Integrin αvβ6 Promotes an Osteolytic Program in Cancer Cells by Upregulating MMP2

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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 αvβ6 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-β1; 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 αvβ6 integrin promotes this type of metastasis. We show for the first time that αvβ6 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 αvβ6 on MMP2 expression and activity is independent of androgen receptor in the analyzed prostate cancer cells. Increased levels of parathyroid hormone–related protein (PTHrP), known to induce osteoclastogenesis, were also observed in αvβ6-expressing cells. However, by using MMP2 short hairpin RNA, we demonstrate that the αvβ6 effect on bone loss is due to upregulation of soluble MMP2 by the cancer cells, not due to changes in tumor growth rate. Another related αv-containing integrin, αvβ5, fails to show similar responses, underscoring the significance of αvβ6 activity. Overall, these mechanistic studies establish that expression of a single integrin, αvβ6, contributes to the cancer cell–mediated program of osteolysis through inducing matrix degradation through MMP2. Our results open new prospects for molecular therapy for metastatic bone disease.

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Introduction

More than 80% of patients with prostate cancer, at autopsy, have metastatic foci in the bone that 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 as 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 with osteolytic metastases, as 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- and 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 (12) as well as αvβ3 (11) have been demonstrated to contribute respectively to osteolytic and osteoblastic lesions.

Overexpression of αvβ6 integrin 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)-TGF-β1 and in terms of signaling responses, facilitates the release of active TGF-β1, which is a prometastatic cytokine (17).

Our data show that the αvβ6 integrin promotes matrix metalloproteinase (MMP) 2 and parathyroid hormone–related protein (PTHrP) upregulation and demonstrates 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

Bovine serum albumin (BSA) was from Sigma-Aldrich, type I collagen and 4,6-diamidino-2-phenylindole (DAPI) were from Invitrogen; LAP-TGF-β1 was from R&D Systems.

We used the following rabbit antibodies against: extracellular signal–regulated kinase 1/2 (ERK1/2) and AKT from Santa Cruz Biotechnology; αvβ6 (a gift of Dr. V. Quaranta, Vanderbilt University, Nashville, TN) for immunohistochemistry; β3 and β5 cytoplasmic domains from Millipore for immunoblotting; MMP2 from Millipore for immunoblotting using tumor lysates or from Cell Signaling Technology for immunoblotting using cell lysates and immunohistochemistry.

We used the following mouse monoclonal antibodies (mAb): 2A1 to β6 for immunoblotting (16, 19) and immunofluorescence; LFMB-14 to osteopontin (OPN) from Santa Cruz Biotechnology; clones AE1 and AE3 to cytokeratin from Millipore. IC10 irrelevant mAb and rabbit immunoglobulin G (IgG; from Sigma) were used as negative controls.

Rabbit anti-mouse Alexa Fluor 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 (Worcester, MA) or Cooperative Human Tissue Network (CHTN, other investigators may have received specimens from these same tissues) and were processed according to Institutional Review Board-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 (Philadelphia, PA). 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 previously (20–22).

Cells and culture conditions

Authentication of the cell lines was provided with their purchase from UroCore Inc. (C4-2B) or American Type Culture Collection (PC3 and RWPE); the cells were used in our laboratory for less than 6 months. Two PC3 sublines, PC3-1 and 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) 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 were performed as detailed in the Supplementary Methods.

Immunofluorescence and confocal microscopy

Immunofluorescence analysis on PC3-1 cells was performed as described previously (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 minutes at 37°C. The sections were blocked with PBS/5% BSA. αvβ6 Staining was performed incubating samples with 2A1 antibody for 1 hour, followed by incubation with Alexa Fluor 488-rabbit anti-mouse antibody 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 previously (25). Chi-square test was used for statistical analysis.

Flow cytometry

Fluorescence-activated cell sorting (FACS) analysis was performed to determine integrin expression by using mAbs: L230 to αv, P1H5 to α2, GoH3 to α6, TS2/16 to β1, AP3 to β3, P1F6 to β5, or 10D5 to β6. IC10 and nonimmune mouse IgG 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 immunoblotting as described before (26). Frozen tumor tissues collected from bone injection sites were homogenized and analyzed by immunoblotting, as described previously (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 transfect 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 αvβ5- or αvβ6-PC3-2 stable transfectants were carried out on isofluorane-anesthetized 4- to 6-week-old male severe combined immunodeficient (SCID) mice (The Jackson Laboratory) by using described techniques (28). Briefly, mice were anesthetized with 0.15 mg ketamine/0.015 mg xylazine intraperitoneally (i.p.) per gram 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; XTec). Tumor cells (1 × 106 in 100 μL of PBS) were slowly injected into the tibia and the incision was closed with 5-0 huprenorphine subcutaneously postoperatively. 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 seconds. 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 analysis of micro-computed tomography (μCT) is described in the Supplementary Methods.

Bones were dissected for fixation in 4% paraformaldehyde for 24 hours 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 hematoxylin and eosin (H&E). The size of αvβ6- and αvβ5-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 used 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.

Figure 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. Magnification, ×40. B, expression of αvβ6 in prostate bone (B) metastases was analyzed by immunohistochemistry using an antibody specific for β6 (left panel). Serial sections were stained using antibodies to IgG (middle) or cytokeratins (CK; right). Two representative examples are shown.
Results

\(\alpha\beta6\) integrin is expressed in human prostate cancer bone metastases and is functionally active in human prostate cancer cells

A detailed analysis of \(\alpha\beta6\) integrin expression in human prostate cancer osteolytic metastases shows that this receptor is detected in 22 of 23 specimens. Representative images of \(\alpha\beta6\) integrin staining performed by immunofluorescence or by immunohistochemistry are shown in Fig. 1A and B, respectively, using an antibody specific for \(\alpha\beta6\). In contrast, a control nonbinding IgG is unreactive (Fig. 1). Consistent with the epithelial origin of metastatic tumor cells, all specimens in this series immunostain positive for cytokeratin expression (Fig. 1B).

Given the above observation, we investigated the functional status of the \(\alpha\beta6\) integrin in human prostate cancer cells upon ligand binding. Our immunofluorescence data show that integrin \(\alpha\beta6\) localizes to focal contacts when PC3-1 cells were allowed to attach to fibronectin (Fig. 2A); the \(\beta6\) integrin subunit, \(\alpha\), was used as a marker for focal contacts. Furthermore, we show that \(\alpha\beta6\) promotes ligand-dependent prostate cancer cell adhesion and migration of PC3-1 cells and that cells transfected with \(\beta6\)-shRNA had reduced activities, whereas \(\beta5\)-shRNA transfecants were not affected (Fig. 2B). The cells were seeded on BSA-, type I collagen-, or LAP-TGF-\(\beta1\)–coated Transwell plates. Parental or sh\(\beta5\) PC3-1 cells attach and consequently migrate on LAP-TGF-\(\beta1\) to a significantly higher extent than sh\(\beta6\) PC3-1 cells.

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Figure 2. \(\alpha\beta6\) expression promotes adhesion and consequently migration. A, expression of \(\beta6\) and \(\alpha\) was observed at the focal contacts in PC3-1 human prostate cancer cells (magnification, \(\times63\)). IgG was used as a negative control (magnification, \(\times40\)). B, adhesion and migration assays were performed using Parental, sh\(\beta5\), or sh\(\beta6\) PC3-1 cells seeded on BSA, type I collagen, or LAP-TGF-\(\beta1\)–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 sh\(\beta5\) and sh\(\beta6\) cells on LAP-TGF-\(\beta1\) are statistically significant. *\(P = 0.004\) and **\(P = 0.002\). C, FACS analysis of different \(\alpha\beta\)-associated \(\beta\)-subunits (\(\beta1\), \(\beta3\), \(\beta5\), and \(\beta6\)) in PC3-1 cells is shown. IgG was used as a negative control antibody (Ab). D, Parental, sh\(\beta5\), or sh\(\beta6\) PC3-1 cells were serum starved for 24 hours and cell lysates were analyzed by SDS-PAGE and probed with an antibody to \(\beta5\) or 2A1 antibody to \(\beta6\). ERK was used as a loading control.
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 by using FACS and immunoblotting analysis. Parental cells express high levels of β6 and β1, moderate levels of β5, and a negligible amount of β3 (Fig. 2C). Immunoblotting 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 medullary 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 seems to be associated with increased osteolysis and decreased

![Figure 3. Specific effect of αvβ6 on osteolytic lesions. A, representative μCT images of bone lesions caused by αvβ6-PC3-2 (top) and αvβ5-PC3-2 cells (bottom) at 2-, 4-, and 8-week time points after intratibial injection. Two representative bones from n = 8 are shown. B, quantification of μCT images for net bone loss in the αvβ6-PC3-2 and αvβ5-PC3-2 bone tumors at 2, 4, and 8 weeks is shown. Quantitation of bone volume from three-dimensional images at a threshold range 220 to 1,000 was performed. *, P < 0.004.](image-url)
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 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 αvβ6 group albeit with no statistical difference in bone volume between the two groups (Fig. 3B). By 4 weeks, these lesions progress toward an osteoblastic phenotype, with woven bone occupying the tumor inoculated region of the tibia and significant increase in tibial bone volume compared with the αvβ6 group. The temporal appearance of the osteoblastic lesions, evident by 4 weeks after inoculation of αvβ5-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 αvβ6 or αvβ5 in mouse tibiae by radiography (Supplementary Fig. S3A). The rate of tumor growth and erosion through the cortical bone is indistinguishable between αvβ6 or αvβ5 transfectants 4 weeks after injection (Supplementary Fig. S3B, low magnification image). These results indicate that the β subunit that associates with αv determines the specific formation of woven bone, as compared with 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.

Figure 4. MMP2 is induced by αvβ6.

A, β6, MMP2, and OPN protein levels (left) and MMP2 activity were analyzed by immunoblotting (IB) or gelatin zymography (Zg; right) in αvβ6- and αvβ5-PC3-2 bone tumors isolated 8 weeks after injection. For MMP2 immunoblotting, intervening lanes have been spliced out. As a positive control for active MMPs, conditioned medium of BPH1 cells was used. B, MMP2 expression (left) and activity (right) in Parental, αvβ5-PC3-2, and two clones of αvβ6-PC3-2 cells were analyzed by immunoblotting (12.5% SDS-PAGE) or Zg, respectively. C, MMP2 expression (left) and activity (right) in Parental, shβ5-, and shβ6-PC3-1 were analyzed by immunoblotting (10% SDS-PAGE) or Zg, respectively. AKT (A) and ERK (A–C) were used as loading controls.
osteolytic effect observed in response to αvβ6 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 αvβ6. Immunohistochemical detection of active osteoclasts by TRAP staining identifies robust bone resorption at 2 weeks in the αvβ6 group, compared with mice injected with αvβ5 transfectants; quantification of TRAP staining indicates a significant increase in osteoclast number in the αvβ6 compared with αvβ5 tumors (Supplementary Fig. S3C).

MMP2 and PTHrP are upregulated upon αvβ6 integrin expression

When analyzed in tumor lysates, MMP2 is induced and found catalytically active in extracts prepared from αvβ6- but not αvβ5-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, αvβ6 expression in PC3-2 cells increases MMP2 at the protein and activity levels compared with αvβ5-expressing PC3-2 cells in vitro (Fig. 4B). Also, we used PC3-1 cells because they express high endogenous levels of αvβ6. In PC3-1 cells, MMP2 expression as well as its activity are reduced significantly upon short hairpin RNA (shRNA)-mediated downregulation of β6 compared with downregulation of β5 (Fig. 4C). Similar results were obtained in another prostate cancer cell line, RWPE, which also expresses high levels of αvβ6 (Supplementary Fig. S4).

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 (36) 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 down-regulation 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 a significant reduction of bone loss, compared with control lesions (Fig. 6C). This phenotype is quantitatively associated with a significant preservation of total bone, and mature bone in MMP2-silenced lesions, as compared with tumors expressing TROP2-shRNA (Fig. 6D).

Because previous studies showed that metastatic prostate cancer contains high levels of androgen receptor (AR; ref. 37), we also evaluated our proposed αvβ6–MMP2 pathway in AR-positive cells in our proposed αvβ6–MMP2 pathway. To perform this study in cells expressing AR, the αvβ6-negative prostate cancer cells, C4-2B, were stably transfected with β6 cDNA. Another αv-associated 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 and B). As analyzed by quantitative real-time PCR (qRT-PCR), MMP2 and PTHrP mRNA levels are also increased in αvβ6-C4-2B transfectants compared with 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 IL-8 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)
Our results show that $\alpha_v\beta_6$ predominantly promotes osteolytic lesion formation without affecting tumor growth in our intratibial model. Finally, while PC3-1 cells ($\alpha_v\beta_6^+$) have been reported to cause aggressive osteolytic lesions upon intrabone injection (36), DU145 and MDA-PCA cells, which lack $\alpha_v\beta_6$ expression (data not shown), fail either to cause bone lesions or cause osteoblastic lesions (38, 39). These results indicate that $\alpha_v\beta_6$ 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 $\alpha_v\beta_6$ in cancer tissues results in enhanced levels of pro-MMPs, specifically MMP2 (40). In another cell type, Morgan and colleagues reported that the $\beta_6$ subunit promotes $\alpha_v\beta_6$-mediated invasion in a MMP9-dependent fashion in vitro (41). In our study, the results seem to be independent of the cell type used and of the expression of AR. 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 toward increased MMP2 activity.

A study by Corey and colleagues shows that administration of zoledronic acid, under prevention or treatment regimens, reduces MMP2 and 9 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 coculture 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, IL-8, osteocalcin and MMP9 remain unaltered. Therefore, in our model, $\alpha_v\beta_6$-mediated osteolysis could result from a cumulative effect of increased MMP2 enzymatic activity, which by degrading ECM facilitates osteoclast-activated bone resorption, and PTHrP secretion (33).

This model is in general agreement with clinical data, implicating MMP2 and 9 as independent predictors of prostate cancer metastasis and MMP2 association with reduced disease-free survival (45). In addition, although a study by Thiolloy and colleagues shows that MMP2 released from the host osteoblasts promotes production of mature bone model. The results also indicate that $\alpha_v\beta_1$ and $\alpha_v\beta_1$ are inactive or poorly active in these cells as minimal bone loss is observed in the Mock groups. Finally, our data indicate that $\alpha_v\beta_6$ has a dominant negative effect on $\alpha_v\beta_3$ as this integrin is expressed in PC3-2 cells and promotes the osteoblastic phenotype of the Mock groups.

Discussion

In this study, we describe a cancer cell-mediated pathway that promotes osteolysis and is mediated by the $\alpha_v\beta_6$ integrin and its downstream effector MMP2.

The present study shows a unique effect of the $\alpha_v\beta_6$ integrin on osteolysis; as $\alpha_v\beta_3$ promotes osteoblastic lesions (11), we propose that the formation of tumor-derived bone lesions might be controlled by the relative expression of $\alpha_v\beta_6$ and $\alpha_v\beta_3$. On the other hand, a different $\alpha_v\beta_1$ integrin, $\alpha_v\beta_5$, does not increase bone loss. Our results also confirm that the observed effects are due to $\beta_6$ expression as other integrin subunits, $\alpha_2$ and $\alpha_6$, known to promote bone lesions, are not affected in our

![Figure 7](image-url). MMP2 is induced upon $\alpha_v\beta_6$ expression in AR$^+$ prostate cancer cells in vitro. A and B, MMP2 protein levels (A) and activity (B) were analyzed by immunoblotting (A) and zymography (Zg, B), respectively, in Parental, vector transfected (Mock), $\alpha_v\beta_6$- or $\alpha_v\beta_3$-transfected C4-2B cells. One clone and one population (pop.) for each $\alpha_v\beta_6$ (#1, #5) or $\alpha_v\beta_3$ (#88, #89) transfected C4-2B cells were used (B). ERK was used as a loading control (A). C, MMP2, PTHrP, and MMP9 mRNA levels in Parental, Mock, $\alpha_v\beta_6$, and $\alpha_v\beta_3$-C4-2B cells were analyzed by qRT-PCR. mRNA expression levels were normalized to GAPDH. *p < 0.05, statistically significant difference in mRNA expression levels between the groups.

with MMP2 expression in human osteolytic disease (Supplementary Fig. S6). Overall, our data establish that MMP2, through its matrix-degrading activity, promotes osteolysis in $\alpha_v\beta_6$-expressing prostate tumors.
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 as 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 (37). 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 proposed 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 antibody or to a peptide that selectively binds αv integrins has been used to enhance the antitumor effect of the drug (49) and a similar approach may prevent osteolysis associated with metastatic prostate cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Disclaimer
The Pennsylvania Department of Health (H.R.) specifically disclaims responsibility for any analyses, interpretations, or conclusions.

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Integrin αvβ6 Promotes an Osteolytic Program in Cancer Cells by Upregulating MMP2

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