melt agarose (ISC BioExpress) and overlaid on a 1-mL base layer of 0.75% agar in six-well plates. The soft agar was overlaid with complete medium (0.5 mL/well), which was changed every other day. After 14 d of incubation, the total number of colonies was quantified by counting 50 fields per well using bright-field optics.

**Luciferase Reporter Assay**

MDA-MB-435 parental, MDA-MB-435/mock, and MDA-MB-435/β4 cells were grown to 70% confluence and transfected with a mixture of the p-NFAT-IL2 and pRL Renilla control luciferase reporter vector using Fugene 6 reagent (Collaborative Research) was diluted with cold water and dried onto each filter overnight at room temperature. After washing with PBS the next day, cells were added to the upper chamber using serum-free DMEM/bovine serum albumin, and LPA was added to the lower chamber as a chemoattractant. The chamber was incubated for 2 h at 37°C with 10% CO2. The cells that attached to the bottom side of the membrane were stained and counted using crystal violet. Assays were done in triplicate and repeated several times.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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