Type I Collagen Receptor ($\alpha_2\beta_1$) Signaling Promotes the Growth of Human Prostate Cancer Cells within the Bone

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Abstract

The most frequent site of prostate cancer metastasis is the bone. Adhesion to bone-specific factors may facilitate the selective metastasis of prostate cancer to the skeleton. Therefore, we tested whether prostate cancer bone metastasis is mediated by binding to type I collagen, the most abundant bone protein. We observed that only bone metastatic prostate cancer cells bound collagen I, whereas cells that form only visceral metastases failed to bind collagen. To confirm the relationship between collagen adhesion and bone metastatic potential, a collagen-binding variant of human LNCaP prostate cancer cells was derived through serial passage on type I collagen ($LNCaP_{col}$). Fluorescence-activated cell sorting analysis showed that $LNCaP_{col}$ cells express increased levels of the integrin collagen I receptor $\alpha_2\beta_1$ compared with LNCaP cells. Antibodies to the $\alpha_2\beta_1$ complex inhibited $LNCaP_{col}$ binding to collagen, confirming that integrins mediated the attachment. Correspondingly, $LNCaP_{col}$ cells displayed enhanced chemotactic migration toward collagen I compared with LNCaP cells, an activity that could be blocked with $\alpha_2\beta_1$ antibodies. To directly test the role of $\alpha_2\beta_1$-dependent collagen binding in bone metastasis, LNCaP and $LNCaP_{col}$ cells were injected into the tibia of nude mice. After 9 weeks, 7 of 13 (53%) mice injected with $LNCaP_{col}$ developed bone tumors, whereas 0 of 8 mice injected with LNCaP cells had evidence of boney lesions. $LNCaP_{col}$ cells were found to express increased levels of the metastasis-promoting RhoC GTPase compared with parental LNCaP. We conclude that collagen I attachment mediated by $\alpha_2\beta_1$ initiates motility programs through RhoC and suggest a mechanism for prostate cancer metastasis to the bone. (Cancer Res 2006; 66(17): 8648-54)

Introduction

A characteristic clinical feature of prostate cancer is the frequency with which it spreads or metastasizes to the bone (reviewed in ref. 1). Recent autopsy data suggest that >80% of all men who die of prostate cancer have metastatic disease within the skeleton, specifically in the trabecular bone of the pelvis, femur, and vertebral bodies (2, 3). This preferential seeding of the skeleton may be mediated, in part, through selective adhesion of prostate cancer cells to bone-specific factors. Identification of these factors may reveal important clues about the mechanism of bone metastasis as well as provide new targets for the prevention of prostate cancer skeletal metastasis.

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Type I collagen is the most abundant protein within the bone, making up >90% of the total protein within this site (4). The most common cell surface receptors for collagen I are integrins. The integrin family is a class of transmembrane adhesion molecules composed of noncovalently linked α and β subunits (reviewed in ref. 5). Each $\alpha\beta$ heterodimer mediates attachment to unique sets of extracellular matrix (ECM) proteins, which for collagen I include integrin pairs $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ (6). Integrin binding is dependent on divalent cations as well as the presence of binding sequences Arg-Gly-Asp or Asp-Gly-Glu-Ala within the ECM protein. Rather than passive anchors for cellular adhesion, the β subunit interacts with signal transduction pathways that mediate, in a cell type-specific manor, proliferation, survival, invasion, and metastasis (7, 8).

Intracellular signaling molecules activated by integrins include focal adhesion kinase (*Fak*) and the nonreceptor tyrosine kinases *Src* and *Fyn*, which lead to ligand-mediated activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase signal transduction pathways (7, 9). Integrin engagement can also stimulate integrin-linked kinase, resulting in cytoskeletal reorganization and signal transduction through the activation of RhoA GTPase (10–12). The Rho GTPases belong to the Ras superfamily of small GTP-binding proteins and act as molecular switches involved in all aspects of cellular motility and invasion, including polarity and cytoskeletal organization but not attachment-dependent growth (12–14). Like Ras, Rho cycles between an inactive GDP-bound and active GTP-bound state that can activate downstream effector proteins (15, 16). RhoC is a Rho GTPase protein implicated in the progression and metastasis of several cancers, including breast, pancreas, and melanoma (17–19). We previously showed that RhoC is present in the PC-3 cell line, which is derived from a bone metastasis, and is responsible for the invasive capabilities of these cells (20). Unlike RhoA, however, RhoC has not previously been shown as a downstream effector of integrin signaling.

Herein, we show that bone metastatic prostate cancer cells both bind to and are chemotactic toward collagen I. Further, cells selected *in vitro* for enhanced binding to collagen from non-tumorigenic LNCaP parental cells are highly motile and acquire the capacity to grow within the bone. Finally, we provide evidence that integrin-mediated activation of RhoC GTPase contributes to the invasive phenotype and suggest a mechanism for prostate cancer metastasis to the bone.

Materials and Methods

Cells. LNCaP human prostate cancer cells were obtained from the American Type Culture Collection (Rockville, MD). C4-2B, an isogenic LNCaP variant capable of spontaneous metastasis to the bone following intraprostatic injection, was obtained from UroCor (Oklahoma City, OK). PC-3M and highly metastatic PC-3M-LN4 and LNCaP-LN3 cells were kind gifts from Dr. Curtis Pettaway (University of Texas M. D. Anderson Cancer Center, Houston, TX; ref. 21). PC-3M was isolated from a liver metastasis

produced by the intrasplenic injection of human PC-3 prostate cancer cells into nude mice. Highly metastatic PC-3M-LN4 cells were generated following the initial intraprostatic injection of PC-3M cells and subsequent orthotopic cycling of resultant lymph node tumor cells four rounds into the prostate of nude mice. LNCaP-LN3 cells were derived through three orthotopic cyclings of lymph node tumors initiated by LNCaP cells. PC-3M, PC-3M-LN4, LNCaP, and LNCaP-LN3 cells were maintained in RPMI 1640 [10% fetal bovine serum (FBS), l mmol/L sodium pyruvate, l× penicillin/streptomycin, 0.l mmol/L nonessential amino acids, 2 mmol/L L-glutamine, and l× vitamin solution (Invitrogen, Carlsbad, CA)]. C4-2B cells were maintained in T-medium [80% DMEM-20% Ham's F12 (Invitrogen), 5 μg/mL insulin, 13.6 pg/mL triiodothyronine, 5 μg/mL transferrin, 0.25 μg/mL biotin, 25 μg/mL adenine (Sigma, St. Louis, MO), 1× penicillin/streptomycin, and 5% FBS]. All cells were shown to be free of *Mycoplasma* by PCR ELISA (Roche, Indianapolis, IN).

Derivation of $LNCaP_{col}$. $LNCaP_{col}$ was derived from heterogenous, parental LNCaP cells by successive panning on type I collagen. Tissue culture plates (100 mm) were coated with 1 $\mu g/cm^2$ type I collagen from rat tail (BD Biosciences, Bedford, MA) in 20 mmol/L acetic acid/PBS for 2 hours at 37°C. LNCaP cells were plated on to collagen-covered dishes at 1×10^5/mL in binding buffer [serum-free RPMI 1640 supplemented with 0.5% bovine serum albumin (BSA)]. After 1 hour, nonadherent cells were removed by washing thrice with binding buffer. Culture medium was added, and remaining collagen-adherent cells were allowed to expand. Once the panned cells had reached 80% confluence, these cells were removed by incubation in 1.0 mmol/L EDTA in PBS for 10 minutes at 37°C and washed and the panning procedure was repeated for a maximum of eight passages.

Integrin profile of LNCaP variant cells. Prostate cancer cells (2×10^5) were incubated with phycoerythrin-conjugated antisera to integrin subunits α_1 (clone SR84, BD PharMingen, San Diego, CA), α_2 (clone AK7, Chemicon, Temecula, CA), α_3 (clone C3 II.1, BD PharMingen), β_1 (clone MAR4, BD PharMingen), or $IgG1_\kappa$ isotype control for 30 minutes at 4°C. Cells were also stained for $\alpha_2\beta_1$ heterodimer using a monoclonal antibody (Chemicon) conjugated to Alexa Fluor 647 (Invitrogen). Labeled cells were washed, fixed with 4% paraformaldehyde, and evaluated on a Coulter FACScan flow cytometer (Beckman Coulter, Fullerton, CA).

***In vitro* attachment assay.** Triplicate wells on a 96-well white opaque tissue culture plate (Costar, Corning, NY) were coated with 1 $\mu g/cm^2$ type I collagen (BD Biosciences), human fibronectin (BD Biosciences), or bovine thyroglobulin (Sigma) for 2 hours at 37°C. Following this incubation, the excess protein was removed by washing once with serum-free medium and plates were blocked with 0.1 mL/well 1% (w/v) BSA in RPMI 1640 for 2 hours at 37°C. Prostate cancer cells were lifted with 1 mmol/L EDTA as above and washed once to binding buffer. Cells (1×10^6/mL) were then labeled with a final concentration of 2 μmol/L calcein AM (Invitrogen) for 10 minutes at room temperature. Calcein AM is a nonfluorescent cell-permeant dye that is converted to green fluorescent calcein by viability cells and was used to fluorescently label cells in our adhesion assays. Labeled cells were washed with binding buffer and diluted to 1×10^5/mL, and 1×10^4 cells were plated to the blocked 96-well plate. Adhesion was allowed to continue for 1 hour at 37°C. Following this incubation, the wells were washed once with binding buffer and medium was replaced with 0.1 mL binding buffer. The total fluorescence per well was measured at 494/517 nm on a SpectraMax Gemini fluorescence plate reader (Molecular Devices, Sunnyvale, CA). The percentage-specific binding to either type I collagen or fibronectin was calculated by subtracting out the mean nonspecific binding to thyroglobulin, BSA, and plastic. To characterize the integrin collagen I receptor on prostate cancer cells, the *in vitro* attachment assay was repeated following a 45-minute, 4°C preincubation of the 1×10^5/mL labeled cell stock with a final concentration of 10 μg/mL of either $\alpha_2\beta_1$ blocking antibody (clone BHA2.1, Chemicon) or GRGDTP peptide (EMD Biosciences, San Diego, CA).

Attachment-dependent growth assay. Tissue culture plates (96 well) were coated with 1 $\mu g/cm^2$ collagen I, fibronectin, or thyroglobulin as above. Cells were then plated at a density of 1.5×10^3/0.2 mL complete medium. The total number of viable cells on one plate was determined every 24 hours by the addition of a final concentration of 1 mmol/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The formazan product was dissolved in DMSO, and absorbencies were read at 570 nm on a SpectraMax Plus plate reader.

***In vitro* migration assay.** Prostate cancer cells were lifted with EDTA and washed to serum-free RPMI 1640. Cells (2.5×10^4/0.5 mL) were plated in duplicate to 3-μm tissue culture inserts that were placed in wells containing medium alone or medium supplemented with 10 μg/mL type I collagen or thyroglobulin. Where shown, a final concentration of 10 μg/mL $\alpha_2\beta_1$ blocking antibody (clone BHA2.1) was added to the cells at plating. After 48 hours, migrating cells were stained with Hema 3 solution (CMS, Houston, TX) and the total number of migrating cells/filter was quantified under ×10 magnification. The data are presented as the mean ± SD of duplicate well from a representative experiment ($n = 4$).

***In vivo* animal model of bone metastasis.** The ability of prostate cancer cells to grow within the bone was evaluated following direct injection into the tibia of male *nu/nu* mice (intratibial injection). Briefly, a 27-gauge needle fitted to a 1 mL syringe was inserted through the knee into the marrow cavity of the tibia. Prostate cancer cells (5×10^5) were then delivered in a volume of 50 μL into the marrow space using a 1 mL syringe with a 27-gauge needle. Evidence of tumor growth was evaluated at day 65 by Faxitron radioscopic scan (Faxitron X-ray Corp., Wheeling, IL). At the experimental end point, injected tibias were removed, fixed in 10% normal-buffered formalin, and decalcified in Cal-Ex II (Fisher Scientific, Hampton, NH). Longitudinal sections of tibias were stained for prostate-specific antigen (PSA) protein by immunohistochemistry using 1:3,000 dilution of rabbit anti-human PSA polyclonal antibody (DAKO, Carpinteria, Ca). The animal protocol was approved by the University of Michigan Institutional Animal Care and Use Committee (Ann Arbor, MI).

RhoC activation assay. Total RhoC protein was detected in whole-cell lysates by Western blot using antisera specific for RhoC (19). The amount of active RhoC was determined by first precipitating active GTP-bound RhoC with glutathione *S*-transferase (GST)–Rhotekin fusion protein and then blotting separated proteins with RhoC-specific antisera (19). Densitometric data are presented as fold increase over parental LNCaP control.

Results

Bone metastatic prostate cancer cells bind type I collagen. As preferential adhesion to bone-specific factors may facilitate bone metastasis, we investigated the ability of prostate cancer cells to bind type I collagen, the most abundant protein within the bone. Two models of prostate cancer were used: PC-3M, a highly metastatic cell line that metastasizes to bone, and LNCaP, a low metastatic cell line that does not metastasize to bone. Both PC-3M and LNCaP have been selected *in vivo* to produce isogenic clones that either metastasize to soft tissue (LNCaP-LN3) or are highly metastatic to the bone (PC-3M-LN4 and C4-2B, respectively; refs. 21, 22). We observed that only those cells that metastasize to the bone could bind type I collagen (PC-3M, PC-3M-LN4, and C4-2B), whereas those cells that form only visceral metastases (LNCaP and LNCaP-LN3) failed to bind collagen I (Fig. 1*A*). All cell types adhered to fibronectin, showing the specificity of this interaction.

To examine the relationship between collagen adhesion and bone metastatic potential, a collagen-binding cell line was derived from LNCaP cells (which routinely fail to bind collagen). This was accomplished through eight cycles in which collagen-adherent cells were selected, expanded, and readhered. The resultant collagen-selected cells ($LNCaP_{col}$) showed a marked increase in adhesiveness to type I collagen, equivalent to bone metastatic C4-2B cells (Fig. 1*B*). Thus, $LNCaP_{col}$ cells provide a powerful tool to dissect the role of collagen I in prostate cancer bone metastasis.

LNCaP variant cells bind collagen I via $\alpha_2\beta_1$. The most widely characterized collagen I receptors are integrins, specifically $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$. Fluorescence-activated cell sorting analysis

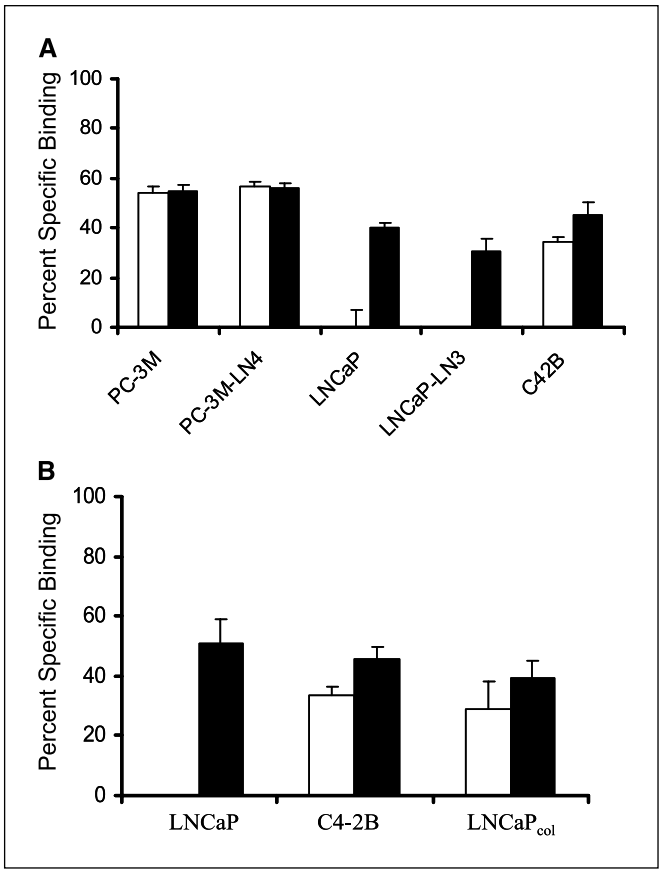

Figure 1. Bone metastatic prostate cancer cells bind type I collagen. *A,* relative attachment of prostate cancer cell lines. Fluorescently labeled cells (1×10^4) were plated in quadruplicate to a 96-well plate that had been coated with 10 μg/mL protein. After 1 hour, the cells were washed and fluorescence was measured. The percentage-specific binding was calculated by subtracting nonspecific binding to plastic or control proteins BSA and thyroglobulin. *Columns,* mean of three separate experiments; *bars,* SD. *B, in vitro* characterization of LNCaP$_{col}$ cells. The ability of LNCaP$_{col}$ cells to bind type I collagen was evaluated through *in vitro* attachment assay as described above. Representative experiment of four. □, collagen type I; ■, fibronectin.

shows that each of LNCaP, LNCaP$_{col}$, and C4-2B expresses an abundant amount of both the α_2 and β_1 integrin subunits (Fig. 2*A, 2* and *4*) but does not express either α_1 or α_3 subunits (Fig. 2*A, 1* and *3*, respectively). To ensure that the α_1 or α_3 antibodies used for our analysis were functional, the antibodies were used to successfully detect α_1 or α_3 protein by flow cytometry in positive control cell lines HeLa (α_1) and MG63 (α_3; data not shown). We further observed that the surface expression of $\alpha_2\beta_1$ heterodimer was increased, an average of 51% in LNCaP$_{col}$ and bone metastatic C4-2B cells compared with LNCaP cells, suggesting that integrin abundance contributes to the increased collagen I binding. To verify that $\alpha_2\beta_1$ is the collagen I receptor on LNCaP cells, *in vitro* attachment assays were done following a pretreatment with a blocking antibody to the $\alpha_2\beta_1$ integrin complex (Fig. 2*B*). The data show that antisera to $\alpha_2\beta_1$ completely blocked the binding of both LNCaP$_{col}$ and C4-2B cells to type I collagen. The blocking was specific for $\alpha_2\beta_1$ integrin, as the antibody failed to interfere with binding to fibronectin. To confirm the role of integrins in the enhanced binding of C4-2B and LNCaP$_{col}$ to collagen I, these experiments were repeated using an RGD-containing peptide (GRGDTP). Similar to $\alpha_2\beta_1$ antibody, RGD peptide blocked the ability of prostate cancer cells to bind collagen I (data not shown).

Collagen I does not increase prostate cancer cell growth but stimulates *in vitro* migration. The preceding data suggest that collagen I binding is associated with the bone metastatic phenotype of prostate cancer cells. To determine whether collagen adhesion affects proliferation, attachment-dependent growth assays comparing the LNCaP series cells were done (Fig. 3*A*). The data show that the growth rates on collagen and plastic are identical for C4-2B and LNCaP$_{col}$, suggesting that collagen binding does not affect cell growth within the LNCaP model.

Fragments of collagen I are chemotactic for various tumor and endothelial cells (23, 24). We therefore investigated whether collagen I could stimulate the chemotactic migration of collagen-binding

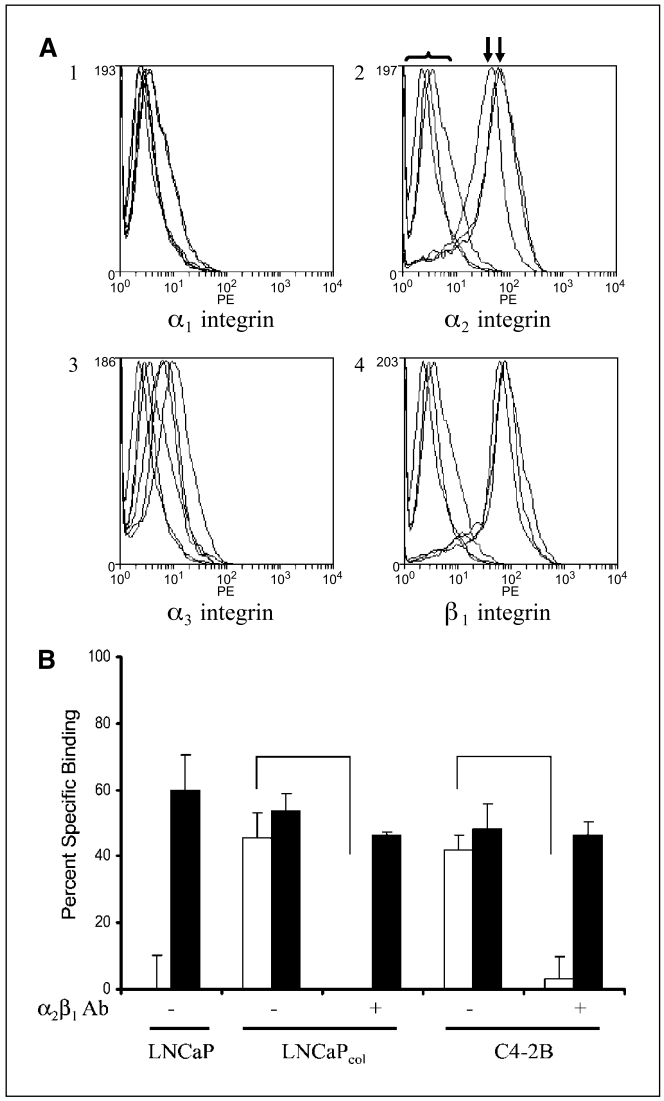

Figure 2. Integrin $\alpha_2\beta_1$ is increased in collagen-binding cells. *A,* flow cytometric analysis. Prostate cancer cells (2×10^5) were incubated with phycoerythrin-conjugated antisera to integrin subunits α_1 (*1*), α_2 (*2*), α_3 (*3*), and β_1 (*4*) for 30 minutes at 4°C. Labeled cells were then washed, fixed, and evaluated on a Coulter FACScan flow cytometer. *Arrows,* α_2 peak for LNCaP and LNCaP$_{col}$/C4-2B, respectively. *B,* LNCaP variant cells bind collagen I via $\alpha_2\beta_1$. *In vitro* attachment assays were repeated following 45-minute preincubation at 4°C with a final concentration of 10 μg/mL of $\alpha_2\beta_1$ blocking antibody. □, collagen type I; ■, fibronectin.

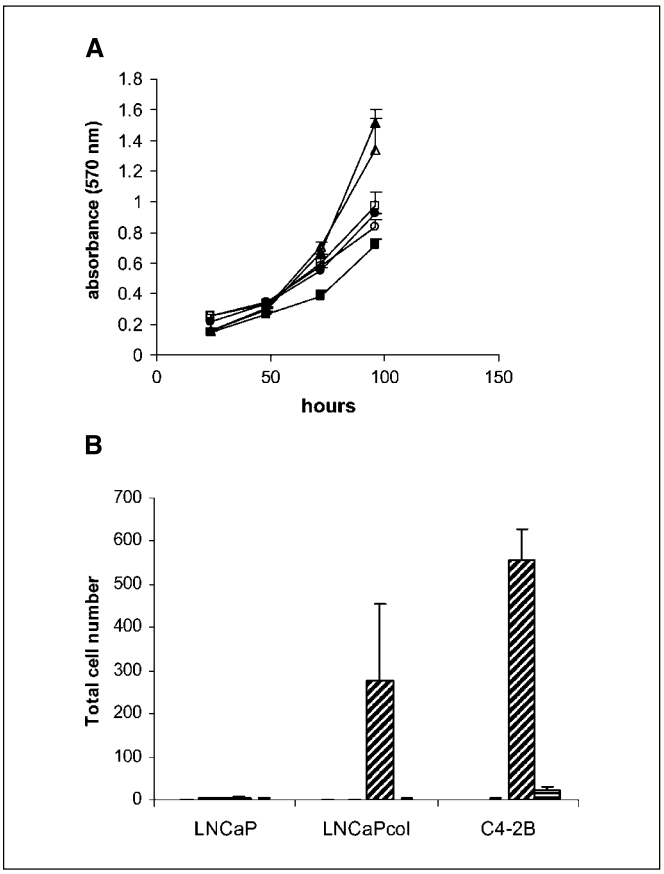

Figure 3. Collagen I promotes chemotaxis but not proliferation of prostate cancer cells. *A,* collagen I binding does not stimulate prostate cancer growth. Cells (1.5×10^5) were plated in triplicate to 96-well plates containing 1 $\mu g/cm^2$ collagen I or plastic. The number of viable cells was determined every 24 hours for 4 days by MTT. *B,* LNCaP cells that bind collagen show increased migration to collagen I. Prostate cancer cells (2.5×10^4) in serum-free medium were plated to 3-mm tissue culture inserts and incubated under the indicated conditions for 48 hours. A final concentration of 10 $\mu g/mL$ of $\alpha_2\beta_1$ blocking antibody was added to the cells at plating as indicated. Following this incubation, migrating cells were stained with Hema 3 solution and the total number of migrating cells/filter was quantified under $\times 10$ magnification. *Columns,* mean ($n = 4$) of duplicate well from a representative experiment; *bars,* SD. —□—, LNCaP on plastic; —■—, LNCaP on collagen; —△—, LNCaPcol on plastic; —▲—, LNCaPcol on collagen; —⊖—, C4-2B on plastic; —●—, C4-2B on collagen; □, no protein; ■, thyroglobulin; ▨, collagen; ▤, collagen + a2b1 Ab.

LNCaP cells ($LNCaP_{col}$ and C4-2B) using modified Boyden chamber assays (Fig. 3*B*). The data show that collagen I did not promote migration of parental LNCaP cells but it significantly stimulated the migration of both $LNCaP_{col}$ and bone metastatic C4-2B cells. This effect was selectively inhibited by antibody-mediated inhibition of $\alpha_2\beta_1$ integrin. These results show that collagen I stimulates chemotaxis through $\alpha_2\beta_1$ and suggest a possible mechanism for the selective invasion of the bone.

Collagen panned cells are capable of growth within the bone. To directly test whether the ability to bind collagen I affects prostate cancer bone metastasis, parental LNCaP and collagen-selected $LNCaP_{col}$ cells were injected into the tibia of male nude mice. Figure 4 shows a representative Faxitron X-ray analysis (Fig. 4, *column 1*) and PSA immunohistochemistry (Fig. 4, *column 2*) of uninjected nude mouse tibia or tibias injected with LNCaP or $LNCaP_{col}$ cells. We found that 53% (7 of 13) of mice injected with collagen I panned cells ($LNCaP_{col}$) show radiographic evidence of mixed osteolytic and osteoblastic lesions (Fig. 4; Table 1). In sharp contrast, none (0 of 8) mice injected with LNCaP cells showed evidence of boney lesions. Histologic analysis of tibias using PSA immunohistochemistry could detect prostate cancer cells within the bone in the absence of radiographically appreciable bone turnover. Thus, a total of 85% of $LNCaP_{col}$-injected tibias had bone lesions, whereas only minimal disease could be detected in LNCaP-injected mice (Fig. 4). In a separate independent experiment, 60% (6 of 10) of mice injected with $LNCaP_{col}$ had PSA-positive tumor burden, whereas none (0 of 10) of the mice injected with LNCaP cells had any detectable tumor (Table 1). A third analysis of $LNCaP_{col}$ cells showed that 50% of mice developed both radiographic and PSA-positive bone lesions (Table 1). Taken together, the data show that collagen I binding in prostate cancer cells confers growth within the bone and suggest that collagen adhesion plays an important role in prostate cancer skeletal metastasis.

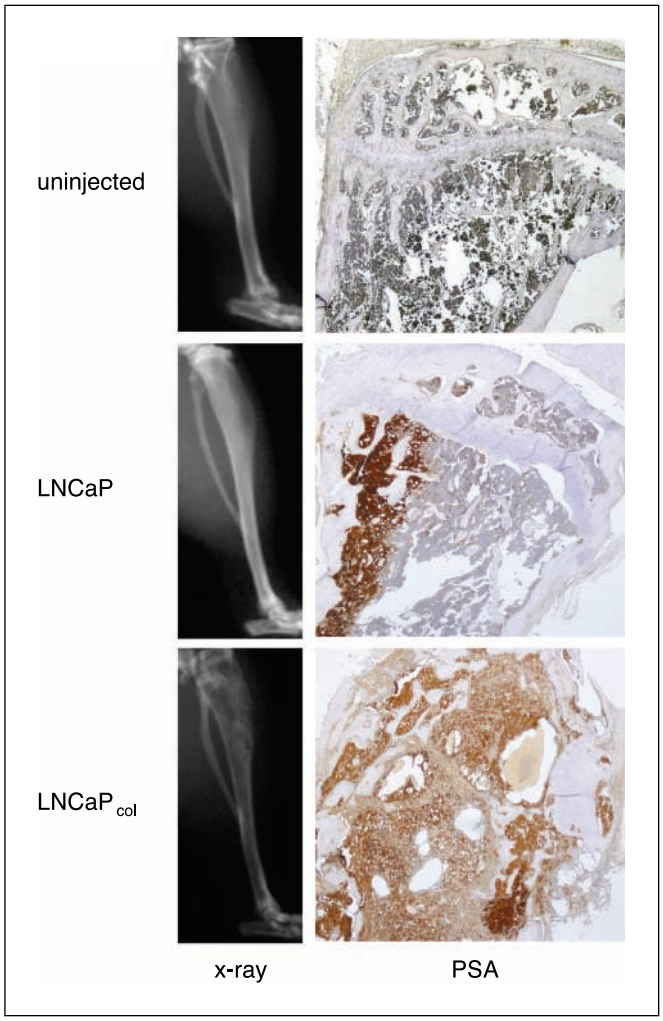

Figure 4. Collagen panned cells are capable of growth within the bone. LNCaP or collagen panned ($LNCaP_{col}$) cells (5×10^5) were injected into the tibia of nude mice. The presence of boney lesions was scored 9 weeks after tumor injection. *Column 1,* representative radiographic images of an uninjected nude mouse tibia or tibias injected with LNCaP or $LNCaP_{col}$ cells, respectively; *column 2,* immunohistochemical staining for PSA in injected tibias. *Brown,* PSA staining.

Table 1. Collagen panned cells are capable of growth within the bone

	Radiographic		Histologic	
	LNCaP	$LNCaP_{col}$	LNCaP	$LNCaP_{col}$
Experiment 1	0/8 (0%)	7/13 (54%)	5/8 (63%)	11/13 (85%)
Experiment 2	0/10 (0%)	0/10 (0%)	0/10 (0%)	6/10 (60%)
Experiment 3	Not done	5/10 (50%)	Not done	5/10 (50%)
Total	0/18 (0%)	12/33 (36%)	5/18 (28%)	22/33 (67%)

NOTE: LNCaP or $LNCaP_{col}$ cells (5×10^5) were injected into the tibia of male nude mice. After 9 weeks, the animals were evaluated for the presence of boney lesions by radiographic analysis and by PSA immunohistochemistry of histologic sections. Data from three independent experiments presented as the number of animals with boney lesions of the total number of animals (percentage of animals with boney lesions).

Activation of RhoC GTPase contributes to collagen-induced migration. The correlation between the collagen-binding phenotype and increased collagen-stimulated migration of both $LNCaP_{col}$ and C4-2B prostate cancer cells suggests that $\alpha_2\beta_1$-mediated signaling induces cellular migration programs. Analysis of integrin-associated signaling pathways revealed that the expression and activity of *Fak* and the *Src* family kinases (*Src*, *Lyn*, and *Yes*) did not differ among $LNCaP_{col}$ or C4-2B cells (data not shown). However, analysis of the Rho pathway showed that both the total and active GTP-bound forms of RhoC GTPase increased 5- to 8-fold in collagen-binding cells ($LNCaP_{col}$ and C4-2B) compared with parental LNCaP cells (Fig. 5*A* and *B*). These data, taken together with our previous published report that RhoC GTPase is required for PC-3 prostate cancer cell invasion (20), suggest that $\alpha_2\beta_1$/collagen I stimulates invasive programs in part through increased RhoC GTPase activity.

Discussion

In order for a tumor cell to form metastases, it must successfully interact with the host microenvironment of the distant organ. This interaction occurs at multiple levels and includes, but is not limited to, adhesive events at the cell surface between the tumor cell and organ-specific endothelial cells or ECM proteins (4, 25). The prominent expression of collagen type I within the bone led us to examine the role of collagen binding in prostate cancer invasion and metastasis of the skeleton.

Recent *in vitro* data suggest an association between $\alpha_2\beta_1$ and prostate cancer skeletal metastasis. For example, studies with human PC-3 prostate cancer cells have shown that these cells bind bone ECM through integrin $\alpha_2\beta_1$ and antibodies to $\alpha_2\beta_1$ block the attachment of primary prostatic epithelial cells to bone marrow stromal cells (26, 27). PC-3 cells grown on type I collagen exhibit increased rates of proliferation, suggesting a functional consequence of collagen binding (28). Further, treatment of PC-3 cells with transforming growth factor-β1 (29, 30), a major bone-derived growth factor, or osteoblast conditioned medium (31) enhanced $\alpha_2\beta_1$ synthesis and adhesion to collagen I. Collectively, these data suggest that type I collagen may have a role in prostate cancer metastasis; however, no direct evidence shows that binding to collagen I or $\alpha_2\beta_1$ expression plays a mechanistic role in prostate cancer bone metastasis.

In the present study, we show that bone metastatic human prostate cancer cells preferentially adhere to collagen I compared with prostate cancer cells that were metastatic to other visceral sites, such as the lymph node. Flow cytometric analysis and blocking studies identified the collagen receptor as $\alpha_2\beta_1$ integrin, a known collagen receptor. Collagen I significantly stimulated the migration of both $LNCaP_{col}$ and bone metastatic C4-2B cells, an effect that could be blocked with antibodies to $\alpha_2\beta_1$. Importantly,

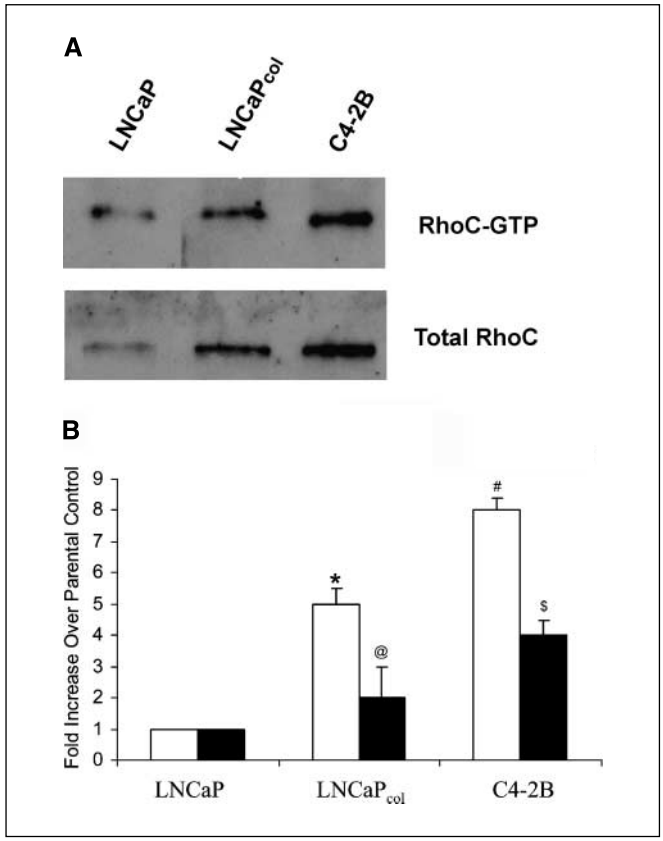

Figure 5. Collagen-binding cells have increased RhoC activity. *A,* Western blot for RhoC protein. *Top,* amount of active, GTP-bound RhoC was determined by first precipitating with GST-Rhotekin fusion protein and then blotting separated proteins with RhoC-specific antisera; *bottom,* total RhoC protein was detected in whole-cell lysates by Western blot using antisera specific for RhoC. *B,* densitometric data are fold increase over parental LNCaP control. *Columns,* mean of four experiments; *bars,* SD. *, $P < 2.6 \times 10^{-8}$ versus LNCaP; @, $P < 0.03$ versus LNCaP; #, $P < 3.1 \times 10^{-9}$ versus LNCaP; $, $P < 5.3 \times 10^{-7}$ versus LNCaP. □, activation; ■, expression.

cells selected for increased collagen I binding ($LNCaP_{col}$) were capable of growth within the bone and induced bone turnover compared with parental LNCaP cells. The acquisition of the bone metastatic phenotype correlated with increased level and activity of RhoC GTPase, a molecule required for prostate cancer invasiveness (20). These data show that collagen I attachment mediated by $\alpha_2\beta_1$ initiates motility programs through RhoC and suggest a mechanism for the selective metastasis of prostate cancer cells to the bone.

LNCaP cells are routinely nontumorigenic in nude mice, and we indeed show that 0 of 18 mice injected with LNCaP cells had evidences of radiographic bone lesions compared with 7 of 13 or 5 of 10 mice injected with $LNCaP_{col}$ cells in two separate experiments (Table 1). However, using very sensitive PSA immunohistochemistry, small tumor deposits in five of eight mice injected with LNCaP cells were detectable in one of two experiments. This is in sharp contrast to $LNCaP_{col}$ cells, which produced tumor detectable by histology in 22 of 33 mice and radiography in 12 of 33 animals (Table 1). The absence of a 1:1 correlation between histologic and radiographic lesions suggests that the prostate cancer tumor volume was insufficient to induce cortical bone destruction. As tumor cells would be detectable by histology before reaching a suitable size to induce osteolysis as measured by X-ray, the reduced numbers of radiographic lesions observed in each cell line in experiment 2 would be explained if the tumors were developing at a slower rate. As these are end point studies, the decreased *in vivo* growth rate likely reflects the condition of the cells or mice at the time of injection. However, despite the variation between the three experiments, the total data support the conclusion that collagen-selected cells produced larger numbers of osteolytic bone tumors than do parental LNCaP cells.

Our data document that expression of a functional collagen I receptor promotes prostate cancer outgrowth within the bone. A direct role of $\alpha_2\beta_1$ signaling in prostate cancer skeletal metastasis suggested by our study is supported by published works, which show that integrins mediate bone-specific metastasis in other tumor systems. For example, small-molecule inhibitors to $\alpha_v\beta_3$ (S247) blocked the metastasis to the bone of human MDA-435 breast cancer cells following intracardiac injection (32). The enforced expression of integrin α_4 into Chinese hamster ovary cells induced experimental metastasis to the bone, which could be blocked by antibodies to α_4 (33). Previous published reports for a role of $\alpha_2\beta_1$ in metastasis, however, are controversial. $\alpha_2\beta_1$ expression in renal cell carcinomas was found to correlate with the malignant phenotype (34), where loss of $\alpha_2\beta_1$ expression in human breast cancer was associated with tumor progression (35). Overexpression of the α_2 subunit in human rhabdomyosarcoma cells led to enhanced experimental metastasis to lung (36), where enforced expression of $\alpha_2\beta_1$ in the same cells slowed postextravasation movement (37). Primary cultures of human ovarian carcinoma and human pancreatic adenocarcinoma cell lines were shown to both bind to and migrate toward collagen I, suggesting that collagen could promote the invasive phenotype but was not directly shown to affect metastasis (38, 39). To our knowledge, this is the first report linking collagen I binding/$\alpha_2\beta_1$ function to prostate cancer outgrowth within the bone.

Based on our findings, $\alpha_2\beta_1$ may represent a useful therapeutic target in prostate cancer bone metastasis. Indeed, neutralizing integrin binding with antibodies or RGD-containing molecules has been shown to reduce spontaneous liver metastasis of human colon cancer cells (40) and disrupt angiogenesis in a severe combined immunodeficient-human model of prostate cancer bone metastasis (41). In addition to antitumor or antiangiogenic effects, $\alpha_2\beta_1$ antagonists might also block prostate cancer cell homing to the bone. Patients with osseous lesions of prostate cancer display breakdown products of collagen I within their blood and urine, specifically deoxypyridinoline, type I collagen cross-linked C-telopeptide, and type 1 procollagen C-propeptide (42). As both collagen I and trimer carboxyl propeptide are potent chemotactic factors for tumor and endothelial cells (23, 24, 38), blocking $\alpha_2\beta_1$ signaling may diminish prostate cancer metastasis to the bone. A final potential effect of $\alpha_2\beta_1$ interference stems from the recent observation that rapid collagen I binding resulting from high $\alpha_2\beta_1$ expression is a marker of prostate cancer stem cells. Within this model, $\alpha_2\beta_1$-expressing cells would be a characteristic of metastatic prostate cancer cells (43, 44). Collectively, these data suggest that $\alpha_2\beta_1$ signaling may be a marker of metastatic prostate cancer cells and a mediator of tumor cell homing to the bone.

The role of $\alpha_2\beta_1$-mediated activation of RhoC GTPase suggests a novel pathway for prostate cancer metastasis to the bone. As collagen I is the most abundant protein in the bone, prostate cancer cells expressing elevated levels of $\alpha_2\beta_1$ would have a selective advantage through RhoC activation. In this report, we show that the collagen I–binding phenotype correlates with both increased total and active amounts of RhoC. Future studies with RhoC short hairpin RNA molecules and RhoC fast cycling mutants will directly test the role of RhoC in prostate cancer skeletal metastasis.

Our data show that bone metastatic prostate cancer cells bind to and migrate toward collagen type I in an $\alpha_2\beta_1$-dependent manner. Likewise, selection for collagen adhesion greatly enhances *in vivo* growth within the bone following intratibial injection. Collectively, these data support a model in which $\alpha_2\beta_1$ integrin-mediated collagen adhesion plays a fundamental role in the ability of prostate cancer cells to metastasize to the bone microenvironment.

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References

1. Keller ET, Zhang J, Cooper CR, et al. Prostate carcinoma skeletal metastases: cross-talk between tumor and bone. Cancer Metastasis Rev 2001;20:333–49.
2. Bubendorf L, Schopfer A, Wagner U, et al. Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. Hum Pathol 2000;31:578–83.
3. Harada M, Iida M, Yamaguchi M, Shida K. Analysis of bone metastasis of prostatic adenocarcinoma in 137 autopsy cases. Adv Exp Med Biol 1992;324:173–82.
4. Buckwalter JA, Glimcher MJ, Cooper RR, Recker R. Bone biology. I. Structure, blood supply, cells, matrix, and mineralization. Instr Course Lect 1996;45:371–86.
5. Felding-Habermann B. Integrin adhesion receptors in tumor metastasis. Clin Exp Metastasis 2003;20:203–13.
6. Gullberg D, Gehlsen KR, Turner DC, et al. Analysis of $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ integrins in cell-collagen

interactions: identification of conformation dependent $\alpha_1\beta_1$ binding sites in collagen type I. EMBO J 1992;11: 3865–73.
7. Miranti CK, Brugge JS. Sensing the environment: a historical perspective on integrin signal transduction. Nat Cell Biol 2002;4:E83–90.
8. Slack-Davis JK, Parsons JT. Emerging views of integrin signaling: implications for prostate cancer. J Cell Biochem 2004;91:41–6.
9. Longhurst CM, Jennings LK. Integrin-mediated signal transduction. Cell Mol Life Sci 1998;54:514–26.
10. Evers EE, van der Kammen RA, ten Klooster JP, Collard JG. Rho-like GTPases in tumor cell invasion. Methods Enzymol 2000;325:403–15.
11. Keely P, Parise L, Juliano R. Integrins and GTPases in tumour cell growth, motility, and invasion. Trends Cell Biol 1998;8:101–6.
12. Price LS, Collard JG. Regulation of the cytoskeleton by Rho-family GTPases: implications for tumour cell invasion. Semin Cancer Biol 2001;11:167–73.
13. Kjoller L, Hall A. Signaling to Rho GTPases. Exp Cell Res 1999;253:166–79.
14. Takai Y, Sasaki T, Matozaki T. Small GTP-binding proteins. Physiol Rev 2001;81:153–208.
15. Geyer M, Wittinghofer A. GEFs, GAPs, GDIs, and effectors: taking a closer (3D) look at the regulation of Ras-related GTP-binding proteins. Curr Opin Struct Biol 1997;7:786–92.
16. Olson MF. Guanine nucleotide exchange factors for the Rho GTPases: a role in human disease? J Mol Med 1996;74:563–71.
17. Clark EA, Golub TR, Lander ES, Hynes RO. Genomic analysis of metastasis reveals an essential role for RhoC. Nature 2000;406:532–5.
18. Suwa H, Ohshio G, Imamura T, et al. Overexpression of the rhoC gene correlates with progression of ductal adenocarcinoma of the pancreas. Br J Cancer 1998;77: 147–52.
19. van Golen KL, Bao L, DiVito MM, et al. Reversion of RhoC GTPase-induced inflammatory breast cancer phenotype by treatment with a farnesyl transferase inhibitor. Mol Cancer Ther 2002;1:575–83.
20. Yao H, Dashner EJ, van Golen CM, van Golen KL. RhoC GTPase is required for PC-3 prostate cancer cell invasion but not motility. Oncogene 2005;25:2285–96.
21. Pettaway CA, Pathak S, Greene G, et al. Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. Clin Cancer Res 1996;2:1627–36.
22. Thalmann GN, Anezinis PE, Chang SM, et al. Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. Cancer Res 1994;54:2577–81.
23. Mundy GR, DeMartino S, Rowe DW. Collagen and collagen-derived fragments are chemotactic for tumor cells. J Clin Invest 1981;68:1102–5.
24. Palmieri D, Camardella L, Ulivi V, Guasco G, Manduca P. Trimer carboxyl propeptide of collagen I produced by mature osteoblasts is chemotactic for endothelial cells. J Biol Chem 2000;275:32658–63.
25. Cooper CR, McLean L, Walsh M, et al. Preferential adhesion of prostate cancer cells to bone is mediated by binding to bone marrow endothelial cells as compared to extracellular matrix components *in vitro*. Clin Cancer Res 2000;6:4839–47.
26. Kostenuik PJ, Sanchez-Sweatman O, Orr FW, Singh G. Bone cell matrix promotes the adhesion of human prostatic carcinoma cells via the $\alpha_2\beta_1$ integrin. Clin Exp Metastasis 1996;14:19–26.
27. Lang SH, Clarke NW, George NJ, Testa NG. Primary prostatic epithelial cell binding to human bone marrow stroma and the role of $\alpha_2\beta_1$ integrin. Clin Exp Metastasis 1997;15:218–27.
28. Kiefer JA, Farach-Carson MC. Type I collagen-mediated proliferation of PC3 prostate carcinoma cell line: implications for enhanced growth in the bone microenvironment. Matrix Biol 2001;20:429–37.
29. Kostenuik PJ, Singh G, Orr FW. Transforming growth factor β upregulates the integrin-mediated adhesion of human prostatic carcinoma cells to type I collagen. Clin Exp Metastasis 1997;15:41–52.
30. Guise TA, Chirgwin JM. Transforming growth factor-β in osteolytic breast cancer bone metastases. Clin Orthop Relat Res 2003;415:S32–8.
31. Knerr K, Ackermann K, Neidhart T, Pyerin W. Bone metastasis: osteoblasts affect growth and adhesion regulons in prostate tumor cells and provoke osteomimicry. Int J Cancer 2004;111:152–9.
32. Harms JF, Welch DR, Samant RS, et al. A small molecule antagonist of the $\alpha(v)\beta_3$ integrin suppresses MDA-MB-435 skeletal metastasis. Clin Exp Metastasis 2004;21:119–28.
33. Matsuura N, Puzon-McLaughlin W, Irie A, et al. Induction of experimental bone metastasis in mice by transfection of integrin $\alpha_4\beta_1$ into tumor cells. Am J Pathol 1996;148:55–61.
34. Anastassiou G, Duensing S, Steinhoff G, et al. *In vivo* distribution of integrins in renal cell carcinoma: integrin-phenotype alteration in different degrees of tumor differentiation and VLA-2 involvement in tumor metastasis. Cancer Biother 1995;10:287–92.
35. Arihiro K, Inai K, Kurihara K, et al. Loss of VLA-2 collagen receptor in breast carcinoma, facilitating invasion and metastasis. Jpn J Cancer Res 1993;84: 726–33.
36. Chan BM, Matsuura N, Takada Y, Zetter BR, Hemler ME. *In vitro* and *in vivo* consequences of VLA-2 expression on rhabdomyosarcoma cells. Science 1991; 251:1600–2.
37. Hangan D, Uniyal S, Morris VL, et al. Integrin VLA-2 ($\alpha_2\beta_1$) function in postextravasation movement of human rhabdomyosarcoma RD cells in the liver. Cancer Res 1996;56:3142–9.
38. Fishman DA, Kearns A, Chilukuri K, et al. Metastatic dissemination of human ovarian epithelial carcinoma is promoted by $\alpha_2\beta_1$-integrin-mediated interaction with type I collagen. Invasion Metastasis 1998;18:15–26.
39. Grzesiak JJ, Bouvet M. The $\alpha_2\beta_1$ integrin mediates the malignant phenotype on type I collagen in pancreatic cancer cell lines. Br J Cancer 2006;94:1311–9.
40. Reinmuth N, Liu W, Ahmad SA, et al. $\alpha_v\beta_3$ integrin antagonist S247 decreases colon cancer metastasis and angiogenesis and improves survival in mice. Cancer Res 2003;63:2079–87.
41. Nemeth JA, Cher ML, Zhou Z, et al. Inhibition of $\alpha(v)\beta_3$ integrin reduces angiogenesis, bone turnover, and tumor cell proliferation in experimental prostate cancer bone metastases. Clin Exp Metastasis 2003;20: 413–20.
42. Berruti A, Dogliotti L, Bitossi R, et al. Incidence of skeletal complications in patients with bone metastatic prostate cancer and hormone refractory disease: predictive role of bone resorption and formation markers evaluated at baseline. J Urol 2000;164:1248–53.
43. Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005;65:10946–51.
44. Collins AT, Habib FK, Maitland NJ, Neal DE. Identification and isolation of human prostate epithelial stem cells based on $\alpha(2)\beta(1)$-integrin expression. J Cell Sci 2001;114:3865–72.