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# Suppression of Integrin α3β1 in Breast Cancer Cells Reduces *Cyclooxygenase-2* Gene Expression and Inhibits Tumorigenesis, Invasion, and Cross-Talk to Endothelial Cells

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## Abstract

Integrin receptors for cell adhesion to extracellular matrix have important roles in promoting tumor growth and progression. Integrin α3β1 is highly expressed in breast cancer cells in which it is thought to promote invasion and metastasis; however, its roles in regulating malignant tumor cell behavior remain unclear. In the current study, we used short-hairpin RNA (shRNA) to show that suppression of α3β1 in a human breast cancer cell line, MDA-MB-231, leads to decreased tumorigenicity, reduced invasiveness, and decreased production of factors that stimulate endothelial cell migration. Real-time PCR revealed that suppression of α3β1 caused a dramatic reduction in expression of the *cyclooxygenase-2* (*COX-2*) gene, which is frequently overexpressed in breast cancers and has been exploited as a therapeutic target. Decreased COX-2 was accompanied by reduced prostaglandin E2 ($PGE_2$), a major prostanoid produced downstream of COX-2 and an important effector of COX-2 signaling. shRNA-mediated suppression of COX-2 showed that it has a role in tumor cell invasion and cross-talk to endothelial cells. Furthermore, treatment with $PGE_2$ restored these functions in α3β1-deficient MDA-MB-231 cells. These findings identify a role for α3β1 in regulating two properties of tumor cells that facilitate cancer progression: invasiveness and ability to stimulate endothelial cells. They also reveal a novel role for COX-2 as a downstream effector of α3β1 in tumor cells, thereby identifying α3β1 as a potential therapeutic target to inhibit breast cancer. *Cancer Res; 70(15); 6359–67. ©2010 AACR.*

## Introduction

Integrins are αβ cell surface receptors that mediate cell adhesion to extracellular matrix. In mammals, 18 α subunits and 8 β subunits can combine to form 24 different integrins with distinct although often overlapping ligand-binding specificities (1). Integrins expressed on tumor cells regulate many processes essential for cancer progression, including proliferation, survival, invasion, and metastasis (2–4). Integrins are therefore attractive targets for anticancer therapeutics, which has lead to preclinical and clinical development of antagonists that target certain integrins (5). However, most of these antagonists alter angiogenesis by targeting endothelial cell integrins (5, 6), and there remains a critical need to identify appropriate integrins to target on tumor cells.

Integrin α3β1 is expressed on many types of cancer cells and can regulate cell functions associated with malignancy (for reviews, see refs. 7, 8). Increased α3β1 has been correlated with breast cancer metastasis (9), and α3β1 regulates matrix metalloproteinase-9 (MMP-9) expression, invasion, and metastatic properties of squamous cell and breast carcinoma cells (9–11). Two major extracellular matrix ligands for α3β1, laminin-332 and laminin-511, are often overexpressed in breast and other carcinomas and have been linked to invasion and metastasis (4, 12, 13). In addition, α3β1 interactions with tetraspanins or other cell surface proteins can also regulate a range of cell functions (reviewed in refs. 7, 8).

Despite evidence that implicates α3β1 in carcinoma progression, little is known about its roles in tumorigenesis or how it regulates malignant cell behavior. To address this question, we used RNA interference (RNAi) to stably downregulate α3β1 in the human breast cancer cell line, MDA-MB-231. We show that suppression of α3β1 leads to reduced tumor growth *in vivo*, reduced invasive potential, and decreased production of soluble factors that stimulate endothelial cell migration. Real-time PCR arrays revealed dramatically reduced expression of the *cyclooxygenase-2* (*COX-2*) gene, which is frequently overexpressed in invasive breast cancers and a known promoter of tumor growth, angiogenesis, invasion, and metastasis

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(14–21). Subsequent analyses identified COX-2 as a mediator of certain tumor cell functions that are attributable to α3β1, including invasion and cross-talk to endothelial cells. As COX-2 has been pursued as a therapeutic target (17), our current findings identify α3β1 as a potential therapeutic target to inhibit breast cancer.

## Materials and Methods

### Cell culture

MDA-MB-231 cells (purchased from the American Type Culture Collection), or the variant line, 4175/TGL (a gift from Dr. Joan Massague', Sloan-Kettering Institute, New York, NY; ref. 22), were cultured in DMEM (BioWhittaker) supplemented with 10% fetal bovine serum (BioWhittaker), 100 U/mL penicillin, 100 μg/mL streptomycin, and L-glutamine (Invitrogen Corp.). Human umbilical vein endothelial cells (HUVEC) were purchased from VEC Technologies and cultured as described (23).

### Short-hairpin RNA–mediated gene suppression

MISSION lentiviral short-hairpin RNA (shRNA) constructs (Sigma) were used to target the human *Itga3* gene (5′-CCGGCGGATGAACATCACAGTGAAACTCGAGTTTCACTGTGATGTTCATCCGTTTTTG-3′) or the *PGTS2* (*COX2*) gene (5′-CCGGCCAGGGCTCAAACATGATGTTCTCGAGAACATCATGTTTGAGCCCTGGTTTTTG-3′); a nontargeting shRNA was used as control (Sigma). Lentiviruses were packaged in 293FT cells as described (11). Viral supernatants were added to MDA-MB-231 cells for 24 to 48 hours, and stably transduced populations were selected in 10 μmol/L puromycin. Separate experiments used a different α3-targeting shRNA to generate a distinct line of α3β1-deficient MDA-MB-231 cells as described (24, 25). For rescue experiments, RNAi effects were overcome by infecting cells with adenovirus that overexpresses human α3 (a gift from Dr. Martin Hemler, Dana-Farber Cancer Institute, Boston, MA) or *lacZ* as described (11).

### Reverse transcription-PCR

Total RNA was isolated using Trizol Reagent (Invitrogen Corp.), then reverse transcribed using First-Strand cDNA Synthesis kit (Promega). PCRs were carried out using PCR REDTaq ready mix (Sigma). Primers and conditions for amplication of α3, vascular endothelial growth factor, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were previously described (11, 26). PCR conditions for COX-2 were as follows: forward primer, 5′-TACAAGCAGTGGCAAAGGC-3′; reverse primer, 5′-AGATCATCTCTGCCTGAGTATCTT-3′; 94°C, 30 seconds; 52°C, 30 seconds; 72°C, 60 seconds; 26 cycles. Signals were visualized using a Bio-Rad FlourS 2000 (Bio-Rad).

### Real-time PCR arrays

Total RNA was isolated using RT2 qPCR-grade RNA Isolation kit. cDNA was synthesized from 1.5 μg RNA using the $RT^2$ First Strand kit (SABioscience). Real-time PCR was performed using $RT^2$ Profiler PCR array system according to the manufacturer's instructions (SABioscience) in an iCycler iQ Multicolor Detection System (Bio-Rad). Four separate experiments were performed using the Breast Cancer & Estrogen Receptor Signaling Pathways array and analyzed using Excel-based PCR Array Data Analysis Templates (SABioscience).

### Immunoblots

Cells were lysed in Cell Lysis Buffer (Cell Signaling Technology), and protein concentrations were determined using the BCA Protein Assay kit (Pierce). Equal protein was assayed by immunoblot using rabbit anti-sera against α3 integrin (1:1,000 dilution), COX-2 (1:200), or extracellular signal-regulated kinase (ERK) (1:1,000), followed by horse radish peroxidase–conjugated goat anti-rabbit IgG (1:5,000). Anti-α3 was previously described (27); other antisera were purchased from Cell Signaling Technology. Chemiluminescence was performed using SuperSignal kit (Pierce).

### Flow cytometry

MDA-MB-231 cells were trypsinized and incubated with 5 μg/mL anti-integrin monoclonal antibody (Chemicon): P1B5 (mouse anti-α3), P1D6 (mouse anti-α5), HB1.1 (mouse anti-β1), 3E1 (mouse anti-β4), GoH3 (rat anti-α6), or normal mouse IgG as control. Secondary antibody was Alexa Fluor 488–conjugated goat anti-rabbit IgG or goat anti-rat IgG (Molecular Probes, Inc.). Cells were fixed in 2% formaldehyde before flow cytometry on a FACSCanto (BD Biosciences). Data from $1 \times 10^4$ cells were analyzed using FlowJo (Tree Star, Inc.).

### Matrigel invasion

MDA-MB-231 derivatives ($8 \times 10^4$ cells) were seeded onto Growth Factor–Reduced Matrigel Invasion Chambers (8 μm pore; BD Biosciences) in complete medium and incubated at 37°C for 18 hours. Filters were fixed in 3.7% formaldehyde; noninvading cells were removed; and invading cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/mL). Images were obtained using an Olympus inverted IX70 microscope equipped with a SensiCam digital camera (Cooke). Cells were counted from three random ×10 fields per well using Image ProPlus (Media Cybernetics). We typically observed approximately 250 to 350 cells per field for control cells under these conditions. Cell invasion was quantified from three independent experiments in which results from duplicate samples were averaged.

### Transwell migration of endothelial cells

Transwell migration was assayed as described (23). Briefly, $5 \times 10^4$ serum-starved HUVECs were seeded onto Transwell inserts (8 μm pore; Costar) coated with 0.2% gelatin. Lower chambers contained serum-free MCDB-131 medium preconditioned for 24 hours by MDA-MB-231 variants in an 80:20 ratio with complete EGM-2 (Lonza). Unconditioned medium was used to establish baseline migration. After 4 hours of migration, cells were fixed in 3.7% formaldehyde and stained with crystal violet; nonmigratory cells were removed; and migrated cells were stained with DAPI and quantified from three random fields

per well as described above. We typically observed 100 to 150 cells per field for control conditions. Data are from three separate experiments in which results from duplicate samples were averaged.

### Cell growth in Matrigel

Single-cell suspensions were prepared in Growth Factor–Reduced Matrigel (~1.5 × $10^3$ cells/40 μL) then submersed in growth medium. Colonies were photographed after 10 days, and mean colony diameter ± SEM was determined for 60 to 100 random colonies for each MDA-MB-231 variant using the ImageJ software as described (25).

### Prostaglandin E2 assay

MDA-MB-231 variants (4 × $10^4$ cells) were cultured on 24-well culture dishes in serum-free medium for 24 hours, then medium was collected and analyzed using the prostaglandin E2 ($PGE_2$) EIA kit-Monoclonal according to the manufacturer's protocol (Cayman Chemical Company). Data are from three separate experiments in which results from triplicate samples were averaged.

### Xenografts in nude mice

For ectopic tumors, MDA-MB-231 cells (2 × $10^6$ cells/200 μL) were injected s.c. into right flanks of NCR nude mice (Taconic). Tumor length ($l$) and width ($w$) were measured using a Vernier caliper, and mean tumor volume was calculated for each test group using the following formula: tumor volume = ($w^2$ × $l$)/2. Tumorigenesis experiments in Supplementary Fig. S2B were performed as described (25). For orthotopic tumors, 2 × $10^6$ cells/50 μL (PBS/Matrigel) were injected into mammary fat pads of nude mice. Tumors were dissected after 35 days, weighed, and photographed. To assess angiogenesis, cryosections (10 μm) were stained with anti-CD31/platelet/endothelial cell adhesion molecule 1 (BD Biosciences) followed by Alexa Flour 594 goat anti-rat IgG (Molecular Probes), and blood vessel density was calculated using IPLab (Scanalytics, Inc.) as described (23). For each test group, CD31 staining area (pixels/field) was averaged from more than ten ×20 fields collected from three or more tumors. To assess proliferation, cryosections were stained with anti-Ki67 rabbit monoclonal antibody (Epitomics), followed by Alexa Flour 594 goat anti-rabbit IgG (Molecular Probes). Proliferation was estimated using IPLab to measure the proportion of DAPI-stained nuclei that also stained positive for Ki67. For each test group, data were averaged from more than six ×10 fields collected from three or more tumors. Apoptosis was assessed using the DeadEnd Fluorometric terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) System according to the manufacturer's instructions (Promega). Images were collected on a Nikon Eclipse 80i using a Spot camera (Diagnostic Instruments).

Experiments performed at Albany Medical College were approved by the Institutional Biosafety Committee and the Institutional Animal Care and Use Committee. Experiments at the University of Birmingham were performed in accordance with institutional and national animal research guidelines.

## Results

### Suppression of integrin α3β1 in breast cancer cells reduces tumorigenesis

As an experimental model for our studies, we used shRNA to generate α3β1-deficient variants of the human breast cancer cell line MDA-MB-231. Parental MDA-MB-231 cells expressed high levels of *α3* mRNA and α3β1 on the cell surface (Fig. 1A), consistent with previous reports (9, 28). *α3* mRNA and α3β1 protein were efficiently suppressed in cells stably expressing an shRNA that targets human α3 [MDA-MB-231/α3(−) cells], but not in cells expressing a nontargeting shRNA [MDA-MB-231/α3(+) cells; Fig. 1A]. MDA-MB-231/α3(−) cells also showed reduced surface expression of the β1 integrin subunit (Supplementary Fig. S1), the sole β subunit partner of α3 (1). The relatively modest reduction in β1 may reflect dimerization of liberated β1 with other endogenous α subunits, as indicated by slightly increased α5β1 (Supplementary Fig. S1). Surface levels of α6 and β4 integrin subunits were slightly decreased in MDA-MB-231/α3(−) cells (Supplementary Fig. S1).

Following s.c. injection into nude mice, MDA-MB-231/α3(−) cells showed dramatically reduced tumor growth over 32 days, compared with MDA-MB-231/α3(+) cells (Fig. 1B). α3β1-deficient MDA-MB-231 cells that were derived independently using a distinct α3-targeting shRNA also showed reduced tumorigenesis, as well as reduced colony formation in Matrigel (Supplementary Fig. S2), confirming that reduced tumor growth was neither an off-target effect of a particular α3-targeting shRNA, nor a peculiarity of a particular MDA-MB-231 laboratory stock. Importantly, similar results were obtained following orthotopic injection into mammary fat pads, in which tumorigenesis was significantly reduced in MDA-MB-231/α3(−) cells compared with MDA-MB-231/α3(+) cells (Fig. 1C, left graph; $P = 0.01$, Mann-Whitney test). Mice injected with α3β1-deficient cells showed reduced tumor initiation (4 of 10) compared with mice injected with control cells (9 of 10), as well as smaller average tumor size. The same trend was observed in a variant of the MDA-MB-231 line, 4175, which grows more aggressively in the mammary fat pad (Fig. 1C, right graph; ref. 22).

Ki67 immunostaining of tumor cryosections indicated a similar proportion of proliferative cells in each test group (Supplementary Fig. S3), and TUNEL staining did not reveal differences in apoptosis (data not shown). Although we cannot rule out the possibility of heterogeneous effects throughout the tumor, these findings indicate that α3β1 deficiency did not dramatically alter overall proliferation or survival of tumor cells, perhaps reflecting instead a role in early tumor cell interactions with stromal elements of the microenvironment that promote initial tumor growth. Consistently, MDA-MB-231/α3(−) tumors appeared less vascularized than MDA-MB-231/α3(+) tumors, and immunohistology with anti-CD31/PECAM confirmed ~2-fold reduction in blood vessel staining in the xenografts from α3-deficient cells (Fig. 1D). These results may reflect a proangiogenic role for α3β1 on

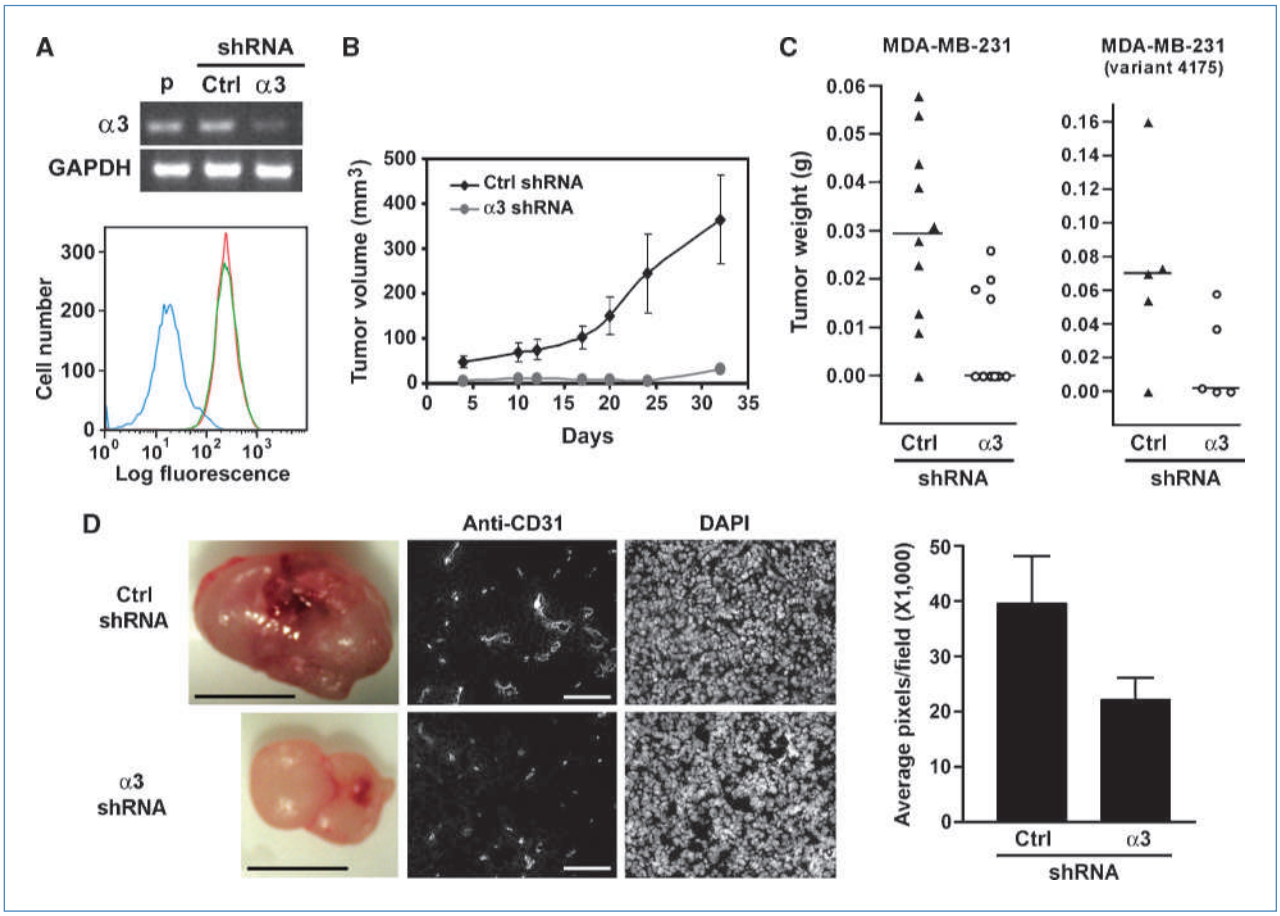


Figure 1. α3β1 regulates tumorigenesis of breast cancer cells. A, parental MDA-MB-231 cells (p), MDA-MB-231/α3(+) cells expressing control shRNA (ctrl), or MDA-MB-231/α3(–) cells expressing α3-targeting shRNA (α3) were assayed by RT-PCR for *α3* mRNA or *GAPDH* mRNA (top). Flow cytometry (bottom) was performed to compare surface levels of α3β1 in parental cells (red line), MDA-MB-231/α3(+) (green line), or MDA-MB-231/α3(–) cells (blue line). B, MDA-MB-231/α3(+) cells (ctrl shRNA) or MDA-MB-231/α3(–) cells (α3 shRNA) were injected s.c. into nude mice, and tumor growth was measured over 32 d. Points, mean ($n > 6$ mice per test group); bars, SEM. C, the same MDA-MB-231 derivatives (left graph, $n = 10$ per test group), or similar derivatives of MDA-MB-231/4175 cells (right graph, $n = 5$ per test group), were injected into mammary fat pads. Column scatter plots show tumor weights with median after 35 d for cells expressing control (▲) or α3-targeting shRNA (○). D, mammary tumors (left; scale bars, 5 mm) were cryosectioned and costained with anti-CD31 and DAPI (right; scale bars, 100 μm). Graph shows relative anti-CD31 staining in tumors from cells expressing control or α3-targeting shRNA; columns, mean ($n > 10$ fields from at least three separate tumors); bars, SEM.

tumor cells, similar to that which we recently described for α3β1 in the epidermis during wound healing (23).

### Integrin α3β1 on breast cancer cells promotes cross-talk to endothelial cells

To test if α3β1 can regulate the production of proangiogenic factors by tumor cells, we compared endothelial cell migration in response to factors secreted by MDA-MB-231 cells that express or lack α3β1. Endothelial cells (HUVECs) were seeded into the upper chambers of Transwell filters, then conditioned culture media from MDA-MB-231/α3(+) or MDA-MB-231/α3(–) cells were added to the lower chambers and tested for effects on HUVEC migration. Medium conditioned by MDA-MB-231/α3(+) cells stimulated HUVEC migration by ~3-fold over basal migration in response to unconditioned medium (Fig. 2A). In contrast, medium conditioned by MDA-MB-231/α3(–)cells failed to induce a migratory response. Furthermore, HUVEC migration was enhanced in conditioned medium from MDA-MB-231/α3(–) cells transduced with adenovirus expressing α3, whereas a control adenovirus did not rescue the response (Fig. 2B and C). These results indicate that α3β1 in breast cancer cells promotes secretion of factors that stimulate endothelial cell migration, an important component of angiogenesis.

### Suppression of integrin α3β1 reduces tumor cell invasion

Increased expression of α3β1 has been correlated with metastatic progression of human breast cancer (9). Consistently, treatment of MDA-MB-231 cells with an antibody that blocks α3β1-mediated adhesion has been shown to reduce invasive potential (9) and arrest in the pulmonary vasculature (10). However, integrin-blocking antibodies may inhibit only a subset of integrin functions, and some may

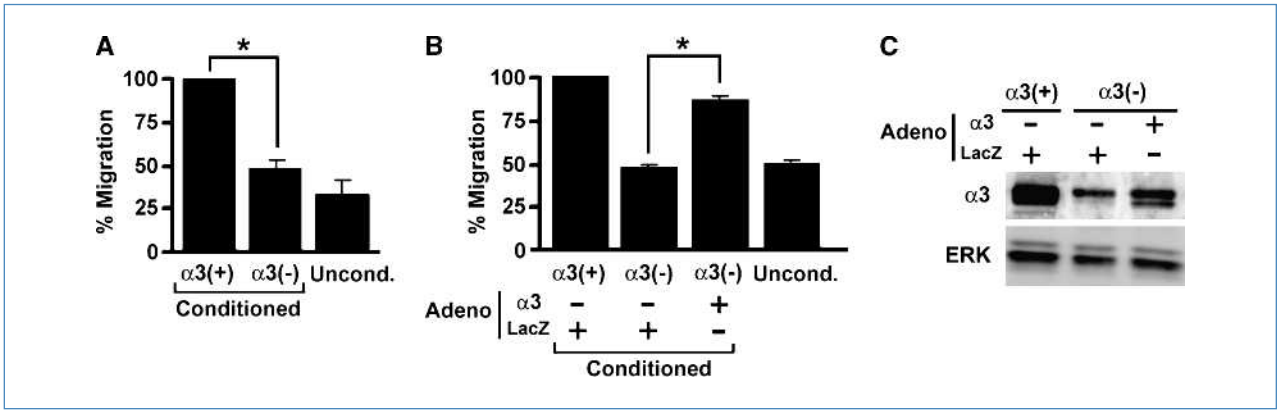


Figure 2. α3β1 in breast cancer cells regulates the secretion of soluble factors that induce endothelial cell migration. A, Transwell migration of HUVECs was compared in response to conditioned medium from MDA-MB-231/α3(+) cells [α3(+)] or MDA-MB-231/α3(–) cells [α3(–)]; unconditioned medium (uncond.) was used to establish baseline migration. Graph shows HUVEC migration as percentage of that in cells treated with medium from MDA-MB-231/α3(+) cells. B, HUVEC migration was assayed as in A, except that conditioned culture medium was collected from MDA-MB-231/α3(+) cells or MDA-MB-231/α3(–) cells transduced with adenovirus expressing α3 (adeno, α3) or LacZ (adeno, LacZ). Columns, mean ($n$ = 3 experiments); bars, SEM; *, $P < 0.004$, two-tailed $t$ test. C, total lysates from MDA-MB-231/α3(+) cells, or MDA-MB-231/α3(–) cells transduced with α3-expressing or LacZ-expressing adenovirus as indicated, were immunoblotted for α3 or ERK as a loading control.

even stimulate certain functions. Therefore, we next tested the effect of shRNA-mediated α3 suppression on cell invasion through Matrigel. MDA-MB-231/α3(–) cells displayed significantly reduced invasion compared with the MDA-MB-231/α3(+) cells (Fig. 3A). Similar results were obtained using the independently derived α3β1-deficient MDA-MB-231 cells described above (data not shown). Exogenous α3 expression restored MDA-MB-231/α3(–) cell invasion (Fig. 3B), indicating that α3β1 promotes an invasive phenotype.

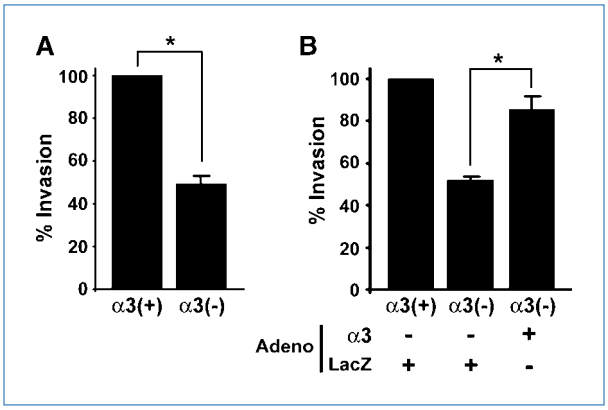


Figure 3. α3β1 regulates breast cancer cell invasion. A, Matrigel assays were performed to compare the invasion between MDA-MB-231/α3(+) and MDA-MB-231/α3(–) cells. Graph shows invasion as percentage of that in MDA-MB-231/α3(+) cells; columns, mean ($n$ = 3 experiments); bars, SEM; *, $P < 0.05$, two-tailed $t$ test. B, Matrigel invasion assays were performed as in A, except that MDA-MB-231/α3(+) cells or MDA-MB-231/α3(–) cells were transduced with adenovirus that expresses either α3 (adeno, α3), or LacZ (*adeno, LacZ*). Data are presented as in A; columns, mean ($n$ = 3 experiments); bars, SEM; *, $P < 0.02$, paired two-tailed $t$ test.

## Integrin α3β1 is required for *COX-2* gene expression

To screen for α3β1-dependent genes that may influence tumor cell function, we used the RT$^2$ Profiler PCR Array (SABiosciences) to compare MDA-MB-231/α3(+) and MDA-MB-231/α3(–) cells for the expression of breast cancer–associated genes. Compiled results from four independent experiments identified several α3β1-dependent differences (Supplementary Table S1). The change of largest magnitude in the α3β1-deficient MDA-MB-231/α3(–) cells was a ~74-fold decrease ($P$ = 0.001) in expression of the *COX-2*/*PTGS2* gene. Clinical studies of COX-2 inhibitors have generated widespread interest in COX-2 as a therapeutic target (17), and roles for COX-2 in tumor growth, angiogenesis, and invasion are well established (15, 17–20). We therefore focused our attention on potential roles for COX-2 in α3β1-mediated tumor cell functions.

Reverse transcription-PCR and immunoblot confirmed that *COX-2* mRNA and protein, respectively, were reduced substantially in MDA-MB-231/α3(–) cells, compared with parental or control shRNA cells (Fig. 4A and B). As a control, we did not detect differences in *VEGF* mRNA by conventional (Fig. 4A) or real-time reverse transcription-PCR (RT-PCR; Supplementary Table S1). Importantly, *COX2* mRNA was also reduced in the independently derived α3β1-deficient MDA-MB-231 cells described above, indicating that this reduction was not an off-target RNAi effect (Supplementary Fig. S4). As an independent measure of COX-2 activity, we assessed MDA-MB-231 culture medium for levels of $PGE_2$, a protumorigenic prostanoid that is produced downstream of COX-2 (17). As predicted, $PGE_2$ levels were decreased substantially in conditioned medium from MDA-MB-231/α3(–) cells, compared with MDA-MB-231/α3(+) or parental cells (Fig. 4C). These findings identify COX-2 as a potential mediator of protumorigenic α3β1 functions.

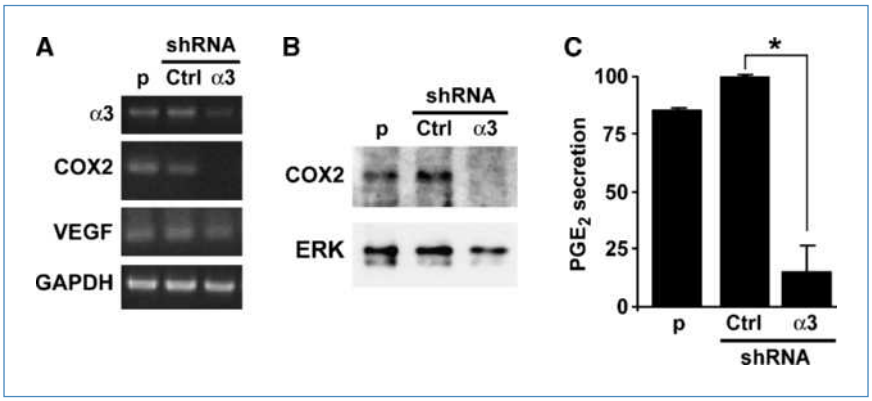


**Figure 4.** α3β1 is required for COX-2 mRNA expression and $PGE_2$ secretion in breast cancer cells. A to C, parental MDA-MB-231 cells (p), MDA-MB-231/α3(+) cells (shRNA, ctrl), or MDA-MB-231/α3(–) cells (shRNA, α3) were cultured for 24 h in serum-free medium. Cells were assayed by (A) RT-PCR for *α3, COX-2*, and *GAPDH* mRNAs, or (B) immunoblot for COX-2 or ERK as a loading control. C, equivalent proportions of conditioned media were assayed for secreted $PGE_2$. Graph shows relative $PGE_2$ levels normalized to that in MDA-MB-231/α3(+) cells (ctrl); columns, mean ($n = 3$ experiments); bars, SEM; *, $P < 0.002$, two-tailed *t* test.

### COX-2 contributes to tumor cell–to–endothelial cell cross-talk and tumor cell invasion

We next used shRNA to stably suppress *COX2* mRNA in MDA-MB-231 cells (Fig. 5A). As expected, COX-2–deficient MDA-MB-231 cells showed reduced tumor growth (Supplementary Fig. S5), consistent with previous reports that COX-2 is necessary for tumorigenesis in xenograft and genetic models (18, 20). Interestingly, HUVECs showed no migration response to conditioned medium from COX-2–deficient MDA-MB-231 (Fig. 5B), implicating COX-2 in tumor cell–to–endothelial cell cross-talk. Tumor cell–derived $PGE_2$ can have both autocrine effects and paracrine effects on endothelial cells (15). Addition of $PGE_2$ directly to endothelial cells did not stimulate migration under our conditions (data not shown), indicating that other factors are required. However, the HUVEC migratory response was restored in conditioned medium from α3β1-deficient MDA-MB-231/α3(–) cells that had been pretreated with $PGE_2$ (Fig. 5C), indicating an autocrine mechanism and is consistent with a previous report that $PGE_2$ strongly induces proangiogenic factors in mammary tumor cells (15). COX-2–deficient cells also showed reduced Matrigel invasion (Fig. 6A), and pretreatment with $PGE_2$ enhanced the invasion of α3β1-deficient MDA-MB-231/α3(–) cells (Fig. 6B). These findings indicate the involvement of COX-2 in two tumor cell functions that we have shown to be regulated by α3β1: (*a*) ability to stimulate angiogenic endothelial cell function and (*b*) invasion.

## Discussion

Although many studies have shown the important roles for integrins in tumor growth and progression (2–4), roles for integrin α3β1 in carcinogenesis remain unclear. In the current study, we show that RNAi-mediated suppression of α3β1 in MDA-MB-231 breast cancer cells leads to decreased tumorigenesis *in vivo* and also inhibits both invasion and production of soluble factors that stimulate endothelial cell migration. In addition, real-time PCR arrays revealed that suppression of α3β1 in MDA-MB-231 cells causes dramatically reduced *COX-2* gene expression (Supplementary Table S1), and RNAi experiments to suppress *COX-2* implicated this gene in the regulation of α3β1-mediated tumor cell functions, including invasion and cross-talk to endothelial cells. Furthermore, treatment of α3β1-deficient cells with $PGE_2$, an important effector of COX-2 signaling, restored both invasive potential and ability to stimulate endothelial cell migration. These findings identify a novel role for COX-2 as a downstream effector of α3β1 in breast cancer cells.

The cyclooxygenases COX-1 and COX-2 control the metabolism of arachidonic acid to prostaglandins (29, 30). Although COX-1 is constitutively expressed in many tissues, COX-2 is induced by proinflammatory or mitogenic stimuli and is upregulated in several human cancers (30). Clinical and preclinical studies strongly support the protumorigenic roles for COX-2 (17). Indeed, the *COX-2* gene is overexpressed in ~40% of invasive breast carcinomas and preinvasive ductal carcinomas *in situ*, and COX-2 promotes breast tumor growth, angiogenesis, and metastasis (14, 29–34). In addition, COX-2–dependent synthesis of $PGE_2$ drives the angiogenic switch during breast cancer progression (15, 29, 33), and numerous pharmacologic and genetic studies support a role for COX-2 in breast cancer (18–20). Importantly, clinical and preclinical studies have supported the development of COX-2 inhibitors as chemopreventative drugs (35, 36). Therefore, our current results suggest the intriguing possibility that suppression of the *COX-2* gene through inhibition of integrin α3β1 could produce similar protective effects.

Our finding that α3β1 was required in breast cancer cells for secretion of factors that stimulate endothelial cell migration is similar to a novel function that we recently described for this integrin during wound healing, in which α3β1 was required in epidermal keratinocytes for secretion of factors that stimulate endothelial cell migration *in vitro* and wound angiogenesis *in vivo* (23). These intriguing similarities suggest a generally important role for α3β1 in promoting communication from the epithelial/tumor cell compartment to the vasculature during normal and pathologic tissue remodeling. Our current findings show that COX-2 may contribute to this effect in tumor cells, consistent with its known ability to induce the expression of proangiogenic factors (15). We have not yet identified α3β1/COX-2–dependent factor(s) that are produced by breast cancer cells to stimulate endothelial cells. However, we have explored the possibility that this effect is mediated by MMP-9; because this MMP is proangiogenic

(37), its induction by extracellular matrix has been linked to COX-2 signaling in some cells (38–40), and we have shown that α3β1 regulates MMP-9 expression in immortalized/transformed keratinocytes (11, 41). Zymography experiments showed that MMP-9 secretion was reduced in α3β1-deficient MDA-MB-231 cells (data not shown), as also reported by another group (9). However, in preliminary experiments, we did not detect reduced MMP-9 in COX-2 knockdown cells, suggesting that MMP-9 production does not require COX-2 in these cells.

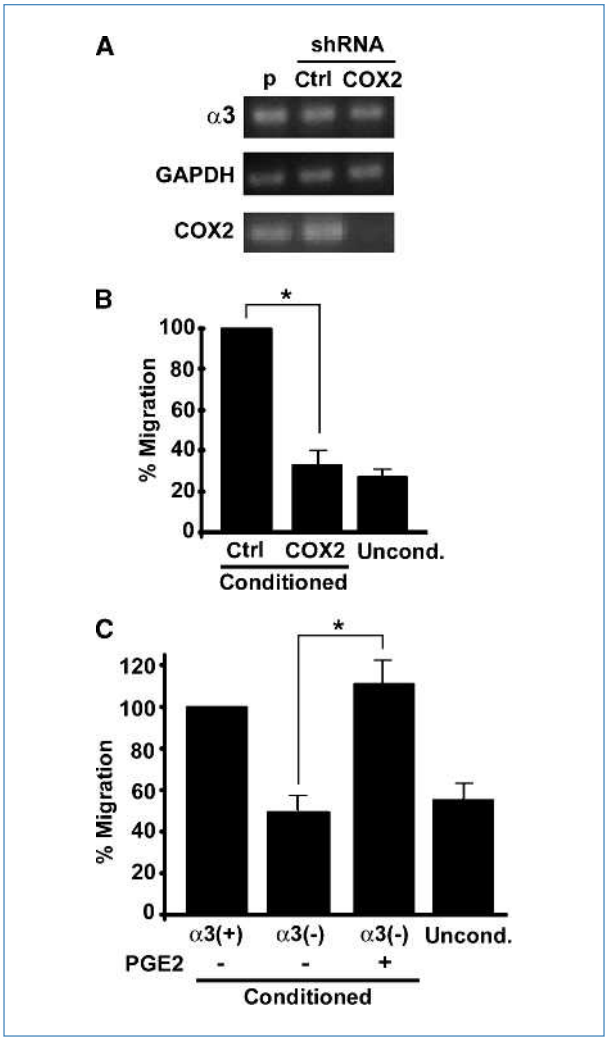


**Figure 5.** COX-2 in breast cancer cells regulates the induction of endothelial cell migration. A, parental MDA-MB-231 cells (p), or cells that stably express nontargeting (ctrl) or COX-2–targeting shRNA (COX2), were assayed by RT-PCR for *COX-2*, *α3*, or *GAPDH* mRNA. B, Transwell assays were performed as in Fig. 2 to compare HUVEC migratory responses to conditioned medium from MDA-MB-231 cells that express control or COX-2–targeting shRNA; uncond, unconditioned medium. Graph shows HUVEC migration as percentage of that in response to medium from control MDA-MB-231 cells. C, HUVEC migration was assayed in response to conditioned medium from MDA-MB-231/α3(+) or MDA-MB-231/α3(–) cells that were either nontreated or pretreated for 24 h with 1 ng/mL $PGE_2$, as indicated. Columns, mean ($n$ = 3 experiments); bars, SEM; *, $P < 0.05$, two-tailed $t$ test.

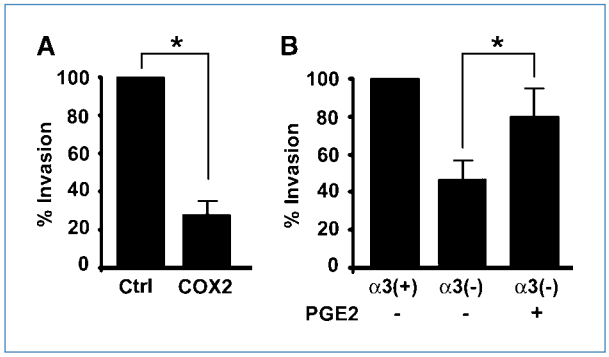


**Figure 6.** COX-2 expression regulates breast cancer cell invasion. A, Matrigel assays were performed as in Fig. 3 to compare the invasion of MDA-MB-231 cells that express control (ctrl) or COX-2–targeting shRNA (COX2). Graph shows invasion as percentage of that in control cells; columns, mean ($n$ = 3 experiments); bars, SEM; *, $P < 0.001$, two-tailed $t$ test. B, invasion assays were performed in MDA-MB-231/α3(+) cells, MDA-MB-231/α3(–) cells, or the latter cells pretreated for 24 h with 1ng/mL $PGE_2$. Graph shows invasion as percentage of that in nontreated MDA-MB-231/α3(+) cells; columns, mean ($n$ = 4 experiments); bars, SEM; *, $P < 0.02$, paired two-tailed $t$ test.

Interestingly, reduced COX-2 expression in α3-silenced MDA-MB-231 cells was not reversed by adenoviral expression of exogenous α3 (data not shown), indicating that this is a stable phenotype and that α3β1-mediated induction of COX-2 occurs through an indirect mechanism. On the other hand, restoring α3 expression in α3-silenced MDA-MB-231 cells did rescue invasion and cross-talk to endothelial cells (Figs. 2 and 3), indicating other α3β1-mediated pathways that can promote these functions independently of COX-2. Consistently, α3-silenced MDA-MB-231 cells also showed changes in other breast cancer–associated genes, including ~22-fold increase in expression of the *CDH1/E-cadherin* gene (Supplementary Table S1). This change is of potential interest because downregulation of E-cadherin is associated with malignancy, and forced expression of E-cadherin suppresses metastatic properties of MDA-MB-231 cells (42). We are currently exploring contributions of this and other α3β1-dependent changes in gene expression to α3β1-mediated tumor cell functions.

To our knowledge, ours is the first study to identify integrin-dependent maintenance of *COX-2* gene expression in tumor cells. Another group showed previously that the noncollagenous domain of type IV collagen, α3(IV)NC1, inhibits hypoxia-induced *COX-2* mRNA expression in endothelial cells by binding to integrin α3β1 on the endothelial cell surface (43). It remains to be determined whether treatment with α3(IV)NC1 would similarly inhibit COX-2 expression in breast cancer cells. However, our current findings that α3β1 enhances, rather than inhibits, COX-2 expression in MDA-MB-231 cells indicate that roles for this integrin differ within distinct cellular compartments of the tumor microenvironment, and they highlight the likely importance of targeting α3β1 specifically on tumor cells to achieve an antiangiogenic effect.

Integrin α3β1 interacts with tetraspanin CD151 on the cell surface (44, 45), and this complex has been shown to regulate cell motility and influence tumorigenic, invasive, and metastatic properties of breast and other carcinoma cells (25, 46–50). RNAi-mediated suppression of CD151 also reduces tumorigenicity of MDA-MB-231 cells (25), suggesting that it may be involved in at least some protumorigenic functions of α3β1. Interestingly, CD151-integrin association was required for tumor cell growth response to factors secreted by endothelial cells (25). Although CD151 on tumor cells also affected vascularization patterns in MDA-MB-231 xenografts, it did not seem to regulate the secretion of factors that induce angiogenic morphology of endothelial cells (25). These findings, together with our current results, suggest that α3β1 and CD151 can each regulate bidirectional communication between tumor cells and endothelial cells, although in some cases, they may do so independently of one another. Future studies will investigate the subset of α3β1-mediated tumor cell functions that require its binding to CD151.

In summary, we have identified a novel role for α3β1 on breast cancer cells in regulating communication with endothelial cells and promoting cell invasion, and we have identified COX-2 as a downstream effector of α3β1. Given the intense focus in recent years on clinical development of COX-2 inhibitors (17), our findings have important implications regarding the potential value of α3β1 as a therapeutic target for breast cancer. The concept of targeting integrins in anticancer therapies is supported by preclinical and clinical development of antagonists of certain integrins (i.e., αvβ3, αvβ5, and α5β1); however, the effects of targeting these integrins result largely from inhibiting their functions on endothelial cells (reviewed in ref. 5). Our current study suggests that targeting α3β1 on breast cancer cells may inhibit both tumor angiogenesis and invasion, in part through suppression of COX-2. Furthermore, because the ability of α3β1 to regulate cell adhesion and MMP expression can also influence carcinoma invasion/metastasis (8–11, 13), targeting α3β1 may have the combinatorial effect of inhibiting multiple tumor cell functions that promote malignant progression.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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