Overexpression of CD9 in Human Breast Cancer Cells Promotes the Development of Bone Metastases

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Abstract. *Background: Bone is a preferred target for circulating metastatic breast cancer cells. We found that the CD9 protein was up-regulated in the B02 osteotropic cell line, derived from the aggressive parental MDA-MB-231 breast cancer cell line. Here, we investigated the putative relationship between CD9 expression and the osteotropic phenotype. Materials and Methods: Overexpression of CD9 was analyzed by immunoblotting in different cell lines. Immunohistochemistry was used to assess CD9 expression in primary tumors and metastatic lesions. In vivo experiments were conducted in mice using a monoclonal antibody against CD9. Results: CD9 overexpression was confirmed in osteotropic cells. CD9 was significantly overexpressed in bone metastases versus primary tumors and visceral metastatic lesions. Finally, in vivo experiments showed that an antibody against CD9 delays homing of B02 cells in bone marrow, slowing down bone destruction. Conclusion: Our study reveals a potential implication of CD9 in the formation of bony metastases from breast cancer cells.*

Disseminated metastases represent the main cause of morbidity and mortality for patients with cancer. The skeleton is a preferred site for the seeding and growth of metastases originating from tumors identified as osteotropic, such as breast, prostate and lung cancer (1). The majority of patients with breast cancer at an advanced, stage experience bone metastasis (2). These secondary lesions are associated with substantial morbidity, including bone pain, pathological

fracture, hypercalcemia, myelopathy and spinal cord compression (3). In this context, identifying the proteins involved in the acquisition of an osteotropic phenotype by cancer cells would represent a major step towards the development of both new prognostic markers and therapeutic improvements.

Cell membrane proteins represent the most likely key actors in the specific tropism of circulating cancer cells. Indeed, membrane proteins control cell–matrix and cell–cell interactions, and therefore participate in cell signaling and adaptation to environment. In this regard, it is not a surprise that many clinical biomarkers and therapeutic targets are cell-surface proteins.

In an attempt to reveal differentially expressed proteins that could be of substantial interest in targeted therapies, we previously used a combination of cell fractionation and biotinylation of membrane proteins to study the membrane proteome of the osteotropic B02 cell line, derived from the highly aggressive MDA-MB-231 breast cancer cell line (4). Our approach actually led to the discovery of proteins that were either up-regulated or down-regulated in the B02 cell line. For instance, we demonstrated a down-regulation of class I human leucocyte antigens (HLAs) in the bone-seeking clone while, on the contrary, some proteins were overexpressed in the osteotropic B02 clone. CD9, also known as motility-related protein 1 (MRP-1), was among the membrane-associated proteins overexpressed in B02 cells. CD9, a member of the tetraspanin family, is a cell-surface glycoprotein that is known to complex with integrins (5) and other tetraspanins to form a 'tetraspanin web' (6). This protein can modulate key events such as cell adhesion, migration, proliferation and growth (7). In bone, it has been shown that CD9 expression in lipid rafts was crucial for cell fusion during osteoclastogenesis (8). A role in the metastatic potential of tumor cells has also already been suggested for this protein. Intriguingly, this protein has been regarded as an important metastasis-inhibitory factor of various types of human cancers, since the loss of CD9 expression in many solid tumors has been shown to be associated with poor

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prognosis, for instance in breast (9, 10), lung (11, 12), endometrial (13), pancreatic (14), colonic (15) and head and neck cancer (16), non-Hodgkin's lymphoma (17), melanoma (18) and esophageal squamous cell carcinoma (19).

The unexpected CD9 overexpression observed in the bone-seeking breast cancer clone led us to further investigate the relationship between CD9 expression and the osteotropic phenotype in human breast cancer.

Overall, our results show that CD9 mediates, at least in part, the homing of cancer cells to bone. It could represent a potential new target for 'intelligent' compounds (*e.g.* antibodies bound to bioactive molecules) aiming at selectively eradicating bone-seeking cells in osteotropic cancer.

Materials and Methods

Cell culture. Different bone-seeking clones were previously generated from the now–well–described invasive cell line MDA-MB-231 (20-22). Peyruchaud *et al.* were able to establish a breast cancer cell line subclone by repeated *in vivo* passages in bone from nude mice using the mouse heart injection model: this osteotropic clone, named MDA-MB-231-B02, is referred to as 'B02' hereafter and is characterized by its unique predilection for bone metastasis (22, 23). MDA-MB-231 and its derivative cell line B02 were used at early passages and routinely cultured until approaching confluency in RPMI-1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (ICN, Asse Relegem, Belgium) at 37°C in a humidified incubator with 5% CO_2.

C4-2B cells are derived from LNCaP cells after several passages through nude mice and aggressive tumors that metastasize to bone (24, 25). The C4-2B cells were maintained in T medium, which consisted of 75% Dulbecco's Modified Eagle's Medium (DMEM), 15% F-12, 100 U/l penicillin G, 100 μg/ml streptomycin, 5 μg/ml insulin, 13.6 pg/ml triiodothyronine, 5 μg/ml apo-transferrin, 0.25 μg/ml d-biotin, 25 μg/ml adenine, supplemented with 10% fetal bovine serum (FBS). The C4-2B cells were kindly provided by E.T. Keller (University of Michigan, Ann Arbor, MI, USA).

For immunocytochemistry experiments, 50×10^6 cells were harvested. The integrity of the pellet was maintained using soft agar. The pellet was then fixed in formalin and embedded in paraffin.

Western blotting. MDA-MB-231, LNCaP cells and their subclones were lysed in RIPA extraction buffer [1% NP40, 0.5% deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS) in 50 mM Tris (pH 7.5), 150 mM NaCl] containing an anti-protease cocktail (Complete®; Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined using the BCA assay (Pierce, Erembodegem-Aalst, Belgium). SDS–polyacrylamide gel electrophoresis (PAGE) was performed under non-reducing conditions. Proteins were separated on 12.5% polyacrylamide gels. Electophoresed proteins were transferred to polyvinylidene difluoride (PVDF) membranes, overnight at 35 V. Membranes were blocked in TBS-Tween (0.1% v/v) with 5% non-fat dried milk, and were incubated with antibodies overnight at 4°C. Equal protein loading and transfer were assessed by the subsequent use of an anti-alpha-tubulin antibody (clone B-5-1-2; Sigma, Merelbeke, Belgium), applied on stripped membranes. Bound antibodies were visualized by incubation of the membranes with ECL chemiluminescent substrate (Amersham, Diegem, Belgium) and exposure to X-ray films (Fuji, Düsseldorf, Germany).

Antibodies. The mouse monoclonal antibody against CD9 used for western blotting (clone MEM-61) was purchased from AbCam (Cambridge, UK). The mouse monoclonal antibody to CD9 used for fluorescence-activated cell sorting (FACS) analyses (clone 72F6) was purchased from Serotec (Düsseldorf, Germany). The mouse monoclonal antibody to CD9 used for *in vivo* experiments (clone Alb6) was purchased from Beckman Coulter (Analis, Belgium).

FACS analyses. Cells were washed twice in phosphate buffered saline (PBS) and incubated with mouse immunoglobulin (IgG, 1 μl per 10^6 cells, for 1 h at 4°C). Cells were then incubated for 1 h at 4°C with fluorescein isothiocyanate (FITC)-conjugated monoclonal secondary antibodies (1/1000 dilution). Cells were acquired using a FacsCalibur flow cytometer and data were analyzed using the CellQuest software (BD Biosciences, Erembodegem, Belgium) and FlowJo software (Tree Star, Ashland, OR, USA).

Patients. Patients with disseminated breast cancer and histologically proven bone metastases were selected from files of the Department of Pathology (Professor J. Boniver, University Hospital of Liège, Belgium). Original slides were reviewed, and the clinical history was obtained. Seven patients with disseminated breast cancer, including bone metastases, were randomly selected for this study.

Six out of the seven patients with breast cancer were treated surgically. At the time of surgery, none of these patients had clinically or scintigraphically detectable bone metastases. The remaining patient with breast cancer presented clinical and/or radiological evidence of bone dissemination, invaded lymph nodes, and pulmonary metastases; no surgical treatment was applied in this case. All patients underwent standard chemotherapy and/or radiotherapy and/or hormonal manipulation as adjuvant and/or neoadjuvant treatments. Tumor estrogen and progesterone receptor status was assessed in five patients. Both receptors were detected in four patients, and were negative in one patient. All patients developed bone metastases (positive bone scan and/or positive radiography and/or clinical evidence). The clinical, pathological, and biological data of the patients are summarized in Table I.

Evaluation of immunohistochemical staining. Mouse monoclonal antibody against CD9 was used for immunohistochemical staining (clone MEM-61; 1:80). Scoring of the intensity of the staining was performed as previously described (26), by two independent observers according to a semi-quantitative arbitrary scale with steps of 0, 1+, 2+, and 3+, where 0 was considered to be no detectable staining, 1+ was considered as weakly-positive staining, 2+ represented moderate staining, and 3+ was considered to be strong staining. When the tumors exhibited heterogeneity in the intensity of the staining, the scoring was assessed according to the staining of the most positive tumor cells, when their estimated percentage represented at least 30% of the total positive tumor cell area.

Statistical analysis. In a primary statistical analysis, tumor specimens were classified into two subgroups according to the intensity of CD9 expression: group 1, low CD9 expression (staining intensity 0 and 1+); group 2, high CD9 expression (staining intensity >1+). Associations between tumor site (primary tumors *versus* skeletal metastases *versus* visceral metastases) and CD9 expression (group 1

Table I. *Clinical, pathological and biological data of breast cancer patients that were investigated in this study.*

Case[#]	Year of birth	Year of breast cancer diagnosis	Lymph node metastasis at diagnosis*	Bone metastasis at diagnosis	Pathology	Lymph node metastasis at surgery[†]	Estrogen receptor	Progesterone receptor
1	1954	1991	p	p	IGC	NA	p	p
2	1919	2002	p	n	IDC	p	p	p
3	1944	1995	p	n	IDC	p	NA	NA
4	1929	1996	n	n	IDC	p	p	p
5	1969	2003	p	n	IDC	p	p	p
6	1949	1992	n	n	IDC	n	n	n
7	1927	1989	p	n	IDC	p	NA	NA

p, Positive; n, negative; NA, not available; IDC, invasive ductal carcinoma; IGC, invasive galactophoric carcinoma; *clinical staging; [†]pathological staging.

versus group 2) were tested with the use of the χ^2 test. In a third analysis, tumor sites in each patient of the data set (primary tumors *versus* skeletal metastases *versus* visceral metastases) were considered as pairs and were tested for differences in CD9 expression. For visceral metastases, the mean level of staining intensity in the different metastatic sites examined was calculated. Comparisons of CD9 expression between the paired tumor sites were then assessed with the use of the Wilcoxon signed-rank test. Statistical tests were two-tailed, and $p<0.05$ was considered statistically significant. The analyses were performed with Statistica software (v8.0; StatSoft, Tulsa, OK, USA). Analysis of osteolytic lesions on radiographs was carried out by performing a non-parametric Mann-Whitney *U*-test.

In vivo experiments. 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 regional Ethical Committee (CREEA, Lyon, France). These studies were monitored on a routine basis by the attending veterinarian to ensure continued compliance with the submitted protocols. Four-week-old female Balb/c homozygous (*nu/nu*) athymic mice were obtained from Charles River (St. Germain sur l'Arbresles, France).

Experimental bone metastases: On day 0, two groups of 10 mice were set up: one group for injection with luciferase-expressing B02 pre-incubated with an optimal concentration of Alb6 monoclonal antibody, and the other group for injection with luciferase-expressing B02 preincubated with an isotype-matched negative control antibody, namely mouse IgG1. Pre-incubation was carried out for 60 min for both groups.

Pre-incubated luciferase-expressing B02 cells (5×10^5 cells /100 μl PBS) were inoculated into the ventral tail artery of anesthetized mice. The extent of bone destruction and tumor burden was assessed by whole-body radiography and bioluminescence intensity, respectively, as previously described (27). Bioluminescence measurements were performed at 0.5 h and 4 h, 1, 7, 14, 21 and 35 days. Osteolytic lesions were detected by radiography at 21, 28 and 35 days [radiographs of B02 tumor-bearing mice were taken and the area of osteolytic lesions was measured, as previously described (27)].

Results

CD9 is a marker of osteotropic cell lines. Searching for potential molecular cell-surface markers that could play a role

in the osteotropic phenotype, we previously identified the CD9 protein as being differentially expressed between the well-known parental invasive breast cancer cell line MDA-MB-231 and its osteotropic cell derivative B02, by using purified membrane protein extracts and a mass spectrometric shotgun approach (4). We confirmed the overexpression of CD9 at the surface of B02 cells using FACS analysis (Figure 1), western blotting (Figure 2A) and immunocytochemistry (Figure 3A and B). These methods clearly showed that the CD9 protein is overexpressed in the B02 subclone, confirming the data obtained in our previous proteomic study (4). We next wondered if CD9 overexpression was restricted to this particular cell line, or if it represented a common feature of osteotropic cell lines. Thus, we investigated the expression levels of CD9 in another osteotropic cell line, originating from another type of cancer, characterized for its high propensity to disseminate to the skeleton, namely prostate cancer. The C4-2B cell line was described by Thalmann *et al.* (24), and derives from the androgen-independent LNCaP subline. These cells display an osteoblastic phenotype, are able to produce hydroxyapatite minerals *in vitro* and stimulate osteoblasts to initiate mineralization in the bone (25). The western blot presented in Figure 2B indicates that CD9 overexpression is also detectable in C4-2B cells and is likely to be a common feature of osteotropic cell lines.

CD9 is overexpressed in bone metastases. We next sought to investigate the CD9 expression levels in a series of paraffin-embedded tissue sections. The expression of CD9 was evaluated by the immunoperoxidase technique in paraffin sections of seven primary breast tumors and their associated bone metastatic lesions. CD9 was detected in the primary tumors of all seven patients with breast cancer, with a cell-surface staining pattern similar to that observed in the paraffin sections of B02 cells (Figure 3B). The immunostaining scoring of the seven primary breast carcinomas and associated metastatic lesions is displayed in Table II. Only one out of the seven primary breast carcinomas expressed high levels of

Table II. *Expression of CD9 in the primary and metastatic lesions of the patients with breast cancer investigated in this study. Scoring of the intensity of the staining was performed according to a semi-quantitative arbitrary scale: 0 was considered as no detectable staining, 1+ was considered as weakly-positive staining, 2+ represented moderate staining, and 3+ was considered to be strong staining.*

Case #	Intensity of immunostaining									
	Primary tumor	Bone	Lung	Liver	Kidney	Thyroid	Uterus	Pancreas	Adrenal gland	Lymph node
1	1	3	2	2.5	1	2		2	2	
2	1	3								
3	1.5	3	2	2			2			
4	1	3	1	1			1			1
5	1	3								
6	1.5	3		2		3				
7	3	3	2	2					1.5	
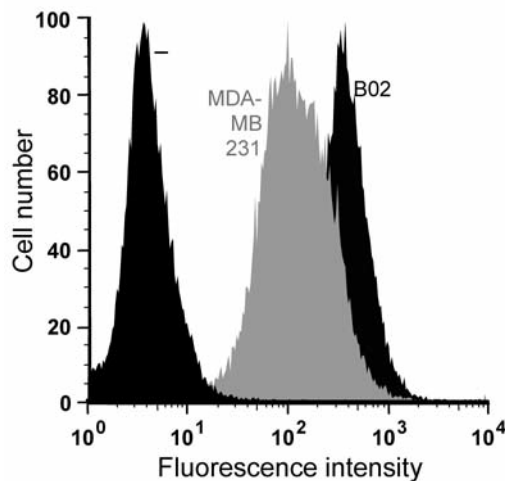
Figure 1. *Fluorescence-activated cell sorting (FACS) analysis of the CD9 protein in MDA-MB-231 and B02 cell lines. MDA-MB-231 and B02 cells were labeled with CD9 antibody (clone 72F6) and analyzed with a FacsCalibur (BD Biosciences). Negative controls (–) are shown in black, B02 in dark grey and MDA-MB-231 in light grey.*

Figure 2. *Western blot analyses. The expression of CD9 was assessed in different cell lines. A: CD9 expression in the highly aggressive MDA-MB-231 breast cancer cell line and its osteotropic derivative, the B02 subclone. B: CD9 expression in the androgen-independent prostate cancer cell line LNCaP and its osteotropic derivative, the C4-2B subclone. CD9 is overexpressed in each osteotropic subclone. The amount of proteins loaded was normalized using an antibody directed against alpha-tubulin.*

CD9 (3+ staining intensity), whereas tumor cells from the remaining primary tumors exhibited on average a 1+ level. Interestingly, positive staining at the surface of cancer cells was seen in all seven associated bone metastases examined (100%), with a staining intensity of 3+ for all cases. The levels of CD9 expression in the bone lesions (illustrated in Figure 3D and H) were found to be significantly higher than those observed in the primary tumors (illustrated in Figure 3C and G; χ^2 test, $p<0.05$; Wilcoxon signed-rank test, $p<0.05$).

We then wondered if CD9 was overexpressed only in bone metastases or if this protein was also overexpressed in other metastatic lesions: we hypothesized that if CD9 was actually associated with an osteotropic phenotype, its expression in other sites of metastasis should not be as high as in bone
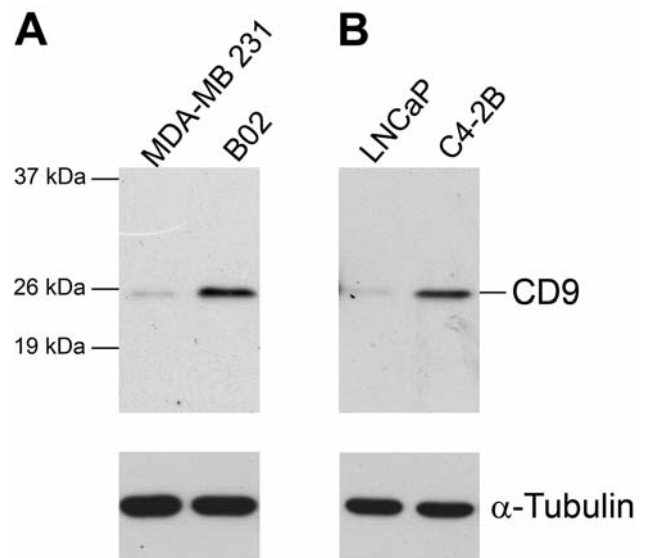
metastases. We therefore stained visceral metastases gathered from the same patients who died of disseminated disease. CD9 expression was evaluated in the following metastatic lesions: lung (Figure 3F and J), liver (Figure 3E), kidney (Figure 3I), thyroid, uterus, pancreas, adrenal glands and lymph nodes. Cancer cells disseminated to these sites also expressed detectable levels of CD9, again with the typical membrane staining pattern. However, with the use of two statistical tests (χ^2 and Wilcoxon signed rank tests), we were
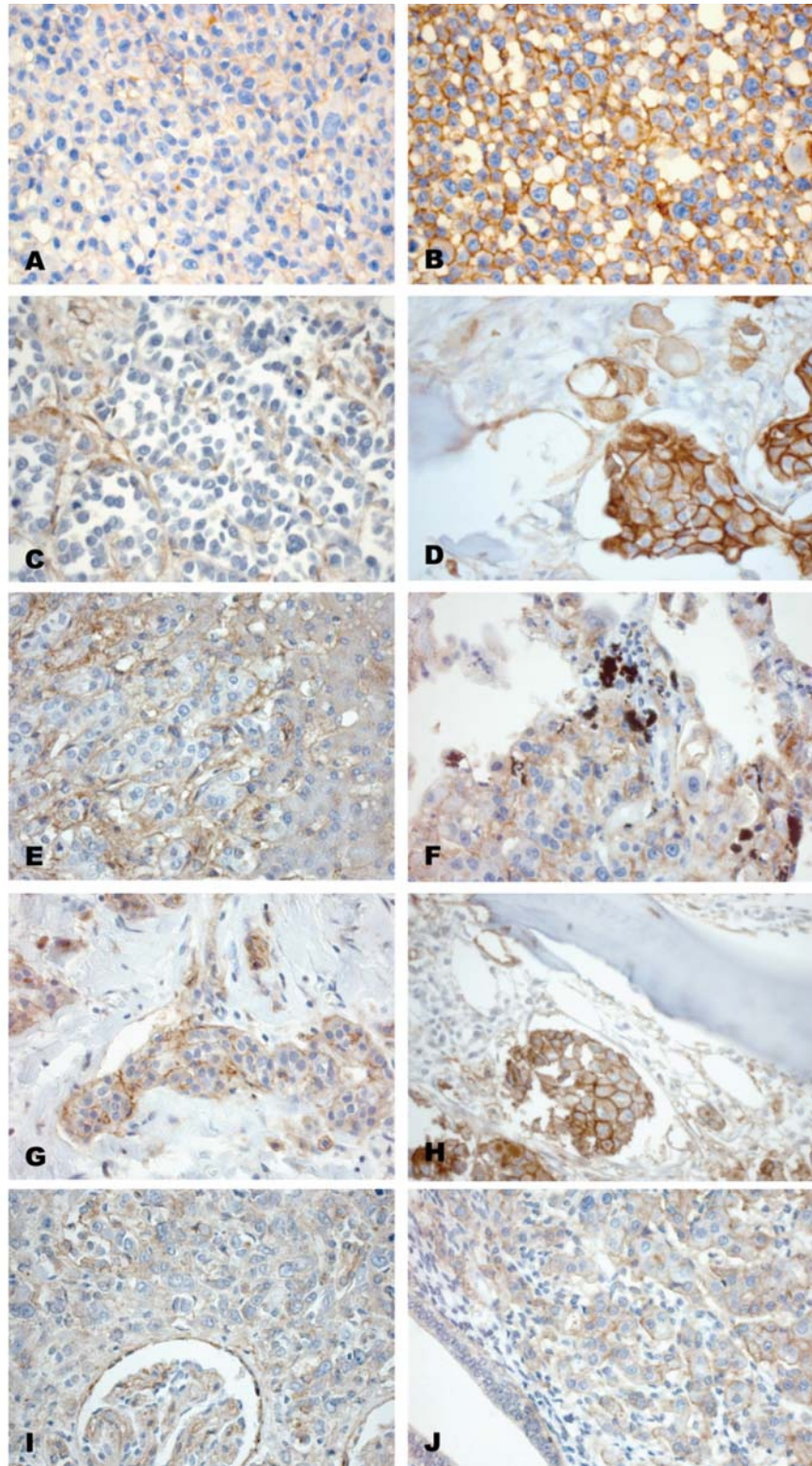
Figure 3. *Immunodetection of CD9. Immunohistochemical detection of CD9 in breast cancer cells, primary tumors, skeletal and visceral metastases, using the the MEM-61 antibody. Centrifuge preparations of MDA-MB-231 (A) and B02 cells (B), revealing a strong membrane staining of the B02 cells. Paraffin-embedded tissue sections of primary tumor (C), and its associated bone metastasis (D) and visceral metastases, hepatic (E) and lung (F). Primary tumor (G), and its associated bone metastasis (H) and visceral metastases, kidney (I) and lung (J).*
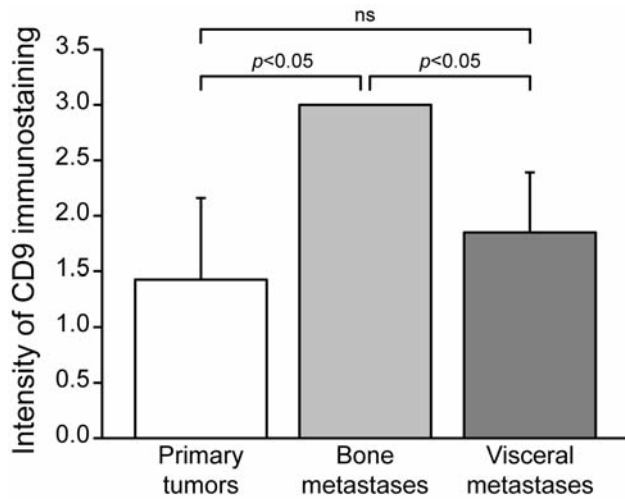
Figure 4. *Intensity of CD9 immunostaining in primary tumors, bone metastases and visceral metastases. Higher levels of CD9 expression were detected in bone metastases than in visceral metastases (Wilcoxon test, p=0.048) or primary tumors (Wilcoxon test, p=0.028); ns, not significant. Data are the mean±standard deviation (SD) of n=7.*

able to show that CD9 expression was significantly higher in bone metastases than in visceral metastases (χ^2 test, $p<0.05$; Wilcoxon signed-rank test, $p<0.01$; Figure 4).

Alb6 antibody to CD9 delays homing of B02 cells to bone marrow. We first determined the optimal concentration of the monoclonal antibody Alb6 for use in *in vivo* experiments. FACS analysis of CD9 expression at the cell surface shows that this optimal concentration is 10 μg/ml (Figure 5). B02 cells were therefore subsequently incubated with this antibody concentration for *in vivo* experiments.

B02 cells, pre-incubated with either monoclonal antibody to CD9 or a negative control from the same isotype, were injected intravenously into anesthetized mice, and animals and were followingly monitored for bioluminescent signals. Figure 6 shows a representative example, for two animals from each group, of the organs where tumor cells accumulated *in vivo* at 0.5 h, 4 h, 1 day and 7, 14, 21 and 35 days. Rapidly (30 min, 4 h), B02 cells were found in lungs, but bioluminescent signals disappeared at 24 h in both groups (with no differences between the control group and mice injected with B02 and anti-CD9). Seven days after the injection of tumor cells, bioluminescent spots appeared on the lower limbs, indicating the site of future bone lesions (although at this stage, radiographic imaging was still negative). Bioluminescent signals increased for all animals in a time-dependent manner, without major differences between groups, both in terms of photon number and incidence of bony metastases. Survival does not appear to be affected by any monoclonal antibody against CD9.

Induced bony metastases were radiographically detected 21 days after the injection of B02 tumor cells. These lesions evolved with time, as shown in Figure 7A. A monoclonal antibody against CD9 modestly but significantly inhibited the progression of bone destruction at 21 days (0.84±0.5 *vs.* 0.37±0.3 mm^2; *i.e.* 56% inhibition) and at 28 days (2.3±1 *vs.* 1.4±0.7 mm^2; *i.e.* 40% inhibition). Bone lesions from the two groups tended to reach similar volumes at 35 days (4.9±1.7 *vs.* 3.9±1.2 mm^2; *i.e.* 20% inhibition, Figure 7B).

Discussion

Tumor dissemination into preferred organs depends on adhesive interactions between tumor cells and organ-specific molecules in vascular beds (28, 29), and also on the interactions between cancer cells and the metastatic environment (30). Since membrane proteins are the primary effectors of these interactions, they are likely to play a fundamental role in the specific tropism of circulating cancer cells. Consequently, up- or down-regulation of membrane proteins involved in reciprocal interactions between circulating invasive cells and the potential metastatic site appears essential for cancer cells to settle. Moreover, membrane proteins represent more than two-thirds of the existing protein targets for drugs (31, 32). They are also preferred potential targets for antibody-based anticancer therapies, which recently emerged as an elegant approach to circumvent the non-specificity of currently delivered anticancer treatments. For these reasons, we previously analyzed the membrane proteome of the bone-seeking B02 cell line with a high-throughput proteomic approach (4). Some membrane proteins were found to be de-regulated, and among the proteins that were up-regulated in the B02 clones, was the CD9 protein. In the present study, we confirmed this overexpression with different techniques. It is noteworthy that CD9 was not found to be either up or down-regulated in our previous study aimed at the comparison of MDA-MB-231 and B02 cell lines at the transcriptomic level (33), despite the presence of CD9 probes in the Affymetrix® HG-U133 GeneChip Array. Thus, it would be interesting to determine whether the increased CD9 protein content is due to increased protein stability or reduced degradation, or due to a loss of RNA *via* degradation involving microRNA. In another study, *CD9* transcripts were found to be two-fold down-regulated in a tumoral *versus* normal pancreatic cell line based on DNA microarray data (34). By using the stable isotope labeling by amino acids in cell culture (SILAC) proteomic approach, these authors demonstrated that the CD9 antigen was differentially overexpressed in the pancreatic cancer secretome, and was also overexpressed at the protein level in pancreatic cancer tissues. These data reinforce the importance of assessing both the transcriptome and the proteome of human cancer cells and tissues.
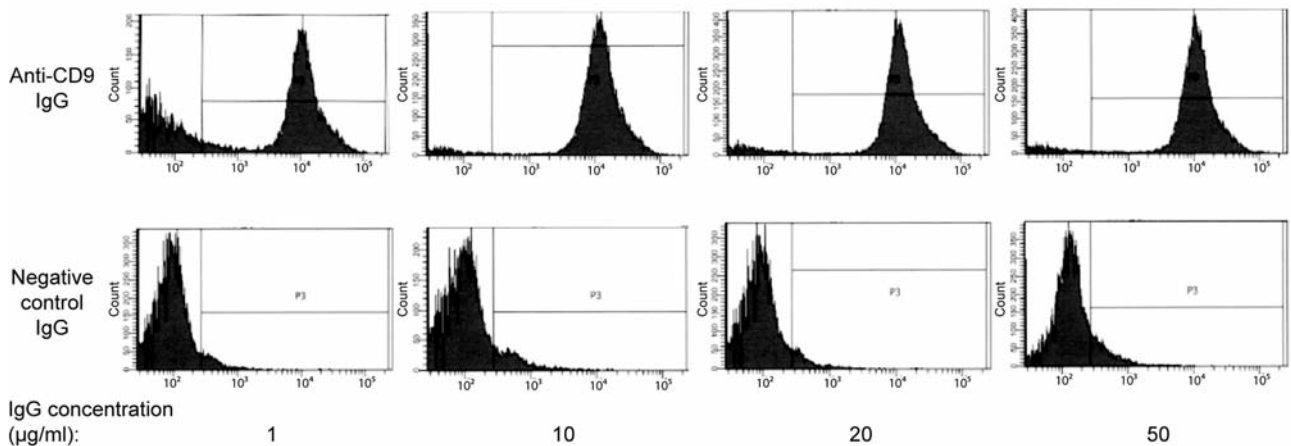
Figure 5. *FACS analysis of B02 cells with an antibody against CD9 or with negative control IgG, used at 1, 10, 20 and 50 μg/ml. FACS analysis of CD9 expression at the B02 cell-surface shows that the optimal concentration is 10 μg/ml. For all in vivo experiments, B02 cells were incubated with this concentration.*
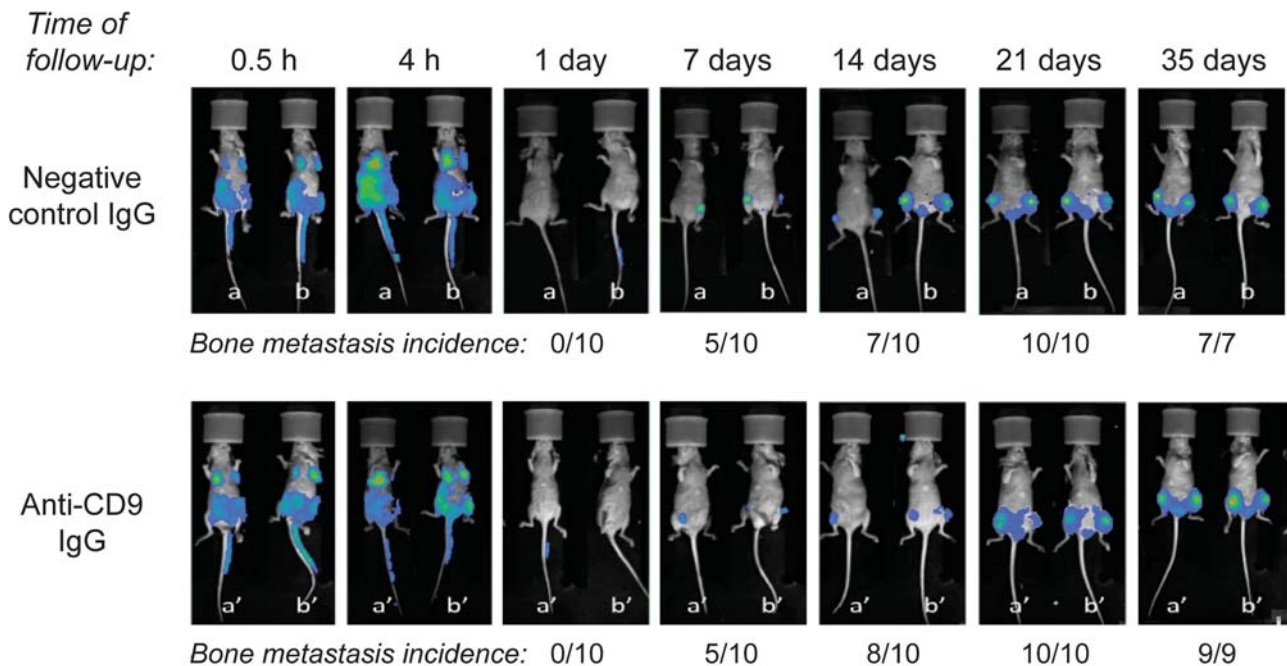

Figure 6. *Luminescent imaging of whole mice after injection of B02 cells into their tail arteries. Luciferase-expressing B02 cells are followed at 30 min, 4 h, 1 day, 7, 14, 21 and 35 days. Bioluminescent images were acquired and the relative light intensity was visualized by pseudocolors. Two representative mice are shown for each time lapse and for each condition (control antibody in the upper panel, CD9 antibody in the lower panel). Bone metastasis incidence is indicated under each condition.*

CD9 is a member of the tetraspanin family, and has been regarded as an important metastasis-inhibitory factor of various types of human cancer. However, CD9 protein expression levels did not provide useful prognostic information regarding human breast cancer (35), or osteosarcoma (36). Moreover, several studies suggest that CD9 could be implicated in tumor–endothelial cell interactions during transendothelial invasion (37, 38), and recent findings indicate that CD9 regulates migration, adhesion and homing of human CD34 hematopoietic stem/progenitor cells in bone marrow (39). Of note, in highly osteotropic multiple myeloma cells, CD9 is i) up-regulated
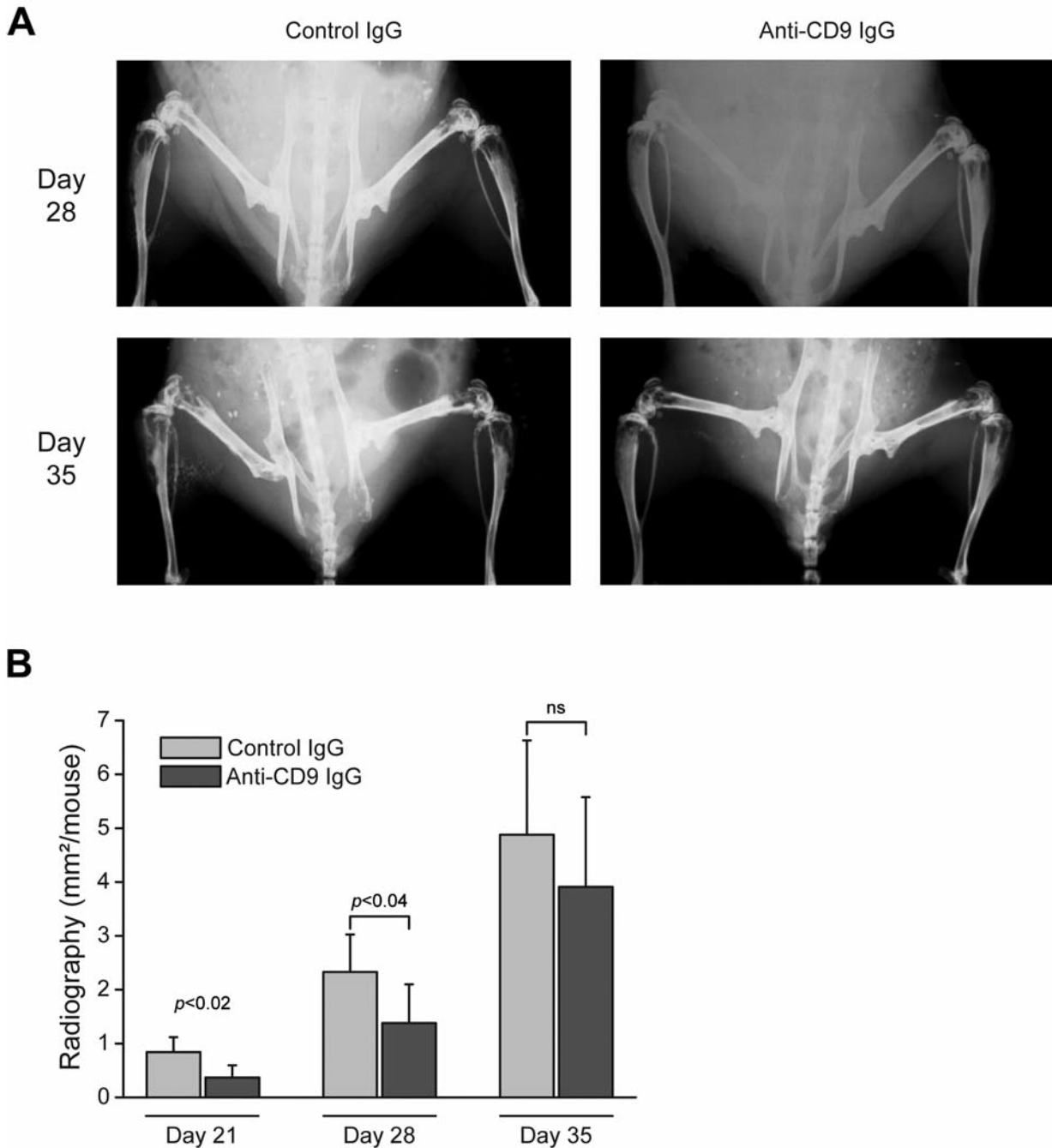
A

B

Figure 7. *Effect of monoclonal antibody against CD9, on osteolytic bone metastasis formation of B02 cells. A: Representative radiographs of hind limbs from metastatic mice bearing B02 cells, 28 and 35 days after injection of B02 breast cancer cells either incubated with a control IgG (left panels) or with anti-CD9 IgG (right panels). B: Histograms showing the mean area±standard deviation (SD) of osteolytic lesions (expressed in mm²) at days 21, 28 and 35; n=10; ns, not significant.*

in vivo following close interaction of the cells with bone marrow endothelial cells, and ii) involved in transendothelial invasion, thus possibly mediating homing and/or spreading of the multiple myeloma cells in bone (40). Thus, the role of CD9 in cancer progression is complex, and in the light of

these results, CD9 overexpression in osteotropic cells (such as B02 and C4-2B cells) is likely to confer a better ability for establishment in the bone microenvironment. We therefore wanted to confirm this hypothesis by injecting B02 cells with or without anti-CD9 blocking antibody. Our results

show that the monoclonal antibody against CD9 modestly, but significantly, inhibited the progression of bone destruction at 21 days (56% inhibition) and 28 days (40% inhibition). At 35 days, the inhibition remained apparent, but only at 20% and the difference was no longer statistically significant. These results could therefore prove that antibody to CD9 slows down homing of B02 cells to bone marrow, most likely delaying subsequent bone destruction resulting from osteoclast activation by tumor cells.

This hypothesis is in agreement with our present results, which clearly demonstrate a significant overexpression of CD9 in the membranes of cancer cells settled in bone metastases, this expression being significantly higher in bony metastases than in visceral ones, or even in primary tumors. Our results are in line with those found in gastric cancer (41), but are opposite to those from many other cancer types. Whether CD9 supports or inhibits invasion essentially depends on the prevailing conditions, and the *in vivo* experiments in literature strongly support the supposition that CD9 may only hamper selective steps of the metastatic cascade (42). Our demonstration that the osteotropic C4-2B clone of the human prostate cancer cell line LNCaP overexpresses CD9 compared to the parent cell line similarly to the osteotropic B02/MDA-MB-231 parental cell line pair strongly suggest that this tetraspanin is involved in the process of development of bone metastases.

To summarize, we demonstrated the overexpression of CD9 in two different osteotropic cancer cell line subclones, and also an overexpression of this protein in cancer cell membranes from bone metastases when compared to visceral ones or their matched primary tumors. The up-regulation of CD9 might be important for the establishment of tumor cells in bone, as was demonstrated for $\alpha V \beta 3$ integrins, which confer breast tumor cells with a greater propensity to metastasize to bone (23) [and which can even promote spontaneous metastasis to bone (43, 44)]. Further studies should help elucidate the exact role of CD9 in bone tropism, and establish whether CD9 could represent a valuable protein for targeting circulating bone-seeking cells originating from osteotropic cancer.

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