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Integrin $\alpha_2\beta_1$ ($\alpha_2\beta_1$) Promotes Prostate Cancer Skeletal Metastasis

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Abstract

Men who die of prostate cancer (PCa) do so because of systemic metastases, the most frequent of which are within the skeleton. Recent data suggest that the colonization of the skeleton is mediated in part by collagen type I, the most abundant protein within the bone. We have shown that enhanced collagen I binding through increased expression of integrin $\alpha_2\beta_1$ stimulated *in vitro* invasion and promoted the growth of PCa cells within the bone. Accordingly, we sought to determine whether $\alpha_2\beta_1$ integrin is a potential mediator of skeletal metastasis. To examine whether $\alpha_2\beta_1$ integrin mediates PCa metastasis, α_2 integrin was over-expressed in low-tumorigenic LNCaP PCa cells or selectively knocked-down in highly metastatic LNCaP_{col} PCa cells. We document that the over-expression of α_2 cDNA stimulated whereas α_2 shRNA inhibited the ability of transduced cells to bind to or migrate towards collagen *in vitro*. Correspondingly, α_2 integrin knock-down reduced the tumor burden of intra-osseous tumors compared to control-transduced cells. To investigate the clinical significance of $\alpha_2\beta_1$ expression in PCa, $\alpha_2\beta_1$ protein was measured in prostatic tissues and in soft tissue and bone metastases. The data demonstrate that $\alpha_2\beta_1$ protein was elevated in PCa skeletal metastases compared to either PCa primary lesions or soft tissue metastases suggesting that $\alpha_2\beta_1$ contributes to the selective metastasis to the bone. Taken together, these data support that $\alpha_2\beta_1$ integrin is needed for the efficient metastasis of PCa cells to the skeleton.

Keywords

prostate cancer; skeleton; metastasis; integrin; collagen

Introduction

Prostate cancer (PCa) is a significant health problem among men in the United States. In 2012, PCa is expected to be the most frequently diagnosed cancer in men and the second leading cause of cancer-related deaths within this group [1]. Of the 28,000 estimated deaths attributable to PCa this year, all will follow the metastatic spread of the disease from the prostate to distant organs including the dura, liver, lung, lymph nodes, and skeleton. The most frequent distant metastases formed by malignant PCa are within the skeleton [2]. Studies of men with progressive castration-resistant, non-metastatic PCa indicate that nearly one half of patients will develop skeletal metastases within two years whereas greater than

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80% of all men who die of PCa will have metastatic disease within the bone, specifically in the trabecular bone of the pelvis, femur, and vertebral bodies [3,4]. Outgrowth of tumors within the bone is quite debilitating resulting in severe pain, fracture, nerve compression/paralysis, and death. Thus to improve the quality of life for PCa patients, a better understanding of the biology of skeletal metastases is needed.

The molecular mechanisms that mediate the preferential metastasis of PCa cells to the skeleton are not well defined. Adhesion to bone-specific factors may facilitate the selective metastasis of PCa cells to the skeleton. Identification of these factors may reveal important clues about the mechanism of bone metastasis as well as provide new targets for the prevention of skeletal metastasis. Collagen type I is a protein factor that is expressed at high levels in the tendon, dermis, and bone. Further, it is the most abundant protein within the bone making up over 90% of the total protein within this site [5]. The broad distribution of collagen I within the bone suggests that it may have a prominent role in skeletal metastasis.

The receptors for type I collagen include integrin ($\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_{11}\beta_1$) and non-integrin receptors (Discoidin Domain Receptor 1 and 2, glycoprotein VI, Leukocyte-associated IG-like receptor-1, and the mannose receptor family); however, the most common cell surface receptors for collagen I are integrins (reviewed in [6,7]). The integrin family is a class of transmembrane adhesion molecules composed of noncovalently linked α and β subunits. Each $\alpha\beta$ heterodimer mediates attachment to a specific set of extracellular matrix proteins, which for collagen I include integrin pairs $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_{11}\beta_1$ [8]. $\alpha_2\beta_1$ integrin is the high affinity receptor for collagen I [9]. It binds to collagen I through the specific amino acid sequence GFOGER (Gly-Phe-HPro-Gly-Glu-Arg) [10] when present in the native triple-helical conformation of the type I collagen fibril.

The integrin β subunit interacts with numerous intracellular signaling molecules including focal adhesion kinase (Fak), integrin-linked kinase, and the non-receptor tyrosine kinase Src (reviewed in [11]). Induction of these molecules through β_1 integrin can mediate cellular proliferation and motility through the activation of mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3-K), or RhoA GTPase [12,13]. The cytoplasmic region of the α_2 integrin subunit can also promote RhoA activation and cell spreading suggesting that both integrin subunits contribute to outside-in signal transduction [14–16]. We have shown that collagen I binding to $\alpha_2\beta_1$ activates the monomeric G-protein RhoC GTPase [17] resulting in PCa cell invasion and migration. Thus, integrin $\alpha_2\beta_1$ can activate multiple signal transduction pathways which may support tumor cell invasion and metastasis.

Published data support a role for $\alpha_2\beta_1$ in the metastasis of PCa cells to the skeleton. For example, antibodies to $\alpha_2\beta_1$ were found to block the attachment of PC-3 PCa to bone matrix [18] whereas treatment with Transforming Growth Factor β_1 [19] or osteoblast conditioned medium [20] enhanced $\alpha_2\beta_1$ synthesis and adhesion to collagen I. We have demonstrated that the ability to bind collagen I is a characteristic of PCa cells isolated from bone vs. soft tissue metastases [21]. To examine the relationship between collagen I adhesion and bone metastatic potential, a collagen I binding variant of human LNCaP PCa cells was derived through serial passage on collagen I. These cells, LNCaP_{col}, displayed a 51% increase in the surface expression of $\alpha_2\beta_1$, bound tightly to collagen I, and were stimulated to invade through collagen I in a $\alpha_2\beta_1$ integrin-dependent manner [21]. When injected directly into the bone, the collagen I binding LNCaP_{col} cells had an increased ability to form osseous lesions compared non-collagen binding LNCaP cells [21]. These data demonstrated that an enhanced ability to bind to collagen I can promote PCa establishment within the bone but does not provide direct evidence that $\alpha_2\beta_1$ expression or signaling mediates PCa bone metastasis. We therefore queried whether $\alpha_2\beta_1$ integrin is a potential mediator of skeletal

metastasis. Herein we provide evidence that bone metastases from PCa patients express elevated levels of $\alpha_2\beta_1$ compared to soft tissue metastases. Further, the selective knock-down of the α_2 subunit in metastatic PCa cells not only blocked the ability of PCa cells to bind to or migrate towards collagen I *in vitro* but also reduced the capability of cells to grow within the bone. These data support the hypothesis that $\alpha_2\beta_1$ integrin is needed for the efficient metastasis of PCa cells to the skeleton.

Materials and Methods

Cells

Human LNCaP PCa cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1x penicillin-streptomycin (Invitrogen, Carlsbad, CA). The isogenic variant LNCaP_{col} PCa cells were derived from LNCaP cells through *in vitro* panning on collagen I as previously described [21]. Both cell lines were engineered to over-express the gene for firefly luciferase through transduction with commercially available lentiviral transduction particles (GenTarget, San Diego, CA). Stable pools of luciferase⁺/RFP⁺ positive cells were selected through treatment with 50 μ g/ml blasticidin. All cells were shown to be free of *Mycoplasma* by the PlasmTest mycoplasma detection method (Invivogen, San Diego, CA).

Generation of α_2 integrin cDNA and shRNA transduced cells

The pLenti6- α_2 plasmid containing the full-length cDNA for human α_2 integrin was the kind gift of Mary Zutter (Vanderbilt University, Nashville, TN). The expression cassette was excised from the plasmid following digestion with SpeI/XhoI and subcloned into the pLenti4/TO/V5-DEST vector (Invitrogen, Carlsbad, CA). Transduction particles were then prepared using the Virapower lentiviral expression system and used to transduce LNCaP-luc PCa cells. Stable pools of luciferase⁺/RFP⁺/ α_2 ⁺ cells were selected with 50 μ g/ml blasticidin and 100 μ g/ml zeocin (Invitrogen). LNCaP-luc cells transduced with the empty pLenti4 vector served as a control for α_2 enforced expression. To achieve α_2 knock-down, the α_2 -specific shRNA set in pLKO was purchased from Sigma-Aldrich (St. Louis, MO). Plasmids were packaged into virus particles according to manufacturer's instructions and used to transduce LNCaP_{col}-luc cells. Stable pools were selected with 50 μ g/ml blasticidin and 1 μ g/ml puromycin (Invivogen, San Diego, CA). Cells transduced with a non-targeting shRNA served as a control for α_2 shRNA expression.

Characterization of α_2 integrin expression

The cell surface expression of α_2 protein was determined by flow cytometry. Briefly, 2×10^5 PCa cells were incubated with a FITC conjugated antisera to integrin α_2 (clone, AK-7, BD Bioscience, San Jose, CA) or IgG_{1 κ} isotype control for 30 minutes at 4°C. Labeled cells were washed, and evaluated on a Coulter FACS Scan flow cytometer (Beckman-Coulter, Fullerton, CA). Analysis of the surface expression of integrin subunits on, α_3 , α_5 , α_6 , β_1 , and $\alpha_v\beta_3$ were conducted as above following incubation with the proceeding purified antibodies and a FITC-conjugated secondary antibody [on, clone SR84; α_3 , clone C3 II1; α_5 , clone IIA1; α_6 , clone GoH3; β_1 , clone MAR4; $\alpha_v\beta_3$ clone 23C6, and FITC anti-mouse IgG each from BD Bioscience, San Jose, CA].

The expression of $\alpha_2\beta_1$ mRNA was measured by quantitative PCR. Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) and 1 μ g RNA reverse transcribed using the reverse transcription kit (Promega, Madison, WI). Integrin α_2 and β_1 specific transcripts were measured on a Roche Lightcycler 480 as previously described [22]. The primers used were as follows: ITGA2-1131F 5' GGGCATTGAAAACACTCGAT 3'; ITGA2-1966R 5'

TCGGATCCCAAGATTTTCTG 3'; β -actin-736F 5' GGA CTTCGAGCAAGAGATGG 3';
 β -actin-969R 5' AGCA CTGTGTTGGCGTACAG 3'; ITGB1 2054F 5'
 CATCTGCGAGTGTGGTGTCT 3'; ITGB1 2262R 5' GGGGTAATTTGTCCCGACTT 3'.

***In vitro* characterization of $\alpha_2\beta_1$ integrin function**

In vitro assays to measure attachment, proliferation, and migration of α_2 transduced cells were performed as described previously [21].

Intratribial injection

Tumor cells (5×10^5 cells/50 μ l) were injected into the tibia of male SCID mice at 7–8 weeks of age as described previously [21]. Tumors were allowed to grow for 9 weeks. Tumor burden was measured by bioluminescent imaging using a Xenogen IVIS imaging system (Xenogen Corporation, Alameda, CA). Animals were then evaluated using Faxitron radiography (Faxitron x-ray Corp, Wheeling, IL). Injected tibiae and contralateral tibiae without tumors were removed and processed for histology as previously described [21].

Intracardiac experimental metastasis model

Male SCID mice at 7–8 weeks of age were injected into the left cardiac ventricle as previously described [23,24]. Nine weeks post tumor cell injection, tumor burden was measured by bioluminescent imaging using a Xenogen IVIS imaging system (Xenogen Corporation, Alameda, CA). Based on our BLI analysis, the liver and mandible were removed from each animal and processed for histology as previously described [21].

cDNA microarray analysis

The ONCOMINE database and gene microarray analysis tool, a repository for published cDNA microarray data (<http://141.214.6.50/oncomine/main/index.jsp>), was explored for mRNA expression of ITGA2 in clinical cases of prostate cancer. Statistical analysis of differences was performed using ONCOMINE algorithms to account for the multiple comparisons among different studies as previously described [25].

Case Selection and Tissue Microarrays

Human primary and metastatic PCa tissues were obtained as part of the PCa research program and University of Washington Medical Center PCa Donor Rapid Autopsy Program. The radical prostatectomy TMA series consisted of 185 evaluable cores taken from 47 total patients; 66 cores of non-neoplastic prostate (from 30 cases), 43 cores of BPH (from 22 cases), and 76 cores of localized PCa (from 30 cases). The PCa metastasis arrays were constructed from soft tissue and bone metastases taken from 42 available autopsies [26]. The arrays comprised 293 evaluable cores of metastases of the liver (18 cases), lymph node (27 cases), and bone (38 cases). Clinical data relating to the 42 autopsy patients is described in [27]. The Institutional Review Board of the University of Washington Medical Center approved all procedures involving human subjects, and all subjects signed written informed consent.

Immunohistochemistry and Evaluation

Five micron sections were deparaffinized and rehydrated. Antigen retrieval was performed in 10 mM Tris buffer (pH 9.0) in a pressure cooker for ten minutes at 20 psi. The slides were then blocked with a 3% H_2O_2 solution followed by an avidin/biotin blocking solution (Vector Laboratories Inc.). After incubation with a 5% chicken/goat/horse serum solution, sections were incubated with mouse anti-human integrin α_2 antibody (20 μ g/mL; cat # MCA2025; AbD Serotec, Raleigh, NC) overnight at 4°C. Negative control slides were incubated with mouse anti-MOPC21 at the same concentration. All slides were then

incubated with biotinylated anti-mouse Ab (BA-2000, Vector Laboratories Inc.), developed using the Vectastain ABC kit (Vector Laboratories Inc.) and stable DAB (Invitrogen Corp.). The slides were counterstained with hematoxylin, dehydrated, and mounted with Cytoseal XYL (Richard-Allan Scientific).

Staining intensity was scored by a genitor-urinary research pathologist (XY) as absent (no staining) [0], weak (faint or fine chromogen deposition) [1], or strong (clear and coarse granular chromogen deposition) [2] as previously described [28]. The percent of positive stained cells was determined from the entire tissue field. For each core, the integrin α_2 protein expression index (EI), the product of staining intensity and the percentage of positive staining, was determined as described previously [29].

Statistical Analysis

A t-test was used to determine statistical significance between groups for continuous covariates. TMA staining intensity was analyzed using a stratified cumulative logistic model where the patient was the stratification factor. The stratification was needed to properly adjust for the correlation of multiple cores from each patient in the model. The type of disease in each core was the lone independent predictor in the model with odds ratios and associated Wald chi-square p-values reported. Analyses were completed using SAS 9.2 (SAS Institute, Cary, NC).

Results

Modulation of α_2 integrin expression alters collagen I-stimulated attachment and migration

We previously demonstrated that LNCaP cells selected *in vitro* for the ability to bind type I collagen gain the capacity to grow within the bone [21]. Relative to LNCaP cells, these bone metastatic LNCaP_{col} PCa cells were found to have increased expression of the collagen I receptor integrin $\alpha_2\beta_1$ compared to LNCaP cells suggesting that $\alpha_2\beta_1$ mediated the formation of PCa bone lesions [21]. To ascertain the role $\alpha_2\beta_1$ in PCa bone metastasis, integrin α_2 was over-expressed in low-tumorigenic LNCaP PCa cells or selectively knocked-down in highly-metastatic LNCaP_{col} PCa cells. A stable pool transduced with small-hairpin RNA (shRNA) molecules that target position 1528 on α_2 integrin reduced α_2 RNA and protein expression 2.4-fold and 31% respectively compared to a non-targeting control shRNA transduced cell line (Figure 1 A and B). In contrast, the forced expression of α_2 integrin cDNA increased α_2 RNA and protein expression of 35-fold and 87% respectively compared to empty vector control transduced cells (Figure 1 D and E). The β_1 integrin subunit was abundantly expressed on both LNCaP and LNCaP_{col} cell but its expression changed in relation to α_2 expression such that it decreased 26% in α_2 shRNA-transduced cells and increased 17% in α_2 cDNA-transduced cells compared to the appropriate control cells (Figure 1 C and F). As the change in the β_1 integrin subunit occurred without a corresponding change in β_1 RNA transcripts (supplemental Figure 1H), the data support that LNCaP cells coordinate the cell surface expression of the α_2 and β_1 subunits at the post-transcriptional level.

Despite the modest change in α_2 integrin expression, shRNA mediated knock-down significantly diminished the ability of α_2 integrin shRNA cells to bind to or migrate towards collagen I (Figure 2, A and B) consistent with our previous studies using α_2 integrin neutralizing antibody [21]. Similarly, α_2 integrin over-expressing cells acquired the ability to bind to and migrate towards collagen I relative to vector control cells (Figure 2, C and D). To confirm that the changes in collagen attachment and migration were a direct result of the modulation of α_2 integrin expression, the surface expression of multiple integrin subunits, including α_1 , α_3 , α_5 , α_6 , and $\alpha_v\beta_3$, was evaluated by flow cytometry (supplemental Figure 1

A–G). The data show that only integrin $\alpha_2\beta_1$ expression was altered following α_2 modulation demonstrating that the changes in collagen I-directed attachment and migration were due to the engineered changes in α_2 integrin. Consistent with these findings, altering the expression of α_2 integrin did not significantly interfere with the ability of PCa cells to attach to fibronectin indicating that α_2 modulation did not affect the expression or activity of other integrins. Taken together, the data show that $\alpha_2\beta_1$ promotes the attachment and motility of human PCa cells *in vitro* suggesting a possible mechanism for the preferential invasion of PCa cells within the skeleton.

Blocking α_2 integrin reduces PCa establishment within the bone

To determine whether $\alpha_2\beta_1$ expression mediates PCa bone metastasis, α_2 over-expressing or knock-down PCa cells were injected into the tibia of male SCID mice. When implanted into the bone, both control shRNA-transduced and α_2 shRNA-transduced LNCaP_{col} cells produced radiologic and histologic bone lesions with equal incidence (100% in each case). However, mice injected with α_2 integrin knock-down cells displayed a 3.2-fold or 69% decrease in osseous tumor burden, as measured by bioluminescence imaging, compared to control shRNA transduced cells (Figure 3A and supplemental Figure 2). The decrease in tumor burden following $\alpha_2\beta_1$ knock-down was likely not a result of decreased PCa cell growth in that α_2 shRNA-transduced cells were found to have equivalent attachment-dependent growth rates compared to control shRNA-transduced cells on plastic or collagen I (supplemental Figure 3) consistent with our published report [21]. The over-expression of α_2 integrin in low-metastatic LNCaP cells increased intra-osseous tumor burden by 20% compared to vector control cells without affecting tumor incidence; however, this change was not statistically significant (Figure 3B). Taken together, these data suggest that $\alpha_2\beta_1$ expression is necessary for the formation of PCa bone lesions but is not itself sufficient to confer this ability to low-tumorigenic LNCaP cells.

We have previously published that collagen I binding to $\alpha_2\beta_1$ increased the activation of RhoC GTPase which may facilitate PCa cell dissemination to the skeleton where collagen I is in abundance [17]. To assess the role of $\alpha_2\beta_1$ expression on PCa cell dissemination, α_2 integrin knock-down or control cells were injected into the left cardiac ventricle of SCID mice to allow systemic delivery of tumor cells. The data show that the intracardiac injection of control shRNA cells produced metastases within the liver and/or mandible in 73% (8/11) of the mice. In contrast, mice injected with α_2 knock-down cells developed metastases at these sites in only 9% (1/11) of the mice, $p=0.0075$ compared to control shRNA injected mice by Fisher's exact test. Consistent with the results obtained following intratibial injection, animals injected with α_2 knock-down cells had reduced tumor burden compared to control shRNA injected mice (Figure 3C, supplemental Figure 4), $p<0.15$. Collectively, these data support that $\alpha_2\beta_1$ integrin is necessary but not sufficient for the establishment of PCa metastases within the bone.

Integrin $\alpha_2\beta_1$ expression correlates with skeletal metastasis in PCa patients

To investigate the clinical significance of $\alpha_2\beta_1$ expression in PCa skeletal metastasis, the Oncomine cDNA microarray repository was explored for differences in α_2 integrin gene expression among PCa metastases. Results from two separate studies demonstrated an increase in the expression of α_2 integrin within PCa skeletal metastases compared to PCa soft tissue lesions, particularly lymph node metastases (Figure 4A) [30,31]. Further, the expression of α_2 integrin message was either equivalent or greater than that expressed within PCa primary lesions suggesting that α_2 integrin may have a mechanistic role in promoting PCa metastasis to the skeleton (Figure 4A). To examine whether α_2 integrin correlates with PCa skeletal metastasis in human patients, the protein expression of $\alpha_2\beta_1$ integrin was evaluated retrospectively in tissue microarrays (TMAs) constructed from

prostatic tissues including non-neoplastic prostate, benign prostatic hyperplasia (BPH), primary PCa, and PCa soft tissue and bone metastases. For each core, the staining intensity (absent, weak, or strong) and the percent of cells expressing $\alpha_2\beta_1$ were determined by a genito-urinary research pathologist. A composite expression index was also calculated which was equal to the product of the staining intensity and percent expression in each core. The data show that the mean $\alpha_2\beta_1$ expression index was increased in PCa skeletal metastases compared to either PCa primary lesions or soft tissue metastases of the liver or lymph node (Figure 4B, Supplemental Figure 5) consistent with the Oncomine data. Analysis of $\alpha_2\beta_1$ staining intensity alone revealed that bone metastases had the highest percent of strong staining samples compared to soft tissue metastases, particularly lymph node metastases (Figure 4C). Using a cumulative logistic model to model the intensity scores between tissue types, lymph node metastases had odds of weaker staining 2.3 times that of bone metastases [Wald confidence limits (1.02, 5.22), p-value, 0.042]. Taken together, these data show that α_2 integrin is frequently over-expressed in PCa skeletal metastases compared to soft tissue metastases.

Our analysis of $\alpha_2\beta_1$ integrin expression on prostatic tissues further revealed that $\alpha_2\beta_1$ protein was highly expressed within normal and benign prostatic hyperplasia samples to a level equivalent to skeletal metastases (Figure 4B & C). The data show that $\alpha_2\beta_1$ expression was decreased in primary PCa lesions compared to normal and benign tissues and was further diminished in PCa soft tissue metastases of the lymph node. The expression of $\alpha_2\beta_1$ within skeletal metastases was increased relative PCa primary lesions and soft tissue metastases back to levels found in non-neoplastic prostate tissue. These data support that integrin $\alpha_2\beta_1$ may have different roles during PCa development and progression that are dependent on the tumor microenvironment.

Discussion

Studies of men with PCa who have a rising PSA, despite androgen deprivation therapy, but no radiographic evidence of metastases indicate that nearly one half of patients will develop skeletal metastases within two years, whereas greater than 80% of all men who die of PCa will be found to have metastatic disease within the skeleton at autopsy [3,4]. Although our knowledge of PCa biology has increased significantly, the mechanism(s) that promote PCa metastasis to the skeleton remain unclear. We and others have published several lines of evidence which suggest that colonization of the skeleton is promoted by collagen type I, the most abundant protein within the bone. Specifically, we have shown that PCa cells selected for collagen I binding become more invasive *in vitro* and acquire the capacity to grow within the bone compared to non-collagen binding parental cells [21]. In the present study, we demonstrate that the collagen I receptor, $\alpha_2\beta_1$, is necessary but not sufficient for the establishment of PCa metastases within the bone. We further document that $\alpha_2\beta_1$ integrin is frequently over-expressed in PCa skeletal metastases compared to soft tissue metastases suggesting that $\alpha_2\beta_1$ is needed for the efficient metastasis of PCa cells to the skeleton.

Our hypothesis that $\alpha_2\beta_1$ facilitates skeletal metastasis is supported by published evidence which shows that collagen I/ $\alpha_2\beta_1$ promote an invasive phenotype in multiple cell types. For example, both intact collagen I and collagen-derived fragments, such as the trimeric form of the carboxyl propeptide, were shown to be potent chemotactic factors for tumor cells and endothelial cells in *in vitro* migration assays [32,33]. We and others have further shown that integrin $\alpha_2\beta_1$ directs the *in vitro* attachment to and migration towards collagen I in multiple cancer types including prostate carcinoma, ovarian carcinoma, pancreatic adenocarcinoma, rhabdomyosarcoma, and osteosarcoma [34–36,21,37,38]. Consistent with these observations, the over-expression of α_2 integrin accelerated the experimental metastasis of melanoma and rhabdomyosarcoma cells [34,39] whereas $\alpha_2\beta_1$ blockade using neutralizing

antibodies reduced tumor dissemination to the liver or lymph node in animal models of melanoma, gastric cancer, and colon cancer [40,41,39]. Our analysis of PCa skeletal metastasis support and extend these observations by demonstrating that $\alpha_2\beta_1$ integrin is necessary but not sufficient for the establishment of PCa metastases within the skeleton.

Whether $\alpha_2\beta_1$ promotes PCa skeletal metastasis in human patients has yet to be proven, however, our TMA data which showed that $\alpha_2\beta_1$ was elevated in osseous vs. visceral metastases of men who died of PCa suggests a role for $\alpha_2\beta_1$ in PCa bone metastasis. These data are consistent with a recent published study of $\alpha_2\beta_1$ protein expression in primary PCa tissues which showed that the percent of $\alpha_2\beta_1$ positive cells in the primary tumor was associated with local invasion and bone metastasis [42]. Further, $\alpha_2\beta_1$ integrin has been identified as a marker of both prostate stem cells and PCa stem cells/tumor initiating cells [43,44]. Correspondingly, the percentage of stem cell-like PCa cells within the prostate was found to have a prognostic impact on the risk of skeletal metastasis [42]. However, a recently published Oncomine survey found that $\alpha_2\beta_1$ expression decreased progressively in PCa primary lesions and metastases compared to non-neoplastic tissue suggesting that $\alpha_2\beta_1$ is a metastasis suppressor in PCa [45]. Although our data support that $\alpha_2\beta_1$ facilitates metastasis in PCa, it is possible that the expression of $\alpha_2\beta_1$ is decreased in primary PCa compared to normal prostate tissue. Immunohistochemical analysis of $\alpha_2\beta_1$ expression in prostatectomy samples and metastases by Bankhoff et al, as well as our own studies, demonstrated that $\alpha_2\beta_1$ expression was decreased in primary lesions vs. normal tissues but was increased in metastases vs. the primary lesion [46]. These observations suggest that $\alpha_2\beta_1$ expression is biphasic during PCa development and progression [47]. It is therefore plausible that during tumor development, $\alpha_2\beta_1$ expression may be reduced in primary PCa cells to promote dissemination but the integrin is re-expressed in circulating tumor cells or is induced in disseminated tumor cells by collagen-rich environments such as the bone to promote metastasis.

A permissive microenvironment is required for successful metastasis (reviewed in [48]). The extracellular matrix is a major component of the microenvironment and may promote tumor development and progression. For example, the presence of a fibrotic, reactive stroma in primary lesions was shown to be associated with shortened biochemical-free recurrence in men with PCa [49]. In human breast cancer, women with high breast density, characterized by enriched stromal collagen I, were at increased risk for local recurrence [50] where patients with fibrotic foci of the breast had a higher risk of developing bone and lymph node metastasis [51]. In preclinical models, the over-expression of collagen I in MMTV transgenic mice accelerated mammary tumor initiation and progression compared to MMTV wild-type mice whereas the induction of fibrosis in the lung through the expression of TGF β_1 was found to enhance the experimental metastasis of indolent mammary tumor cells [52,53]. Collectively, the data show that a collagen-rich, fibrotic environment contributes to tumor initiation and metastasis.

In summary, we demonstrated that $\alpha_2\beta_1$ integrin mediates PCa migration towards collagen I and that its expression is necessary but not sufficient for the establishment of PCa metastases within the bone. We further document that $\alpha_2\beta_1$ is frequently over-expressed in PCa skeletal metastases compared to soft tissue metastases from PCa patients. These data support a model in which collagen I/ $\alpha_2\beta_1$ promote a metastatic phenotype in PCa cells to mediate the preferential metastasis of PCa cells to the skeleton. These studies provide a strong rationale for the development of $\alpha_2\beta_1$ as a therapeutic target for the treatment of PCa skeletal metastases and thus have the potential to reduce both the incidence and mortality associated with metastatic PCa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Modulation of α_2 integrin mRNA and protein following stable expression of specific shRNA oligonucleotides or α_2 cDNA

A, D) **Quantitative PCR.** Total RNA was isolated from stable pools of LNCaP_{col}-luc control shRNA-transduced, LNCaP_{col}-luc α_2 integrin shRNA-transduced, LNCaP-luc empty vector-transduced, and LNCaP-luc α_2 cDNA-transduced cells. The RNA was reverse transcribed and α_2 integrin mRNA transcripts evaluated on a Roche LightCycler 480 according to manufacturer's instructions. Shown is the mean $\pm$ the standard deviation of duplicate experiments, $p < 0.05$ by t-test. B, C, E, F) **Flow cytometry.** The surface expression of the α_2 and β_1 integrin subunits was quantified using a Coulter FACS Scan flow cytometer following treatment of cells with α_2 or β_1 integrin-specific FITC-conjugated antibodies. Shown is a representative histogram of four independent experiments.

Figure 2. α_2 integrin knock-down suppresses whereas α_2 integrin over-expression increases collagen I-stimulated attachment and migration

A, C) *In vitro* attachment assay. 1×10^4 fluorescently labeled cells were plated in triplicate to a 96 well plate that had been coated with 10 μ g/ml protein. After 1 hour, the cells were washed and fluorescence measured. The percent specific binding was calculated by subtracting non-specific binding to plastic or control protein BSA. Shown is the mean $\pm$ the standard deviation of four separate experiments, $p < 0.05$ by t-test. B, D) *In vitro* migration assay. 2.5×10^4 PCa cells in serum-free medium were plated to 3 mm tissue culture inserts and placed in wells containing 0 or 10 μ g/ml collagen I. Following a 48 hour incubation, migrating cells were stained and the total number of migrating cells per five high power fields was quantified under 20 $\times$ magnification. The data is presented as the mean $\pm$ standard deviation of duplicate well from a representative experiment, $p < 0.05$ by t-test.

Figure 3. Blocking α_2 integrin reduces PCa establishment within the bone

A) Intratibial injection. Control shRNA-transduced or α_2 integrin shRNA-transduced LNCaP_{col}-luc cells (5×10^5 cells/50 μ l) were directly injected into the right proximal tibia of anesthetized male SCID mice. Tumors were allowed to grow for 9 weeks. Tumor burden was measured using bioluminescent imaging following the injection of luciferin. The data are presented as mean number of photons/sec $\pm$ the standard error, 12 mice/group. *p<0.05 compared to control shRNA transduced cells by t-test. B) Intratibial injection. LNCaP-luc empty vector-transduced or LNCaP-luc α_2 cDNA-transduced cells were injected as described in A. The data are presented as mean number of photons/sec $\pm$ the standard error, 12 mice/group. C) Intracardiac injection. Control shRNA-transduced or α_2 integrin shRNA-

transduced LNCaP_{col}-luc cells were injected into the left cardiac ventricle (2×10^5 cells/0.1 ml) to establish bone metastases. Tumor burden was measured after 9 weeks using bioluminescent imaging following the injection of luciferin. Shown is the mean number of photons/sec $\pm$ the standard error of hepatic or skeletal metastases.

Figure 4. Integrin $\alpha_2\beta_1$ expression correlates with skeletal metastasis in PCa patients.

A) α_2 RNA expression within human PCa metastases. The ONCOMINE gene microarray database [25] was explored for differences in α_2 integrin mRNA expression between human PCa soft tissue or skeletal metastases. See inset image for graph key. B & C) Analysis of α_2 integrin protein expression in prostatic tissues. Tissue microarrays constructed from samples of non-neoplastic prostate tissue, BPH, primary PCa lesions, and soft tissue and skeletal metastases were stained for α_2 integrin expression using routine immunohistochemistry as outlined in the Methods section. B) Plot of the mean $\pm$ the 95% confidence limits of α_2 integrin expression index vs. tissue type. C) Plot of α_2 integrin staining intensity from Figure 4B. Shown is the percent of positive cores with each intensity - absent, weak, or strong. Wald chi-square $p < 0.042$ bone vs. lymph node metastasis.