NEOPLASIA
www.neoplasia.com

Volume 10 Number 8 August 2008 pp. 797–803 797

Type I Collagen Receptor ($\alpha_2\beta_1$) Signaling Promotes Prostate Cancer Invasion through RhoC GTPase[1]

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Abstract

The most frequent site of metastasis in human prostate cancer (PCa) is the bone. Preferential adhesion of PCa cells to bone-specific factors may facilitate the selective metastasis of the skeleton. The most abundant protein within the skeleton is type I collagen. We previously demonstrated that PCa cells selected *in vitro* for collagen I binding ($LNCaP_{col}$) are highly motile and acquired the capacity to grow within the bone compared to nontumorigenic LNCaP parental cells. Treatment with $\alpha_2\beta_1$-neutralizing antibodies selectively blocked collagen-stimulated migration, suggesting that integrin signaling mediates PCa migration. To elucidate the mechanism of collagen-stimulated migration, we evaluated integrin-associated signaling pathways in non–collagen-binding LNCaP parental cells and in collagen-binding isogenic C4-2B and $LNCaP_{col}$ PCa cells. The expression and activity of RhoC guanosine triphosphatase was increased five- to eightfold in collagen-binding $LNCaP_{col}$ and C4-2B cells, respectively, compared to parental LNCaP cells. RhoC activation was selectively blocked with antibodies to $\alpha_2\beta_1$ where treatment with a small hairpin RNA specific for RhoC suppressed collagen-mediated invasion without altering the PCa cells' affinity for collagen I. We conclude that the ligation of $\alpha_2\beta_1$ by collagen I activates RhoC guanosine triphosphatase, which mediates PCa invasion, and suggests a mechanism for the preferential metastasis of PCa cells within the bone.

Neoplasia (2008) 10, 797–803

Introduction

Selective adhesion of tumor cells to organ-specific protein factors may influence organ-specific metastasis. The most abundant protein within the bone is collagen type I, which comprises greater than 90% of the total protein within the skeleton [1]. Prostate cancer (PCa) metastasizes to the bone, specifically the pelvis, femur, and vertebral bodies, in greater than 80% of patients [2]. We have previously demonstrated that human PCa cells isolated from bone lesions bound type I collagen with high affinity compared to PCa cells isolated from soft tissue metastases [3]. Further, cells selected *in vitro* for collagen I binding from nonmetastatic LNCaP PCa cells displayed increased surface expression of the integrin collagen I receptor $\alpha_2\beta_1$, exhibited increased collagen-mediated migration, and acquired the ability to grow within the bone [3]. These cells, $LNCaP_{col}$, were found to have increased levels of active RhoC guanosine triphosphatase (GTPase), thus suggesting that collagen I/$\alpha_2\beta_1$ signaling mediates bone metastasis of PCa cells through RhoC activation.

RhoC GTPase is one of three Rho isoforms that belong to the Ras superfamily of small guanosine triphosphate (GTP) binding proteins [4–6]. The members of this family cycle between an inactive guanosine diphosphate (GDP)–bound and active GTP-bound states that stimulate downstream effector proteins [7,8]. The Rho GTPases

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[1]Supported in part by National Cancer Institute Grants P01 CA093900 and R01 CA071672 (E.T.K.), National Institutes of Health, and Department of Defense Prostate Cancer Research Program W81XWH-04-1-0225 and W81XWH-05-1-0005 (K.L.v.G.).
Received 13 March 2008; Revised 12 May 2008; Accepted 13 May 2008

DOI 10.1593/neo.08380

participate in the formation of contractile actin/myosin filaments and therefore act as molecular switches involved in all aspects of cell morphogenesis and cellular motility. Due to their involvement in motility, Rho GTPases have been implicated in tumor progression and metastasis. RhoC, in particular, was shown to participate in the metastasis of several cancers including breast, pancreas, and melanoma [9–11]. We previously demonstrated that RhoC was expressed in bone metastatic PC-3 PCa cells and was responsible for the invasive capabilities of these cells [12].

The mechanism through which RhoC GTPase becomes activated in metastatic cancer cells is unclear. Integrin engagement can stimulate integrin-linked kinase resulting in cytoskeletal reorganization and signal transduction through the activation of the RhoA GTPase [13–15]. Unlike RhoA, however, RhoC has not been previously shown as a downstream effector of integrin signaling. In the present study, we demonstrate that RhoC is activated on integrin $\alpha_2\beta_1$ engagement and regulates the collagen I–mediated invasion of PCa cells. Activation of RhoC invasive programs after collagen I binding suggests a mechanism for the preferential metastasis of PCa cells to the skeleton where collagen I is in abundance.

Materials and Methods

Cells

LNCaP human prostate cancer cells were obtained from the American Type Culture Collection (Rockville, MD). LNCaP cells routinely fail to bind collagen type I in *in vitro* attachment assays and are considered nontumorigenic in nude mice. $LNCaP_{col}$ is an isogenic variant of LNCaP PCa cells that was derived through successive panning on type I collagen [3]. $LNCaP_{col}$ cells have a high affinity for collagen I with corresponding increases in $\alpha_2\beta_1$ integrin expression, collagen-mediated migration, and *in vivo* growth in bone [3]. Cells were maintained in RPMI-1640 medium [10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 1× penicillin–streptomycin, 0.1 mM nonessential amino acids, 2 mM L-glutamine, and 1× vitamin solution (Invitrogen, Carlsbad, CA)] in a 90% air, 10% CO_2 atmosphere at 37°C.

In Vitro *Invasion Assay*

PCa cells were grown to 75% confluence in T-75 tissue culture flasks, harvested using nonenzymatic cell dissociation buffer (Sigma, St. Louis, MO), and washed twice with Hank's balanced salt solution to serum-free growth medium. Cells (2.75×10^5/0.25 ml) were plated in duplicate to collagen I–coated, 3-μm Transwell inserts (BD Biosciences, San Jose, CA). The lower chamber of the Transwell contained normal growth medium with 10% serum. Transwells were incubated for 16 hours at 37°C in a 10% CO_2 incubator. Nonmigrated cells were removed from the upper chamber, and invaded cells were stained with 1% methylene blue and counted under a light microscope. For integrin blocking experiments, cells were harvested and resuspended in a final concentration of 10 μg/ml $\alpha_2\beta_1$ blocking antibody (clone BHA2.1; Chemicon, Temecula, CA) or $IgG_{1\kappa}$ isotype control (BD Biosciences). Data are presented as mean ± SD of duplicate wells from a representative experiment ($n = 4$).

In Vitro *Monolayer Wound Assay*

Glass coverslips, 25 mm in diameter, were coated with 1 μg/cm^2 type I collagen (BD Biosciences) or with human fibronectin (BD Biosciences) for 2 hours at 37°C. After this incubation, the coverslips were washed three times with serum-free medium to remove excess protein and were placed in triplicate in six-well plates (Griener, VWR, Bridgeport, NJ). PCa cells were harvested as previously mentioned and were resuspended in a final concentration of 1×10^5 cells/ml. Approximately 1 ml of cell suspension was added to coverslips and allowed to grow to confluence, approximately 2 days later. Once the cells had reached confluence, a sterile 1000-μl pipette tip was used to wound the cell monolayers across the length of the coverslip. The ability of the cells to migrate on the matrix proteins and fill in the scratch was followed for 16 hours.

Generation of Small Hairpin RNA–Expressing $LNCaP_{col}$ Cells

Small hairpin RNA (shRNA) expression vector for RhoC was obtained from Open Biosystems (Huntsville, AL). Lentivirus particles containing the shRNA were generated by the University of Michigan Vector Core and used to transduce $LNCaP_{col}$ cells. Individual shRhoC clones were selected, and expression of the RhoC and homologous RhoA GTPases were determined by semiquantitative reverse transcription–polymerase chain reaction using Fast SYBR GreenER (Roche, Indianapolis, IN) on an Evocycler (Denville Scientific, Metuchen, NJ). RhoC protein expression was determined by Western blot analysis as previously described [12].

RhoC Activation Assay

The RhoC GTPase activation assay was performed using a modification of the G-LISA RhoA absorbance-based activation assay (Cytoskeleton, Denver, CO). Briefly, LNCaP cells were grown to 75% confluence in 100-mm dishes and then serum-starved for 24 hours by incubating in serum-free medium. On the day of the assay, cells were harvested using nonenzymatic cell dissociation buffer (Sigma), washed twice in Hank's balanced salt solution, and resuspended in serum-free medium to a final concentration of 1×10^6 cells/ml. For each condition, 5×10^5 cells in a final volume of 2 ml were placed in 15-ml conical tubes and 0 or 10 μg/ml $\alpha_2\beta_1$ blocking antibody (Chemicon) or $IgG_{1\kappa}$ isotype control (BD Biosciences) was added and incubated for 15 minutes at 37°C with rocking. After this incubation, 1 μg/ml human collagen I (Sigma) was added, and the cells were incubated for an additional 15 minutes at 37°C with rocking. At the end of the incubation period, all cells were centrifuged gently, washed twice with ice-cold phosphate-buffered saline (PBS), and resuspended in 65 μl of G-LISA lysis buffer. Protein lysates were transferred to ice-cold 1.5-ml centrifuge tubes and clarified by centrifuging at 10,000 rpm for 2 minutes. Protein concentrations were determined using the Precision Red Advance Protein Assay (Cytoskeleton), and 1.0 mg/ml protein was used for the GTPase activation assay as per manufacturer's recommendations with the exception that a RhoC-specific antibody [11] was substituted for the supplied RhoA antibody. In preliminary experiments, the RhoC antibody was titrated to yield optimal signal against 5 ng/well recombinant RhoC-GTPγS protein (a nonhydrolyzable form of GTP bound to RhoC). A 1:50 dilution of the primary antibody and 1:250 dilution of the horseradish peroxidase (HRP)–conjugated secondary antibody were found to produce a RhoC-specific signal comparable to the pan-Rho–specific signals produced by a pan-Rho antibody (Millipore, Billerica, MA). After antibody and HRP detection reagent incubation, signals were detected on a Benchmark Plus microplate spectrophotometer at 490 nm (Bio-Rad Laboratories, Hercules, CA). Data analysis was performed using Prism 4.01 (GraphPad, San Diego, CA).

Binding Assays

Binding assays were performed by seeding 5000 cells in 100 μl of normal growth medium into fibronectin- or collagen I–precoated 96-well plates (BD Biosciences). At the 0, 5, 15, 30, and 60 minute time points, wells were washed thrice with PBS, normal growth medium was replaced, and 50 μl of 5 mg/ml MTT (Sigma) was added. After 2 hours, the MTT-containing medium was removed, the cells were lysed, and the formazan dye was solublized with the addition of 100 μl of DMSO (Sigma). Absorbance values of the lysates were determined on a microplate reader at 530 nm.

Immunofluorescence and Immunohistochemistry

$LNCaP_{Col}$ cells expressing either a scrambled shRNA control (shScr) or shRNA to Rhoc (shRhoC) were grown on collagen I–coated coverslips (BD Biosciences) until 70% confluence, serum-starved for 16 hours, stimulated with 10% FBS for 60 minutes, rinsed three times with 1× PBS, pH 7.4, and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. After fixation, cells were rinsed three times with 1× PBS and blocked with 5% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in 1× PBS, pH 7.4, with 0.3% Triton X-100 for 1 hour at room temperature. Immunofluorescence was performed using fluorescein isothiocyanate–phalloidin (Invitrogen Molecular Probes) in a humidified chamber. After rinsing with PBS, cells were treated with antifade (Invitrogen, Carlsbad, CA), mounted onto SuperPlus slides (Corning, Corning, NY) with Gel Mount (Biomedia, Foster City, CA), and sealed with clear nail polish. Immunofluorescence was performed on a scanning laser confocal microscope (Zeiss, Thornwood, NY) housed in the University of Delaware Department of Biological Sciences.

For immunohistochemistry, tibia sections were warmed to 60°C for 15 minutes, placed in xylene twice for 5 minutes, transferred to 100% ethanol twice for 5 minutes, rehydrated in decreasing concentrations of ethanol for 3 minutes each, and rinsed with ddH_2O. Antigen retrieval was performed by simmering sections in citrate buffer, pH 6.0, for 10 minutes in a microwave followed by a 5-minute incubation with H_2O_2 to block endogenous peroxidases. Primary antibodies, rabbit anti-RhoC (1:150) [16], anti–prostate-specific antigen (PSA, 1:500), or IgG control were diluted in 5% BSA/PBS and incubated overnight at 4°C. After this incubation, the sections were treated for 10 minutes with a biotin link and streptavidin–nickel. Colorimetric visualization was performed with the addition of DAB–peroxidase substrate solution for 10 minutes followed by rinsing. Slides were counterstained with 2% w/v methyl green in 0.1 M NaOAc, pH 4.2, coversliped using Clear-Mount (EMS, Hatfield, PA), and sealed with clear nail polish. Imaging was performed on a fluorescence microscope (Axioscope; Zeiss) at a magnification of ×10.

Results

Blocking $\alpha_2\beta_1$ Decreases Collagen-Mediated Invasion

Fragments of type I collagen were shown to induce chemotactic migration of several tumor cell lines and endothelial cells [17,18]. We sought to define the role of $\alpha_2\beta_1$ integrin in collagen-stimulated directional and random motility using nonmetastatic parental LNCaP PCa cells and bone metastatic $LNCaP_{col}$ cells. $LNCaP_{col}$ cells were derived from LNCaP cells and have elevated $\alpha_2\beta_1$ expression and collagen I attachment [3]. Therefore, when plated on collagen I, $LNCaP_{col}$ cells were stimulated to invade three-fold over parental LNCaP cells, which had a low collagen-stimulated invasion ($P < .0001$; Figure 1*A*). Pretreating cells with a neutralizing antibody to the $\alpha_2\beta_1$ complex decreased the invasion of both LNCaP and $LNCaP_{col}$ cells compared to IgG isotype control antibody treated cells ($P < .003$ and $P < .0001$, respectively; Figure 1*A*). Further, treatment with neutralizing $\alpha_2\beta_1$ antibody decreased $LNCaP_{col}$ collagen-stimulated invasion to that of basal LNCaP cells, indicating that the invasion was mediated by $\alpha_2\beta_1$. The ability to induce migration was specific to collagen I/$\alpha_2\beta_1$ signaling. *In vitro* monolayer wound assays demonstrated that $LNCaP_{col}$ cells plated on collagen were stimulated to migrate into the cleared region within 16 hours where cells plated on plastic or fibronectin failed to migrate (Figure 1*B*). These data suggest that the signal transduction pathways activated by integrins differ depending on the

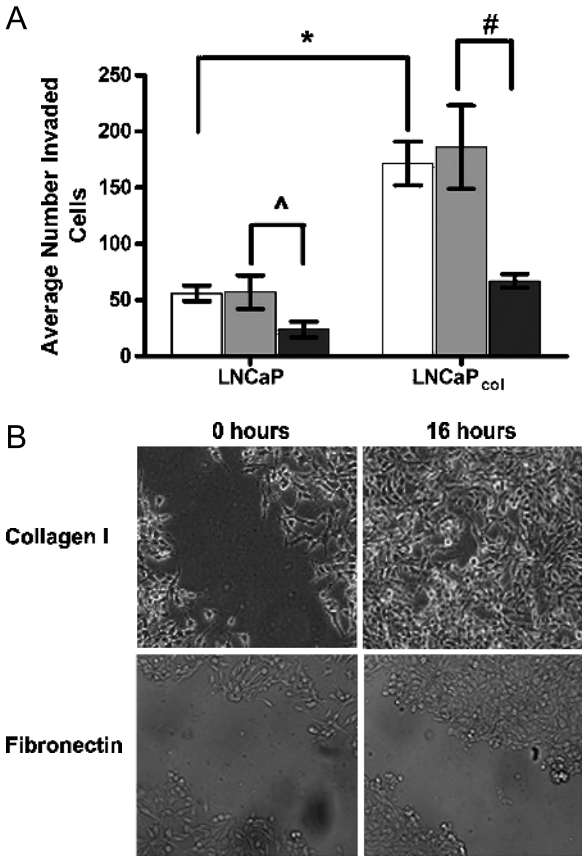

Figure 1. Blocking $\alpha_2\beta_1$ decreases collagen-mediated invasion. (A) *In vitro* invasion through collagen I. PCa cells (2.75×10^5) in serum-free medium were plated to 3-μm collagen I–coated inserts and placed in wells containing 10% FBS growth medium for 16 hours. A final concentration of 10 μg/ml of $\alpha_2\beta_1$ blocking antibody or IgG control was added to the cells at plating as indicated. After this incubation, migrating cells were stained with methylene blue, and the total number of migrating cells/filter was quantified under a magnification of ×10. Data are presented as mean ± SD of duplicate wells from a representative experiment ($n = 4$). *White bars* indicate untreated; *light gray bars*, IgG control; *dark gray bars*, $\alpha_2\beta_1$ blocking antibody–treated cells: $^*P < .001$ compared to LNCaP cells; $^{\wedge}P < .003$ and $^{\#}P < .001$ compared to isotype control cells. (B) *In vitro* monolayer migration wound assay. Cells (1×10^6/ml) were plated to collagen I– or fibronectin-coated coverslips and allowed to grow to confluence. At that time, a channel was scraped in the monolayers, and the ability of the cells to migrate on the matrix proteins was followed for 16 hours.

integrin pairs involved and that the ligation of $\alpha_2\beta_1$ specifically stimulates invasive programs in PCa cells.

Blocking $\alpha_2\beta_1$ Decreases RhoC GTPase Activation

In vitro invasion assays suggest that $\alpha_2\beta_1$-mediated signaling induces cellular migration programs. Analysis of integrin-associated signaling pathways revealed that the expression and activity of *Fak* and the *Src* family kinases (*Src*, *Lyn*, and *Yes*) did not differ within $LNCaP_{col}$ cells (data not shown). We have previously demonstrated using immunoblot analysis that both the active and total amounts of RhoC GTPase were increased in collagen I–binding $LNCaP_{col}$ and C4-2B PCa cells compared to parental LNCaP cells [3]. To enable accurate quantification of RhoC activity, an ELISA-based colorimetric assay for RhoA activity was modified to evaluate GTP-bound or active RhoC protein in $LNCaP_{col}$ cell lysates. RhoC GTPase activity was optimally detected with a 1:50 dilution of a RhoC-specific primary antibody (Figure 2). Treatment of $LNCaP_{col}$ cells in suspension with soluble type I collagen increased the active form of RhoC protein sixfold compared to untreated cells ($P < .0001$; Figure 3*A*). Binding of $LNCaP_{col}$ to fibronectin did not increase RhoC activation (data not shown). Pretreatment with neutralizing antibody to $\alpha_2\beta_1$ specifically and selectively reduced type I collagen activation of RhoC protein by fivefold in $LNCaP_{col}$ cells compared to IgG-treated collagen-stimulated cells, confirming that $\alpha_2\beta_1$ mediated the observed increase in RhoC, $P < .0028$ (Figure 3*B*). To our knowledge, this is the first report to demonstrate that $\alpha_2\beta_1$ integrin ligation by collagen I stimulates the activation of RhoC GTPase.

Blocking RhoC Decreases Collagen-Mediated Invasion

To directly test whether RhoC mediates collagen-stimulated invasion of PCa cells, an shRNA specific to RhoC were transfected into $LNCaP_{col}$ cells and the effect on collagen I–mediated invasion was measured. Western blot analysis confirmed the complete loss of total

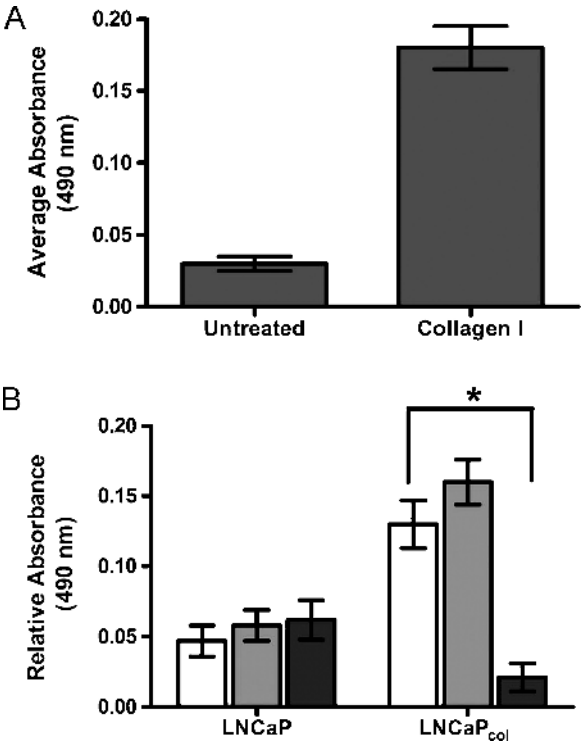

Figure 3. Blocking $\alpha_2\beta_1$ decreases RhoC GTPase activation. (A) Collagen stimulation increases RhoC activation. $LNCaP_{col}$ cells were treated in suspension with 1 μg/ml type I collagen for 15 minutes before lysis and were analyzed for RhoC. Data are presented as the fold increase over parental LNCaP control. (B) Blocking $\alpha_2\beta_1$ integrin (dark gray bars) decreases active RhoC compared to untreated (white bars) or IgG control (light gray bars). Experiments in A were repeated in the presence of 10 μg/ml neutralizing antibody to $\alpha_2\beta_1$ or IgG control and were evaluated for active RhoC protein ($n = 4$), $^*P < .0028$.

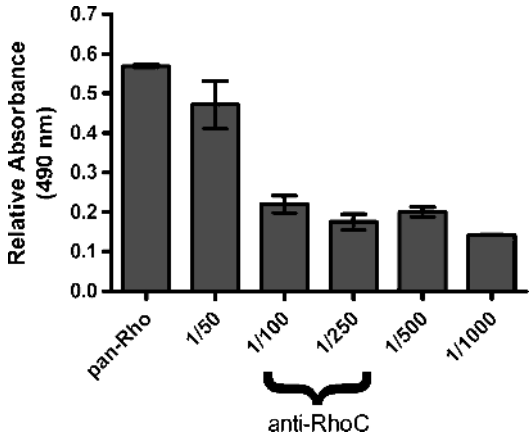

Figure 2. Optimization of RhoC activation assay. The G-LISA RhoA, B, and C absorbance-based GTPase activation assay was optimized for detecting active RhoC GTPase. Recombinant RhoC protein was labeled with nonhydrolyzable GTPγS, and 5 ng of protein was used per assay well. A 1:250 dilution of pan-Rho antibody was compared with decreasing antibody dilutions of a polyclonal RhoC-specific antibody, and a 1:250 dilution of an HRP-conjugated antirabbit secondary antibody. Absorbance was read at 490 nm, and data are presented as the mean ± SD of triplicate wells from a representative experiment ($n = 3$).

RhoC protein expression in the shRhoC cells relative to untransfected and shcontrol $LNCaP_{col}$ cells (Figure 4*A*). As previously reported, no effect was seen on the expression of RhoA GTPase (data not shown) [19]. Reducing RhoC protein expression with shRNA decreased *in vitro* invasion from 75% to 50% compared to untransfected and shcontrol $LNCaP_{col}$ cells, respectively, thus confirming that RhoC mediates collagen I–stimulated invasion ($P < .005$; Figure 4*B*).

Evidence from other laboratories suggest that Rho GTPase activation can lead to a refinement in the affinity of integrin binding to substrates such as type I collagen [20]. To determine whether the decreased invasiveness of shRhoC-transfected cells was due to a reduced affinity for type I collagen, collagen I binding assays were performed (Figure 4*C*). shRhoC-transfected cells bound both collagen I and fibronectin comparably to shcontrol cells, demonstrating that the affinity for collagen I was unaffected by RhoC knock-down at 60 minutes ($P = .3679$ and $P = .5472$, respectively). However, distinct changes in cell morphology due to alterations in the actin cytoskeleton were apparent (Figure 4*D*). Inhibition of RhoC expression led to a profound transition from an epithelial morphology to a spindle-like morphology reminiscent of what was observed in PC-3 PCa cells transfected with a dominant-negative RhoC [12].

We previously demonstrated that the $LNCaP_{col}$ cells could grow in the tibias of nude mice [3]. To examine whether RhoC expression

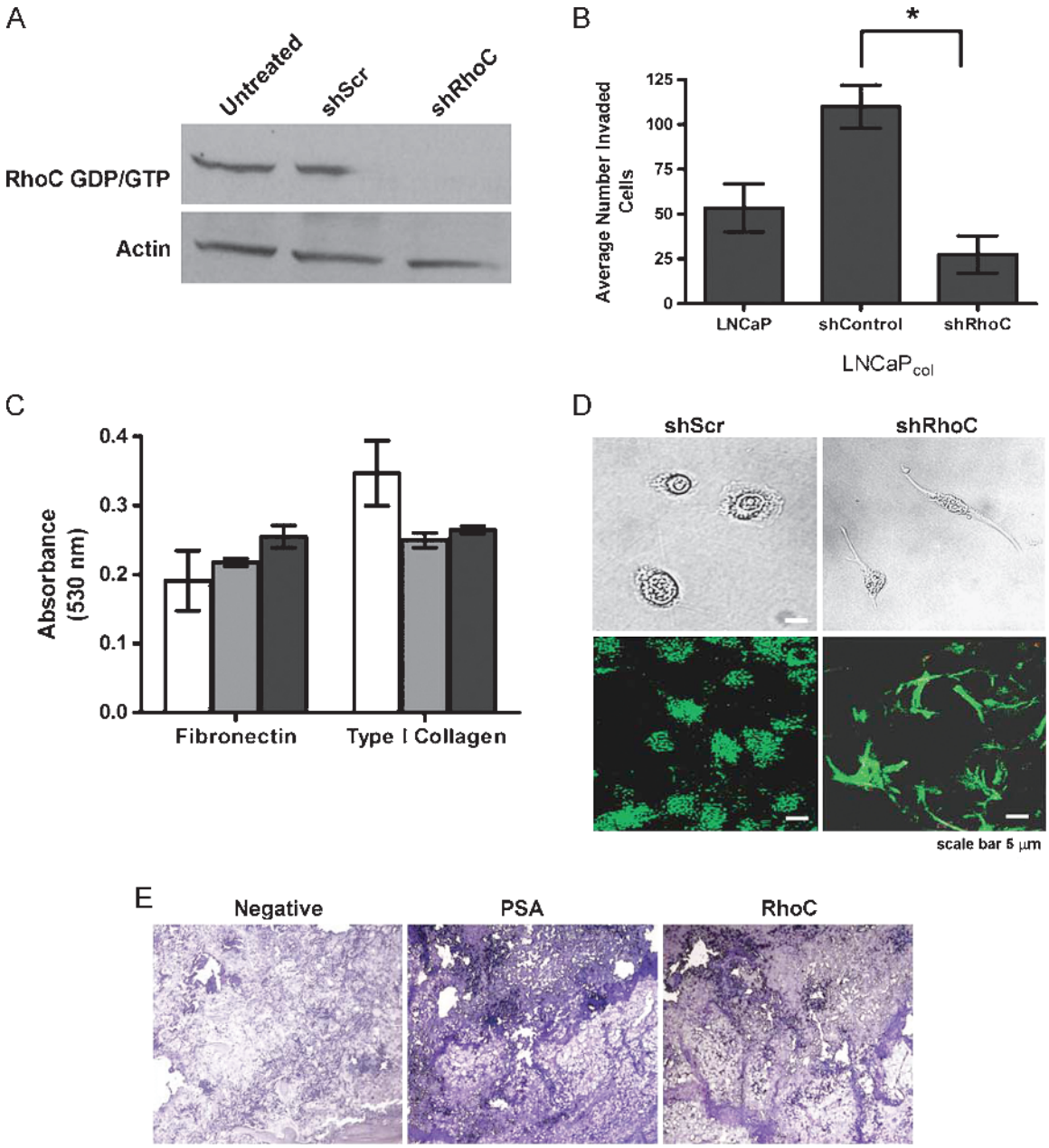

Figure 4. Blocking RhoC decreases collagen-mediated invasion. (A) Western blot for RhoC protein. RhoC protein expression was determined by Western blot analysis as previously described [12]. (B) shRNA to RhoC decreases collagen-stimulated invasion. Human LNCaP and LNCaP$_{col}$ PCa cells were viral-transduced with shRNA molecules RhoC. Transduced cells were plated to collagen-coated invasion chambers, and the extent of invasion was measured after 16 hours. Shown are means ± SD of duplicated wells from a representative assay ($n = 3$), *$P < .005$. (C) RhoC knock-down does not alter collagen attachment. Untransfected (white bars), shScr control (light gray bars), and shRNA RhoC–transfected cells (dark gray bars) were plated to fibronectin- or collagen I–coated wells and were allowed to attach for 15, 30, and 60 minutes, respectively, before thorough washing. The number of adherent cells was determined by MTT staining ($P < .3670$ and $P < .5472$, respectively). (D) RhoC knock-down alters cell morphology and actin cytoskeleton. Control shScr and shRhoC LNCaP$_{col}$ cells were plated on collagen I–coated coverslips, stained with fluorescein isothiocyanate–phalloidin, fixed, and mounted. Shown are phase contrast and immunofluorescent images taken using a confocal microscope at a magnification of ×40. Images are representative of three separate experiments. (E) PSA and RhoC expression overlap in PCa bone lesions. Bone tumors generated after intratibial injection were stained for PSA, to identify PCa cells, and RhoC GTPase by standard immunohistochemistry. Shown are representative lesions of 20.

correlated with the formation of PCa bone lesions, tibias obtained from this published study were stained for PSA, to mark PCa cells, and RhoC. All PSA-positive bone lesions, whether produced by LNCaP (not shown) or LNCaP$_{col}$ cells, stained positive for RhoC GTPase protein expression (Figure 4*E*). Viewed together, the data show that blocking $\alpha_2\beta_1$ decreases RhoC GTPase activation, which reduces collagen I–stimulated invasion. As type I collagen is the major protein component of the bone, these observations suggest a possible mechanism for the selective metastasis of PCa cells to the skeleton.

Discussion

Patients with PCa who die of their disease do so because of the metastatic spread of the tumor cells from the prostate to distant organ sites [21]. A prerequisite for metastatic spread is the ability of the tumor cell to reorganize the cytoskeleton to allow cellular motility. The Rho GTPases comprise a large family of monomeric GTP-binding proteins that are required for cytoskeletal rearrangement [15,22,23]. These proteins are overexpressed in multiple cancers and are linked to enhanced metastatic potential [4–6]. In particular, the expression of RhoC GTPase is associated with poor prognosis in breast and lung cancers [24,25]. Overexpression of RhoC in human mammary epithelial cells and murine lung cancer cells significantly enhanced the metastasis of transfected cells [24]. Inhibition of RhoC GTPase function within these cells through the stable expression of a dominant-negative protein decreased *in vitro* invasion and experimental metastasis *in vivo* [12,24,25]. Consistent with its role in motility, knock-out of RhoC in PyV-mT transgenic mice did not affect tumor development but led to the inhibition of metastasis by decreasing the motility and survival of metastatic cells [26]. The mechanism through which RhoC is thought to regulate motility is through the activation of both the mitogen-activated protein kinase (MAPK) and PI-3 kinase (PI3K) pathways. Specifically, RhoC affects tumor cell motility and invasion through the activation of MAPK where RhoC GTPase activation of PI3K affects attachment independent of growth [27]. The mechanism by which RhoC becomes activated in tumor cells, however, is yet unclear.

Integrins are a large family of proteins, which mediate adhesion to extracellular matrix proteins and link the extracellular matrix to the cytoskeleton [28]. Integrins have been shown to control cell survival, proliferation, differentiation, and metastasis through the activation of intracellular signaling molecules including Src, Fak, MAPK, and PI3K [29–32]. We have previously shown that PCa cells isolated from the bone both adhered to and were chemotactic toward to type I collagen compared to cells isolated from soft tissue lesions [3]. The increased adherence and migration was attributed to increased surface expression of the integrin collagen receptor $\alpha_2\beta_1$ as neutralizing antibodies to $\alpha_2\beta_1$ blocked collagen-mediated properties [3]. In the present study, we report that $\alpha_2\beta_1$ directs collagen I–stimulated invasion through an increased activity of the RhoC GTPase. We found that not only did the basal levels of total and active RhoC increase in collagen-binding cells compared to control cells but also that treatment with collagen I increased the amount of active RhoC protein within collagen-binding cells. Treatment with neutralizing antibodies to $\alpha_2\beta_1$ decreased both active RhoC levels and collagen-stimulated *in vitro* invasion. Transfection with shRNA specific to RhoC blocked collagen-stimulated invasion demonstrating that $\alpha_2\beta_1$ signaling mediates collagen invasion through the up-regulation of RhoC. We further report that matrix-stimulated motility was specific to type I collagen in that cells plated on fibronectin failed to migrate. These data are consistent with previously published reports, which showed that the migration of MDCK cells was inhibited on fibronectin but stimulated when plated on collagens by signaling through the Rho family member Rac GTPase [33].

The activation of RhoC GTPase through collagen I/$\alpha_2\beta_1$ signal transduction suggests a novel pathway for PCa metastasis to the bone. As the bone is rich in collagen I, PCa cells that express high levels of $\alpha_2\beta_1$ would be stimulated to invade the bone through RhoC activation. Collectively, these data support a model in which $\alpha_2\beta_1$ integrin-mediated collagen adhesion plays a fundamental role in the ability of PCa cells to metastasize to the bone microenvironment.

Acknowledgments

The authors thank Stephen Zins (University of Michigan) and Linda Sequeira (University of Delaware) for their technical assistance. The authors also thank the members of the Prostate Cancer Working Group at the University of Delaware for insightful discussions during the preparation of the paper.

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