Published in final edited form as:

Mol Cancer Res. 2013 April ; 11(4): 405–417. doi:10.1158/1541-7786.MCR-12-0551.

Targeting Constitutively Activated β_1 Integrins inhibits Prostate Cancer Metastasis

Yu-Chen Lee^{1,*}, Jung-Kang Jin^{2,8,*}, Chien-Jui Cheng^{6,*}, Chih-Fen Huang^{1,7}, Jian H. Song², Miao Huang¹, Wells S. Brown⁴, Sui Zhang³, Li-Yuan Yu-Lee⁵, Edward T. Yeh³, Bradley W. McIntyre⁴, Christopher J. Logothetis², Gary E. Gallick^{2,8}, and Sue-Hwa Lin^{1,2,8}

¹Department of Molecular Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

²Department of Genitourinary Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

³Department of Cardiology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

⁴Department of Immunology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

⁵Department of Medicine, Baylor College of Medicine, Houston, Texas 77030

⁶Department of Pathology, College of Medicine, Taipei Medical University, Taipei, Taiwan

⁷Department of Pharmacy at National Taiwan University Hospital, Taipei, Taiwan

⁸The Program in Cancer Metastasis, The University of Texas Graduate School of Biomedical Sciences at Houston

Abstract

Disseminated prostate cancer (PCa) cells must survive in circulation for metastasis to occur. Mechanisms by which these cells survive are not well understood. By immunohistochemistry of human tissues, we found that levels of β_1 integrins and integrin-induced autophosphorylation of FAK (pFAK-Y397) are increased in PCa cells in primary PCa and lymph node metastases, suggesting that β_1 integrin activation occurs in metastatic progression of PCa. A conformation-sensitive antibody, 9EG7, was used to examine β_1 integrin activation. We found that β_1 integrins are constitutively activated in highly metastatic PC3 and PC3-mm2 cells, with less activation in low metastatic LNCaP and C4-2B4 cells. Increased β_1 integrin activation as well as the anoikis resistance in PCa cells correlated with metastatic potential in vivo. Knockdown of β_1 integrin abrogated anoikis resistance in PC3-mm2 cells. In agreement with β_1 integrin activation, PC3-mm2 cells strongly adhered to type I collagen and fibronectin, a process inhibited by the β_1 integrin neutralizing antibody mAb 33B6. mAb 33B6 also inhibited the phosphorylation of β_1 integrin downstream effectors, focal adhesion kinase (FAK) and AKT, leading to a 3-fold increase in PC3-mm2 apoptosis. Systemic delivery of mAb 33B6 suppressed spontaneous metastasis of PC3-mm2 from the prostate to distant lymph nodes following intra-prostatic injection and suppressed metastasis of PC3-mm2 to multiple organs following intra-cardiac injection. Thus,

Correspondence: Sue-Hwa Lin, Department of Molecular Pathology, Unit 89, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Phone: 713-794-1559; Fax: 713-834-6084; slin@mdanderson.org; or Gary E. Gallick, Department of Genitourinary Medical Oncology, Unit 18-4, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Phone: 713-563-4919; Fax: 713-792-4198; ggallick@mdanderson.org.

*equal contribution

There are no conflicts of interests among all the authors.

constitutively activated β_1 integrins play a role in survival of PC3-mm2 cells in circulation and represent a potential target for metastasis prevention.

Keywords

prostate cancer; metastasis; integrin; extracellular matrix; collagen; fibronectin

Introduction

Prostate cancer (PCa) is the second most commonly diagnosed malignancy in men (1). While organ-confined PCa is often successfully treated by surgery, effective treatment for metastatic PCa is lacking. PCa cells metastasize to multiple sites including bone, lymph node, lung, liver, soft tissues, and the adrenal gland, with lymph nodes and bone being the most common sites (2). For PCa cells to colonize distant organs, cells that dislodge from the prostate need to survive in the circulation before colonizing metastatic sites (3). Although tumors release cancer cells into the circulation frequently (4, 5), the metastatic process is not efficient, as many disseminated cells do not survive in circulation (4, 6). Thus, survival of tumor cells in the circulation is one of the rate-limiting steps in metastasis (7). Understanding the mechanisms that sustain the survival of disseminated PCa cells will be critical for developing strategies for metastasis prevention.

The integrin family of adhesion molecules mediates cell-extracellular matrix (ECM) interactions and signaling, which are essential for normal cell adhesion, migration, and ECM assembly. The integrins consist of 24 glycoprotein heterodimers, composed of combinations of 18 α and 8 β subunits, which bind to specific subsets of ECM ligands (8). These receptors provide the binding specificity for ECM and transmit signals to promote diverse cellular responses including adhesion, survival and migration (8). Integrin family proteins have been shown to play a role in the survival of tumor cells (9, 10). Integrins may also mediate interactions between tumor cells and the ECM in the vasculature during the extravasation process, before tumor cells reach their metastatic sites. In PCa cells, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha v\beta 1$, $\alpha IIb\beta 3$, and $\alpha v\beta 3$ integrins are expressed (11). These integrins allow PCa cells to interact with multiple ECMs, including type 1 collagen, laminin and fibronectin (12). Scott et al. (13) showed that the binding of PC3 cells to human bone marrow endothelial cells was inhibited by β_1 integrin antibody, but not by antibodies to other integrins, suggesting that the attachment of PC3 cells to bone marrow endothelium is primarily mediated by β_1 integrins. However, a possible role for integrin activation in the promotion of metastasis had heretofore not been examined.

Integrins are activated by both outside-in and inside-out mechanisms. Outside-in signaling is triggered by integrin binding to its ECM ligand, leading to integrin multimerization and recruitment of signaling and cytoskeletal molecules (14–16). Inside-out signaling occurs when cytoplasmic signals, rather than extracellular ECM ligands, trigger integrin activation (17). Inside-out signaling induces a conformational change of integrins and increases their affinity for ECM ligands (8, 18–20), providing prompt signaling upon encountering ECM proteins. The two mechanisms may be mutually regulatory, as inside-out activation may facilitate outside-in activation and vice-versa.

In this study, we demonstrated that constitutive inside-out integrin activation and signaling occur in prostate cells of high metastatic potential and plays a critical role in the survival of PCa cells, thereby enhancing PCa metastasis. We further showed that inhibition of β_1 integrin-ECM interaction by an anti- β_1 integrin neutralizing antibody significantly reduces the metastasis of PCa cells to lymph nodes and bone.

Materials and Methods

Cell lines and reagents

PC3 and LNCaP cell lines were obtained from ATCC. The PC3-mm2 cell line is a highly metastatic cell line derived from PC3 cells through in vivo passages in mice (21) (gift of Dr. Isaiah J. Fidler, MD Anderson Cancer Center). The C4-2B4 is a LNCaP subline (22), kindly provided by Dr. Robert Sikes, University of Delaware. PC3-mm2, LNCaP, and C4-2B4 cell lines were confirmed by fingerprinting. The anti- β_1 integrin monoclonal antibody mAb 33B6 was generated previously (23). Anti- β_1 integrin antibodies mAb MAR4, 9EG7, and 7F10 were purchased from Chemicon, BD Pharmingen and Novocastra, respectively. Anti-pFAK-Y397 and Alexa Fluor 647 conjugated anti-mouse or anti-rat antibodies were purchased from Invitrogen. Anti-total FAK, AKT, pAKT (Ser473), PARP and cleaved PARP antibodies were purchased from Cell Signaling. SCID mice were purchased from Jackson Laboratory (Bar Harbor, ME). All experimental procedures involving animals were performed in compliance with institutional and governmental requirements and approved by M. D. Anderson's Animal Care and Use Committee.

Flow cytometry for β_1 integrin expression

Cells (1×10^6) were plated on fibronectin-coated plates for 15 min, detached with trypsin, and incubated with β_1 integrin antibodies mAb, MAR4 or 9EG7 for 1 hour at 4°C, followed with Alexa Fluor 647-conjugated anti-mouse or rat antibody for 1 hr, respectively. The fluorescence intensity of the cells was measured by a BD FACSCantoII flow cytometer (BD Biosciences). Cells incubated with mouse or rat normal IgG were used as controls.

FAK and AKT phosphorylation, PARP cleavage, and apoptosis assays

PC3-mm2 cells plated on collagen-coated plate were treated with control IgG or mAb 33B6 for 24 h. The apoptotic PC3-mm2 cells were detected with sulforhodamine 101-annexin V and DEVD-NucView 488 caspase-3 substrate using NucView™ 488 dual apoptosis assay kit (Biotium). Cell lysates were used in immunoblots with antibodies against total and or cleaved PARP (Cell Signaling), phospho-FAK-Y397-the autophosphorylation site resulting from activated integrins-Cell Signaling), or phospho-AKT (Ser473) and total AKT (Cell Signaling).

Anoikis assay

LNCaP, C4-2B4, PC3, and PC3mm2 cells were detached by 2mM EDTA, washed with medium, and resuspended in serum-free medium at 8×10^4 cells/ml. Fourteen ml of cell suspension was added in a 15 ml conical tube with vent cap, and rotated in an incubator to prevent cell aggregation. For counting live and dead cells, 500 μ l of cell suspension was incubated with 2 μ M Calcein AM (BD Biosciences) for 30 min at 37°C followed with 4 μ M propidium iodide (BD Biosciences) for 30 min at 37°C. Cells were counted using a fluorescence microscope. To detect apoptotic cells, the cell suspension was centrifuged and washed with PBS, fixed by cold methanol for 30 min on ice and washed again. Cells then were resuspended in PBS with 2 mg/ml Ribonuclease A (Sigma, St. Louis, MO) and 50 μ M propidium iodide for 30 min on ice, and analyzed by flow cytometry.

β_1 -integrin knockdown

A β_1 integrin viral plasmid (pLKO.1-puro (Sigma, St. Louis, MO) harboring shRNA 5'-GCCCTCCAGATGACATAGAAA-3') was used to produce lentivirus. A second, non-targeting sequence 5'-GCGCGATAGCGCTAATAATTT-3' cloned in the same vector (Sigma) was used as a control plasmid. To make lentivirus, the viral plasmid was co-transfected with the packaging plasmid pCMV-dR8.2 dvpr and the envelope plasmid

pCMV-VSVG (gifts from Dr. Yutong Sun, MD Anderson Cancer Center) into 293FT cells using Lipofectamine 2000 (Invitrogen). Viral supernatant was collected after 48–72 hrs and centrifuged at 20,000 rpm for 2hrs to pellet virus particles, which were then resuspended in RPMI medium. PC3-MM2 cells were infected by incubating them with virus particles in the presence of 8 $\mu\text{g}/\text{ml}$ of polybrene (Sigma) for 24 hrs, and then medium was replaced with 5 $\mu\text{g}/\text{ml}$ puromycin for one week to select stable $\beta 1$ integrin knockdown cells. Cells with decreased $\beta 1$ integrins have remained stable for five passages.

Adhesion assay

PC3-mm2 cells (5×10^4 cells/100 μL) pre-labeled with 1 μM calcein AM (Invitrogen) were mixed with equal volume of 20 $\mu\text{g}/\text{mL}$ mouse IgG, the anti- $\beta 1$ integrin mAb 33B6, or medium only, and seeded onto 96-well plates that were pre-coated with 20 $\mu\text{g}/\text{mL}$ collagen-I, fibronectin, or BSA. The plates were incubated for 30 min at 37°C; the wells were washed with PBS twice and the cells that adhered to the plate were quantified by measuring the fluorescence intensity at 485/528 nm in each well on a Synergy HT fluorescent plate reader (BioTek).

Parallel plate flow chamber detachment assay

The parallel plate flow detachment assay was performed as described previously (24). In brief, fibronectin (10 $\mu\text{g}/\text{mL}$) or collagen-I (50 $\mu\text{g}/\text{mL}$ in 0.1M NaHCO_3) was immobilized onto plastic slides. The slides were blocked with 2% BSA before placing into a parallel plate flow chamber. PC3-mm2 cells (2×10^6) treated with or without mAb 33B6 (5 $\mu\text{g}/\text{mL}$) for 5 min at 37°C were injected into the flow chamber and allowed to settle on the slide for 10 min. A computer controlled syringe pump (Harvard Apparatus) was used to apply an increasing linear gradient of shear flow to the adhered cells for 300 seconds and the number of cells remaining on the slide was recorded. Shear stress calculations were determined as described (24).

In vitro spreading assay

PC3-mm2 cells (5×10^4) were treated with 20 $\mu\text{g}/\text{mL}$ mouse IgG, or 20 $\mu\text{g}/\text{mL}$ anti- $\beta 1$ integrin antibody, or control buffer, seeded onto coverslips coated with collagen-I (20 $\mu\text{g}/\text{mL}$), fibronectin or BSA (20 $\mu\text{g}/\text{mL}$), incubated for 1h, and fixed with 4% formaldehyde. Cell morphology was examined under a microscope.

Migration assay

Falcon HTS FluoroBlok inserts were coated with collagen-I, fibronectin or BSA at a density of 5 $\mu\text{g}/\text{cm}^2$ overnight. Cells (10^5 cells in 0.3 mL/insert) in serum free RPMI medium containing 20 $\mu\text{g}/\text{mL}$ mouse IgG or mAb 33B6 were seeded on the inserts and incubated at 37°C for 24 h. The migrated cells were labeled with 1 μM calcein AM and counted.

Apoptosis Assay

PC3-mm2 cells plated on collagen-coated plate were treated with control IgG or mAb 33B6 for 24 h. The apoptotic PC3-mm2 cells were detected with sulforhodamine 101-annexin V and DEVD-NucView 488 caspase-3 substrate using NucView™ 488 dual apoptosis assay kit (Biotium, Hayward, CA). Cell lysates were used in immunoblots with antibody against cleaved PARP.

Proliferation assay

Cells were mixed with 20 $\mu\text{g}/\text{mL}$ mAb 33B6, mouse IgG, or buffer and plated onto the collagen, fibronectin, or BSA-coated plate. Cells were incubated at 37°C for 1–4 days. Cell proliferation was determined by viable cell counting.

Intracardiac, intra-prostatic injection and bioluminescence imaging of mice

PC3-mm2 cells were transduced with a retrovirus containing luciferase (Luc) and GFP genes as described previously (25). PC3-mm2-Luc cells were mixed with control IgG or mAb 33B6 at 30 μ g/mouse for 30 min before injecting into the left ventricle (1×10^6 cells/mouse) or the prostate (0.5×10^6 cells/mouse) of male SCID mice. Mice were injected with control or anti- β_1 integrin antibody (30 μ g/mouse or 1 mg/kg) before cell inoculation and twice per week thereafter. Tumor growth was monitored weekly using bioluminescence imaging. Images were acquired and analyzed with an IVIS Imaging System (Xenogen).

Immunohistochemical analysis

Formalin-fixed, paraffin-embedded PCa tissue samples from prostate and lymph node metastases obtained from the PCa tissue bank (supported by a SPORE award to The University of Texas M.D. Anderson Cancer Center) were immunostained with mAb 7F10 or anti-pFAKY397 according to the manufacturer's recommended procedures. The immunostaining was considered positive when more than 10% of the tumor cells were immunoreactive.

Statistical analyses

Two-tailed, paired Student's t test was used for statistical analyses. A P-value of less than 0.05 was considered statistically significant. Data are expressed as the means $\pm$ SD unless otherwise specified. The Chi-square test was used for immunohistochemical analysis.

Results

β_1 integrin expression and activation in human PCa specimens

To assess whether integrin activation occurs in metastatic progression in human PCa, we examined the expression of β_1 integrins in human specimens from normal prostate glands, localized PCa, and lymph node metastases. Immunohistochemical analysis showed that in normal prostate glands, β_1 -integrins are expressed on the basal cell layer and localized at the basal cell/stromal interface, where integrins interact with ECM (Fig. 1A). In PCa specimens, where basal cells are absent, β_1 integrins were detected in the membranes in 13 of 20 (65%) primary PCa (Fig. 1A, and Table 1A), suggesting that β_1 integrins were upregulated in PCa cells. In lymph node metastases, β_1 integrins were detected in PCa cells in 13 of 18 (72%) lymph node specimens (Fig. 1A and Table 1A). The difference in expression between the normal prostate and primary PCa or lymph node metastasis is significant ($p < 0.001$ by Chi-square test). However, there was no correlation between expression of β_1 integrins and the Gleason score, and no significant difference in expression of β_1 integrins between primary PCa and lymph node metastasis was observed (Table 1A).

To examine whether integrin activation occurs in PCa progression, human PCa specimens were examined for the autophosphorylation of FAK at Y397, which results from integrin activation (9). Of the 16 PCa specimens that were evaluated, seven showed positive staining for pFAK-Y397 (Fig. 1B and Table 1B). Of the 12 lymph node metastases, seven showed positive staining for pFAK-Y397 (Fig. 1B and Table 1B). Together, these results suggest that activation of β_1 integrins occurs during PCa progression.

β_1 integrin activation in PCa cell lines

To examine whether activation of β_1 integrins plays a role in PCa metastasis, we determined β_1 integrin expression and activation in several available PCa cell lines, including the lymph node derived LNCaP and its castration-resistant variant C4-2B4, as well as the bone-derived PC3 and its metastatic variant PC3-mm2 cells. LNCaP and C4-2B4 are tumorigenic,

however, with low metastatic potential when implanted orthotopically or intracardially. PC3 and PC3-mm2 are tumorigenic and highly metastatic, exhibiting high incidence of metastasis in both spontaneous and experimental metastasis models (25, 26). Using anti- β_1 integrin mAb MAR4, FACS analysis showed that all four cell lines expressed high levels of β_1 integrins (Fig. 2A). Next, we examined integrin activation in these cell lines. Western blot showed that the levels of phosphorylation of pFAK397 are higher in PC3 and PC3-mm2 than LNCaP and C4-2B4 (Fig. 2B), suggesting that integrin signaling is activated in PC3 and PC3-mm2 cells. We further used the conformation-sensitive antibody 9EG7 (27–29) to examine whether β_1 integrins in these cells were present in the activated conformation. 9EG7 bound to PC3 and PC3-mm2 strongly (Fig. 2C). In contrast, 9EG7 showed modest binding to LNCaP and C4-2B4 (Fig. 2C). These observations suggest that β_1 integrins are present in an activated conformation only in cell lines with high metastatic potential.

β_1 integrin activation correlates with anoikis resistance in PCa cell lines

Next, we examined whether increased β_1 integrin activation in PCa cells correlated with cell survival by using an anoikis assay. Single cells from PCa cell lines were placed in suspension with constant rotation for 24 and 48 hrs to prevent aggregation-induced outside to inside integrin aggregation and the number of viable and dead cells determined. As shown in Fig. 2D, PC3-mm2 has the highest number of viable cells at 48h, while LNCaP and C4-2B4 cells did not survive in these anoikis conditions. The decreases in viable cell number corresponded to the increases in dead cell number (Fig. 2D). Analysis of nuclear integrity by propidium iodide staining showed that the increased dead cell number in anoikis sensitive cell lines correlated with an increase in the sub-G1 fractions (Fig. 2E), suggesting that these cells had undergone apoptosis. Consistent with this result, western blot analysis showed a correlation between anoikis sensitivity and increases in PARP cleavage (Fig. 2F). These observations indicate that the ability of PCa cells to survive in anoikis conditions correlates with the activation status of β_1 integrins and also the metastatic potential of these cell lines.

Silencing of β_1 integrins in PC3-mm2 cells by expression of shRNA abrogates anoikis resistance

To examine the causal effect of β_1 integrins on PCa cell survival, we used stable expression of shRNA to knockdown β_1 integrins in PC3-mm2 cells. As shown in Fig. 3A, transfection of β_1 integrin-specific shRNA reduced the expression of β_1 integrins by more than 90%, while control shRNA did not have significant effects on β_1 integrin expression. To examine the effects on integrin-mediated survival pathways, phosphorylation of FAK397 and AKT473 were examined. Knockdown of integrin greatly reduces pFAK397 and pAKT473 phosphorylation (Fig. 3A). Further, the β_1 integrin knockdown cells in suspension are sensitized to anoikis (Fig. 3B), with a concomitant increase in PARP cleavage (Fig. 3C). These results suggest that β_1 integrins play a critical role in the survival of highly metastatic PC3-mm2 cells.

Effects of mAb 33B6 on β_1 integrin-mediated cell adhesion and migration

We reasoned that PC3 and PC3-mm2 cells have increased inside-to-outside activation of β_1 integrins, leading to increased ECM binding affinity, which increases the adhesion, migration, and survival of the tumor cells during extravasation. Thus, we examined the effects of mAb 33B6, a β_1 integrin-neutralizing antibody, on ECM-mediated adhesion, migration, and apoptosis of PC3-mm2 cells. In a static binding assay, PC3-mm2 adhered to type I collagen or fibronectin coated dishes efficiently compared to non-coated or BSA-coated plates (Fig. 4A) and the adhesion was inhibited by mAb 33B6 but not by control IgG (Fig. 4A). The β_1 integrin-mediated adhesion was further examined under flow conditions using the parallel plate flow detachment assay that mimics tumor cells in circulation. mAb

33B6 led to the detachment of PC3-mm2 cells from collagen or fibronectin-coated surface when compared to control IgG (Fig. 4B). When plated on type I collagen or fibronectin coated plates, PC3-mm2 cells showed a flattened morphology with lamellipodia-like projections, but remained rounded when plated on BSA coated plates (Fig. 4C). The cell spreading on collagen or fibronectin-coated plates was inhibited by mAb 33B6 (Fig. 4C). We further examined the effect of mAb 33B6 on the migration of PC3-mm2 cells. Little migration of PC3-mm2 cells was observed on the control BSA-coated wells, but the migratory activity was significantly stimulated by type 1 collagen or fibronectin (Fig. 4D). The ECM-induced PC3-mm2 migration was significantly inhibited by mAb 33B6 but not by control IgG ($P < 0.05$). In addition, treatment of PC3-mm2 cells with mAb 33B6 led to the inhibition of FAK and AKT phosphorylation (Fig. 4E). This treatment led to an approximate 3-fold increase in annexin V staining and caspase 3 activation compared to IgG-treated control cells (Fig. 4F). Western blotting showed a 2–3 fold increase in PARP cleavage upon treatment with mAb 33B6 (Fig. 4F), suggesting that blocking PC3-mm2 interaction with ECM resulted in an increase in PC3-mm2 cell apoptosis. Because mAb 33B6 induced ~3% apoptosis in PC3-mm2 cells (Fig. 4F) and reduced migration of these cells to ~10–20% relative to that of untreated control cells, the effects of mAb 33B6 on PC3-mm2 cell migration are not due to its effect on cell viability. However, ECM inhibition did not affect the proliferation of PC3-mm2 cells on type I collagen, fibronectin or BSA-coated plates (data not shown). mAb 33B6 also did not have significant effect on the proliferation of PC3-mm2 cells in serum-free medium (data not shown). Together, these results demonstrate that mAb 33B6 inhibits ECM-mediated binding, spreading, migration, and survival of PC3-mm2 cells.

Inhibition of PC3-mm2/ECM interaction decreases lymph node metastasis in vivo

Next, we examined the role of PCa/ECM interaction in the ability of PCa cells to metastasize to lymph nodes. Dislodged PCa cells that survive in the circulation usually metastasize first to lymph nodes adjacent to the prostate, then to distant lymph nodes (30, 31). We used the intra-prostatic injection model that mimics spontaneous metastasis in vivo and examined whether inhibition of PC3-mm2/ECM interaction would block the metastasis of PC3-mm2 cells from the prostate to the lymph node. Luciferase-labeled PC3-mm2 cells were implanted intraprostatically into SCID mice and the mice were treated with or without mAb 33B6 twice per week. Tumor growth in the prostate and its metastases in the lymph nodes were monitored using bioluminescence. At 2-weeks post-injection, we observed that PC3-mm2 had metastasized from the prostate to multiple lymph nodes in the control IgG injected mice (Fig. 5A). In contrast, no obvious metastases in distant lymph nodes were detected in mice treated with mAb 33B6 (Fig. 5A). At 3-weeks post-injection, mice were killed and tumors were harvested from the primary (prostate) and metastatic (lymph nodes) sites. In the control IgG-treated mice, PC3-mm2 cells metastasized to both regional and distant lymph nodes, based on bioluminescence imaging and post-mortem tissue harvesting (Fig. 5A). In the mAb 33B6-treated mice, PC3-mm2 cells mainly metastasized to regional lymph nodes (Fig. 5A). Upon postmortem dissection, the presence of tumor cells in the lymph nodes was further confirmed by histology. The incidence of lymph node metastasis from intraprostatic injection of PC3-mm2 cells was 100%. An average of 7.2 ± 2.6 lymph node metastases versus 2.3 ± 1.8 ($p < 0.001$) was detected in the IgG-treated and mAb 33B6-treated mice, respectively (Fig. 5B). The decreases in tumor metastases to distant lymph nodes in mAb 33B6-treated group are not due to tumor size as one of the mice in mAb 33B6-treated group produced a large tumor but exhibited metastasis only to the local lumbar lymph node (data not shown). In addition, the average tumor sizes were similar between the control and mAb 33B6-treated groups (Fig. 5C). These results indicate that inhibition of interaction of PC3-mm2 cells with the ECM limits the ability of tumor cells to metastasize to distant lymph nodes.

Inhibition of PC3-mm2/ECM interaction on disseminated PC3-mm2 cells in vivo

Next, we examined whether blocking PC3-mm2/ECM interaction by mAb 33B6 affects the metastatic growth of disseminated PC3-mm2 cells. We mimicked the hematogenous dissemination of cancer cells by injecting luciferase-labeled PC3-mm2 cells intracardially. Bioluminescence imaging was used to examine the metastases of PC3-mm2 in vivo. PC3-mm2 cells were considered disseminated into the circulation if bioluminescence signals were detected throughout the whole-body (Fig. 6A). Bioluminescence signals subsided one day post-injection (data not shown) and the signals started to appear at multiple organ sites at 7 days post injection (Fig. 6A). Tumor volumes in the control IgG-treated group exhibited an exponential growth at multiple organ sites at 2- and 3-weeks post injection (Fig. 6A). In contrast, the growth of tumors in the mAb 33B6-treated mice was significantly decreased (Fig. 6A). Quantification of the tumor sizes based on bioluminescence intensity showed no significant difference in tumor volume between the control and mAb 33B6-treated mice at 7 days post tumor injection; however, the average tumor size was significantly smaller at 14 days, with mAb 33B6-treated tumor burden only 7% of that in the control group ($p < 0.003$). At 20 days, the average tumor burden of anti- β_1 integrin-treated mice was 6% of those of control mice ($p < 5 \times 10^{-5}$) (Fig. 6B). We also examined the tumor burden in femurs/tibias and found that the tumor sizes were also significantly reduced in the mice treated with mAb 33B6 (Fig. 6C). These in vivo data are consistent with the in vitro data, which together indicate that activation of β_1 -integrins increases tumor cell survival and extravasation, resulting in increased tumor metastasis to distant metastatic sites.

Discussion

Our studies suggest that constitutive activation of β_1 integrins plays a critical role in PCa metastasis. Metastasis is a multi-step process and failure to complete any one of these steps will prevent tumor cell colonization at distant sites (4, 32). Two of the key steps for PCa cells to successfully metastasize are survival in the circulation and extravasation of the cells from the circulation into the bone microenvironment. Our results suggest that constitutively activated integrins in PCa cells contribute to their metastatic potential by increasing their resistance to anoikis-induced cell death during tumor dissemination and enhancing their interactions with ECM during extravasation. A model that summarizes the in vitro and in vivo results is shown in Fig. 6D. We suggest that in highly metastatic PCa cells, β_1 integrins are present in an activated conformation through an inside-out activation mechanism, which increases its ligand binding affinity. Upon binding with ECM, β_1 integrins are further activated by an outside-in mechanism that results in integrin clustering and activation of a downstream signaling cascade that enhances the metastatic potential of PCa cells (Fig. 6D). Our in vitro and in vivo findings are further corroborated with the observations that in human prostate specimens, where not only was β_1 integrin expression upregulated, but its downstream signaling molecule FAK was also activated (pFAK397) in prostate tumors and lymph node metastases, suggesting that this mechanism is clinically relevant. Our studies thus identify constitutive activation of β_1 integrins as one of the critical mechanisms in the metastatic progression of PCa and suggest that specifically targeting activated β_1 integrins or integrin signaling may inhibit PCa metastasis.

β_1 integrin isoforms

β_1 integrins contain several alternatively spliced variants. For the human β_1 subunit, at least five different cytoplasmic variants, i.e. β_{1A} , β_{1B} , β_{1C} , β_{1C-2} , β_{1D} have been identified (33). Among the five known β_1 integrin cytoplasmic variants, β_{1A} and β_{1C} have been shown to differentially affect prostate cell functions (34). The β_{1A} variant was shown to act as a stimulator of cell proliferation, whereas the β_{1C} variant acted as an inhibitor of proliferation. Goel et al. (35) further demonstrated that β_{1C} expression increases the levels of an

extracellular matrix protein thrombospondin 1, which inhibits angiogenesis. While β_{1A} protein is ubiquitously expressed, β_{1C} protein levels are reduced in neoplastic prostate epithelium (34). PCa cell lines, including PC3, LNCaP and DU145, mainly express β_{1A} integrins (35). Thus, the β_1 integrins activated in PC3-mm2 cells are the β_{1A} variants.

Inside-out integrin activation

The mechanism(s) by which β_1 integrins are activated in PC3 and PC3-mm2 cells are not known. Integrins are normally expressed in an inactive state and can be activated through inside-out or outside-in mechanisms. While the ECM ligand-induced outside-in integrin activation increases the avidity (36, 37), inside-out integrin activation increases its ligand binding affinity, cell adhesion, migration, and ECM assembly (8, 18–20). The activation of β_1 integrins in PC3-mm2 cells is likely through an inside-out mechanism, which induces conformational changes of β_1 integrins in the absence of ECM ligands (17). Diverse mechanisms have been shown to contribute to the inside-out activation, which include interaction of the integrin cytoplasmic domain with its regulatory molecules (20, 38), biochemical modification of the integrin molecule, and regulation by extracellular growth factors (39). Talin binding to the cytoplasmic domain of integrins is one of the mechanisms that leads to inside-out integrin activation (20, 38). Sakamoto et al. (40) showed that talin1 overexpression increased PCa cell adhesion, migration and resistance to anoikis, and talin1 levels were higher in metastatic tissue compared with primary prostate tumors. Talin1 overexpression was also observed in aggressive oral squamous cell carcinomas (41). These observations suggest that increase in talin1 levels may play a role in the inside-out activation of β_1 integrins in PC3-mm2 cells. However, we did not detect differences in talin1 expression among C4-2B4 and PC3-mm2 cells by Western blot (data not shown). Extracellular growth factors have been shown to activate integrins (39). In breast cancer cells, Chandrasekaran et al. (42) showed that insulin-like growth factor-1 and CD98 regulate $\alpha_3\beta_1$ integrin activation. Interestingly, Chatterjee et al. (43) showed that the chemokine CCL2 increased β_1 -integrin activation in PC3 cells. Both IGF-1 and CCL2 have been shown to be upregulated during PCa progression (44, 45), suggesting that these or other growth factors may regulate activation of β_1 integrins in PC3-mm2 cells. Delineating the mechanisms that lead to inside-out activation of β_1 integrins in PC3-mm2 cells may provide additional strategies for metastasis prevention. Further study on the mechanisms of integrin activation and mechanisms by which β_1 integrins become constitutively activated in highly metastatic PCa cells are warranted.

Targeting activated β_1 -integrins as anti-metastatic therapy

While our study and several other studies showed that blocking integrin/ECM interactions is effective in inhibiting tumor growth (35, 46, 47) or metastasis (11, 48) in mouse tumor models, ECM proteins are also involved in many cellular activities essential for normal cell functions, raising the possibility of high toxicity with anti-integrin therapy. However, our studies showed that β_1 integrins are activated in highly metastatic but not in low metastatic PCa cells or normal prostate epithelial cells. Thus, an anti- β_1 antibody that specifically inhibits the “activated conformation” of β_1 integrins may increase target specificity in selectively targeting only activated integrins. Consistent with such an idea, a monoclonal antibody that targets a tumor-specific, conformationally exposed epitope of wild-type EGFR (mAb 806) has recently been developed for the purpose of reducing the side-effect from anti-EGFR treatments (49). Whether it is possible to generate such a conformation-specific antibody for therapeutic application remains to be seen. On the other hand, an antibody against α_v integrin (DI17E6) is being tested in a clinical trial for the treatment of progressive castrate-resistant PCa with bone metastasis (50). α_v integrin is expressed in a number of human cancers, including PCa (11), as well as cells in the tumor environment, e.g. osteoclasts, osteoblasts, and angiogenic blood vessels. A preliminary report suggests

that DI17E6 was well tolerated (50). Completion of this study will reveal whether blocking tumor cell/ECM interaction is feasible for cancer treatments. Because metastasis is the major cause of cancer mortality, there is a strong interest in developing “anti-metastatic” therapies, especially in PCa, as the disease is often caught early and there is nearly a 10-year “window” during which anti-metastatic therapy would be useful as “secondary prevention.” In addition to anti-integrin antibodies, several “anti-metastatic” therapies are also being developed, including the use of anti N-cadherin (51), anti-cadherin 11 (25) antibodies.

PCa cell/ECM interactions at distant metastatic sites

Besides occurring in circulation, PCa/ECM interactions have been shown to be involved in the progression of cancer in their metastatic sites (4, 52, 53). Type I collagen is the most abundant ECM in bone, raising the possibility that β_1 integrins may play a role in metastatic progression of PCa in bone. Indeed, Kostenuik et al. (54) showed that bone cell matrix promotes the adhesion of human PC3 cells via $\alpha_2\beta_1$ integrins. Hall et al. (55) showed that LNCaP cells selected for binding to collagen exhibited increased levels of the $\alpha_2\beta_1$ integrins and increased tumorigenesis in bone when the cells were injected into mouse tibia. In this study, we found that mAb 33B6 reduced tumor volume in bone when PC3-mm2 cells were injected intracardially. Whether mAb 33B6 also inhibits PC3-mm2 cell/bone matrix interaction requires further investigation.

Metastatic progression of PCa significantly impacts the survival of men with PCa. Thus, there is an urgent need to identify strategies for the prevention or treatment of metastasis. β_1 integrin antibody has been previously shown to inhibit breast (46) or prostate (35) tumor growth at subcutaneous sites. Our studies provide new evidence that constitutively activated β_1 integrins play a role in the metastasis process and our pre-clinical studies suggest that strategies that inhibit activation of β_1 integrins may be developed for preventing PCa metastasis.

Acknowledgments

This work was supported by grants from the NIH including CA111479, P50 CA140388 and CA16672, the Prostate Cancer Foundation, DOD PC093132 and PC080847, Cancer Prevention and Research Institute of Texas (CPRIT RP110327).

References

1. Siegel R, Ward E, Brawley O, Jemal A. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J Clin.* 2011; 61:212–36. [PubMed: 21685461]
2. Shah RB, Mehra R, Chinnaiyan AM, Shen R, Ghosh D, Zhou M, et al. Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res.* 2004; 64:9209–16. [PubMed: 15604294]
3. Fidler IJ. Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes memorial award lecture. *Cancer Res.* 1990; 50:6130–8. [PubMed: 1698118]
4. Zetter BR. The cellular basis of site-specific tumor metastasis. *N Engl J Med.* 1990; 322:605–12. [PubMed: 2406604]
5. Husemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E, et al. Systemic spread is an early step in breast cancer. *Cancer Cell.* 2008; 13:58–68. [PubMed: 18167340]
6. Nguyen DX, Bos PD, Massague J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer.* 2009; 9:274–84. [PubMed: 19308067]
7. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer.* 2002; 2:563–72. [PubMed: 12154349]

8. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell*. 2002; 110:673–87. [PubMed: 12297042]
9. Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY. Control of adhesion-dependent cell survival by focal adhesion kinase. *J Cell Biol*. 1996; 134:793–9. [PubMed: 8707856]
10. Guo W, Giancotti FG. Integrin signalling during tumour progression. *Nat Rev Mol Cell Biol*. 2004; 5:816–26. [PubMed: 15459662]
11. Fornaro M, Manes T, Languino LR. Integrins and prostate cancer metastases. *Cancer Metastasis Rev*. 2001; 20:321–31. [PubMed: 12085969]
12. Giancotti FG. Complexity and specificity of integrin signalling. *Nat Cell Biol*. 2000; 2:E13–4. [PubMed: 10620816]
13. Scott LJ, Clarke NW, George NJ, Shanks JH, Testa NG, Lang SH. Interactions of human prostatic epithelial cells with bone marrow endothelium: binding and invasion. *Br J Cancer*. 2001; 84:1417–23. [PubMed: 11355957]
14. Miranti CK, Brugge JS. Sensing the environment: a historical perspective on integrin signal transduction. *Nat Cell Biol*. 2002; 4:E83–90. [PubMed: 11944041]
15. Assoian RK, Schwartz MA. Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression. *Curr Opin Genet Dev*. 2001; 11:48–53. [PubMed: 11163150]
16. Giancotti FG, Tarone G. Positional control of cell fate through joint integrin/receptor protein kinase signaling. *Annu Rev Cell Dev Biol*. 2003; 19:173–206. [PubMed: 14570568]
17. Ginsberg MH, Du X, Plow EF. Inside-out integrin signalling. *Curr Opin Cell Biol*. 1992; 4:766–71. [PubMed: 1419055]
18. Coppolino MG, Dedhar S. Bi-directional signal transduction by integrin receptors. *Int J Biochem Cell Biol*. 2000; 32:171–88. [PubMed: 10687952]
19. Giancotti FG, Ruoslahti E. Integrin signaling. *Science*. 1999; 285:1028–32. [PubMed: 10446041]
20. Shattil SJ, Kim C, Ginsberg MH. The final steps of integrin activation: the end game. *Nat Rev Mol Cell Biol*. 2010; 11:288–300. [PubMed: 20308986]
21. Huang SF, Kim SJ, Lee AT, Karashima T, Bucana C, Kedar D, et al. Inhibition of growth and metastasis of orthotopic human prostate cancer in athymic mice by combination therapy with pegylated interferon-alpha-2b and docetaxel. *Cancer Res*. 2002; 62(20):5720–6. [PubMed: 12384530]
22. Wu TT, Sikes RA, Cui Q, Thalmann GN, Kao C, Murphy CF, et al. Establishing human prostate cancer cell xenografts in bone: induction of osteoblastic reaction by prostate-specific antigen-producing tumors in athymic and SCID/bg mice using LNCaP and lineage-derived metastatic sublines. *Int J Cancer*. 1998; 77:887–94. [PubMed: 9714059]
23. Bednarczyk JL, Wygant JN, Szabo MC, Molinari-Storey L, Renz M, Fong S, et al. Homotypic leukocyte aggregation triggered by a monoclonal antibody specific for a novel epitope expressed by the integrin beta 1 subunit: conversion of nonresponsive cells by transfecting human integrin alpha 4 subunit cDNA. *J Cell Biochem*. 1993; 51:465–78. [PubMed: 7684386]
24. Mitchell JS, Brown WS, Woodside DG, Vanderslice P, McIntyre BW. Clustering T-cell GM1 lipid rafts increases cellular resistance to shear on fibronectin through changes in integrin affinity and cytoskeletal dynamics. *Immunol Cell Biol*. 2009; 87:324–36. [PubMed: 19139760]
25. Chu K, Cheng CJ, Ye X, Lee YC, Zurita AJ, Chen DT, et al. Cadherin-11 promotes the metastasis of prostate cancer cells to bone. *Mol Cancer Res*. 2008; 6:1259–67. [PubMed: 18708358]
26. Park SI, Zhang J, Phillips KA, Araujo JC, Najjar AM, Volgin AY, et al. Targeting SRC family kinases inhibits growth and lymph node metastases of prostate cancer in an orthotopic nude mouse model. *Cancer Res*. 2008; 68:3323–33. [PubMed: 18451159]
27. Bazzoni G, Shih DT, Buck CA, Hemler ME. Monoclonal antibody 9EG7 defines a novel beta 1 integrin epitope induced by soluble ligand and manganese, but inhibited by calcium. *J Biol Chem*. 1995; 270:25570–7. [PubMed: 7592728]
28. Lenter M, Uhlig H, Hamann A, Jenö P, Imhof B, Vestweber D. A monoclonal antibody against an activation epitope on mouse integrin chain beta 1 blocks adhesion of lymphocytes to the endothelial integrin alpha 6 beta 1. *Proc Natl Acad Sci U S A*. 1993; 90:9051–5. [PubMed: 7692444]

29. Byron A, Humphries JD, Askari JA, Craig SE, Mould AP, Humphries MJ. Anti-integrin monoclonal antibodies. *J Cell Sci.* 2009; 122:4009–11. [PubMed: 19910492]
30. Hall MA, Kwon S, Robinson H, Lachance PA, Azhdarinia A, Ranganathan R, et al. Imaging prostate cancer lymph node metastases with a multimodality contrast agent. *Prostate.* 2012; 72:129–46. [PubMed: 21538422]
31. Burton JB, Johnson M, Sato M, Koh SB, Mulholland DJ, Stout D, et al. Adenovirus-mediated gene expression imaging to directly detect sentinel lymph node metastasis of prostate cancer. *Nat Med.* 2008; 14:882–8. [PubMed: 18622403]
32. Fidler IJ. Critical determinants of metastasis. *Semin Cancer Biol.* 2002; 12:89–96. [PubMed: 12027580]
33. Fornaro M, Languino LR. Alternatively spliced variants: a new view of the integrin cytoplasmic domain. *Matrix Biol.* 1997; 16:185–93. [PubMed: 9402008]
34. Moro L, Perlino E, Marra E, Languino LR, Greco M. Regulation of beta1C and beta1A integrin expression in prostate carcinoma cells. *The Journal of biological chemistry.* 2004; 279:1692–702. [PubMed: 14585844]
35. Goel HL, Moro L, Murphy-Ullrich JE, Hsieh CC, Wu CL, Jiang Z, et al. Beta1 integrin cytoplasmic variants differentially regulate expression of the antiangiogenic extracellular matrix protein thrombospondin 1. *Cancer Res.* 2009; 69:5374–82. [PubMed: 19549894]
36. Longhurst CM, Jennings LK. Integrin-mediated signal transduction. *Cell Mol Life Sci.* 1998; 54:514–26. [PubMed: 9676571]
37. Ginsberg MH, Partridge A, Shattil SJ. Integrin regulation. *Curr Opin Cell Biol.* 2005; 17:509–16. [PubMed: 16099636]
38. Tadokoro S, Shattil SJ, Eto K, Tai V, Liddington RC, de Pereda JM, et al. Talin binding to integrin beta tails: a final common step in integrin activation. *Science.* 2003; 302:103–6. [PubMed: 14526080]
39. Anthis NJ, Campbell ID. The tail of integrin activation. *Trends Biochem Sci.* 2011; 36:191–8. [PubMed: 21216149]
40. Sakamoto S, McCann RO, Dhir R, Kyprianou N. Talin1 promotes tumor invasion and metastasis via focal adhesion signaling and anoikis resistance. *Cancer Res.* 2010; 70:1885–95. [PubMed: 20160039]
41. Lai MT, Hua CH, Tsai MH, Wan L, Lin YJ, Chen CM, et al. Talin-1 overexpression defines high risk for aggressive oral squamous cell carcinoma and promotes cancer metastasis. *J Pathol.* 2011; 224:367–76. [PubMed: 21547905]
42. Chandrasekaran S, Guo NH, Rodrigues RG, Kaiser J, Roberts DD. Pro-adhesive and chemotactic activities of thrombospondin-1 for breast carcinoma cells are mediated by alpha3beta1 integrin and regulated by insulin-like growth factor-1 and CD98. *J Biol Chem.* 1999; 274:11408–16. [PubMed: 10196234]
43. Chatterjee M, Sequeira L, Jenkins-Kabaila M, Dubyk CW, Pathak S, van Golen KL. Individual rac GTPases mediate aspects of prostate cancer cell and bone marrow endothelial cell interactions. *J Signal Transduct.* 2011; 2011:541851. [PubMed: 21776386]
44. Soultz N, Karyotis I, Delakas D, Spandidos DA. Expression analysis of peptide growth factors VEGF, FGF2, TGFβ1, EGF and IGF1 in prostate cancer and benign prostatic hyperplasia. *Int J Oncol.* 2006; 29:305–14. [PubMed: 16820871]
45. Zhang J, Patel L, Pienta KJ. CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis. *Cytokine Growth Factor Rev.* 2009; 21:41–8. [PubMed: 20005149]
46. Park CC, Zhang H, Pallavicini M, Gray JW, Baehner F, Park CJ, et al. Beta1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and in vivo. *Cancer Res.* 2006; 66:1526–35. [PubMed: 16452209]
47. Cress AE, Rabinovitz I, Zhu W, Nagle RB. The alpha 6 beta 1 and alpha 6 beta 4 integrins in human prostate cancer progression. *Cancer and Metastasis Reviews.* 1995; 14:219–28. [PubMed: 8548870]

48. Sroka IC, Anderson TA, McDaniel KM, Nagle RB, Gretzer MB, Cress AE. The laminin binding integrin $\alpha 6 \beta 1$ in prostate cancer perineural invasion. *J Cell Physiol.* 2010; 224:283–8. [PubMed: 20432448]
49. Gan HK, Burgess AW, Clayton AH, Scott AM. Targeting of a Conformationally Exposed, Tumor-Specific Epitope of EGFR as a Strategy for Cancer Therapy. *Cancer research.* 2012; 72:2924–30. [PubMed: 22659454]
50. Wirth M, Heidenreich A, Gschwend JE, Gil T, Zastrow S, Laniado M, et al. A multicenter phase I study of DI17E6, a novel de-immunized monoclonal antibody to human αv integrins, in progressive castrate-resistant prostate cancer (CRPC) with bone metastases after chemotherapy. *J Clin Oncol.* 2011; 29(suppl):abstr e15060.
51. Tanaka H, Kono E, Tran CP, Miyazaki H, Yamashiro J, Shimomura T, et al. Monoclonal antibody targeting of N-cadherin inhibits prostate cancer growth, metastasis and castration resistance. *Nat Med.* 2010; 16:1414–20. [PubMed: 21057494]
52. Malanchi I, Santamaria-Martinez A, Susanto E, Peng H, Lehr HA, Delaloye JF, et al. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature.* 2012; 481:85–9. [PubMed: 22158103]
53. Oskarsson T, Acharyya S, Zhang XH, Vanharanta S, Tavazoie SF, Morris PG, et al. Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. *Nature medicine.* 2011; 17:867–74.
54. Kostenuik PJ, Sanchez-Sweatman O, Orr FW, Singh G. Bone cell matrix promotes the adhesion of human prostatic carcinoma cells via the $\alpha 2 \beta 1$ integrin. *Clin Exp Metastasis.* 1996; 14:19–26. [PubMed: 8521612]
55. Hall CL, Dai J, van Golen KL, Keller ET, Long MW. Type I collagen receptor ($\alpha 2 \beta 1$) signaling promotes the growth of human prostate cancer cells within the bone. *Cancer Res.* 2006; 66:8648–54. [PubMed: 16951179]

A β_1 integrins

B p-FAK^{Y397}

Figure 1. Expression of β_1 integrins and pFAK-Y397 in human PCa specimens

(A) Immunohistochemical staining of β_1 integrins in PCa specimens. In normal prostate gland, β_1 integrins are expressed on the basal cell layer (arrow). In PCa specimens, β_1 integrins were localized on the membranes of tumor cells (arrow). In lymph node metastasis, β_1 integrins are expressed on the membranes of metastatic cancer cells in lymph node (arrow). (B) Immunohistochemical staining of pFAK-Y397 in PCa specimens. Seven of 16 PCa specimens showed positive staining for pFAK-Y397 (right panel), while nine specimens were negative (left panel). Seven of 12 lymph node metastasis specimens showed positive staining for pFAK-Y397 (right panel), while the rest of five specimens were negative (left panel).

Figure 2. β_1 integrin activation correlates with anoikis resistance in PCa cell lines
 (A) FACS using anti-total β_1 -integrin antibody mAb MAR4. LNCaP, C4-2B4, PC3, and PC3-mm2 cells showed similar levels of binding with mAb MAR4. (B) Western blot of cell lysates for the expression of FAK and phosphorylated FAK^{Y397}. FAK is phosphorylated at Y397 in PC3 and PC3-mm2 cells, but not LNCaP or C4-2B4 cells. (C) FACS using conformation sensitive anti- β_1 -integrin antibody mAb 9EG7. High percentage of PC3 and PC3-mm2 showed specific binding with the conformation-sensitive mAb 9EG7, while LNCaP and C4-2B4 showed modest binding. (D) Cells were grown in anoikis condition as described in Materials and Methods. Viable and dead cell numbers were determined by Trypan Blue exclusion and propidium iodide staining, respectively. PC3 and PC3-mm2 are more resistant to anoikis-induced cell death than LNCaP and C4-2B4 cells. (E) FACS of propidium iodide labeled cells after the cells were grown in anoikis conditions for 24 or 48 hrs. LNCaP and C4-2B4 have higher sub-G1 fraction than PC3 or PC3-mm2 cells. (F) Western blot analysis of PARP and cleaved PARP of PC3 or PC3-mm2 grown under anoikis conditions for various lengths of times.

Figure 3. Knockdown of $\beta 1$ -integrins reduces FAK phosphorylation at Y397 and anoikis-resistance of PC3-mm2 cells

A stable PC3-mm2 cell line was derived in which $\beta 1$ -integrins are reduced by expression of shRNA as described in Materials and Methods. (A) Immunoblotting of $\beta 1$ -integrins, pFAK 397 and Total FAK, pAKT473 and total AKT. (B) Cells were grown in anoikis condition as described in Materials and Methods. Viable and dead cell numbers were determined by Trypan Blue exclusion. (C) Western blot analysis of PARP and cleaved PARP of PC3 or PC3-mm2 grown under anoikis conditions for various lengths of times.

Figure 4. Effect of mAb 33B6 on ECM-mediated cell adhesion, migration, apoptosis in vitro
 (A) mAb 33B6 inhibits PC3-mm2 adhesion to ECM. PC3-mm2 cells were plated on type I collagen, fibronectin, or BSA coated plates in the presence or absence of mAb 33B6 and the fractions of cells that bound to the coated plates were determined by the fluorescence intensity. (B) Measurement of PC3-mm2 binding to fibronectin or type I collagen under flow. PC3-mm2 (2×10^6) cells treated with or without mAb 33B6 were injected into the flow chambers coated with type I collagen or fibronectin as described in Materials and Methods. Shear stress calculations were determined every 50 seconds. (C) mAb 33B6 inhibits PC3-mm2 spreading on extracellular matrix. Binding of PC3-mm2 to type I collagen or fibronectin, but not BSA, leads to a flattened cell shape, which was inhibited by mAb 33B6. (D) mAb 33B6 inhibits PC3-mm2 migration on type I collagen or fibronectin. *,

p<0.05. (E) Lysates from PC3-mm2 cells treated with mAb 33B6 or control IgG for 24 hrs were immunoblotted with antibody against phospho-FAK, FAK, or phospho-AKT or AKT. The phosphorylated versus non-phosphorylated FAK or AKT ratios were determined by densitometry. The experiments were repeated twice. (F) PC3-mm2 cells were treated with mAb 33B6 or control IgG for 24 hrs and apoptosis was determined by staining with sulforhodamine 101-Annexin V and caspase 3 substrate DEVD-NucView 488. Cell lysates were immunoblotted with antibody against cleaved PARP. The cleaved PARP and actin ratios were determined by densitometry and the average of two experiments was shown.

Figure 5. Inhibition of PC3-mm2 metastasis to lymph node in vivo by mAb 33B6

Luciferase-labeled PC3-mm2 cells (1×10^6) were injected into the prostate of SCID mice. Mice were treated with IgG or mAb 33B6 (1 mg/kg) before tumor cell inoculation and twice a week subsequently by intra-peritoneal injection. Bioluminescence imaging of mice was performed weekly afterwards to monitor tumor growth and metastasis in vivo. (A) Bioluminescence imaging of mice at 2-weeks post-injection. At termination, the prostates were removed before bioluminescence imaging. Arrows point to tumors in lymph nodes. (B) Average numbers of lymph node $\pm$ SD metastases detected at the termination of the study. (C) Average tumor sizes $\pm$ SD in the prostates at the termination of the study. *, $p < 0.05$.

Figure 6. Inhibition of disseminated PC3-mm2 cell growth in vivo by mAb 33B6

Luciferase-labeled PC3-mm2 cells (1×10^6) were injected into the left ventricle of SCID mice. Mice were treated with IgG or mAb 33B6 (1 mg/kg) before tumor cells inoculation and twice a week subsequently by intra-peritoneal injection. (A) Bioluminescence imaging of mice was performed within 30 min of injection and weekly afterwards to monitor tumor growth in vivo. (B) Overall tumor burden was recorded as photons per second. Average photon numbers $\pm$ SE of the control and mAb 33B6-treated mice are presented. (C) Tumor burden in the femurs of the control and mAb 33B6-treated mice. * $p < 0.05$ (D) Model of activated β_1 integrins enhancing PCa metastasis. In highly metastatic PCa cells, β_1 integrins are present in an activated conformation through an inside-out activation mechanism, which increases its ligand binding affinity. Upon binding with ECM, β_1 integrins are further

activated by an outside-in mechanism that results in integrin clustering and activation of a downstream signaling cascade that enhances the metastatic potential of PCa cells.

Table 1
Immunohistochemical staining of human PCa specimens for β_1 integrins and pFAK-Y397 expression.

(A) β_1 integrin expression in human PCa samples.					
Specimens	Total No. Samples	No. of Samples (Gleason Score)	β_1 integrins Positive (%)		P Value *
Normal prostate (Luminal cells)	20			0/20 (0%)	
Primary PCa	20	5 (6)	3/5 (60%)	13/20 (65%)	P<0.001
		7 (7)	4/7 (57%)		
		2 (8)	2/2 (100%)		
		6 (9)	4/6 (67%)		
Lymph node metastases	18	N/A	13/18 (72%)		P<0.001

(B) pFAK-Y397 expression in human PCa samples.					
Specimens	Total No. Samples	No. of Samples (Gleason Score)	pFAK-Y397 Positive (%)		P Value *
Normal prostate (Luminal cells)	16			0/16 (0%)	
Primary PCa	16	4 (6)	0/4 (0%)	7/16 (44%)	P<0.007
		7 (7)	4/7 (57%)		
		2 (8)	1/2 (50%)		
		3 (9)	2/3 (67%)		
Lymph node metastases	12	N/A	7/12 (58%)		P<0.001

Abbreviation: N/A, not applicable.
* From chi square tests.