Tumor $\alpha_v\beta_3$ Integrin Is a Therapeutic Target for Breast Cancer Bone Metastases

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Abstract

In breast cancer bone metastasis, tumor cells stimulate osteoclast-mediated bone resorption, and bone-derived growth factors released from resorbed bone stimulate tumor growth. The $\alpha_v\beta_3$ integrin is an adhesion receptor expressed by breast cancer cells and osteoclasts. It is implicated in tumor cell invasion and osteoclast-mediated bone resorption. Here, we hypothesized that the therapeutic targeting of tumor $\alpha_v\beta_3$ integrin would prevent bone metastasis formation. We first showed that, compared with mock-transfected cells, the i.v. inoculation of $\alpha_v\beta_3$-overexpressing MDA-MB-231 breast cancer cells in animals increased bone metastasis incidence and promoted both skeletal tumor burden and bone destruction. The direct inoculation of $\alpha_v\beta_3$-overexpressing transfectants into the tibial bone marrow cavity did not however enhance skeletal tumor burden and bone destruction, suggesting that $\alpha_v\beta_3$ controls earlier events during bone metastasis formation. We next examined whether a nonpeptide antagonist of $\alpha_v\beta_3$ (PSK1404) exhibits meaningful antitumor effects in experimental breast and ovarian cancer bone metastasis. A continuous PSK1404 treatment, which inhibited osteoclast-mediated bone resorption in an animal model of bone loss, substantially reduced bone destruction and decreased skeletal tumor burden. Importantly, a short-term PSK1404 treatment that did not inhibit osteoclast activity also decreased skeletal tumor burden and bone destruction. This dosing regimen caused a profound and specific inhibition of bone marrow colonization by green fluorescent protein, $\alpha_v\beta_3$-expressing tumor cells *in vivo* and blocked tumor cell invasion *in vitro*. Overall, our data show that tumor $\alpha_v\beta_3$ integrin stands as a therapeutic target for the prevention of skeletal metastases. [Cancer Res 2007;67(12):5821–30]

Introduction

Bone metastases are common complications of breast cancer (1). Most often osteolytic, or, to a lesser extent, osteoblastic or mixed, bone metastases can be fatal or may rapidly impede the quality of life (1). Bone-residing breast cancer cells do not directly destroy bone (1, 2). Instead, they secrete molecules, such as parathyroid hormone-related protein, interleukins (IL-6, IL-8, and IL-11), and prostaglandins that stimulate the activity of bone-resorbing cells (osteoclasts), leading to osteolysis (1, 3–6). These observations (1–6) have provided the rationale for using bisphosphonates (as inhibitors of osteoclast-mediated bone resorption) in the treatment of breast cancer patients with bone metastases (1). Yet, these treatments are only palliative and do not provide a life-prolonging benefit to patients with advanced disease. Molecular mechanisms involved in bone colonization by breast cancer cells need therefore to be understood to develop new therapies directed toward early bone metastatic processes.

Integrins constitute a family of cell surface receptors that are heterodimers composed of noncovalently associated α and β subunits (7). Some studies support the concept that integrins mediate metastasis in bone (8–12). Bone colonization by prostate cancer cells is mediated by $\alpha_2\beta_1$ integrin (8). The expression of $\alpha_v\beta_3$ integrin by breast cancer cells has been also associated with bone metastasis (9–12). For instance, by *in vivo* selection of MDA-MB-231 breast cancer cells, we have isolated a cell subpopulation (called B02) that only metastasizes to bone and constitutively overexpresses $\alpha_v\beta_3$ integrin (9). Similarly, the *de novo* expression of $\alpha_v\beta_3$ in 66cl4 breast cancer and Chinese hamster ovary (CHO) ovarian cancer cells that metastasize to lungs, but not to bone, is sufficient to promote their dissemination to bone (9, 10). Finally, $\alpha_v\beta_3$ integrin cooperates with bone sialoprotein (BSP) and matrix metalloproteinase-2 (MMP-2) in promoting osteotropic cancer cell invasion (11, 12). These observations (9–12) were in line with a previous study showing that bone-residing breast cancer metastases express elevated levels of $\alpha_v\beta_3$ integrin compared with primary breast carcinomas (13). Osteoclasts also express $\alpha_v\beta_3$ integrin (14), and selective inhibitors of $\alpha_v\beta_3$ have been shown to inhibit osteoclast-mediated bone resorption in animal models of osteoporosis (15, 16) and malignant osteolysis (17, 18). For instance, the treatment of animals with an anti-β_3 antibody blocks the formation of osteolytic lesions caused by PC-3 prostate cancer cells that do not express $\alpha_v\beta_3$ integrin (17). The preventive treatment of animals bearing MDA-MB-435 breast cancer cells with a peptidomimetic inhibitor of $\alpha_v\beta_3$ also reduces bone destruction (18).

Here, we present *in vivo* evidence that tumor $\alpha_v\beta_3$ integrin participates in the development of experimental breast cancer bone metastases and that a selective $\alpha_v\beta_3$ nonpeptide antagonist not only inhibits osteoclast-mediated bone resorption in animal models of bone metastasis but also blocks bone colonization by $\alpha_v\beta_3$-expressing cancer cells.

Materials and Methods

Immunohistochemistry. Eight pairs of human primary breast carcinomas and their bone metastases were selected from the tumor bank of the

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Centre Léon Bérard (Lyon, France). The tumor and metastatic material was fixed in Bouin Hollande and then embedded in paraffin. Four-micrometer-thick tissue sections were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked in a sterile water solution containing 5% hydrogen peroxide. Tissue sections were then pretreated in a citrate buffer (pH 6.0) for 50 min at 95°C in a water bath. After antigen retrieval in citrate buffer, tissue sections were incubated for 1 h at room temperature with mouse monoclonal antibody (mAb) SZ21 directed against the β_3 integrin subunit [1:200 dilution in PBS containing 2 mg/mL bovine serum albumin (BSA); Chemicon]. After washing, tissue sections were incubated with a biotinylated secondary antibody bound to a streptavidin-peroxidase conjugate (LSAB+ kit, DAKO), and the signal was developed with diaminobenzidine. Tissue sections were then counterstained with hematoxylin, dehydrated, and mounted.

Scoring of β_3 integrin immunostaining in breast cancer tissue specimens. The immunostaining intensity was evaluated independently by two investigators (I.T. and S.G.). The intensity of the staining was scored arbitrarily as follows: negative (−), weak (1+), moderate (2+), and strong (3+). In case of disagreement between examiners, slides were reviewed and a consensus opinion was obtained.

Cell lines and transfection. Human MDA-MB-231 breast carcinoma cells were obtained from the American Type Culture Collection. Characteristics of CHO-$\beta_3$wt cells, stably transfected to *de novo* express $\alpha_v\beta_3$ integrin, mock-transfected CHOdhfr cells, and MDA-MB-231/B02 cells, constitutively overexpressing integrin $\alpha_v\beta_3$, were described elsewhere (9). CHO-$\beta_3$wt, CHOdhfr, and MDA-MB-231/B02 cell lines were stably transfected to express green fluorescent protein (GFP) as described previously (19). MDA-MB-231 cells were transfected with a full-length human β_3 cDNA (20) with the use of TransFast (Promega) to overexpress $\alpha_v\beta_3$ integrin in these cells. Stable transfectants were selected with geneticin (1 mg/mL for 4 weeks). Selection of the clones was obtained after growing the cells for 2 weeks in the presence of puromycin (2 μg/mL). Two transfectants overexpressing $\alpha_v\beta_3$ integrin (clones #30.1 and #14.3) and one transfectant expressing the empty vector (clone EV#1.4) were used in the present study. Transfectants and cell lines were routinely cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS; Bio-Media) and 1% (v/v) penicillin/streptomycin (Life Technologies) at 37°C in a 5% CO_2 incubator.

Reverse transcription, standard, and quantitative PCR. Total RNA from MDA-MB-231 transfectants was extracted using Total RNA Isolation System (Promega). cDNA was synthesized using Moloney murine leukemia virus-1 (Promega). Primers for human β_3 integrin subunit were designed from the *β3* gene (National Center for Biotechnology Information accession number J02703) using nucleotides 1995-2016 as the forward primer and nucleotides 2259-2237 as the reverse primer. PCRs were run using a program consisting of 40 cycles of 95°C for 15 s, 64°C for 6 s, and 72°C for 15 s. Human β_3 mRNA was quantified by real-time PCR using the Master SYBR Green I kit (Roche Diagnosis). The fluorescence data were quantitatively analyzed by using serial dilution of control samples included in each reaction to produce a standard curve. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was analyzed in parallel to confirm the use of equal amount of cDNAs in each reaction. Results were expressed as the ratio of *β3* to *GAPDH* gene expression in each transfectant.

Antibodies and nonpeptidic $\alpha_v\beta_3$ integrin antagonist. Function-blocking mouse mAb LM609 directed against $\alpha_v\beta_3$ integrin and mouse mAbs directed against β_3 integrin subunit (clone SZ21) and $\alpha_v\beta_5$ (clone P1F6) and $\alpha_v\beta_6$ (clone E7P6) integrins were purchased from Chemicon. Phycoerythrin-conjugated antimouse IgG and mouse mAbs directed against α_1 (clone HP2B6), α_2 (clone Gi9), α_3 (clone M-KID2), α_4 (clone HP2/1), α_5 (clone SAM-1), α_6 (clone GoH3), β_1 (clone K20), and β_4 (clone ASC-3) integrin subunits were purchased from Coulter/Immunotech. Mouse myeloma mAb MOPC21 was obtained from ICN Pharmaceuticals.

Nonpeptidic $\alpha_v\beta_3$ integrin antagonist PSK1404 has been described among a new series of highly potent and selective RGD peptidomimetic $\alpha_v\beta_3$ antagonists that contain acylguanidines as an arginine replacement (21). PSK1404 is selective for $\alpha_v\beta_3$ (IC_{50} for fibrinogen binding to human recombinant $\alpha_{IIb}\beta_3$ integrin, 10,000 nmol/L; IC_{50} for vitronectin binding to human recombinant $\alpha_v\beta_3$ integrin, 2 nmol/L; ref. 22).

Western immunoblotting. The immunodetection of the β_3 integrin subunit in MDA-MB-231 transfectants was done as described previously (9).

Flow cytometry analysis. Cell surface expression of integrins by MDA-MB-231 transfectants was analyzed via a Galaxy flow cytometer (DAKO) as described previously (9).

Cell adhesion, invasion, and proliferation assays. All of the different experimental procedures were essentially as described (6, 9). Briefly, tumor cells resuspended in RPMI 1640 containing 1% (v/v) BSA (0.12×10^6 cells/mL) were plated in 100-mm bacteriologic Petri dishes dotted with BSP. After a 3-h incubation at 37°C, nonadherent cells were removed, and adherent cells were fixed, stained, and counted via microscope. Cell invasion experiments were done using Bio-Coat cell migration chambers (Becton Dickinson) coated with basement membrane Matrigel (30 μg/filter). Tumor cells resuspended in RPMI 1640 containing 1% (v/v) BSA were added to the upper chamber (5×10^4 cells/0.5 mL), and the chemoattractant (10% FBS) was placed in the lower chamber (0.75 mL/well). After a 24-h incubation at 37°C, the noninvading cells were removed and the invading cells on the under surface of the 8-μm-diameter pore size filter were fixed, stained, and counted via microscope. For cell proliferation experiments, tumor cells resuspended in complete RPMI 1640 were seeded in 96-well plates (500 cells/100 μL/well). After a 24-h incubation at 37°C, growing cells were washed and further cultured in complete medium for 5 days. Cell proliferation was measured spectrophotometrically at 550 nm using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (9).

Animals. All procedures involving animals, including housing and care, method of euthanasia, and experimental protocols, were conducted in accordance with a code of practice established by the local ethical committee (CREEA, Lyon, France). These studies were monitored on a routine basis by the attending veterinarian to ensure continued compliance with the proposed protocols. Four-week-old female BALB/c homozygous (*nu/nu*) athymic mice and sham-operated or ovariectomized wild-type (WT) BALB/c mice of 4 weeks of age were obtained from Charles River.

Animal models of bone metastasis. Bone metastasis experiments in animals were conducted as described previously (6, 9, 19). Briefly, MDA-MB-231 transfectants overexpressing integrin $\alpha_v\beta_3$ (3×10^5 cells in 100 μL PBS) were inoculated into the tail vein of anesthetized nude mice. Alternatively, MDA-MB-231/B02 (5×10^5 cells in 100 μL PBS) or CHO-$\beta_3$wt cells (10^6 cells in 100 μL PBS) were inoculated i.v. into animals. Radiographs of anesthetized animals were taken weekly with the use of MIN-R2000 films (Kodak) in an MX-20 cabinet X-ray system (Faxitron X-ray Corp.). The area of osteolytic lesions was measured using a Visiolab 2000 computerized image analysis system (Explora Nova) and the extent of bone destruction per animal was expressed in square millimeter as described previously (6, 9, 19). Metastatic animals were killed by cervical dislocation at day 21 (CHO-$\beta_3$wt cells), day 30 (MDA-MB-231/B02 cells), or day 42 (MDA-MB-231 transfectants) after tumor cell inoculation, and bones were collected for histologic analysis.

Animal model of intraosseous tumorigenesis. For intraosseous tumor xenograft experiments in mice, a small hole was drilled with a 30-gauge sterile needle through the tibial plateau with the knee flexed. Using a new sterile needle fitted to a 50-μL sterile Hamilton syringe (Hamilton Co.), a single-cell suspension (3×10^4 cells in 30 μL PBS) of $\alpha_v\beta_3$-overexpressing MDA-MB-231 cells (clone #30.1) or mock-transfected cells (clone EV#1.4) was then carefully injected in the bone marrow cavity. The progression of osteolytic lesions was monitored by radiography as described above. Animals were killed 42 days after tumor cell inoculation, and bones were collected for histologic analysis.

Animal models of homing of GFP-expressing tumor cells to bone. MDA-MB-231/B02 cells (5×10^5 in 100 μL PBS) and CHO-$\beta_3$wt and mock-transfected CHO cells (10^6 cells in 100 μL PBS) that had been stably transfected with the gene encoding GFP were injected into the tail vein of anesthetized animals. Tumor-bearing animals (that did or did not receive a short-term treatment with PSK1404; see below) were killed 7 days after tumor cell inoculation. None of these mice had radiographic evidence of bone destruction on the day they were killed. For each mouse, long bones were collected and the bone marrow was flushed out. Bone marrow cells

were then analyzed by flow cytometry for the detection of GFP-expressing cells.

PSK1404 treatment protocols. For the treatment of animals, we used a continuous or a short-term treatment protocol with nonpeptide $\alpha_v\beta_3$ integrin antagonist PSK1404.

In the continuous treatment protocol, PSK1404 administration (10 mg/kg, twice daily, s.c.) to BALB/c nude mice was initiated at the time of tumor cell inoculation and continued until day 21 (CHO-$\beta_3$wt), day 30 (MDA-MB-231/B02), or day 42 (MDA-MB-231 transfectants). The progression of osteolytic lesions was monitored weekly by radiography. In addition, sham-operated and ovariectomized BALB/c mice (used here as an animal model of osteoporosis) received a similar PSK1404 dosing regimen for 30 days to evaluate, in the absence of tumor cells, the effect of the drug on ovariectomy-induced bone loss. At the end of each protocol, animals were killed by cervical dislocation and bones were collected for histologic analysis.

In the short-term treatment protocol, PSK1404 administration (10 or 30 mg/kg, twice daily, s.c.) to animals was initiated 1 day before tumor cell inoculation. Two days after tumor cell inoculation, the administration of PSK1404 was discontinued, and the nude mice were left untreated until the end of the protocols (day 21 for CHO-$\beta_3$wt cells, day 30 for MDA-MB-231/B02 cells, and day 42 for MDA-MB-231 transfectants). The progression of osteolytic lesions was monitored weekly by radiography. Sham-operated and ovariectomized mice also received a short-term treatment, to evaluate the effect of PSK1404 on ovariectomy-induced bone loss. At the end of each protocol, animals were killed by cervical dislocation and bones were collected for histologic analysis.

Bone histology. Hind limbs from animals were fixed and embedded in methylmethacrylate. Seven-micrometer sections of undecalcified long bones were then cut with a microtome (Polycut E, Reichert-Jung) and stained with May-Grunwald-Giemsa or Goldner's trichrome. Histologic

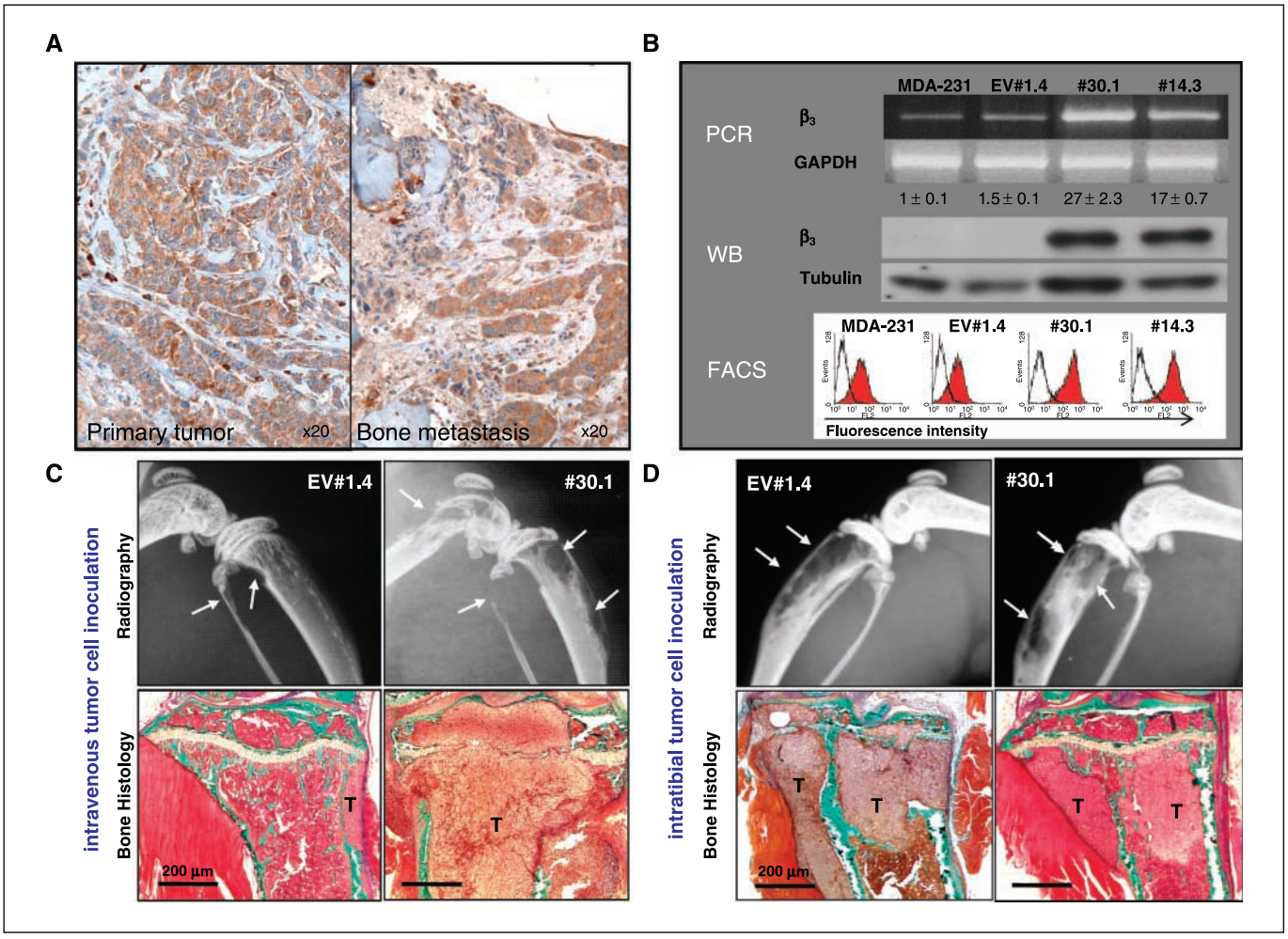

Figure 1. Tumor $\alpha_v\beta_3$ integrin expression and bone metastasis. *A,* immunohistochemical detection of β_3 integrin subunit in the primary breast carcinoma and bone metastasis of the same patient. Magnification, ×20. Immunohistochemistry was done using β_3-specific, mouse mAb SZ21. Almost all breast cancer cells were strongly positive for β_3 integrin protein expression. *B,* overexpression of $\alpha_v\beta_3$ in MDA-MB-231 breast cancer cells. *PCR*: β_3 and GAPDH mRNA expression in parental MDA-MB-231 cells, mock-transfected MDA-MB-231 cells (EV#1.4), and $\alpha_v\beta_3$-overexpressing #30.1 and #14.3 cells. Reverse transcription-PCR fragments were separated on a 2% agarose gel and stained with ethidium bromide. Numbers correspond to real-time PCR quantification data of the β_3 mRNA copy number relative to that of GAPDH mRNA for each clone (mean ± SD; $P < 0.001$ for #30.1 and #14.3 clones when compared with parental and EV#1.4 cells). Western blotting (*WB*): cell extracts (40–50 μg proteins/lane) were electrophoresed on a 7% Laemmli SDS-polyacrylamide gel under nonreducing conditions and then transferred to polyvinylidene difluoride membranes and immunoblotted with mAbs against human β_3 integrin (clone SZ21) or tubulin (used as an internal control for equal protein loading). Fluorescence-activated cell sorting (*FACS*): FACS analysis for cell surface expression of $\alpha_v\beta_3$ in parental MDA-MB-231 cells, EV#1.4 cells, and clones #30.1 and #14.3. The detection was done using mouse mAb LM 609 that recognizes the $\alpha_v\beta_3$ complex (*red histograms*). Background fluorescence was detected with isotype-matched negative control mouse mAb MOPC21 (*white histograms*). *Y axis,* the number of cells per channel (events); *X axis,* the relative fluorescence intensity in arbitrary units (log scale). *C,* radiography and bone histology of metastatic legs, 42 d after i.v. inoculation of EV#1.4 or #30.1 cells (3×10^5 cells per animal). There was a marked increase in the extent of osteolytic lesions (*arrows*) and skeletal tumor burden (*T*) in mice bearing #30.1 cells. *Green,* bone; *Red,* bone marrow and tumor cells (*T*). *D,* intraosseous growth of EV#1.4 and #30.1 cells. Cell suspensions (3×10^4 cells) were injected intratibially. Forty-two days after tumor cell inoculation, the extent of bone destruction and skeletal tumor burden was the same in animals bearing EV#1.4 or #30.1 tumors. *Bar,* 200 μm.

analyses were done on longitudinal medial sections of tibial metaphysis by using a Visiolab 2000 computerized image analysis system as described previously (6, 9, 19).

Statistical analysis. All data were analyzed with the use of StatView software (version 5.0; SAS Institute, Inc.). Statistical analyses were carried out by doing an unpaired Student's *t* test or ANOVA followed by a Fisher's protected least significant difference test. *P* values <0.05 were considered statistically significant.

Results

Expression of β_3 integrin subunit in pairs of human primary breast carcinomas and their matching bone metastases. As a first step toward evaluating tumor $\alpha_v\beta_3$ integrin in breast cancer bone metastasis, we did immunohistochemistry on the primary breast carcinoma and bone metastases of the same patient using mAb SZ21 directed against the β_3 integrin subunit. Eight pairs of primary breast tumors and their matching bone metastases were studied. As shown in Fig. 1*A*, all of the matching primary breast and metastatic tumors expressed β_3, and the immunostaining was always homogenous (80–90% of the tumor cells were positive for β_3). The scoring of β_3 staining intensity in pairs of primary tumors and bone metastatic specimens showed a moderate-to-strong staining in tumor cells from seven of eight patients, whereas a weak staining of tumor cells was observed in the primary tumor and matching bone metastatic lesion from one patient. As opposed to what has been reported previously by Liapis et al. (13), we did not notice any increase in the β_3 immunostaining intensity of bone-residing breast cancer cells when compared with that observed in tumor cells from matched primary carcinomas.

Overexpression of $\alpha_v\beta_3$ integrin in breast cancer cells increases the incidence and formation of osteolytic lesions in animals. Because immunohistochemical data indicated a possible role of $\alpha_v\beta_3$ integrin in the pathogenesis of breast cancer bone metastases, human MDA-MB-231 breast cancer cells were stably transfected to overexpress $\alpha_v\beta_3$ integrin. Two transfectants (clones #30.1 and #14.3) were selected based on their specific and high expression of β_3 subunit mRNA (real-time PCR) and protein (Western blotting) when compared with that observed in mock-transfected (clone EV#1.4) and parental MDA-MB-231 cells (Fig. 1*B*). The flow cytometry analysis of clones #30.1 and #14.3 for the cell surface expression of $\alpha_v\beta_3$ integrin confirmed Western blot data (Fig. 1*B*) and showed that $\alpha_v\beta_3$ integrin overexpression did not modify the cell surface expression levels of $\alpha_v\beta_5$, $\alpha_v\beta_6$, α_1, α_2, α_3, α_5, α_6, β_1, and β_4 integrins (Supplementary Fig. S1).

MDA-MB-231 transfectants were then injected into the tail vein of animals to examine the contribution of $\alpha_v\beta_3$ integrin in the development of bone metastases *in vivo*. The overexpression of $\alpha_v\beta_3$ in breast cancer cells was associated with a higher bone metastasis incidence in animals (Table 1). The follow-up by radiography of metastatic animals bearing $\alpha_v\beta_3$-overexpressing tumors also showed a 2-fold increase of bone destruction compared with that of mice bearing EV#1.4 tumors (Fig. 1*C*; Table 1).

Histomorphometric analysis of hind limbs with metastases showed that animals bearing $\alpha_v\beta_3$-overexpressing tumors had statistically significantly lower ratios for bone volume relative to tissue volume (BV/TV), indicating a higher bone destruction compared with mice bearing mock-transfected EV#1.4 tumors (Fig. 1*C*; Table 1). The skeletal tumor burden relative to soft tissue volume ratio (TB/STV) in animals bearing $\alpha_v\beta_3$-overexpressing tumors was also markedly increased compared with mice bearing EV#1.4 tumors (Fig. 1*C*; Table 1). In sharp contrast, when EV#1.4 and #30.1 cells were directly injected into the tibial bone marrow cavity, mice bearing EV#1.4 or #30.1 cells had a similar extent of bone destruction [11.3 ± 1.1 mm^2 ($n = 5$) and 7.5 ± 3.1 mm^2 ($n = 5$), respectively] and skeletal tumor burden (Fig. 1*D*). The larger bone metastatic lesions in animals bearing $\alpha_v\beta_3$-overexpressing tumors were therefore not directly related to $\alpha_v\beta_3$ integrin overexpression but an indirect result of the greater number of $\alpha_v\beta_3$-expressing tumor cells residing in the bone marrow that stimulated osteoclast-mediated bone resorption.

All of the transfectants proliferated at a similar rate *in vitro*, irrespective of $\alpha_v\beta_3$ integrin expression (Supplementary Fig. S2*A*). In contrast, there was a 2- to 3-fold increase in the attachment of #30.1 and #14.3 cells to BSP when compared with that observed with EV#1.4 and parental MDA-MB-231 cells (Supplementary Fig. S2*B*). Similarly, there was a substantial gain in invasion of #30.1 and #14.3 cells when compared with EV#1.4 and parental cells (Supplementary Fig. S2*C*). The increased invasion of $\alpha_v\beta_3$-expressing transfectants was specifically and statistically significantly inhibited by anti-$\alpha_v\beta_3$ antibody LM609 when compared with that observed with negative control antibody MOPC21 and anti-$\alpha_v\beta_5$ antibody P1F6 (Supplementary Fig. S2*D*).

Effect of a nonpeptide $\alpha_v\beta_3$ integrin antagonist (PSK1404) on bone metastasis formation caused by $\alpha_v\beta_3$-expressing cancer cells. Because osteoclasts express $\alpha_v\beta_3$ integrin (14), we

Table 1. Effect of $\alpha_v\beta_3$ overexpression on breast cancer bone metastasis formation

Cell line*	Radiography		Histomorphometry	
	Incidence (%)	Osteolytic lesions (mm^2/mouse)	BV/TV (%)	TB/STV (%)
EV#1.4	4/6 (67)	3.4 ± 1.4	17.8 ± 6.6 ($n = 6$)	18 ± 3 ($n = 6$)
#30.1	10/12 (83)	7.7 ± 1.8 †	5.5 ± 3.5 ($n = 8$) ‡	75 ± 25 ($n = 8$) ‡
#14.3	4/5 (80)	7.4 ± 0.6 †	4.8 ± 2 ($n = 5$) ‡	84 ± 2 ($n = 5$) ‡

NOTE: All measurements were made 42 d after tumor cell inoculation. Data are expressed as the mean ± SD of two independent experiments. *n* is the number of legs with bone metastasis.

*EV#1.4, mock-transfected MDA-MB-231 breast cancer cells; #30.1 and #14.3, MDA-MB-231 transfectants overexpressing $\alpha_v\beta_3$.

†$P < 0.05$, when compared with EV#1.4 cells using unpaired Student's *t* test.

‡$P < 0.01$, when compared with EV#1.4 cells using unpaired Student's *t* test.

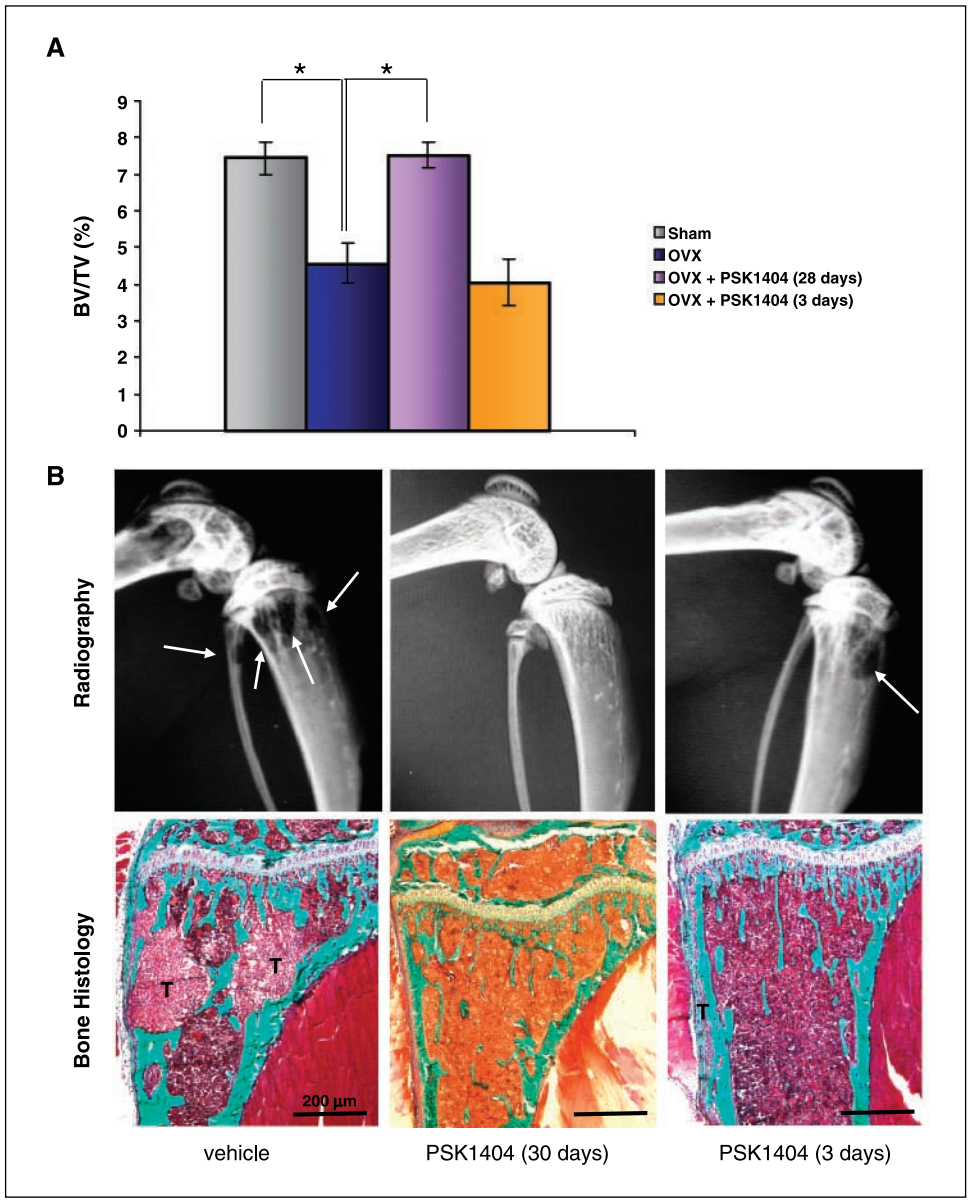

Figure 2. Effect of a continuous or short-term treatment with $\alpha_v\beta_3$ integrin antagonist PSK1404 on ovariectomy-induced bone loss and bone metastasis formation. *A,* ovariectomized (*OVX*) animals were left untreated or treated with PSK1404 using a dosing regimen (10 mg/kg) given s.c. twice daily for 28 or 3 d. Histomorphometric analysis of legs revealed that the bone volume relative to tissue volume was significantly decreased in ovariectomized animals. A continuous treatment (28 d) with PSK1404 completely prevented bone loss associated with ovariectomy, as opposed to a short-term PSK1404 treatment (3 d). *, $P < 0.001$ using unpaired Student's *t* test. *B,* animals inoculated with B02 breast cancer cells were treated continuously with PSK1404 (10 mg/kg, twice daily, s.c.) or the vehicle from the time of tumor cell inoculation until day 30. Alternatively, PSK1404 administration was initiated 1 d before B02 tumor cell inoculation and discontinued 2 d later. Radiographic and histologic analyses were done 30 d after tumor cell inoculation. A continuous or a short-term PSK1404 treatment substantially reduced bone destruction and skeletal tumor burden.

first assessed the antiresorptive potency of PSK1404 in a mouse model of bone loss caused by ovariectomy. Histomorphometric measurement of tibial metaphyses from placebo-treated ovariectomized wild-type BALB/c mice showed a bone loss, as judged by the marked reduction of the BV/TV ratio (40% reduction) compared with that of sham-operated animals (Fig. 2*A*). Bone loss induced by ovariectomy was completely prevented on continuous treatment of ovariectomized animals with PSK1404, when using a dosing regimen of 10 mg/kg, given s.c., twice daily for 28 days (Fig. 2*A*). In contrast, a 3-day treatment with PSK1404 at a dose of 10 mg/kg (twice daily) did not prevent bone loss (Fig. 2*A*).

Next, we validated the antiresorptive effect of PSK1404 in animal models of bone metastasis caused by #30.1 breast cancer cells, B02 breast cancer cells, or CHO-$\beta_3$wt ovarian cancer cells. PSK1404 (at a dose of 2×10 mg/kg/d, given continuously until the end of the protocols) significantly reduced the extent of osteolytic lesions in the three different animal models of bone metastasis, as judged by radiography (Fig. 2*B*; Table 2). Histomorphometric analysis confirmed radiographic analysis and showed that bone destruction observed in animals bearing #30.1, B02, or CHO-$\beta_3$wt cells was substantially reduced on PSK1404 treatment (Fig. 2*B*; Table 2). A PSK1404 treatment also markedly reduced skeletal tumor burden in animals when compared with the vehicle (Fig. 2*B*; Table 2).

We then examined whether a 3-day treatment with PSK1404, which did not prevent bone loss in ovariectomized animals (Fig. 2*A*), inhibits bone metastasis formation. Compared with the vehicle, a 3-day PSK1404 treatment statistically significantly decreased the extent of bone destruction and skeletal tumor burden in animals bearing B02 or CHO-$\beta_3$wt tumors, whereas it had no inhibitory effect in animals bearing #30.1 tumors (Fig. 2*B*; Table 3).

Effect of a short-term treatment with nonpeptide $\alpha_v\beta_3$ integrin antagonist PSK1404 on bone marrow colonization by $\alpha_v\beta_3$-expressing cancer cells. The detection of fluorescence expressed by tumor cells is a highly sensitive method to detect the early development of bone metastasis (19). Our use of B02 or

Table 2. Effect of a continuous treatment with nonpeptide $\alpha_v\beta_3$ integrin antagonist PSK1404 on the formation of experimental bone metastases

Cell line*	Radiography		Histomorphometry	
	Osteolytic lesions (mm^2/mouse)		BV/TV (%)	
	Vehicle	PSK1404	Vehicle	PSK1404
#30.1	7.5 ± 1.7 (n = 5)	2.9 ± 2.2† (n = 6)	4.3 ± 2.5 (n' = 5)	15.5 ± 6† (n' = 6)
B02	8.1 ± 3.5 (n = 15)	0.2 ± 0.5† (n = 8)	4.8 ± 1.1 (n' = 15)	8.8 ± 1.7‡ (n' = 8)
CHO-$\beta_3$wt	7.3 ± 2.9 (n = 13)	0.7 ± 0.8‡ (n = 8)	2.3 ± 0.3 (n' = 13)	12 ± 3.4‡ (n' = 8)

NOTE: All measurements were made at the end of the protocols. Data are expressed as the mean ± SD. n is the number of metastatic animals. n' is the number of legs with bone metastasis.

*#30.1, MDA-MB-231 breast cancer cells stably transfected to overexpress $\alpha_v\beta_3$; B02, bone metastatic clone of MDA-MB-231 cells constitutively overexpressing $\alpha_v\beta_3$ (9); CHO-$\beta_3$wt, CHO cells stably transfected to *de novo* express $\alpha_v\beta_3$ (9). Animals inoculated with each of the different cell lines received a treatment with PSK1404 (10 mg/kg) or the vehicle, given s.c., twice daily from the day of tumor cell inoculation to the end of the protocol.

†$P < 0.05$, when compared with vehicle-treated animals using unpaired Student's t test.

‡$P < 0.001$, when compared with vehicle-treated animals using unpaired Student's t test.

CHO-$\beta_3$wt cells that expressed a stably transfected gene encoding GFP (19) allowed us to examine whether mice treated for 3 days with PSK1404 displayed evidence of GFP-expressing cancer cells in the bone marrow on day 7 after tumor cell inoculation, at which time there was no radiographic evidence of osteolytic lesions (data not shown). We assumed that the detection of fluorescent cells by flow cytometry was an indication that cancer cells were present in the bone marrow.

Forward and side scatter (FSC/SCC) dot plot analysis of bone marrow cells of hind limbs from vehicle-treated animals that had been inoculated with B02/GFP cells showed two distinct cell populations, whereas a single bone marrow cell population was observed when using naive mice that had not been inoculated with tumor cells (Fig. 3*A*). Measurement of the fluorescence intensity in the bone marrow cell population of hind limbs from vehicle-treated animals showed that 15.5% of the bone marrow cells corresponded to B02/GFP cells (Fig. 3*B*). By contrast, the bone marrow cell population of hind limbs from mice treated with PSK1404 had only 1.8% of B02/GFP cells (Fig. 3*B*). There was an 88% reduction in B02/GFP tumor burden on PSK1404 treatment. Compared with the vehicle, PSK1404 also substantially decreased CHO-$\beta_3$wt/GFP tumor burden by 95% (8.7% versus 0.4% of the bone marrow cell population; Fig. 3*C*), whereas it had no inhibitory effect on the tumor burden of GFP-expressing parental CHO cells that do not express $\alpha_v\beta_3$ (2.6% versus 3.4% of the bone marrow cell population; Fig. 3*D*). CHO-$\beta_3$wt/GFP tumor burden in the bone marrow of hind limbs from vehicle-treated animals was 3.3-fold higher than that of mice bearing CHO/GFP cells, further indicating that $\alpha_v\beta_3$ integrin promoted bone colonization by cancer cells.

Table 3. Effect of a short-term treatment with nonpeptide $\alpha_v\beta_3$ integrin antagonist PSK1404 on the formation of experimental bone metastases

Cell line*	Radiography		Histomorphometry	
	Osteolytic lesions (mm^2/mouse)		BV/TV (%)	
	Vehicle	PSK1404	Vehicle	PSK1404
#30.1	7.5 ± 1.7 (n = 5)	6 ± 3.1 (n = 4)	4.3 ± 2.5 (n' = 5)	n.d.
B02	8.1 ± 3.5 (n = 15)	3.5 ± 4.2† (n = 7)	4.8 ± 1.1 (n' = 15)	12.7 ± 4.1‡ (n' = 7)
CHO-$\beta_3$wt	7.3 ± 2.9 (n = 13)	1.3 ± 0.6‡ (n = 6)	2.3 ± 0.3 (n' = 13)	11.3 ± 1.4‡ (n' = 6)

NOTE: All measurements were made at the end of the protocols. Data are expressed as the mean ± SD. n is the number of metastatic animals. n' is the number of legs with bone metastasis.

Abbreviation: n.d., not done.

*#30.1, MDA-MB-231 breast cancer cells stably transfected to overexpress $\alpha_v\beta_3$; B02, bone metastatic clone of MDA-MB-231 cells constitutively overexpressing $\alpha_v\beta_3$ (9); CHO-$\beta_3$wt, CHO cells stably transfected to *de novo* express $\alpha_v\beta_3$ (9). Animals inoculated with each of the different cell lines received a treatment with PSK1404 (10 mg/kg) or the vehicle, given s.c., twice daily for 3 d.

†$P < 0.05$, when compared with vehicle-treated animals using unpaired Student's t test.

‡$P < 0.001$, when compared with vehicle-treated animals using unpaired Student's t test.

Table 2. Effect of a continuous treatment with nonpeptide $\alpha_v\beta_3$ integrin antagonist PSK1404 on the formation of experimental bone metastases (Cont'd)

Histomorphometry			
TB/STV (%)		Tumor area (mm^2)	
Vehicle	PSK1404	Vehicle	PSK1404
87 ± 4 (*n*' = 5)	24 ± 34 † (*n*' = 6)	1.98 ± 0.09 (*n*' = 5)	0.44 ± 0.61 ‡ (*n*' = 6)
62.2 ± 8.7 (*n*' = 15)	4.7 ± 0.6 ‡ (*n*' = 8)	1.41 ± 0.19 (*n*' = 15)	0.11 ± 0.22 ‡ (*n*' = 8)
80.8 ± 25 (*n*' = 13)	0.3 ± 0.5 ‡ (*n*' = 8)	1.84 ± 0.57 (*n*' = 13)	0.05 ± 0.09 ‡ (*n*' = 8)

To investigate the molecular mechanisms by which a short-term PSK1404 treatment inhibited bone marrow colonization by $\alpha_v\beta_3$-expressing cancer cells, we examined whether PSK1404 inhibits tumor cell invasion, which is an early event in the formation of bone metastases (1, 11). The pharmacokinetic of a single dose (10 mg/kg) of PSK1404 revealed that the peak plasma concentration was ~3,700 ng/mL (time to maximum concentration, 0.5 h) and that plasma levels in animals 1 and 6 h after drug administration were 500 and 10 ng/mL, respectively. PSK1404 concentrations ranged between 2 and 200 ng/mL were therefore chosen for *in vitro* tumor cell invasion experiments. PSK1404 dose dependently inhibited B02 and CHO-$\beta_3$wt cell invasion, with half-maximal inhibitory concentration (IC_{50}) values of 1.9 and 2.2 ng/mL, respectively (Supplementary Fig. S3*A*). By contrast, PSK1404 was less potent at inhibiting #30.1 tumor cell invasion (IC_{50}, 25 ng/mL; Supplementary Fig. S3*A*).

Integrin $\alpha_v\beta_3$ also plays an important role in tumor angiogenesis, and the treatment of mice with an anti-β_3 antibody reduces angiogenesis in experimental prostate cancer bone metastases (17). The inhibitory effect of PSK1404 on capillary-like tube formation was therefore tested. As shown in the Supplementary Fig. S3*B*, PSK1404 dose dependently inhibited angiogenesis *in vitro*, with a IC_{50} value of 11 ng/mL.

Discussion

Different molecular mechanisms are responsible for the propensity of breast cancer cells to metastasize to bone. The chemokine receptor CXCR4 controls the metastatic destination of breast cancer cells in certain organs (lungs, liver, and bone marrow) where its ligand, the chemokine CXCL-12, is produced in high quantity (23). Bone-derived cytokine receptor activator of nuclear factor-κB ligand (RANKL) has been involved recently in the bone tropism of RANK-expressing cancer cells (24). There is also a growing body of evidence from preclinical research showing that $\alpha_v\beta_3$ integrin expression by tumor cells is associated with bone metastasis formation (9–12). For instance, by *in vivo* selection of MDA-MB-231 breast cancer cells, we have isolated a cell subpopulation (called B02) that only metastasizes to bone and constitutively overexpresses $\alpha_v\beta_3$ integrin (9). Similarly, the *de novo* expression of $\alpha_v\beta_3$ in 66cl4 breast cancer and CHO ovarian cancer cells that metastasize to lungs, but not to bone, is sufficient to promote their dissemination to bone (9, 10). Our present results show that $\alpha_v\beta_3$ integrin overexpression in MDA-MB-231 breast cancer cells enhanced bone metastasis incidence in animals. Finally, bone-derived growth factors released from resorbed bone may enhance the bone metastatic potential of $\alpha_v\beta_3$-expressing cancer cells. For instance, transforming growth factor β (TGFβ; a bone-derived growth factor) stimulates $\alpha_v\beta_3$ expression by MDA-MB-435 breast cancer cells and the blockade of TGFβ signaling reduces the incidence of MDA-MB-435 skeletal metastases in animals (25). Interestingly, CXCL-12 and RANKL also stimulate $\alpha_v\beta_3$ integrin expression in prostate cancer cells and osteoclasts, respectively (26, 27). It is therefore possible that tumor $\alpha_v\beta_3$ acts in concert with CXCR4, RANK, and/or TGFβ receptors to promote the bone tropism of breast cancer cells.

We surmise that tumor $\alpha_v\beta_3$ participates in the bone tropism of breast cancer cells in mediating early metastatic steps. This assumption is supported by our finding that the i.v. inoculation of $\alpha_v\beta_3$-expressing tumor cells promoted skeletal tumor burden compared with mock-transfected cells, whereas a similar extent of tumor burden was observed when either of these tumor cell lines was inoculated directly into the tibial bone marrow cavity. Moreover, we found that $\alpha_v\beta_3$ integrin overexpression specifically

Table 3. Effect of a short-term treatment with nonpeptide $\alpha_v\beta_3$ integrin antagonist PSK1404 on the formation of experimental bone metastases (Cont'd)

Histomorphometry			
TB/STV (%)		Tumor area (mm^2)	
Vehicle	PSK1404	Vehicle	PSK1404
1.98 ± 0.09 (*n*' = 5)	n.d.		
62.2 ± 8.7 (*n*' = 15)	6.4 ± 1.1 ‡ (*n*' = 7)	1.41 ± 0.19 (*n*' = 15)	0.12 ± 0.02 ‡ (*n*' = 7)
80.8 ± 25 (*n*' = 13)	4.3 ± 0.7 ‡ (*n*' = 6)	1.84 ± 0.57 (*n*' = 13)	0.08 ± 0.01 ‡ (*n*' = 6)

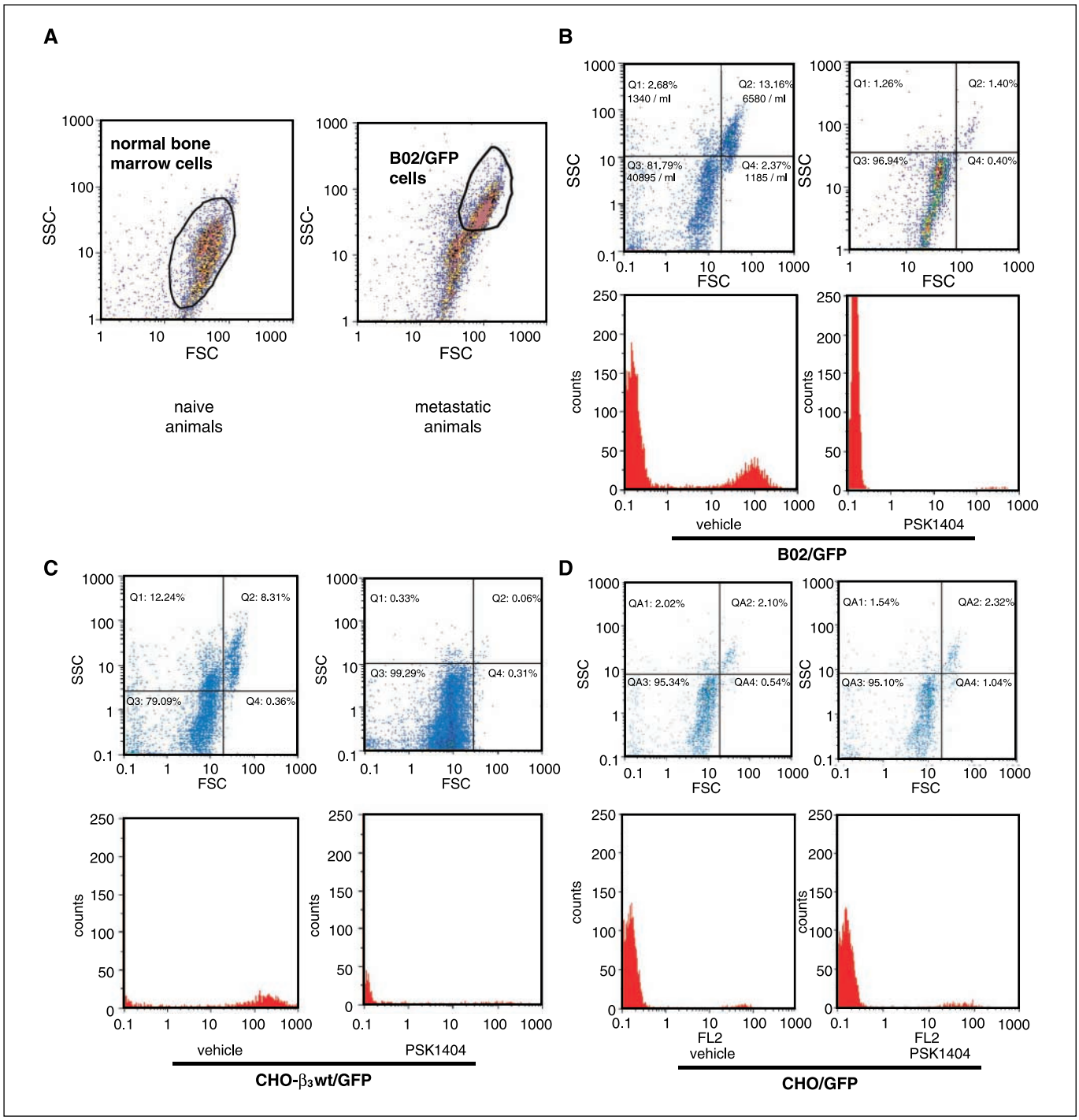

Figure 3. Flow cytometry analysis of bone marrow cells isolated from vehicle- and PSK1404-treated animals bearing GFP-expressing tumor cells. *A,* FSC/SCC dot plots of bone marrow cells isolated from naive and metastatic animals. FSC/SSC variables were set using bone marrow cells from naive animals that did not receive tumor cells. A single-cell population is observed. In contrast, two cell populations are observed in the bone marrow from metastatic animals. *B* to *D,* animals receiving a 3-day treatment with PSK1404 or the vehicle were sacrificed 7 d after i.v. inoculation of either GFP-expressing B02 breast cancer cells that constitutively overexpress $\alpha_v\beta_3$ (B02/GFP), GFP-expressing CHO ovarian cancer cells stably transfected to express $\alpha_v\beta_3$ (CHO-$\beta_3$wt/GFP), or GFP-expressing CHO ovarian cancer cells that do not express $\alpha_v\beta_3$ (CHO/GFP). Bone marrow cells were then analyzed by flow cytometry to detect the presence of fluorescent tumor cells. *Top,* FSC/SSC dot plots of bone marrow cells from metastatic animals. In metastatic animals, the normal bone marrow cell population is located in quadrant 3 (*Q3*), whereas tumor cells are in quadrants 2 and 4 (*Q2* and *Q4*). Percentage of cells present in each quadrant. *Bottom,* flow cytometry histograms of bone marrow cells. *Y axis,* the number of cells per channel (events); *X axis,* the relative fluorescence intensity in arbitrary units (log scale).

increased the adhesiveness and invasiveness of MDA-MB-231 cells *in vitro*. Our results are in accordance with the observation that tumor $\alpha_v\beta_3$ integrin mediates the attachment of MDA-MB-231 cells to extracellular bone matrices (28) and cooperates with BSP and MMP-2 in promoting osteotropic cancer cell invasion *in vitro* (11, 12). In addition, a similar increased adhesiveness and invasiveness has been reported previously for $\alpha_v\beta_3$-expressing 66cl4 and CHO cells (9, 10). Thus, invasion and adhesion could be

critical for bone colonization by $\alpha_v\beta_3$-expressing cancer cells. Additional $\alpha_v\beta_3$-dependent mechanisms, such as tumor cell arrest during blood flow (29), may also be involved in bone metastasis formation. We therefore reasoned that a therapeutic approach that targets tumor $\alpha_v\beta_3$ could be an effective way to minimize bone colonization by breast cancer cells.

We used a short-term preventive therapy with a nonpeptide $\alpha_v\beta_3$ integrin antagonist (PSK1404) to investigate whether it has the potential to inhibit bone metastasis formation. Notably, this short-term dosing regimen of PSK1404 did not inhibit bone resorption in ovariectomized animals, a feature that allowed us to uncouple the direct effects of PSK1404 to $\alpha_v\beta_3$-expressing tumor cells from osteoclast-mediated effects. We found that PSK1404 treatment of animals caused a profound and specific inhibition of the bone marrow colonization by $\alpha_v\beta_3$-expressing B02 and CHO tumor cells. As discussed previously, bone colonization by cancer cells likely involves early metastatic processes, such as tumor cell invasion. In this respect, PSK1404 blocked tumor cell invasion *in vitro* at concentrations that can be achieved in the plasma of metastatic animals, suggesting that a short-term preventive regimen of PSK1404 could inhibit tumor cell invasion *in vivo*. Moreover, it is interesting to notice that a short-term dosing regimen of PSK1404 not only decreased skeletal tumor burden but also reduced bone destruction in animals bearing $\alpha_v\beta_3$-expressing B02 or CHO tumor cells. These results are in accordance with the observation that tumor cells stimulate osteoclast-mediated bone resorption (1–6). They suggest that a short-term therapy with PSK1404 inhibits bone colonization by tumor cells, which in turn decreases the production of tumor-derived factors that are required for osteoclast-mediated bone resorption, thereby leading to a reduction of bone destruction.

Aside from our observation that a nonpeptide $\alpha_v\beta_3$ integrin antagonist may directly interfere with bone colonization by tumor cells, we used a continuous therapy with PSK1404 to determine whether this integrin antagonist also had the potential to directly inhibit osteoclast-mediated bone destruction. We found that PSK1404, at a dose that inhibited osteoclast-mediated bone resorption in ovariectomized animals, drastically reduced the formation of osteolytic lesions caused by three different $\alpha_v\beta_3$-expressing cancer cell lines. A continuous therapy with PSK1404 was even more effective than a short-term therapy for reducing bone destruction and skeletal tumor burden in animals bearing $\alpha_v\beta_3$-overexpressing MDA-MB-231 breast cancer cells (clone #30.1). Our results are reminiscent of those obtained in a preventive study on animals bearing MDA-MB-435 breast cancer cells, in which a peptidomimetic inhibitor of $\alpha_v\beta_3$ (S247) reduces bone destruction and skeletal tumor burden (18). Likewise, the treatment of animals with an anti-β_3 antibody blocks the formation of osteolytic lesions caused by PC-3 prostate cancer cells that do not express $\alpha_v\beta_3$ integrin (17). Thus, these results (this study and refs. 17, 18) highlight the importance of osteoclast $\alpha_v\beta_3$ integrin in mediating malignant osteolysis. These results are also in accordance with the "vicious cycle" theory (1), in which tumor cells stimulate osteoclast-mediated bone resorption and bone-derived growth factors released from resorbed bone stimulate tumor growth. They suggest, as observed previously for osteoclast inhibitors bisphosphonates and osteoprotegerin (30, 31), that the blockade of bone resorption by PSK1404 (S247 or the anti-β_3 antibody) most probably deprives tumor cells of bone-derived growth factors that are required for tumor growth. Additional mechanisms, such as the inhibition of tumor angiogenesis, may also be involved in reducing skeletal tumor burden. Endothelial cell $\alpha_v\beta_3$ integrin is known to play a key role in tumor angiogenesis (32). We found that PSK1404 inhibited the formation of capillary-like tubes *in vitro*. The treatment of mice with an anti-β_3 antibody also reduces angiogenesis in experimental prostate cancer bone metastases (17). Thus, a continuous regimen of PSK1404 may enable multiple inhibitory effects on cancer cells, endothelial cells, and osteoclasts, leading to inhibition of bone metastasis formation.

In conclusion, our study shows that $\alpha_v\beta_3$ integrin not only plays a causal role in the bone colonization by metastatic cells but also stands as a therapeutic target for the prevention of bone metastases.

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