Integrin αvβ6 promotes an osteolytic program in cancer cells by upregulating MMP2

Anindita Dutta1,2, Jing Li1,3, Huimin Lu1,2, Jacqueline Akech1,3, Jitesh Pratap1,3, Tao Wang4, Brad J. Zerlanko1,2, Thomas J. FitzGerald4, Zhong Jiang5, Ruth Birbe1,6, John Wixted1,7, Shelia M. Violette8, Janet L. Stein1,9, Gary S. Stein1,9, Jane B. Lian1,9 and Lucia R. Languino1,2

1Prostate Cancer Discovery and Development Program; 2Department of Cancer Biology, Thomas Jefferson University, Philadelphia, PA, USA; 3Department of Cell Biology, 4Department of Radiation Oncology and 5Department of Pathology, University of Massachusetts Medical School, Worcester, MA, USA; 6Department of Pathology, Thomas Jefferson University, Philadelphia, PA, USA; 7Department of Orthopedics University of Massachusetts Medical School, Worcester, MA, USA; 8Biogen Idec, Inc., Cambridge, MA, USA; 9Department of Biochemistry, The University of Vermont, Burlington, VT, USA.

A. Dutta and J. Li share first co-authorship of this article. Jitesh Pratap’s present address: Department of Anatomy and Cell Biology, Rush University Medical Center, Chicago, IL 60612.

Financial Support: This work was supported by the following grants: NIH R01 CA89720 (L.R. Languino); PO1 CA140043 (L.R. Languino, G.S. Stein and J.B. Lian); Our Danny Cancer Research Foundation P0001000330000 (T. Wang) and P6001000830000 (J. Li); NIH S10 RR023540 (J. B. Lian) and R37DE012528 ARRA Merit Award NCI PO1 CA82834 (G.S. Stein and J.B. Lian). This project is also funded, in part, under a Commonwealth University Research Enhancement Program grant with the Pennsylvania Department of Health (H.R.). The Department specifically disclaims responsibility for any analyses, interpretations or conclusions. Histology core resources were supported by NIDDK grant DK32520 and the Kimmel Cancer Center Cancer Histology Resources supported in part by NCI grant P30CA56036, which also supports the Kimmel Cancer Center Cancer Genomics Resource used in this study.

Corresponding Author: Lucia R. Languino, 233 S. 10th Street, Philadelphia, PA 19107, 215-503-3442, Lucia.Languino@jefferson.edu.

Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 2014 American Association for Cancer Research.
Running Title: αvβ6 integrin – mediated osteolysis.

Keywords: integrins, osteolysis, prostate cancer, bone volume, matrix metalloproteinases.

Disclosure of Potential Conflicts of Interest: Shelia Violette is an employee and shareholder of Biogen, Cambridge, MA. The other authors do not have any conflicts of interests.

Word count: 5,694

Number of Figures: 7; Supplementary Figures: 6.
ABSTRACT

The molecular circuitries controlling osseous prostate metastasis are known to depend on the activity of multiple pathways, including integrin signaling. Here, we demonstrate that the $\alpha_v\beta_6$ integrin is upregulated in human prostate cancer bone metastasis. In prostate cancer cells, this integrin is a functionally active receptor for fibronectin and latency associated peptide-TGF$\beta$; it mediates attachment and migration upon ligand binding and is localized in focal contacts. Given the propensity of prostate cancer cells to form bone metastatic lesions, we investigated whether the $\alpha_v\beta_6$ integrin promotes this type of metastasis. We show for the first time that $\alpha_v\beta_6$ selectively induces matrix metalloproteinase 2, MMP2, in vitro in multiple prostate cancer cells, and promotes osteolysis in vivo in an immunodeficient mouse model of bone metastasis through upregulation of MMP2, but not MMP9. The effect of $\alpha_v\beta_6$ on MMP2 expression and activity is independent of androgen receptor in the analyzed prostate cancer cells. Increased levels of PTHrP, known to induce osteoclastogenesis, were also observed in $\alpha_v\beta_6$ expressing cells. However, using MMP2 shRNA, we demonstrate that the $\alpha_v\beta_6$ effect on bone loss is due to upregulation of soluble MMP2 by the cancer cells, not to changes in tumor growth rate. Another related $\alpha_v$-containing integrin, $\alpha_v\beta_5$, fails to show similar responses, underscoring the significance of $\alpha_v\beta_6$ activity. Overall, these mechanistic studies establish that expression of a single integrin, $\alpha_v\beta_6$, contributes to the cancer cell-mediated program of osteolysis by inducing matrix degradation through MMP2. Our results open new prospects for molecular therapy of metastatic bone disease.

INTRODUCTION

Over 80% of prostate cancer patients, at autopsy, have metastatic foci in the bone which constitute an important negative prognostic factor for end-stage malignancy (1). When present in the bone, metastatic prostate cancer cells produce osteolytic (2, 3), in addition to the well characterized osteoblastic, lesions (4). Both types of histopathology often occur in the same bone area, but the molecular underpinnings of such mixed lesion formation and the effector molecules participating in this dynamic process are still largely elusive. Prostate cancer osteolytic metastases cause rapid disease progression since rapid degradation of bone by osteoclasts provides space for the tumor cells to grow (5). In contrast, the osteoblastic nature of bone metastases contributes to a slower progress of the disease as compared to osteolytic metastases, since the initial increase in bone volume could limit the space available to cancer cells, and therefore help to confine tumor growth.
Likely mediators/activators of this osteolytic pathway include members of the integrin family of cell surface receptors and their extracellular matrix (ECM) ligands together with the backdrop of a complex bone microenvironment affected by a plethora of regulatory cytokines (3, 6, 7). Integrins are transmembrane receptors that comprise an α- subunit and a β- subunit and are known to be deregulated as prostate cancer progresses to advanced stages (8). In this context, there is now compelling evidence that signals originating from integrin ligand binding orchestrate key mechanisms of tumor progression, including cell survival, adhesion, proliferation, gene expression and modulation of migratory/invasive phenotypes (8). These properties are exploited in prostate cancer especially as it progresses to an advanced disease status (8). Although expression of integrins in human prostate cancer bone metastasis has never been shown, a causal role for integrins in this type of lesion has been reported: αvβ1, αvβ3, αvβ5 or α2β1 have been shown to promote tumor growth in bone (9-11), and α6β1 (10, 12) as well as αvβ3 (11) have been demonstrated to contribute respectively to osteolytic and osteoblastic lesions.

Overexpression of αvβ6 integrin in human cancer correlates with metastasis in endometrial and colon carcinoma patients (13) and has been reported to promote the metastatic potential of HT29 colon cancer cells (13). αvβ6, an epithelial-specific integrin, is an ideal target, yet to be validated, for therapeutic intervention in metastatic disease (14), as this molecule is largely undetectable in most normal tissues but abundantly expressed in primary malignancies (15-18). Furthermore, this integrin has been shown to bind latency associated peptide (LAP)-TGFβ1 and in terms of signaling responses, facilitates the release of active TGFβ1 which is a pro-metastatic cytokine (17).

Our data show that the αvβ6 integrin promotes matrix metalloproteinase (MMP) 2 and Parathyroid hormone-related protein (PTHrP) upregulation and demonstrate the interplay between integrin expression and bone remodeling mechanisms in intraosseous metastatic prostate cancer. We have established that expression of a single integrin, αvβ6, is sufficient to execute a cancer cell-mediated program of osteolysis centered on upregulation of MMP2, as well as of PTHrP, and consequent increased MMP2 catalytic activity that completes the final matrix degradation stage of osteolytic bone disease.

**MATERIALS AND METHODS**

**Reagents and Antibodies**

BSA was from Sigma-Aldrich, type I Collagen and 4', 6-diamidino-2-phenylindole (DAPI) were from Invitrogen; LAP-TGFβ1 was from R&D Systems.
We used the following rabbit Abs against: ERK1/2, and AKT from Santa Cruz Biotechnology; αvβ6 (a gift of Dr. Quaranta, Vanderbilt University) for immunohistochemistry; β3 and β5 cytoplasmic domains from Millipore for immunoblotting (IB); MMP2 from Millipore for IB using tumor lysates or from Cell Signaling for IB using cell lysates and immunohistochemistry.

We used the following mouse (m)Abs: 2A1 to β6 for IB (16, 19) and immunofluorescence; LFMb-14 to osteopontin (OPN) from Santa Cruz Biotechnology; clones AE1 and AE3 to CK from Millipore. IC10, irrelevant mAb and rabbit IgG (from Sigma) were used as negative controls.

Rabbit-anti-mouse Alexa 488 secondary antibody for immunofluorescence was from Invitrogen.

Human Tissue Specimens
Fourteen human bone biopsies and one additional specimen, dissected from a bone metastasis, of patients with osteolytic prostate cancer were obtained from the Department of Pathology, University of Massachusetts Medical School or Cooperative Human Tissue Network (CHTN, other investigators may have received specimens from these same tissues) and were processed according to Institution-approved protocols. Thirteen human bone biopsies and three specimens, dissected from prostate cancer bone metastases (all were osteolytic) were obtained from the Department of Pathology, Thomas Jefferson University. Two human osteolytic prostate cancer bone biopsies were obtained from CHTN. The specimens were formalin-fixed and paraffin-embedded.

Immunohistochemistry
Immunohistochemical staining was performed as described (20-22).

Cells and culture conditions
Authentication of the cell lines was provided with their purchase from UroCore Inc. (C4-2B, Oklahoma City, OK) or ATCC (PC3 and RWPE); the cells were used in our laboratory for less than 6 months. Two PC3 sublines: PC3-1 & PC3-2, previously designated PC3-H and PC3-L respectively (23), were used: PC3-1 (PC3-H) are positive for β6 expression and PC3-2 (PC3-L) are negative for β6 expression (23). BPH1 cells were provided by Dr. Simon W. Hayward (Vanderbilt University, TN) and authenticated as previously described (24). C4-2B and BPH1 cells were maintained in RPMI supplemented with 5% FBS, 2 mmol/L glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin.

Viral Constructs and Cell Transfection
Constructs and transfection of PC3-1, PC3-2 and C4-2B cells was performed as detailed in the
Supplementary Methods.

**Immunofluorescence and Confocal Microscopy**

Immunofluorescence analysis on PC3-1 cells was performed as described (25). To perform immunofluorescence on tissue sections, antigen retrieval was performed on rehydrated formalin-fixed paraffin-embedded sections from human prostate cancer osteolytic samples by incubation in pepsin for 20 min at 37°C. The sections were blocked with PBS / 5% BSA. αvβ6-staining was performed incubating samples with 2A1 Ab for 1 hour, followed by incubation with Alexa Fluor 488-Rabbit anti mouse Ab for 30 minutes. Slides were analyzed on an inverted confocal microscope (LSM510, Carl Zeiss). DAPI was used for nuclear staining.

**Cell Adhesion and Migration Assays**

Cell Adhesion and Migration Assays were performed as described (25). Chi-Square test was used for statistical analysis.

**Flow Cytometry**

FACS analysis was performed to determine integrin expression using mAbs: L230 to αv, PIH5 to α2, GoH3 to α6, TS2/16 to β1, AP3 to β3, P1F6 to β5 or 10D5 to β6. IC10 and non-immune mIgG were used as negative controls.

**Immunoblotting**

PC3-1, PC3-2 and C4-2B cell lysates were prepared, separated by SDS-PAGE gel and analyzed by IB as described before (26). Frozen tumor tissues collected from bone injection sites were homogenized and analyzed by IB, as described (20).

**Gelatin Zymography**

Serum-free culture media collected from Parental, Mock, αvβ6 or αvβ3-expressing C4-2B cells, and from αvβ6-PC3-2 cells stably transfected with shMMP2 or shTROP2 were analyzed by gelatin zymography as described before. Serum-free conditioned medium from BPH1 was used as control for MMP2 and MMP9 (27).

**Intratibial Injection**
Animal studies were conducted in accordance with approved Institutional Animal Care and Use Committee protocols and the NIH Guide for the Care and Use of Laboratory Animals. Tibia intramedullary injections of Mock-PC3-2 cells or \( \alpha v\beta 5 \)- or \( \alpha v\beta 6 \)-PC3-2 stable transfectants were carried out on isofluorane-anesthetized 4- to 6-week-old male SCID mice (The Jackson Laboratory) by using described techniques (28). Briefly, mice were anesthetized with 0.15 mg ketamine/0.015 mg xylazine IP per g body weight. A medial parapatellar incision was created and a needle was placed in the intramedullary canal of the tibia, by aid of fluoroscopy (XiScan 1000-1, XiTec, East Windsor CT). Tumor cells (1x10^5 in 100 μl of PBS) were slowly injected into the tibia and the incision was closed with 5-0 chromic suture (Ethicon Inc, Somerville, NJ). Mice were given 0.1 mg/kg buprenorphine subcutaneously post-operatively. The formation of osteolytic lesions in bones was assessed by radiography using a Faxitron MX-20 (Faxitron X-ray). Bone radiographs were collected on X-Omat TL film (Kodak) using an exposure of 25 kV for 60 s. Lytic areas from epiphysis to metaphysis were quantitated by using ImageJ software, a public domain Java image processing program inspired by NIH Image. Triplicate values were measured and the averages ± SD are shown.

**Micro-Computed Tomography and Bone Histology**

A detailed of Micro-Computed Tomography (\( \mu \)CT) is described in the Supplementary Methods.

Bones were dissected for fixation in 4% paraformaldehyde for 24 hr and either demineralized in 18% EDTA for paraffin embedding or embedded in methyl methacrylate for examination of sections of mineralized tissues. Bone sections were stained with HE. The size of \( \alpha v\beta 6 \)- and \( \alpha v\beta 5 \)-PC3-2 bone tumors, isolated 8-weeks after injection, was evaluated visually by Drs. Languino, Lian and Li using a Zeiss Axioskop 40 and AxioCam HRC camera. The software utilized was AxioVision Rel. 4.7.

**Quantitative Real-Time PCR analysis:** RNA isolated from tumors using Trizol reagent was performed as previously described (29). The sequences for the primers used for human mRNA transcripts for PCR amplification are detailed in the Supplementary Methods.

**Statistical analysis**

Statistical significance between datasets was calculated using t-test and all graphs were generated using Microsoft Excel. The error bars were calculated and represented in terms of mean ± SD. A two-sided p-value of less than 0.05 was considered statistically significant.

**RESULTS**
αvβ6 Integrin Is Expressed in Human Prostate Cancer Bone Metastases and is Functionally Active in Human Prostate Cancer Cells

A detailed analysis of αvβ6 integrin expression in human prostate cancer osteolytic metastases shows that this receptor is detected in twenty-two of thirty-three specimens. Representative images of αvβ6 integrin staining performed by immunofluorescence or by immunohistochemistry are shown in Fig. 1A and 1B respectively using an Ab specific for αvβ6. In contrast, a control non-binding IgG is unreactive (Fig. 1). Consistent with the epithelial origin of metastatic tumor cells, all specimens in this series immunostain positive for cytokeratin (CK) expression (Fig. 1B).

Given the above observation, we investigated the functional status of the αvβ6 integrin in human prostate cancer cells upon ligand binding. Our immunofluorescence data show that integrin αvβ6 localizes to focal contacts when PC3-1 were allowed to attach to fibronectin (Fig. 2A); the α partner of the β6 integrin subunit, αv, was used as marker for focal contacts. Furthermore, we show that αvβ6 promotes ligand-dependent prostate cancer cell adhesion and migration of PC3-1 cells and that cells transfected with β6-shRNA had reduced activities whereas β5-shRNA were not affected (Fig. 2B). The cells were seeded on BSA, type I Collagen or LAP-TGFβ1 coated transwell plates. Parental or shβ5 PC3-1 cells attach and consequently migrate on LAP-TGFβ1 at a significantly higher extent than shβ6 PC3-1 cells. On the other hand, Parental, shβ6 PC3-1 and shβ5 PC3-1 cells migrated equally well on type I Collagen (Fig. 2B). Characterization of the cells used in these experiments was performed using FACS and IB analysis. Parental cells express high levels of β6 and β1, moderate levels of β5 and a negligible amount of β3 (Fig 2C). IB analysis confirms successful downregulation of the β6 and β5 subunits in PC3-1 transfectants (Fig 2D). Overall, these results indicate that αvβ6 is functionally active in human prostate cancer cells.

αvβ6 Integrin Promotes Early Onset of Osteolytic Lesions

To elucidate a potential causal role for αvβ6 in promoting metastatic bone disease, we used PC3-2 cells stably transfected with β6 or control vector (Mock). To prove the specificity of the effect of β6, we selected clones of αvβ6 stably transfected PC3-2 cells which did not show changes in expression of other integrins, such as αv, α2, α6, β1, β3 or β5 molecules, as evaluated by FACS analysis (Supplementary Fig. S1). Injection of PC3-2 transfectants expressing αvβ6 into the tibial medullar cavity of immunocompromised mice gives rise to extensive osteolysis, with complete loss of trabecular bone at the site of inoculation followed by erosion of the cortical bone, resulting in tumor invasion into
the surrounding muscle (Supplementary Fig. S2A). In contrast, intratibial injection of control transfectants (Mock) is associated with only mild osteolysis and characterized by irregular patterns of woven bone tissue suggesting that both mixed osteolytic and osteoblastic lesions occur under these conditions. The extent of osteolytic lesions was quantified from radiograph images of darkened areas of individual bone lesions, representing the absence of mineralized bone (Supplementary Fig. S2B). This analysis confirmed that αvβ6 expressing cells significantly increase and sustain the osteolytic disease compared to Mock cells. To further validate that β6 is driving the osteolytic disease, lysates were prepared from the tumors excised from the limbs from each group (β6 and mock); the results show that osteolysis correlates with high β6 protein levels (Supplementary Fig. S2C), whereas there is no difference in the expression of α2, α6 or β3 integrin subunits (data not shown). In these experiments, tumor cell expression of αvβ6 appears to be associated with increased osteolysis and decreased formation of woven bone, as compared to Mock transfectants (Supplementary Fig. S2D). Histologic analysis of limb lesions shows extensive loss of trabecular and cortical bone in tumor specimens generated by αvβ6 integrin or Mock transfectants (Supplementary Fig. S2D). However, mock transfectants exhibit more new woven bone formation. These osteoblastic lesions initiate within the tumor mass at the margins of tumor (T) and cortical bone (CB) (NB; Supplementary Fig. S2D). Because some woven bone is observed in the αvβ6 group, this suggests competency for osteoblastic lesions; however, αvβ6 may be inducing osteolytic mechanisms that readily degrade the woven bone. Overall, these data show that extensive loss of trabecular and cortical bone in tumor specimens is generated by αvβ6 integrin expressing cells.

The Effect of αvβ6 Integrin on Osteolysis is Specific

To determine whether the integrin-mediated causal role in osteolysis is specific for αvβ6, we injected PC3-2 cells stably transfected with a related integrin αvβ5 (30) in mouse tibiae, and characterized the associated tumor lesions by quantitative μCT (Fig. 3A).

Tumors generated in the αvβ6 group promote extensive osteolytic lesions from 2- to 8-weeks (Fig. 3), consistent with the data presented above. Conversely, bone lesions generated by αvβ5-expressing transfectants are predominantly osteoblastic like the PC3-2-Mock transfectants. In the αvβ6 group, bone erosion starts at 2-weeks and continues with minimal replacement by woven bone until 8-weeks with extensive loss of bone volume (BV) at comparable time points (Fig. 3B). In contrast, limbs injected with αvβ5-expressing cells exhibit an initial, minor osteolytic response at 2-weeks, as observed with the
\(\alpha\beta_6\) group albeit with no statistical difference in BV between the two groups (Fig. 3B). By 4-weeks, these lesions progress towards an osteoblastic phenotype, with woven bone occupying the tumor inoculated region of the tibia and significant increase in tibial BV compared to the \(\alpha\beta_6\) group. The temporal appearance of the osteoblastic lesions, evident by 4-weeks after inoculation of \(\alpha\beta_5\)-expressing cells, exhibits disorganized woven bone within the medullary cavity as well as within the tumor growing on the periosteal side of the eroded cortex (Fig. 3A).

We also examined PC3-2 cells stably transfected with \(\alpha\beta_6\) or \(\alpha\beta_5\) in mouse tibiae by radiography (Supplementary Fig. S3A). The rate of tumor growth and erosion through the cortical bone is indistinguishable between \(\alpha\beta_6\) or \(\alpha\beta_5\) transfectants 4-weeks after injection (Supplementary Fig. S3B, low magnification image). These results indicate that the \(\beta\) subunit that associates with \(\alpha\) determines the specific osteolytic effect observed in response to \(\alpha\beta_6\) expression. Analogous to normal bone remodeling (31) prostate tumor cells secrete factors that facilitate the coupling between osteoclast resorptive activity and bone formation due to osteoblast-like activity (6, 7). Therefore, we examined whether osteoclast activity is induced by \(\alpha\beta_6\). Immunohistochemical detection of active osteoclasts by TRAP staining identifies robust bone resorption at 2-weeks in the \(\alpha\beta_6\) group, compared to mice injected with \(\alpha\beta_5\) transfectants; quantification of TRAP staining indicates a significant increase in osteoclast number in the \(\alpha\beta_6\) compared to \(\alpha\beta_5\) tumors (Supplementary Fig. S3C).

**MMP2 and PTHrP Are Upregulated upon \(\alpha\beta_6\) Integrin Expression**

When analyzed in tumor lysates, MMP2 is induced and found catalytically active in extracts prepared from \(\alpha\beta_6\)- but not \(\alpha\beta_5\)-expressing tumors (Fig. 4A). In contrast, the levels of OPN, a molecule described to mediate prostate cancer cell adhesion and migration in bone (32), remain constant in both groups (Fig. 4A). Consistent with this *in vivo* observation, \(\alpha\beta_6\) expression in PC3-2 cells increases MMP2 at protein and activity levels compared to \(\alpha\beta_5\)-expressing PC3-2 cells *in vitro* (Fig. 4B). Also, we used PC3-1 cells because they express high endogenous levels of \(\alpha\beta_6\). In PC3-1 cells, MMP2 expression as well as its activity is reduced significantly upon shRNA-mediated downregulation of \(\beta_6\) compared to downregulation of \(\beta_5\) (Fig. 4C). Similar results were obtained in another prostate cancer cell line, RWPE, which also expresses high levels of \(\alpha\beta_6\) (Supplementary Fig. S4).

To identify \(\alpha\beta_6\) targets related to the tumor phenotype in bone, we screened a panel of markers in PC3-2 cells expressing \(\beta_6\) for potential expression of genes associated with osteolytic or osteoblastic
lesions (Fig. 5) (23, 33-35). mRNA levels of the following factors were not changed: MMP9, Interleukin-8 (IL8), osteocalcin (OC), dickkopf WNT signaling pathway inhibitor 1 (DKK1), receptor activator of nuclear factor kappa-B ligand (RANKL), runt-related transcription factor 2 (Runx2), vascular endothelial growth factor (VEGF), secreted frizzled-related protein 1 (SFRP1), lymphoid enhancer-binding factor 1 (LEF1) and transcription factor 4 (TCF4). Conversely, mRNA levels of MMP2 and PTHrP, consistently upregulated in αvβ6-PC3-2 tumors (Fig. 5A) and cells (Fig. 5B).

MMP2 Mediates Osteolysis Caused by αvβ6 Integrin Expression

We investigated whether MMP2 activity induced by αvβ6-expressing tumors significantly contributed to the osteolytic lesions, as the causal role of PTHrP in mediating the vicious cycle of osteolytic disease and tumor growth in bone (41) is well established. We generated stable PC3-2 transfectants expressing MMP2-shRNA or a negative control shRNA directed against TROP2. In these experiments, shRNA-mediated downregulation of MMP2 causes dramatic suppression of prostate cancer osteolytic lesions in the intratibial model of metastatic disease (Fig. 6A). Zymographic analysis shows successful reduction of MMP2 activity upon shRNA-mediated downregulation (Fig. 6B). Consistent with these findings, MMP2 silencing also results in significant reduction of bone loss, compared to control lesions (Fig. 6C). This phenotype is quantitatively associated with significant preservation of total bone, and mature bone in MMP2-silenced lesions, as compared with tumors expressing TROP2-shRNA (Fig. 6D).

Since previous studies showed that metastatic prostate cancer contains high levels of androgen receptor (AR) (36), we also evaluated the role of AR in our proposed αvβ6-MMP2 pathway. To perform this study in cells expressing AR, the αvβ6 negative prostate cancer cells, C4-2B, was stably transfected with β6 cDNA. Another αv-associatied integrin αvβ3 was used as a control. FACS analysis shows successful transfection of β6 or β3 (Supplementary Fig. S5). MMP2 expression and activity are consistently found to be induced upon αvβ6, but not αvβ3, expression in C4-2B cells (Fig. 7A, B). As analyzed by qRT-PCR, MMP2 and PTHrP mRNA levels are also increased in αvβ6-C4-2B transfectants compared to Parental cells, Mock or αvβ3-C4-2B transfectants (Fig. 7C). Reproducible results were obtained using one clone and one population for each transfectant. DKK1, RANKL and IL8 levels were undetectable in these cells (data not shown).

Given the above observations in vivo and in vitro, we analyzed 11 human prostate cancer mediated osteolytic specimens to study the expression of αvβ6 and MMP2. Our results show that αvβ6 positively correlates (r=0.6787, P=0.0048) with MMP2 expression in human osteolytic disease (Supplementary
Fig. S6). Overall, our data establish that MMP2, through its matrix degrading activity, promotes osteolysis in αvβ6-expressing prostate tumors.

**DISCUSSION**

In this study, we describe a cancer cell-mediated pathway that promotes osteolysis and is mediated by the αvβ6 integrin and its downstream effector MMP2.

The present study shows a unique effect of the αvβ6 integrin on osteolysis; since αvβ3 promotes osteoblastic lesions (11), we propose that the formation of tumor-derived bone lesions might be controlled by the relative expression of αvβ6 and αvβ3. On the other hand, a different αv integrin, αvβ5, does not increase bone lysis. Our results also confirm that the observed effects are due to β6 expression since other integrin subunits, α2 and α6, known to promote bone lesions, are not affected in our bone model. The results also indicate that α2β1 and α6β1 are inactive or poorly active in these cells since minimal bone loss is observed in the Mock groups. Finally, our data indicate that αvβ6 has a dominant negative effect on αvβ3 since this integrin is expressed in PC3-2 cells and promotes the osteoblastic phenotype of the Mock groups.

Our results show that αvβ6 predominantly promotes osteolytic lesion formation without affecting tumor growth in our intratibial model. Finally, while PC3-1 cells (αvβ6+) have been reported to cause aggressive osteolytic lesions upon intrabone injection (37), PC3-2, MDA-PCa-2b and DU145 cells which lack αvβ6 expression (data not shown), fail either to cause bone lesions or osteoblastic lesions respectively (38, 39). These results indicate that αvβ6 expression correlates with the osteolytic phenotype of the cell type analyzed.

Our results highlight a new specific function of integrins in upregulating MMP2 which consequently causes osteolysis. As in our report, a previous study has shown that expression of αvβ6 in cancer tissues results in enhanced levels of pro-MMPs, specifically MMP2 (40). In another cell type, Morgan et al. reported that the β6 subunit promotes αvβ6-mediated invasion in a MMP9-dependent fashion in vitro (41). In our study, the results appear to be independent of the cell type used and of the expression of androgen receptor. It remains to be investigated whether MMP2 enzymatic activity is maintained by the balance between MMP2 and its natural inhibitor, tissue inhibitor of metalloproteinase 2 (TIMP2). Reduced levels of TIMP2 expression, which result in activation of pro-MMP2 (42), in conjunction with the observed increase in MMP2 protein levels, may conceivably further shift the MMP2/TIMP2 ratio towards increased MMP2 activity.
A study by Corey et al. shows that administration of zoledronic acid, under prevention or treatment regimens, reduces MMP2 and MMP9 expression which correlates with suppression of osteolysis caused by PC3 cells in SCID mice (43). The mechanistic requirements of this pathway have not been completely delineated, but a role for MMPs, including MMP2 and MMP9 in increasing the number of osteoclasts with concomitant bone resorption, has been shown (44). Inhibition of MMP activity has been shown to prevent mineralized bone breakdown induced by the addition of PC3 prostate cancer cells to an in vitro co-culture system with bone organs (44) and to prevent osteoclast recruitment within bone metastases. In our study, mRNA levels of an osteolytic factor PTHrP, which induces osteoclastogenesis when released during the bone remodeling process, are also found to be increased, whereas the levels of other osteolytic factors such as DKK1, IL8, OC and MMP9 remain unaltered. Therefore, in our model, αvβ6-mediated osteolysis could result from a cumulative effect of increased MMP2 enzymatic activity, which by degrading extracellular matrix facilitates osteoclast-activated bone resorption, and PTHrP secretion (33).

This model is in general agreement with clinical data, implicating MMP2 and MMP9 as independent predictors of prostate cancer metastasis and MMP2 association with reduced disease-free survival (45). In addition, although a study by Thiolloy et al. shows that MMP2 released from the host osteoblasts promotes production of mature osteoclasts (46), our system implicates a mechanism in which MMP2 is released from cancer cells rather than host cells. In addition, it is conceivable that since a lack of MMP2 leads to a reduced number of osteoclasts as shown in MMP2−/− mice (47), the cancer cell-mediated signaling supplements the need of the local environment to indirectly activate osteolytic pathways (47). A potential role of integrins in the osteolytic pathway had not been previously investigated. Our study provides new insights into the functions of tumor cells and integrins in the process that occurs in metastatic osteolysis (1, 3).

The role of AR is important in bone metastasis given a recent report that MDV3100, an AR antagonist, stabilizes bone disease (48) and most metastatic androgen-independent prostate cancers express high levels of AR gene transcripts (36). However, in our model, the presence of AR neither alters nor is required for αvβ6-mediated induction of MMP2.

Designing new therapeutic approaches for prostate cancer based on inhibiting integrin function or integrin downstream signaling offers novel strategies to cure this cancer. In our model, we propose that increased expression of αvβ6 causes upregulation of MMP2 and consequently promotes osteolysis. Thus, by inhibiting αvβ6/MMP2 signaling pathways we are likely to prevent bone metastasis associated with advanced prostate cancer. Conjugation of a drug or toxin to an Ab or to a peptide that selectively
binds $\alpha_v$ integrins has been used to enhance the anti-tumor effect of the drug (49) and a similar approach may prevent osteolysis associated with metastatic prostate cancer.

**AUTHORS’ CONTRIBUTIONS**

Conception and Design: J.L., A.D., T.J.F, J.B.L. and L.R.L.


Writing of the Manuscript: J.L., A.D., J.L.S., G.S.S., J.B.L. and L.R.L.

Materials: Z.J., R.B. and S.V.

Supervision of Study: L.R.L.

**ACKNOWLEDGEMENTS**

We thank Drs. C. Fedele, A. Sayeed and M. Trerotola for constructive discussion. We also thank Drs. L.W. Chung for providing PC3-1 cell lines; A. Cress for antibody to $\alpha_6$; I. Hart for pBabe-$\beta_6$ integrin construct; S. Hayward for BPH1 cells; C. Hsieh and Qin Liu for statistical analysis; V. Quaranta for antibody to $\beta_6$. We appreciate Tiziana DeAngelis for immunofluorescence analysis; Cheryl Morris assistance for tissue procurement; Jennifer Colby for intratibia injection procedures; Stacy Russell and Sadiq Hussain for imaging and bone histological analyses in the University of Massachusetts $\mu$-CT Core.

**REFERENCES**


18. Garlick DS, Li J, Sansoucy B, Wang T, Griffith L, FitzGerald TJ, et al. \(\gamma_6\alpha_6\) integrin expression is induced in the POET and PTEN\(^{0\text{c-}}\) mouse models of prostatic inflammation and prostatic adenocarcinoma Am J Transl Res. 2012;4:165-74.


FIGURE LEGENDS

Fig. 1. αvβ6 is expressed in human prostate cancer bone metastases
A. Prostate cancer osteolytic metastases were stained for αvβ6 by immunofluorescence. The panels show two representative images of αvβ6 immunostaining in prostate cancer bone (B) metastases. DAPI was used for nuclear staining. 40X magnification. B, Expression of αvβ6 in prostate bone (B) metastases was analyzed by immunohistochemistry using an Ab specific for β6 (left panel). Serial sections were stained using Abs to IgG (middle panel) or cytokeratins (CK, right panel). Two representative examples are shown.

Fig. 2. αvβ6 expression promotes adhesion and consequently migration
A, Expression of β6 and αv was observed at the focal contacts in PC3-1 human prostate cancer cells (63X magnification). IgG was used as a negative control (40X magnification). B, Adhesion and migration assays were performed using Parental, shβ5 or shβ6 PC3-1 cells seeded on BSA, type I Collagen or LAP-TGFβ1-coated transwell chambers respectively. The experiment was repeated at least three times, and similar results were observed. The results from a representative experiment are shown in the bar graph. Error bars, SEM. The differences between adhesion and migration, of shβ5
and shβ6 cells on LAP-TGFβ1 are statistically significant. *, P=0.004 and **, P=0.002. C, FACS analysis of different αv associated β-subunits (β1, β3, β5, β6) in PC3-1 cells is shown. IgG was used as a negative control Ab. D, Parental, shβ5 or shβ6 PC3-1 cells were serum starved for 24 hr and cell lysates were analyzed by SDS-PAGE and probed with an Ab to β5 or 2A1 Ab to β6. ERK was used as a loading control.

**Fig. 3. Specific effect of αvβ6 on osteolytic lesions**

A, Representative μ-CT images of bone lesions caused by αvβ6-PC3-2 (upper panels) and αvβ5-PC3-2 cells (lower panels) at 2-, 4- and 8-week time points after intratibial injection. Two representative bones from n=8 are shown. B, Quantification of μ-CT images for net bone loss in the αvβ6-PC3-2 and αvβ5-PC3-2 bone tumors at 2-weeks, 4-weeks and 8-weeks is shown. Quantitation of bone volume from 3D images at a threshold range 220-1000 was performed. P<0.004.

**Fig. 4. MMP2 is induced by αvβ6**

A, β6, MMP2 and OPN protein levels (left panels) and MMP2 activity were analyzed by IB or gelatin zymography (Zg, right panel) in αvβ6- and αvβ5-PC3-2 bone tumors isolated 8-weeks after injection. For MMP2 IB, intervening lanes have been spliced out. As a positive control for active MMPs, conditioned medium of BPH1 cells was used. B, MMP2 expression (left panels) and activity (right panels) in Parental, αvβ5-PC3-2 and two clones of αvβ6-PC3-2 cells were analyzed by IB (12.5 % SDS-PAGE) or Zg respectively. C, MMP2 expression (left panels) and activity (right panels) in Parental, shβ5- and shβ6-PC3-1 were analyzed by IB (10% SDS-PAGE) or Zg respectively. AKT (A) and ERK (A-C) were used as loading controls.

**Fig. 5. αvβ6 expression selectively upregulates MMP2 and PTHrP**

A, mRNA levels of osteolytic (DKK1, IL8, MMP2, MMP9, OC, PTHrP, RANKL, Runx2, SFRP1, VEGF) and osteoblastic factors (LEF1, Runx2, SFRP1, TCF4) in αvβ6- and αvβ5-PC3-2 bone tumors were analyzed 8-weeks after injection by qRT-PCR. B, MMP2, PTHrP, MMP9, DKK1, RANKL and IL8 mRNA levels were analyzed in αvβ6- and αvβ5-PC3-2 cells by qRT-PCR. mRNA expression levels were normalized to GAPDH. * indicates statistically significant differences in mRNA expression levels between the two groups.
Fig. 6. MMP2 mediates αvβ6-induced osteolysis in vivo

A, Comparison of μCT images from αvβ6-PC3-2-shMMP2 (upper panels) and -shTROP2 (lower panels) groups of tibiae 4-weeks after injection (n=5 mice per group). A representative image of each group showing front and back side of the tibia, scanned at 10 μm resolution reveals osteolysis in the shTROP2 group, although osteolysis was not detectable in the shMMP2 group. B, MMP2 activity was analyzed in the culture supernatant of αvβ6-PC3-2-shMMP2 and αvβ6-PC3-2-shTROP2 cells by gelatin zymography (Zg). Serum-free culture medium from BPH1 cells was used as a control for active MMPs. C, Bone loss in tumor tibiae. As a reference point for quantitating the inhibited osteolytic disease by shMMP2, the contralateral tibia of each mouse was imaged and BV quantitated at the 275-1000 threshold based on mature high density bone (H.D.). Bone loss was calculated by subtracting the BV of the tumor bearing limb from the BV of the normal tibia and the difference is presented as a % loss of the contralateral limb. n=3, αvβ6-PC3-2 control group; n=5, shMMP2 group; and n=5, shTROP2 group. D, Calculations of BV include three hundred 10 μm slices from the beginning growth plate performed at two thresholds 175-1000 to include woven bone and 275-1000 to encompass the mature bone. A group of mice (n=3) injected with the αvβ6-PC3-2 cell line (Control) was included for comparison to evaluate the effect of the shTROP2 or shMMP2.

Fig. 7. MMP2 is induced upon αvβ6 expression in AR+ prostate cancer cells in vitro. A-B, MMP2 protein levels (A) and activity (B) were analyzed by IB (A) and zymography (Zg, B) respectively in Parental, vector transfected (#A, Mock), αvβ6- or αvβ3-transfected C4-2B cells. One clone and one population (pop.) for each αvβ6 (#1, #9) or αvβ3 (#88, #89) transfected C4-2B cells were used (B). ERK was used as a loading control (A). C, MMP2, PTHrP, MMP9 mRNA levels in Parental, Mock, αvβ6 and αvβ3-C4-2B cells were analyzed by qRT-PCR. mRNA expression levels were normalized to GAPDH. * indicates statistically significant difference in mRNA expression levels between the groups.
Figure 2

A

Ab: $\beta_6$  $\alpha_v$  IgG

PC3-1 cells

B

(A) Attached cells (OD562 nm)

- Parental
- sh$\beta_5$
- sh$\beta_6$

(B) Migrated/Attached cells (%)

- LAP-TGF$\beta_1$
- Type I Collagen
- BSA

PC3-1

C

PC3-1 cells

- IgG
- $\beta_6$
- $\beta_5$
- $\beta_3$
- $\beta_1$

D

PC3-1 cell lysate

- ERK

- sh$\beta_5$
- sh$\beta_6$
**Figure 3**

A

<table>
<thead>
<tr>
<th>2wks</th>
<th>4wks</th>
<th>8wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
</tbody>
</table>

PC3-2

![Images showing bone structures at different times](image)

$\alpha_{\nu}\beta_6$ $\alpha_{\nu}\beta_5$

B

![Bar chart showing bone volume](image)

<table>
<thead>
<tr>
<th>Bone Volume (mm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
</tr>
<tr>
<td>6</td>
</tr>
</tbody>
</table>

$\alpha_{\nu}\beta_5$-PC3-2 $\alpha_{\nu}\beta_6$-PC3-2

* Significant difference
Figure 4

A

IB

PC3-2 Tumor lysate

β6
MMP2
OPN
AKT
AKT

control
αvβ5
αvβ6

BPH1
PC3-2
Tumor
lysate

Zg
MMP9
MMP2

B

IB

PC3-2

Parental
αvβ5
αvβ6

Pro-MMP2
Active MMP2

ERK

Active MMP2

cell lysate
culture supernatant

C

IB

PC3-1

Parental
shβ5
shβ6

Pro-MMP2
Active MMP2

ERK

Active MMP2

cell lysate
culture supernatant
Figure 6

A

4wks

shMMP2

αvβ6-PC3-2

shTROP2

B

Zg

MMP9

MMP2

αvβ6-PC3-2

BPH1

C

HD Bone Volume loss (%)

shMMP2  32.92%

shTROP2  29.90%

Control  16.77%

D

Bone Volume (mm3)

Total bone (175-1000) Mature bone (275-1000)

Control (n=3) shMMP2 (n=5) shTROP2 (n=5)

* p<0.01  ** p<0.009  *** p<0.002
Integrin $\alpha v \beta 6$ promotes an osteolytic program in cancer cells by upregulating MMP2

Anindita Dutta, Jing Li, Huimin Lu, et al.

Cancer Res  Published OnlineFirst January 2, 2014.