American Journal of Pathology, Vol. 169, No. 1, July 2006

DOI: 10.2353/ajpath.2006.051152

Tumorigenesis and Neoplastic Progression

Role of the Integrin-Binding Protein Osteopontin in Lymphatic Metastasis of Breast Cancer

Alison L. Allan,*† Rosamma George,‡§ Sharon A. Vantyghem,*†‡ Mark W. Lee,‡§ Nicole C. Hodgson,§ C. Jay Engel,*†§ Ron L. Holliday,§ David P. Girvan,§ Leslie A. Scott,§ Carl O. Postenka,* Waleed Al-Katib,* Larry W. Stitt,*§ Toshimitsu Uede,** Ann F. Chambers,*†‡ and Alan B. Tuck*†‡§

From the London Regional Cancer Program, Departments of Oncology,† Pathology,‡ and Surgery, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada; the London Health Sciences Centre,§ London, Ontario, Canada; and the Division of Molecular Immunology,** Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan*

Although a primary route of breast cancer metastasis is believed to be via lymphatics, the molecular factors involved are poorly understood. We hypothesized that one such factor may be the integrin-binding protein osteopontin (OPN), and we investigated this clinically and experimentally. In breast cancer patients undergoing sentinel lymph node biopsy, OPN levels were significantly higher in lymph node metastases than in the primary tumor ($P < 0.001$). To test the functional contribution of OPN to lymphatic metastasis and to determine whether the RGD (Arg-Gly-Asp) integrin-binding sequence of OPN is important for this process, we transfected wild-type OPN or mutant OPN (lacking the RGD sequence) into MDA-MB-468 human breast cancer cells. *In vitro*, cells overexpressing OPN demonstrated increased anchorage-independent growth in soft agar ($P = 0.001$) and increased RGD-dependent adhesion ($P = 0.045$). Following mammary fat pad injection of nude mice, cells overexpressing OPN showed increased lymphovascular invasion, lymph node metastases, and lung micrometastases at earlier time points ($P = 0.024$). Loss of the RGD region partially abrogated this effect in the lymphatics ($P = 0.038$). These novel findings indicate that OPN is a key molecular player involved in lymphatic metastasis of breast cancer, potentially by affecting RGD-mediated adhesive interactions and by enhancing the establishment/persistence of tumor cells in the lymphatics. *(Am J Pathol 2006, 169:233–246; DOI: 10.2353/ajpath.2006.051152)*

Breast cancer is a leading cause of morbidity and mortality in women,[1] mainly due to the propensity of primary breast tumors to metastasize to regional and distant sites such as lymph node, lung, liver, brain, and bone.[2,3] Cancer cells may leave the primary tumor by one of two routes: either through hematogenous dissemination via blood circulation or by dissemination via the lymphatic system. Over the past decade or so, numerous studies have demonstrated that hematogenous metastasis is a complex process involving the escape of cancer cells from the primary tumor into the bloodstream (intravasation), survival in the circulation, arrest and extravasation into the secondary site, and initiation and maintenance of growth to form clinically detectable metastases.[2–7] However, despite advances in our understanding of hematogenous metastasis, the process and mechanisms of lymphatic metastasis remain relatively understudied and poorly understood.

The lymphatic system is largely considered to be the primary route for early dissemination of breast cancer, and the histopathological identification of tumor deposits in the axillary lymph node is one of the most powerful prognostic indicators for this disease.[8–10] Until recently, the standard treatment for patients with operable breast cancer included the dissection of multiple axillary lymph nodes. However, in many cases, the removed lymph nodes are found to be free of metastases, indicating that some patients may have been subjected unnecessarily to the risks and morbidity associated with this procedure. A

Supported by the Canadian Breast Cancer Research Alliance (grant 15323 to A.F.C. and A.B.T.) and by the Ontario Cancer Research Network (grant 04-MAY-00089 to A.F.C. and A.B.T.). A.L.A. is the recipient of an H.L. Holmes Award from the National Research Council of Canada. S.A.V. is supported by a Rethink Breast Cancer Career Development Award. A.F.C. is a Canada Research Chair in Oncology.

Accepted for publication March 23, 2006.

Address reprint requests to Alison L. Allan, Ph.D., Department of Oncology, Schulich School of Medicine and Dentistry, University of Western Ontario, London Regional Cancer Program, 790 Commissioners Road East, London, Ontario N6A 4L6, Canada. E-mail: alison.allan@lhsc.on.ca.

less invasive method for the assessment of lymph node status involves biopsy of the sentinel lymph node, a practice that is increasingly being used in the surgical setting.[11–13] As the node that primarily drains the tumor and is most likely to harbor metastatic disease, the sentinel node is highly predictive of the histopathology of the remaining lymphatic basin and has been shown to accurately predict axillary lymph node status in up to 98% of cases.[11,12,14–16] Tumor deposits (≥2 mm) present in lymph nodes are a sign that the tumor has spread beyond the confines of the breast, and this knowledge has important (but not fully understood) clinical implications in making treatment decisions.[17,18] Furthermore, the molecular mechanisms by which lymph node metastasis occurs require further elucidation. The identification of factors that functionally contribute to this process therefore has the potential to both provide a greater understanding of the biology of lymphatic metastasis in breast cancer, as well as to contribute to improved clinical management of the disease.

We hypothesize that one such factor may be the integrin-binding protein osteopontin (OPN). Clinical studies have demonstrated that OPN is overexpressed by many human cancers.[19–31] In particular, we and others have found that OPN levels are elevated in the blood[20,22,23] and primary tumors[19,23,24,27,32] of patients with breast cancer, and in some cases this has been correlated with poor prognosis.[22–24,32] The OPN protein backbone contains several highly conserved structural elements, including heparin- and calcium-binding domains, a thrombin-cleavage site, and an RGD (Arg-Gly-Asp) integrin-binding domain.[33,34] It is therefore not surprising that OPN can interact with a diverse range of factors, including cell-surface receptors (integrins and CD44), secreted proteases (matrix metalloproteinases and urokinase-type plasminogen activator), and growth factor/receptor pathways (transforming growth factor α/epidermal growth factor receptor, hepatocyte growth factor/Met, and vascular endothelial growth factor (VEGF)). These complex signaling interactions can result in changes in gene expression, which ultimately lead to alterations in cell properties involved in malignancy such as adhesion, migration, invasion, survival, angiogenesis, and metastasis.[35–45] However, a direct functional link between OPN and lymphatic metastasis has not previously been demonstrated.

Recently, we have described a novel human breast cancer cell line (468LN) that preferentially metastasizes to the lymphatics in a rapid and aggressive manner. Interestingly, preliminary molecular characterization indicated that OPN was one of the most highly up-regulated genes in the 468LN cell line relative to the weakly metastatic MDA-MB-468 parental cell line, suggesting that OPN may play a role in lymphatic metastasis.[46] We have also previously shown that OPN-induced changes in breast cancer cell behavior *in vitro* can occur by integrin-dependent mechanisms and that these OPN/integrin interactions occur via a conserved RGD sequence in the OPN protein backbone.[35,38,41,47] We were therefore interested in determining whether OPN could functionally contribute to lymphatic metastasis and also whether the RGD integrin-binding region was important for this process. In the current study, we investigated the significance of OPN in lymphatic metastasis of breast cancer using both clinical and experimental approaches.

Materials and Methods

Patients

Matched samples of primary tumor and lymph node tissue were obtained from 106 female patients undergoing sentinel lymph node biopsy for breast cancer at the London Health Sciences Centre, under a protocol approved by the institutional tissue committee. Patient demographics are summarized in Table 1.

Histopathology and Immunohistochemistry

Formalin-fixed, paraffin-embedded tumor and lymph node samples were subjected to standard staining with hematoxylin and eosin (H&E) and staining with an immunoperoxidase technique for OPN essentially as described previously.[24,31] Immunostaining was performed using a streptavidin-biotin complex method (DakoCytomation Inc., Mississauga, ON, Canada). The primary antibody used was the monoclonal antibody mAb53, which was generated in our laboratory and has previously been shown to efficiently and specifically detect human OPN protein via an epitope in the vicinity of the RGD region of the protein.[24,31,48,49] The chromogen used was aminoethylcarbazol, and slides were counterstained with Meyer's hematoxylin. Stained slides were evaluated by light microscopy in a blinded fashion by two independent pathologists. The overall amount of staining for OPN was determined using a semiquantitative system based on staining intensity (I) and proportion/extent (E) of stained tumor cells as previously described,[23,24,50] where overall amount of staining = I + E = 0 to 8 (0 for negative staining and 2 to 8 for positive staining ranges).

Plasmids and Site-Directed Mutagenesis

The wild-type OPN expression vector was previously generated by cloning the full-length human OPN cDNA (from plasmid OP-10)[51] into the expression plasmid pcDNA3 (Invitrogen, Carlsbad, CA) as described elsewhere.[36] The control plasmid consisted of the unmodified pcDNA3 plasmid. A mutant OPN expression vector in which the RGD integrin-binding sequence was deleted was generated by site-directed mutagenesis of the wild-type OPN/pcDNA3 expression plasmid using the Gene-Editor Mutagenesis System (Promega, Madison, WI) as per the manufacturer's instructions, with a 50-mer mutagenic oligonucleotide specific for introducing the RGD deletion (ΔRGD) (5′-TTGACCTCAGTCCATAAACCA-CACTGCCATCATATGTGTCTACTGTGGGG-3′). Following the site-directed mutagenesis procedure, plasmid

Table 1. Descriptive Characteristics of the Patient Population

Characteristic	*N*	Range	Median	Standard deviation
Age at surgery (years)	106	29 to 82	57	11.98
Tumor size (cm)	106	0.2 to 8.5	1.7	1.38
Characteristic		*N*		%
Tumor type				
No Special Type invasive		98		92.45
Lobular (classic)		2		1.89
Lobular (pleomorphic)		1		0.94
Tubular (pure)		2		1.89
Metaplastic		2		1.89
Mucinous (pure)		1		0.94
Scarff-Bloom-Richardson tumor grade				
1 or I		35		33.33
2 or II		43		40.95
3 or III		27		25.71
Unknown		1		
Lymphovascular invasion				
Negative		73		70.87
Positive		30		29.13
Unknown		3		
Estrogen/progesterone receptor				
Negative		18/17		17.82/16.83
Positive		83/84		82.18/83.17
Unknown		5/5		
SLN status				
Negative		67		63.21
Positive		39		36.79
ALN status				
Negative		74		83.15
Positive		15		16.85
None removed		17		
No. of positive SLN				
0		67		63.21
1		34		32.08
2		4		3.77
3		1		0.94
No. of positive ALN				
0		74		83.15
1		7		7.87
2		5		5.62
4		2		2.25
6		1		1.12
None removed		17		
Total positive nodes (SLN + ALN)				
0		64		60.38
1		25		23.58
2		10		9.43
3		3		2.83
4 to 7		4		3.77

DNA was isolated and screened by sequence analysis to confirm that the RGD region had been deleted.

Cell Culture and Transfections

The tumorigenic, weakly metastatic MDA-MB-468 human breast cancer cell line (a kind gift from Dr. Janet Price, University of Texas, M.D. Anderson Cancer Center, Houston, TX)[52,53] was maintained in α-minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO). Stable transfections were performed using the Lipofectamine 2000 reagent (Invitrogen) as per the manufacturer's guidelines. For selection and maintenance of stable transfectants, active Geneticin (G418-sulfate; Invitrogen) was added to the medium at 500 μg/ml (a concentration previously determined to give 100% death of parental MDA-MB-468 cells). Colonies were allowed to develop, were then isolated, grown as clonal cell populations, and screened for transgene expression by Northern blot analysis. To control for clonal heterogeneity, pooled populations were created by combining several positive clonal cell populations. The specific cell populations generated were as follows: one control vector-transfected MDA-MB-468 pooled population consisting of six clones (468-CON), one wild-type OPN-transfected MDA-MB-468 pooled population consisting of six clones (468-OPN), and one mutant OPN (ΔRGD)-transfected pooled population consisting of six clones (468-ΔRGD).

Northern Blot Analysis

RNA was isolated from cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. RNA (10 μg per sample) was run on a 1.1% agarose gel with 6.8% formaldehyde and capillary-transferred to a nylon GeneScreen Plus membrane (PerkinElmer Life Sciences, Boston, MA). Blots were probed with denatured oligolabeled ^{32}P-dCTP cDNA probes using an oligolabeling kit (Amersham Biosciences, Baie d'Urfé, QC, Canada) according to the manufacturer's recommendations. The OPN probe used was a full-length human OPN cDNA (1493 bp) generated using *Eco*RI digestion of the OP-10 plasmid.[51] To confirm even loading of lanes, human 18S rRNA from the p100D9 plasmid (a kind gift from Dr. D.T. Denhardt, Rutgers University, Piscataway, NY) was also used.

Western Blot Analysis

Conditioned media were prepared by plating 5×10^5 cells/100-mm plate in regular growth media and incubating for 18 hours at 37°C, 5% CO_2. Media was replaced with 3 ml of serum-free OPTI-minimal essential medium media (Invitrogen), and plates were incubated for a further 24 hours at 37°C, 5% CO_2. Conditioned media from each plate were collected, centrifuged to remove cell debris, and concentrated 10-fold by ultracentrifugation in Centricon-30 miniconcentrators (Amicon Inc., Beverly, MA). Each corresponding plate was trypsinized, and a cell count was performed to allow appropriate correction for loading in cell equivalents. For each cell line, a normalized volume of conditioned media equivalent to 1×10^5 cells was subjected to standard protein electrophoresis in 12% sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA). After transfer, gels were stained with Coomassie Blue to confirm equal loading and transfer efficiency. The primary antibody used was the 5A1 mouse monoclonal antibody against human OPN. This antibody has been shown to specifically and efficiently detect human OPN via an epitope near the N terminus end of the protein,[49] hence detecting both wild-type and mutant forms of the OPN protein. The secondary antibody used was an anti-mouse horseradish peroxidase conjugate (Sigma-Aldrich), and the OPN protein was visualized using an enhanced chemiluminescence system (Roche Applied Sciences, Laval, QC, Canada).

Flow Cytometry

Primary antibodies used for flow cytometry were unconjugated mouse anti-human monoclonal antibodies directed against integrin β_1 (clone P5D2), integrin $\alpha_v\beta_5$ (cloneP1F6), integrin β_3 (clone PM6/13), and integrin $\alpha_9\beta_1$ (clone Y9A2) (Chemicon International Inc., Temecula, CA). The negative IgG isotype control was a mouse primary IgG_1 antibody (Cedarlane Laboratories Ltd., Hornby, ON, Canada). In all cases, the secondary antibody used was goat anti-mouse IgG (IgG) conjugated to R-phycoerythrin (Chemicon International). Cells were incubated with primary antibodies (1 μg/10^6 cells) for 1 hour at 4°C on a rotating plate, washed twice with phosphate-buffered saline (PBS) plus 2% fetal bovine serum, and incubated with the secondary antibody (0.5 μg/10^6 cells) for 1 hour at 4°C on a rotating plate in the dark. Samples were analyzed using a Beckman-Coulter EPICS XL-MCL flow cytometer.

Cell Growth Assays

To assess and compare the growth patterns of the different cell lines under standard (ie, anchorage-dependent) culture conditions, cells were plated at a density of 5.0×10^4 cells/60-mm plate ($n = 3$ for each time point) and maintained in regular growth media. Every 48 hours for 7 days, triplicate cultures were trypsinized, stained with trypan blue, and counted for viable cells using a hemocytometer. The doubling time of each cell population was estimated during the exponential growth phase according to the equation $T_d = 0.693t/\ln(N_t/N_0)$, where t is time (in hours), N_t is the cell number at time t, and N_0 is the cell number at initial time.

The ability of the different cell lines to survive and grow under anchorage-independent culture conditions was tested and compared using a soft agar assay. In preparation for the assay, 60-mm dishes were coated with 1% agarose (Bioshop, Burlington, ON, Canada) in normal growth media and allowed to set for 1 hour at room temperature. Cell solutions (1.0×10^4 cells/60-mm plate) were prepared using 0.4% agarose in normal growth media and plated on top of the 1% agarose base layer ($n = 4$ for each time point). Normal growth media (2 ml) was added on top of the cell layer, and the media was changed every 3–4 days for 4 weeks. At the end of the assay, media was removed from the plates, and cells were fixed in 10% neutral-buffered formalin (EM Sciences, Gladstone, NJ) for 12 hours. Five fields of view were counted for each dish, and the mean number of colonies per field and the mean colony diameter were calculated and compared.

Cell Adhesion Assays

Cells were plated onto sterile 96-well nontissue culture plates (Titertek; Flow Laboratories Inc., McLean, VA) treated with either 5 μg/ml human vitronectin (Sigma-Aldrich) or PBS (negative control), using 1×10^4 cells/well in triplicate wells for each treatment. Vitronectin was chosen because it is a ligand for $\alpha_v\beta_1$ and $\alpha_v\beta_5$, two integrins that are known to interact with OPN (via the RGD region) and are expressed by MDA-MB-468 cells. For antibody blocking experiments, cells were preincubated on a rotating plate with saturating concentrations (as determined by preliminary titration experiments) of mouse monoclonal antibodies against human integrin β_1 (clone P5D2), integrin $\alpha_v\beta_5$ (cloneP1F6; Chemicon), or a negative control IgG_1 isotype primary mouse antibody (Cedarlane) for 15 minutes at 37°C before plating. Plates

were incubated for 5 hours at 37°C, the medium was then removed, and nonadhered cells were rinsed away with three gentle washes of PBS. Adhered cells were fixed using fresh 2% glutaraldehyde for 20 minutes, then washed with PBS and stained using Harris' hematoxylin for 10 minutes. The staining was intensified using a 1% solution of ammonia water, and the blue-staining cells were counted using a light microscope. Five fields of view were counted for each well, and the mean number of cells per field was calculated and compared.

In Vivo *Tumorigenicity and Metastasis Assays*

Cells were prepared in sterile PBS at a concentration of 1×10^6 cells/100 μl. Pooled populations of MDA-MB-468 cells stably transfected with the control vector (468-CON), wild-type OPN (468-OPN), or mutant OPN (468-ΔRGD) were injected into the second thoracic mammary fat pad of 7- to 8-week-old female athymic nude (*nu*/*nu*) mice as described elsewhere,[41,46,52] using 1×10^6 cells/mouse and 20 to 26 mice/treatment group. Primary tumor growth was evaluated biweekly by measurement with calipers in two perpendicular dimensions, and the tumor volume was estimated using the formula, [volume = $0.52 \times (\text{width})^2 \times (\text{length})$], for approximating the volume (mm^3) of an ellipsoid.

Because parental MDA-MB-468 cells have been shown to be only weakly metastatic,[52] we considered the possibility that spontaneous metastases would not have adequate time to develop before the primary tumor grew to such a size that required sacrifice of the animals for humane purposes. Therefore, mice were either subjected to surgery at ~12 weeks after injection to remove the primary tumor (mean tumor volume = ~1500 mm^3) or sacrificed at 12 weeks and assessed for metastatic burden. In the surgery group, spontaneous metastases were given time to develop for a further 12 weeks, and mice were sacrificed 24 weeks after injection and assessed for metastatic burden. Tumor resection surgeries were performed as described previously.[54] All surgical procedures were done under general anesthesia using a ketamine/xylazine mixture administered by intraperitoneal injection.

At end-point, animals were euthanized and necropsies performed. Tissues and organs (including lymph nodes, lung, liver, spleen, pancreas, kidneys, ovaries, brain, and any other tissues of abnormal appearance) were examined superficially for evidence of gross macroscopic metastases before processing for histology. Primary tumors and mouse tissues collected at necropsy were fixed in 10% neutral-buffered formalin before processing. Tissues were embedded in paraffin wax, sectioned (4 μm thick), and subjected to standard H&E staining. Stained slides were evaluated by light microscopy in a blinded fashion by two independent pathologists to observe histopathological characteristics and to determine micrometastatic involvement.

Animal care and surgical procedures were conducted in accordance with the recommendations of the Canadian Council on Animal Care, under a protocol approved by the University of Western Ontario Council on Animal Care.

Statistical Analysis

For clinical studies, logistic regression methods were used to determine the effect of various independent factors on (sentinel or axillary) node positivity. The Wilcoxon two-sample test and Spearman rank correlation were used to determine the effect of various independent factors on the number of (sentinel, axillary, or total) nodes. The association between OPN in the primary tumor and OPN in the (sentinel or axillary) lymph node was evaluated using the Wilcoxon signed rank test, and the association between OPN in the primary tumor and (sentinel or axillary) lymph node positivity (involvement by metastatic carcinoma) was examined using a two-sided Wilcoxon two-sample test.

For experimental studies, statistical analyses were performed using Sigma-Aldrich Stat analysis software (Access Softek Inc., San Rafael, CA). For *in vitro* experiments, statistical differences in means between groups were determined by a Student's *t*-test. For *in vivo* animal studies, a two-sided *z*-test was used to compare proportions. Differences between means were determined using the Student's *t*-test when groups passed both a normality test and an equal variance test. When this was not the case, the Mann-Whitney rank-sum test was used. In all cases, P values of ≤ 0.05 were regarded as being statistically significant.

Results

Factors Associated with Sentinel Lymph Node and Axillary Lymph Node Status in Patients Undergoing Sentinel Lymph Node Biopsy for Breast Cancer

Histopathological analysis was performed in a group of 106 patients undergoing sentinel lymph node (SLN) biopsy for breast cancer. Of the 106 patients, 17 underwent SLN biopsy only, and the other 89 patients had both SLN biopsy and concomitant or subsequent axillary lymph node (ALN) dissection. The demographics of this patient population are presented in Table 1. Primary tumor size and lymphovascular invasion were significantly associated with both SLN and ALN positivity (SLN: $P = 0.007$, ALN: $P = 0.032$), whereas age, Scarff-Bloom-Richardson tumor grade, and estrogen or progesterone receptor status were not. Similarly, primary tumor size and lymphovascular invasion were significantly associated with total number (SLN plus ALN) of lymph nodes involved ($P < 0.001$), whereas age, tumor grade, and hormone (estrogen or progesterone) receptor status were not. The number of positive SLNs was significantly associated with both ALN positivity ($P < 0.001$) and the number of ALNs involved ($P < 0.001$).

OPN Is Overexpressed in Lymph Node Metastases of Breast Cancer Patients

Immunohistochemical analysis of OPN expression was performed on the primary tumors of 64 lymph node-negative

Table 2. OPN Expression Scores in Primary Tumors and Lymph Nodes of Breast Cancer Patients

	Primary tumors		
	Lymph node-negative cases	Lymph node-positive cases	Lymph node metastases
No. of cases analyzed	64	42	34
Mean OPN score*	3.26	3.60	5.09†
Standard deviation	2.41	2.30	1.56

Tumor and lymph node samples were subjected to immunohistochemistry for OPN as described in the Materials and Methods.
*OPN scores were determined using a semiquantitative system based on staining intensity (I) and proportion/extent (E) of stained tumor cells as described elsewhere.[23,24,50] Overall amount of staining = I + E = 0 to 8 (0 for negative staining and 2 to 8 for positive staining ranges).
†Mean OPN score was significantly higher in the lymph node metastases of lymph node-positive patients than in the primary tumor ($P < 0.001$).

cases and 42 lymph node-positive cases and on the lymph node metastases of 34 cases. OPN staining was scored using the semiquantitative system[23,24,50] described in Materials and Methods. Mean OPN levels were significantly higher in lymph node metastases than in the primary tumor ($P < 0.001$) (Table 2 and Figure 1, C and D). Furthermore, there was a trend toward increased OPN levels in the primary tumors of patients with lymph node-positive versus lymph node-negative disease (Table 2), although this difference was not significant within the power of the present study ($P = 0.694$). Staining for OPN expression in normal breast tissue (negative control) was focal and faint (Figure 1A). Dystrophic calcifications in the primary tumor and follicles/germinal centers and perifollicular areas in the lymph node (where present) showed strong OPN staining (Figure 1, B and D, respectively) and served as an internal positive control. OPN staining localized to calcifications or normal surrounding cells was excluded from quantification, and

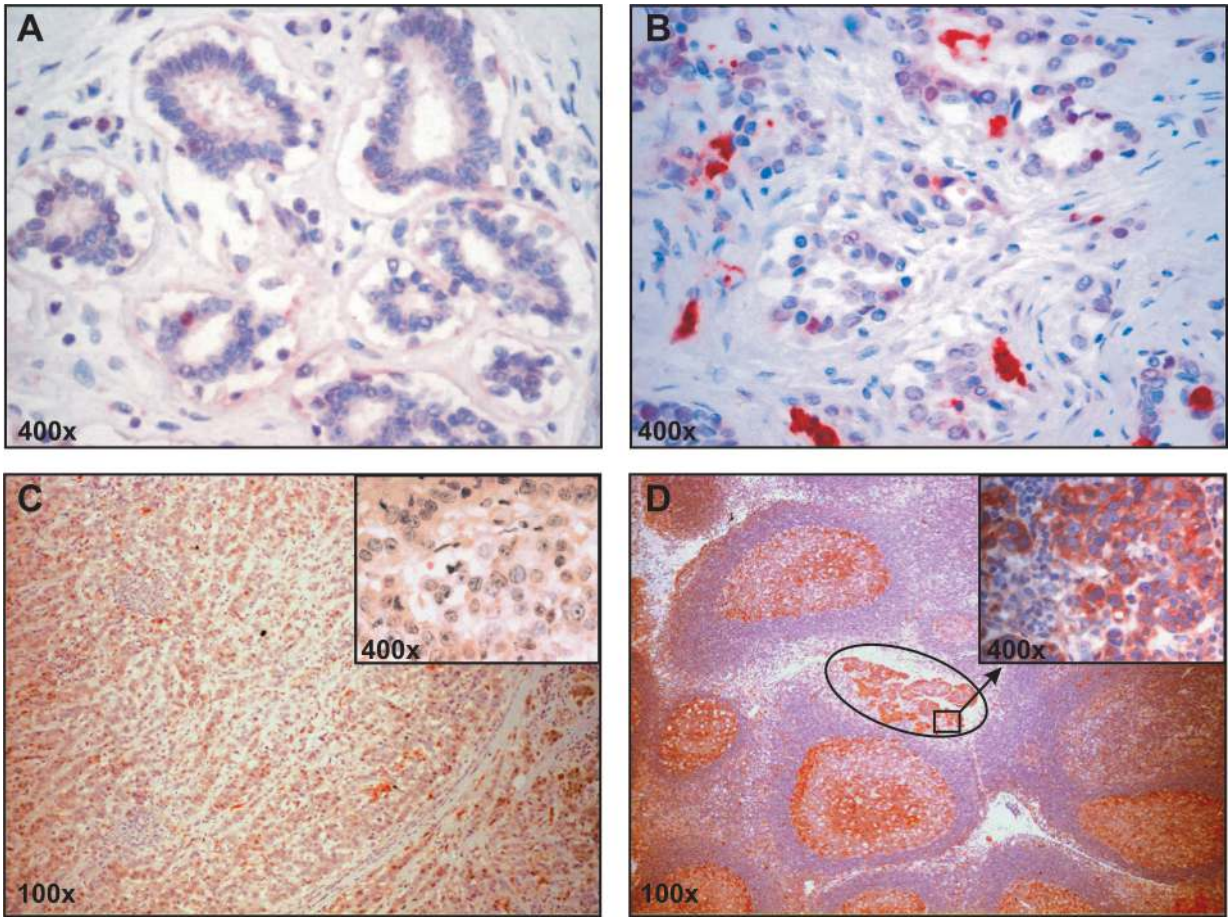

Figure 1. Immunohistochemical analysis of OPN expression in primary tumors and lymph node metastases of breast cancer patients undergoing sentinel lymph node biopsy. Formalin-fixed, paraffin-embedded tumor and lymph node samples were subjected to an immunoperoxidase technique as described in the Materials and Methods. The primary antibody used was the monoclonal antibody mAb53. Immunostained slides were evaluated by light microscopy in a blinded fashion by two independent pathologists using a semiquantitative scoring system based on staining intensity (I) and proportion/extent (E) of stained tumor cells as described elsewhere.[23,24,50] The overall amount of staining = I + E = 0 to 8 (0 for negative staining and 2 to 8 for positive staining ranges). **A:** OPN expression in normal breast tissue. **B:** OPN expression in breast tissue with dystrophic calcification. **C** and **D:** OPN expression in representative primary tumor (OPN score = 6) (**C**) and matched lymph node metastases (OPN score = 8) (**D**) from an individual lymph node-positive patient. In **D**, OPN expression can be observed both in tumor cells (outlined representative region in main micrograph and **inset**) and in the follicles/germinal centers and perifollicular areas of the surrounding lymph node. Only OPN staining localized to tumor cells was quantified. Magnification: ×400 (**A** and **B** and **insets** in **C** and **D**); ×100 (main micrographs **C** and **D**).

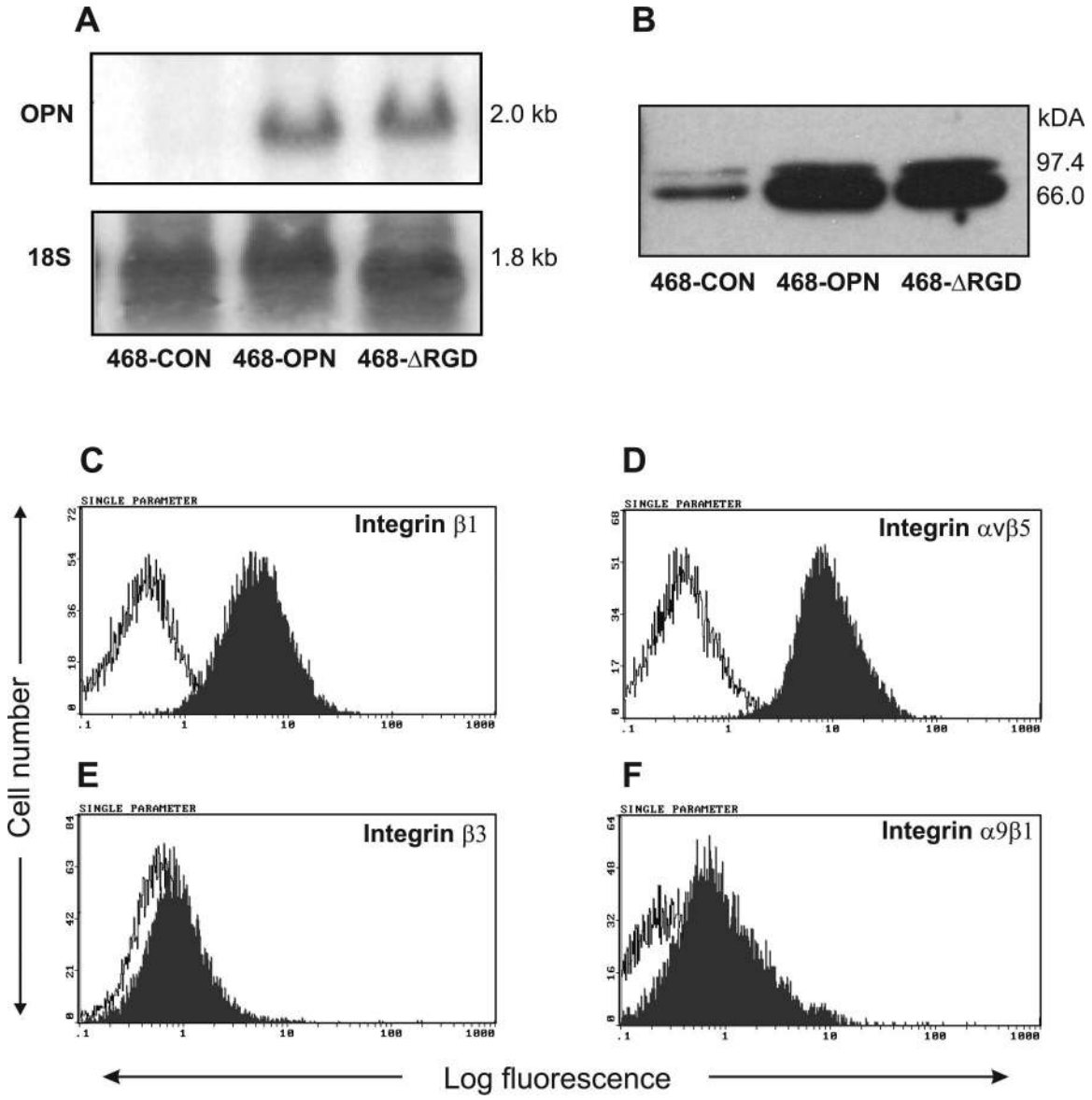

Figure 2. OPN and integrin expression of 468-CON, 468-OPN, and 468-ΔRGD cell lines. **A:** Expression of OPN mRNA was assessed by Northern blot analysis of 10 μg of total RNA/cell line. A cDNA probe specific for human OPN was radiolabeled with ^{32}P-dCTP and hybridized to the membrane. A radiolabeled cDNA probe specific for the 18S subunit was used as a loading control. **B:** Expression of secreted OPN in conditioned media (CM) as assessed by Western blot analysis. A normalized volume of CM equivalent to 1×10^5 cells was loaded into each lane, and equal loading was confirmed by Coomassie Blue staining (data not shown). Two typical forms of OPN were detected: high molecular weight (97 kd) and lower molecular weight (66 kd).[36,46] **C–F:** Flow cytometry analysis of the expression of various cell-surface integrin receptors by the 468-CON cell line. Cultured cells were incubated with integrin-specific antibodies (**filled profiles**) or with a nonspecific isotype control primary antibody (**open profiles**). Filled profiles represent surface expression of integrin β_1 (**C**), integrin $\alpha_v\beta_5$ (**D**), integrin β_3 (**E**), and integrin $\alpha_9\beta_1$ (**F**). No difference in integrin expression was seen among 468-CON, 468-OPN, 468-ΔRGD, or parental MDA-MB-468 cell lines (data not shown). Analyses were performed on RNA, conditioned media, or cells isolated from three separate experiments and gave similar results.

only that OPN staining confined to the tumor cells themselves was quantified.

OPN and Integrin Expression in Transfected MDA-MB-468 Cells

Based on our clinical observations that OPN is associated with lymphatic metastasis of breast cancer, we were next interested in determining if OPN plays a functional role in promoting this process and also whether the RGD integrin-binding sequence in the OPN backbone is involved. To test this, MDA-MB-468 breast cancer cells were stably transfected to overexpress wild-type OPN, mutant OPN (lacking an RGD sequence), or an empty vector control. The resulting cell lines (468-OPN, 468-ΔRGD, and 468-CON) were characterized for their OPN mRNA and protein expression using Northern and Western blot analysis, respectively.

Relative to control (468-CON), 468-OPN and 468-ΔRGD cells were confirmed to have elevated and approximately equivalent levels of OPN mRNA (Figure 2A) and protein (Figure 2B). We also characterized and compared 468-CON, 468-OPN, and 468-ΔRGD cells

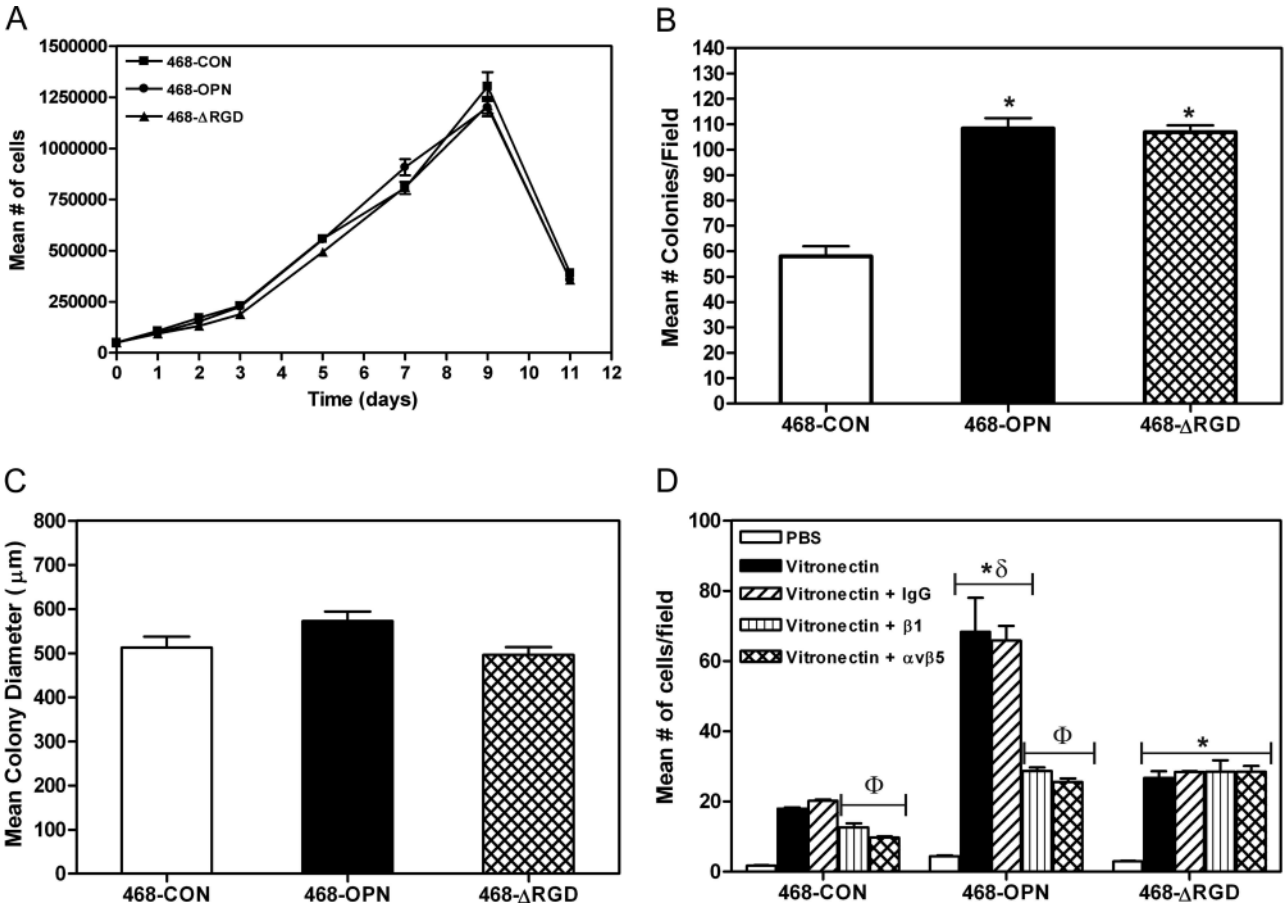

Figure 3. Differences in cell growth and cell adhesion *in vitro*. **A:** Cell growth of 468-CON (■), 468-OPN (●), and 468-ΔRGD (▲) in standard culture conditions over time. Data are presented as the mean ± SE. **B** and **C:** Anchorage-independent cell growth of 468-CON (**white bars**), 468-OPN (**black bars**), and 468-ΔRGD (**hatched bars**) in 0.4% soft agar. Cells (1.0×10^4 cells/60 mm plate; $n = 4$ for each cell line) were allowed to grow for 4 weeks. Plates were quantified with regards to the mean number of colonies per field (**B**) and the mean colony diameter (**C**) using 5 fields of view per plate. Data are presented as the mean ± SE. * Significantly different from 468-CON cells ($P < 0.001$). **D:** Cell adhesion of 468-CON, 468-OPN, and 468-ΔRGD to PBS (**white bars**; negative control) or 5 μg/ml vitronectin (**black bars**) in the presence or absence of blocking antibodies to RGD-dependent integrins ($\beta 1$; **vertical striped bars** and $\alpha_v\beta_5$; **hatched bars**) or an IgG negative isotype control (**diagonal striped bars**). Nontissue culture 96-well dishes were used, and cells (1×10^4 cells/well; $n = 3$ for each treatment) were allowed to adhere for 5 hours. Adhered cells were quantified by manual counting of five fields of view per well. Data are presented as the mean ± SE. * Significantly different from corresponding treatment of 468-CON cells ($P < 0.05$); δ significantly different from corresponding treatment of 468-ΔRGD cells ($P < 0.05$); Φ significantly different from respective IgG control ($P < 0.05$). Experiments were repeated three times and gave similar results.

with regards to their expression of various cell-surface integrins known to interact with OPN.[55–57] Flow cytometric analysis revealed that MDA-MB-468 cells express integrins β_1 and $\alpha_v\beta_5$ at moderate to strong levels but express only minimal amounts of integrins β_3 and $\alpha_9\beta_1$ (Figure 2, C–F). The integrin profile did not vary significantly among the three transfected cell lines or the parental cell line (data not shown). Analyses were performed on RNA, conditioned media, or cells isolated from three separate experiments and gave similar results.

Expression of OPN Increases in Vitro *Anchorage-Independent Growth and RGD-Dependent Cell Adhesion of MDA-MB-468 Cells*

In vitro assays were used to compare 468-CON, 468-OPN, and 468-ΔRGD cells for differences in cell growth, both in standard (anchorage-dependent) and soft-agar (anchorage-independent) culture conditions (Figure 3, A–C). No significant differences were observed in growth kinetics or population doubling time between the three cell lines under standard culture conditions (Figure 3A). However, cells overexpressing OPN showed an enhanced ability to survive/grow in soft agar, forming significantly more colonies than control ($P = 0.001$). This OPN-mediated survival/growth was not dependent on the presence of RGD region, because the 468-ΔRGD cells formed approximately the same number of colonies as the 468-OPN cells (Figure 3B). The mean diameter of the colonies formed was not significantly different between the three cell lines (Figure 3C).

In vitro assays were also used to compare 468-CON, 468-OPN, and 468-ΔRGD cells for differences in cell adhesion to vitronectin, a ligand for the RGD-dependent integrins ($\alpha_v\beta_1$ and $\alpha_v\beta_5$) expressed by MDA-MB-468 cells (Figure 3D). 468-OPN cells were found to be signif-

icantly more adherent to vitronectin than either 468-CON ($P = 0.033$) or 468-ΔRGD ($P = 0.049$) cells. Adhesion of both 468-OPN ($P = 0.019$) and 468-CON ($P = 0.045$) cells was significantly reduced in the presence of blocking antibodies to β_1 or $\alpha_v\beta_5$. Adhesion of 468-ΔRGD cells was not affected by the addition of integrin-blocking antibodies. Assays were performed three times and gave similar results.

Expression of OPN Increases in Vivo *Lymphovascular Invasion and Lymphatic Metastasis via Involvement of the RGD Integrin-Binding Domain*

To compare *in vivo* tumorigenicity and spontaneous metastatic ability to the lymphatics and elsewhere, 468-CON, 468-OPN, and 468-ΔRGD cells were injected into the mammary fat pad of female nude mice. Primary tumors were allowed to develop, and at 12 weeks after injection, mice were either subjected to surgery to remove the primary tumor or sacrificed and assessed for metastatic burden. Mice in the surgery group were sacrificed 24 weeks after injection and assessed for metastatic burden.

Mice in all groups displayed 100% tumor take, and there was no significant difference in primary tumor latency period (time to reach a size of 500 mm^3) or primary tumor doubling time between groups (Figure 4A). In mice that did not receive surgery (12-week endpoint), overexpression of wild-type OPN significantly increased the incidence of lymphovascular vessel invasion at the site of the primary tumor ($P = 0.003$; Figure 4, B and C), gross metastasis to the axillary lymph node ($P = 0.024$; Figure 5A), micrometastasis to the axillary lymph node ($P = 0.013$; Figure 5, A and C), and micrometastasis to the lung ($P = 0.004$; Figure 5, A and D) compared to control. OPN-induced gross metastases in the axillary lymph node were only observed in the 468-OPN group and not in the 468-ΔRGD group ($P = 0.024$). Although an increased incidence of micrometastases in the axillary lymph node and micrometastases in the lung was noted in the 468-OPN group relative to the 468-ΔRGD group, no statistically significant difference was achieved ($P = 0.551$; Figure 5A). In mice that received surgery (24-week endpoint), there was no significant difference observed in the incidence of metastases in the axillary lymph node or the lung between the three groups. However, overexpression of wild-type OPN did significantly increase the incidence of gross metastasis to distant (peritracheal) lymph nodes compared to control ($P < 0.001$), and this OPN-mediated lymphatic metastasis was significantly reduced in the absence of the RGD sequence ($P = 0.038$; Figure 5B). Data were compiled from two individual animal experiments, which gave comparable results.

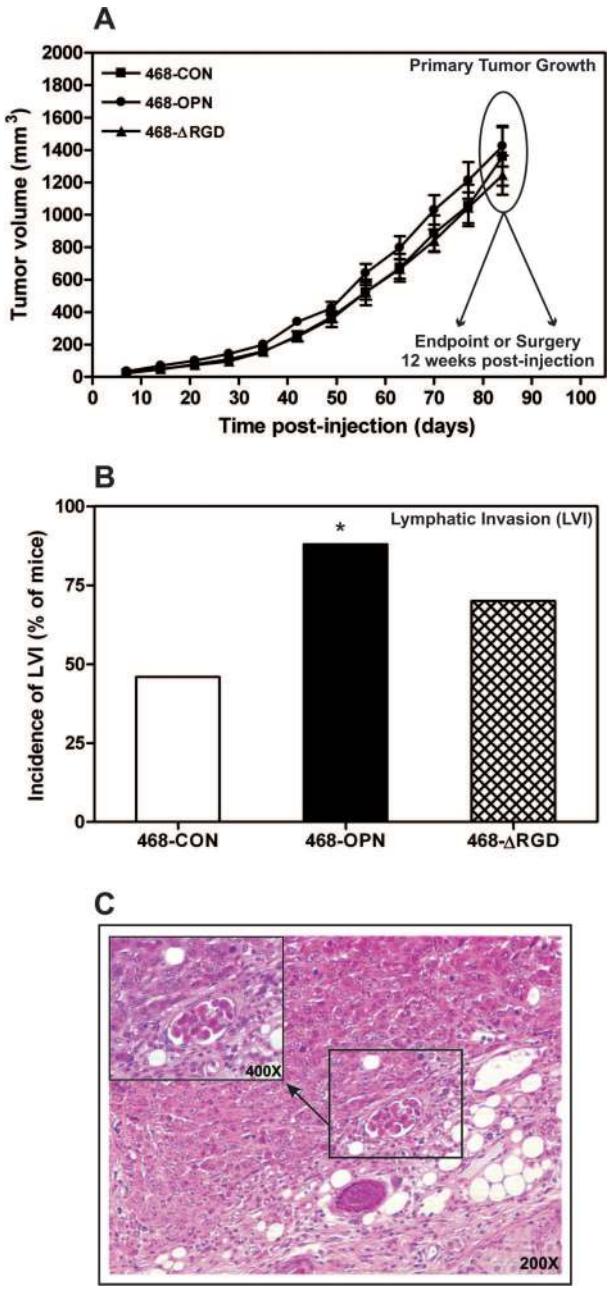

Figure 4. *In vivo* tumorigenicity and invasive behavior of 468-CON, 468-OPN, and 468-ΔRGD cells. **A:** Primary tumor growth kinetics. 468-CON (■), 468-OPN (●), or 468-ΔRGD (▲) cells were injected into the second thoracic mammary fat pad of female athymic nude (*nu/nu*) mice using 1×10^6 cells/mouse and 20–26 mice/treatment group. Tumors were measured in two dimensions twice a week for 12 weeks using standard calipers, and the tumor volume was estimated as described in the Materials and Methods. At 12 weeks after injection, mice were either subjected to surgery to remove the primary tumor or sacrificed and assessed for metastatic burden. **B:** Incidence of lymphovascular invasion at the site of the primary tumor in mice injected with 468-CON (**white bars**), 468-OPN (**black bars**), or 468-ΔRGD (**hatched bars**) cells. Tissue sections from primary tumors were subjected to histochemical staining with H&E and assessed for morphology and the incidence of lymphovascular invasion in a blinded fashion by two independent pathologists. *, Significantly different from 468-CON ($P = 0.003$). **C:** Representative H&E section showing lymphovascular invasion (**arrow**) at the site of the primary tumor. Magnification: ×200 (main micrograph); ×400 (**inset**).

Discussion

The clinical importance of lymphatic metastasis has been recognized for over 200 years, and oncologists have long based their management strategies on this knowledge.[9,58] However, very little is known about the mechanistic details of this process. Once considered to be

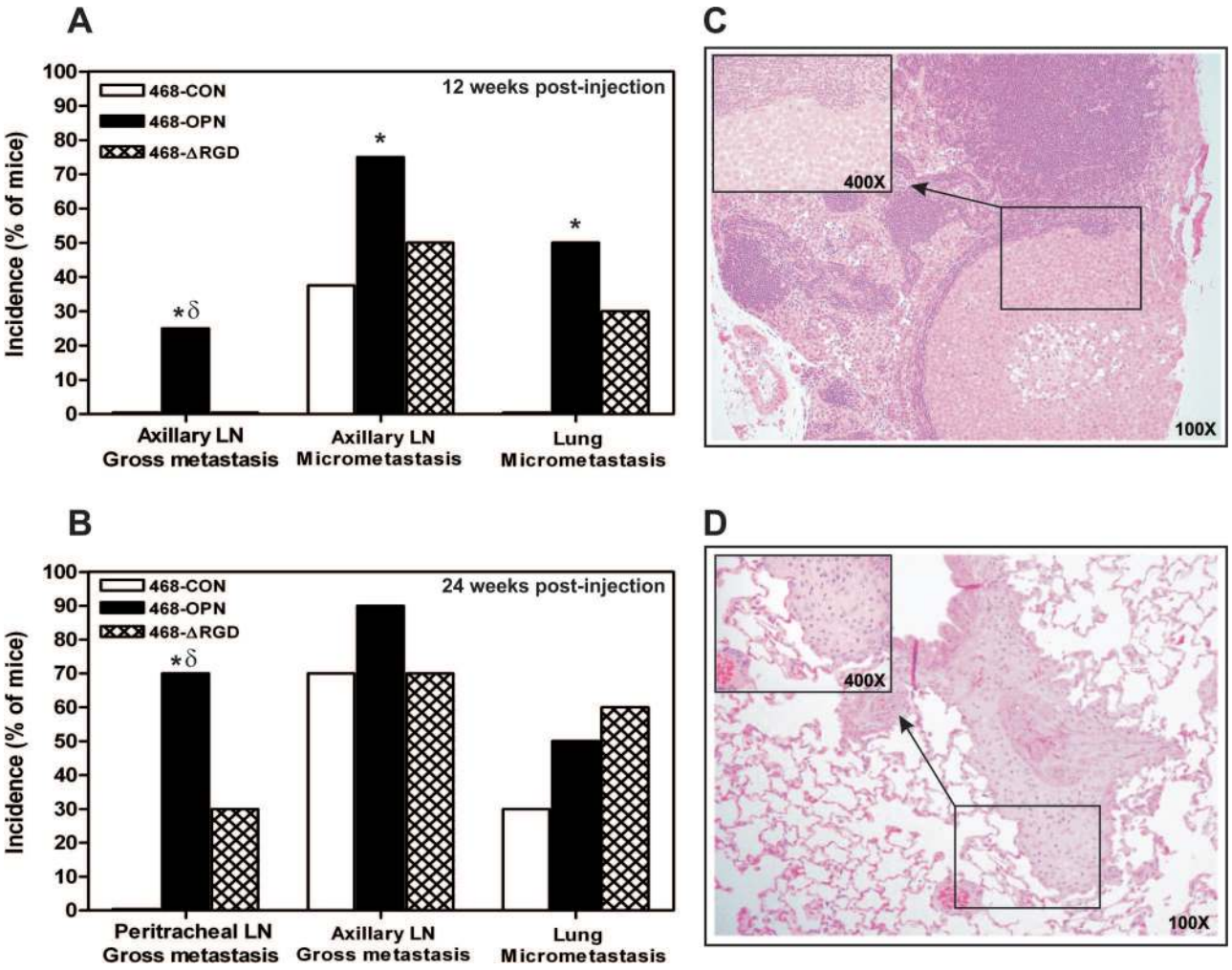

Figure 5. *In vivo* spontaneous metastasis of 468-CON, 468-OPN, and 468-ΔRGD cells. Cells were injected into the second thoracic mammary fat pad of female athymic nude (*nu/nu*) mice using 1×10^6 cells/mouse and 20–26 mice/treatment group. At 12 weeks after injection, mice were either subjected to surgery to remove the primary tumor or sacrificed and assessed for metastatic burden. Mice in the surgery group were sacrificed 24 weeks after injection and assessed for metastatic burden. Tissue sections from regional and distant organs, including lymph nodes and lungs, were subjected to histochemical staining with H&E. The incidence of metastasis was determined in a blinded fashion by two independent pathologists. **A:** Spontaneous metastasis of 468-CON (**white bars**), 468-OPN (**black bars**), or 468-ΔRGD (**hatched bars**) cells at 12 weeks after injection (no surgery group; $n = 10$–16 mice/treatment group). **B:** Spontaneous metastasis of 468-CON (**white bars**), 468-OPN (**black bars**), or 468-ΔRGD (**hatched bars**) cells at 24 weeks after injection (surgery group; $n = 10$ mice/treatment group). * Significantly different from 468-CON ($P = 0.024$); δ significantly different from 468-ΔRGD ($P = 0.038$). **C** and **D:** Representative H&E sections showing lymph node micrometastases (**C**) or lung micrometastases (**D**). Magnification: ×100 (main micrographs); ×400 (**inset**).

simply a passive drainage system, the lymphatics are now emerging as an active contributor to the dissemination of tumor cells from a primary tumor, and the identification of molecular factors involved in lymphatic circulation and lymphatic metastasis has become the subject of intense experimental investigation.[59] Proteins such as the hyaluronan receptor LYVE-1,[60] members of the VEGF family (VEGF-C, VEGF-D, and VEGFR3),[61,62] and the integrin $\alpha_9\beta_1$[63] have all been identified as markers of lymphatic vessels and/or of lymphangiogenesis and in some cases have also been linked to lymphatic metastasis.[64–66] Over the past several years, the secreted phosphoprotein OPN has been investigated extensively by ourselves and others as a key player in breast cancer progression. Given that OPN exerts its effect on tumor cell behavior via molecular interactions with other factors, including integrins and growth factor/receptor pathways,[35–44] we hypothesized that OPN might also be important in lymphatic metastasis. The present study provides, for the first time, clinical and experimental evidence that OPN is not only associated with breast cancer metastasis to the lymph nodes but also functionally contributes to this process.

Our patient population consisted of 106 breast cancer patients undergoing sentinel lymph node biopsy for breast cancer, 89 of which had both sentinel lymph node biopsy and concomitant or subsequent axillary lymph node dissection. Of the factors examined, primary tumor size and lymphovascular invasion were shown to be significantly associated with both SLN and ALN involvement. SLN positivity was also significantly associated with ALN involvement, and a false-negative rate of only 1.9% was observed. These findings are in keeping with those previously published[11,12,14–16,67–71] and indicate the accuracy of sentinel lymph node biopsy in determining lymph node status of most patients.

Our observation that there is increased OPN expression in the lymph node metastases of breast cancer patients compared to the primary tumor indicates that OPN may have a role in the establishment of lymphatic metastases. This increased expression of OPN may be a reflection of an increased propensity of OPN-expressing tumor cells to metastasize to the lymph nodes, and the observed trend toward increased OPN expression in the primary tumors of lymph node-positive versus lymph node-negative patients suggests that there might be a possible role of OPN in predicting this. Additionally or alternatively, it is possible that the lymph node microenvironment may induce or enhance expression of osteopontin in breast cancer cells. Several growth factors and cytokines that affect the PEA3/ets or AP-1 transcription factors can alter OPN expression. Some of these factors (ie, interleukin-1β and tumor necrosis factor-α) are present in the lymph node microenvironment, especially in situations of reactive or neoplastic involvement of the lymph node,[34,45,72–74] and may in fact be responsible for the observed increase in OPN in lymph node metastases. Finally, because both T cells and macrophages are also known to produce (and secrete) OPN, a certain proportion of the tumor cell OPN present may have been taken up from the lymph node environment. Although further investigation using a larger sample population is needed to determine the potential clinical value of tumor cell OPN (at the primary site or in lymph node metastases), our findings clearly demonstrate that an increased OPN level is associated with lymphatic metastasis.

We used a complementary experimental approach to examine the functional implications of OPN expression in lymphatic metastasis of breast cancer. Our findings indicate that overexpression of OPN in MDA-MB-468 breast cancer cells has no effect on standard growth *in vitro* but does lead to increased colony formation in soft agar. Although the 468-OPN cells were able to establish a greater number of colonies relative to control, the size of the colonies was not different, suggesting that OPN may confer more of a survival advantage rather than a growth advantage in soft agar. Overexpression of OPN in MDA-MB-468 breast cancer cells also resulted in increased *in vitro* cell adhesion. *In vivo*, overexpression of OPN did not affect primary tumor growth but did increase lymphovascular invasion at the site of the primary tumor and metastasis to regional and distant sites. Interestingly, the effect of OPN seemed to be most marked in the lymphatics and at earlier endpoints. At 12 weeks after injection, overexpression of OPN resulted in a significant increase in the incidence of both gross and microscopic axillary lymph node metastases, as well as microscopic lung metastases. In contrast, although there was a trend toward increased metastasis to the axillary lymph node and lung in the presence of OPN at the later endpoint (24 weeks after injection), the early "advantage" provided by OPN was not as apparent. The exception to this was the incidence of gross metastasis to distant lymph nodes, which only occurred in the presence of wild-type OPN. These experimental findings complement our clinical observations and support the idea that OPN expression is important for the establishment (and perhaps persistence) of metastases in the lymphatics. The results of the *in vitro* experiments and the observation that OPN had no significant effect on primary tumor growth suggest that this OPN-mediated establishment of metastases in the lymphatics may be due (at least in part) to an increased capacity for survival and increased adhesive interactions, rather than to an increased growth advantage.

Growing evidence indicates that one of the major mechanisms by which OPN influences cell behavior is via interactions with various integrins, including $\alpha_v\beta_3$, $\alpha_9\beta_1$, $\alpha_v\beta_5$, $\alpha_v\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_4\beta_7$.[33,34,44,45] Integrins are a family of cell-surface receptors that mediate adhesion to components of the extracellular matrix and/or to other cells.[75] OPN can interact with integrins in either an RGD-dependent or RGD-independent manner to activate cellular signaling pathways that allow OPN to mediate cell-cell and cell-matrix interactions.[49,76,77] We have previously shown that breast cancer cells can adhere to, migrate toward, or invade through basement membrane in response to exogenous OPN *in vitro*[35,36,38,40,47] and that these effects are mediated in part by the RGD sequence.[35,47] However, the importance of the RGD domain in endogenously produced OPN and its effect on breast cancer cell behavior *in vitro* and *in vivo* have not previously been examined.

Results from the present study demonstrate that, relative to cells expressing wild-type OPN, cells expressing a mutant form of OPN lacking an RGD domain show a reduced capacity for cell adhesion *in vitro*. Concordantly, adhesion of cells expressing wild-type OPN was also reduced in the presence of blocking antibodies to the RGD-dependent integrins β_1 and $\alpha_v\beta_5$. Given that all transfected cell lines (468-CON, 468-OPN, and 468-ΔRGD) showed similar integrin profiles, our findings suggest that the observed changes in cell adhesion are reliant on the interaction between OPN and RGD-dependent integrins and not simply on changes to integrin or OPN expression alone. Further studies are required to determine whether this interaction directly or indirectly influences the activation of integrins and/or of specific downstream cell signaling pathways that are responsible for the changes in adhesive cell behavior.

In vivo, we observed that OPN-mediated lymph node metastasis was partially abrogated in the absence of the RGD region, suggesting that this domain is important for more rapid/aggressive metastatic progression in the lymphatics. This is supported by the observation that the RGD region influenced both the progression from microscopic to gross metastases in the axillary lymph node (earlier endpoint), as well as the progression from local node to distant node involvement (later endpoint). However, the fact that some OPN-mediated effects were still observed in the lymphatics even in the absence of the RGD region suggests that both RGD-dependent and RGD-independent mechanisms may be involved in this process. Interestingly, deletion of the RGD region did not have a significant effect on reducing OPN-mediated lung metastases, suggesting that OPN may interact with different RGD-independent integrins in the lung microenvironment versus the lymphatic microenvironment and/or interact with nonintegrin receptors such as CD44[77,78] to

promote metastasis. The role of the RGD integrin-binding site of OPN in the clinical situation is not as readily examinable, because it would require a natural situation in which the RGD domain of OPN was deleted in a subgroup of patients. This type of spontaneous mutation (of the OPN RGD domain) has not previously been reported in the literature. However, based on our findings that the RGD integrin-binding site is important for lymphatic metastasis in experimental models, future studies are aimed at investigating the association and colocalization of several specific integrins with OPN in patient samples and mouse models to further elucidate the mechanistic, functional, and therapeutic implications of OPN/integrin interactions in lymphatic metastasis.

In summary, the combined clinical and experimental studies presented here strongly suggest that OPN is a key molecular player involved in lymphatic metastasis of breast cancer, potentially by affecting RGD-mediated adhesive interactions between tumor cells and their microenvironment and enhancing the ability of tumor cells to establish themselves and persist in the lymphatics. These are novel and important findings, because this is the first study to demonstrate the functional involvement of OPN in lymphatic metastasis. Furthermore, the role of the RGD integrin-binding domain in the OPN backbone has not previously been demonstrated in the context of breast cancer metastasis *in vivo*. Clarification of the molecular requirements for metastasis to lymph nodes and the translation of this knowledge into the clinic could have important implications for patient management and prognosis and may also lead to novel therapeutic approaches to inhibit this process.

Acknowledgments

We thank Michael Keeney and the London Health Sciences Centre Flow Cytometry Unit for their help in performing and analyzing the flow cytometry studies.

References

1. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, Thun MJ: Cancer statistics, 2005. CA Cancer J Clin 2005, 55:10–30
2. Chambers AF, Groom AC, MacDonald IC: Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer 2002, 2:563–572
3. Pantel K, Brakenhoff RH: Dissecting the metastatic cascade. Nat Rev Cancer 2004, 4:448–456
4. Fidler IJ: 7th Jan Waldenstrom Lecture. The biology of human cancer metastasis. Acta Oncol 1991, 30:668–675
5. MacDonald IC, Groom AC, Chambers AF: Cancer spread and micrometastasis development: quantitative approaches for in vivo models. BioEssays 2002, 24:885–893
6. Woodhouse EC, Chuaqui RF, Liotta LA: General mechanisms of metastasis. Cancer 1997, 80:1529–1537
7. Swartz MA, Skobe M: Lymphatic function, lymphangiogenesis, and cancer metastasis. Microsc Res Tech 2001, 55:92–99
8. Fisher B, Bauer M, Wickerham DL, Redmond CK, Fisher ER, Cruz AB, Foster R, Gardner B, Lerner H, Margolese R, Poisson R, Shibata H, Volk H: Relation of number of positive axillary nodes to the prognosis of patients with primary breast cancer. An NSABP update. Cancer 1983, 52:1551–1557
9. Foster RS, Jr: The biologic and clinical significance of lymphatic metastases in breast cancer. Surg Oncol Clin N Am 1996, 5:79–104
10. McGuire WL: Prognostic factors for recurrence and survival in human breast cancer. Breast Cancer Res Treat 1987, 10:5–9
11. Sakorafas GH, Geraghty J, Pavlakis G: The clinical significance of axillary lymph node micrometastases in breast cancer. Eur J Surg Oncol 2004, 30:807–816
12. Sakorafas GH, Tsiotou AG: Sentinel lymph node biopsy in breast cancer. Am Surg 2000, 66:667–674
13. White RL, Jr., Wilke LG: Update on the NSABP and ACOSOG breast cancer sentinel node trials. Am Surg 2004, 70:420–424
14. Giuliano AE, Kirgan DM, Guenther JM, Morton DL: Lymphatic mapping and sentinel lymphadenectomy for breast cancer. Ann Surg 1994, 220:391–398, discussion 398–401
15. Giuliano AE, Dale PS, Turner RR, Morton DL, Evans SW, Krasne DL: Improved axillary staging of breast cancer with sentinel lymphadenectomy. Ann Surg 1995, 222:394–399, discussion 399–401
16. Giuliano AE, Jones RC, Brennan M, Statman R: Sentinel lymphadenectomy in breast cancer. J Clin Oncol 1997, 15:2345–2350
17. Singletary SE, Allred C, Ashley P, Bassett LW, Berry D, Bland KI, Borgen PI, Clark GM, Edge SB, Hayes DF, Hughes LL, Hutter RV, Morrow M, Page DL, Recht A, Theriault RL, Thor A, Weaver DL, Wieand HS, Greene FL: Staging system for breast cancer: revisions for the ed 6 of the AJCC Cancer Staging Manual. Surg Clin North Am 2003, 83:803–819
18. Thor A: A revised staging system for breast cancer. J Breast 2004, 10(Suppl 1):S15–S18
19. Brown LF, Papadopoulos-Sergiou A, Berse B, Manseau EJ, Tognazzi K, Perruzzi CA, Dvorak HF, Senger DR: Osteopontin expression and distribution in human carcinomas. Am J Pathol 1994, 145:610–623
20. Fedarko NS, Jain A, Karadag A, Van Eman MR, Fisher LW: Elevated serum bone sialoprotein and osteopontin in colon, breast, prostate, and lung cancer. Clin Cancer Res 2001, 7:4060–4066
21. Agrawal D, Chen T, Irby R, Quackenbush J, Chambers AF, Szabo M, Cantor A, Coppola D, Yeatman TJ: Osteopontin identified as lead marker of colon cancer progression, using pooled sample expression profiling. J Natl Cancer Inst 2002, 94:513–521
22. Singhal H, Bautista DS, Tonkin KS, O'Malley FP, Tuck AB, Chambers AF, Harris JF: Elevated plasma osteopontin in metastatic breast cancer associated with increased tumor burden and decreased survival. Clin Cancer Res 1997, 3:605–611
23. Tuck AB, O'Malley FP, Singhal H, Tonkin KS, Harris JF, Bautista D, Chambers AF: Osteopontin and p53 expression are associated with tumor progression in a case of synchronous, bilateral, invasive mammary carcinomas. Arch Pathol Lab Med 1997, 121:578–584
24. Tuck AB, O'Malley FP, Singhal H, Harris JF, Tonkin KS, Kerkvliet N, Saad Z, Doig GS, Chambers AF: Osteopontin expression in a group of lymph node negative breast cancer patients. Int J Cancer 1998, 79:502–508
25. Hotte SJ, Winquist EW, Stitt L, Wilson SM, Chambers AF: Plasma osteopontin: associations with survival and metastasis to bone in men with hormone-refractory prostate carcinoma. Cancer 2002, 95:506–512
26. Schorge JO, Drake RD, Lee H, Skates SJ, Rajanbabu R, Miller DS, Kim JH, Cramer DW, Berkowitz RS, Mok SC: Osteopontin as an adjunct to CA125 in detecting recurrent ovarian cancer. Clin Cancer Res 2004, 10:3474–3478
27. Coppola D, Szabo M, Boulware D, Muraca P, Alsarraj M, Chambers AF, Yeatman TJ: Correlation of osteopontin protein expression and pathological stage across a wide variety of tumor histologies. Clin Cancer Res 2004, 10:184–190
28. Saeki Y, Mima T, Ishii T, Ogata A, Kobayashi H, Ohshima S, Ishida T, Tabunoki Y, Kitayama H, Mizuki M, Katada Y, Asaoku H, Kitano M, Nishimoto N, Yoshizaki K, Maeda M, Kon S, Kinoshita N, Uede T, Kawase I: Enhanced production of osteopontin in multiple myeloma: clinical and pathogenic implications. Br J Haematol 2003, 123:263–270
29. Le QT, Sutphin PD, Raychaudhuri S, Yu SC, Terris DJ, Lin HS, Lum B, Pinto HA, Koong AC, Giaccia AJ: Identification of osteopontin as a prognostic plasma marker for head and neck squamous cell carcinomas. Clin Cancer Res 2003, 9:59–67
30. Pan HW, Ou YH, Peng SY, Liu SH, Lai PL, Lee PH, Sheu JC, Chen CL, Hsu HC: Overexpression of osteopontin is associated with intrahe-

patic metastasis, early recurrence, and poorer prognosis of surgically resected hepatocellular carcinoma. Cancer 2003, 98:119–127
31. Chambers AF, Wilson SM, Kerkvliet N, O'Malley FP, Harris JF, Casson AG: Osteopontin expression in lung cancer. Lung Cancer 1996, 15:311–323
32. Rudland PS, Platt-Higgins A, El-Tanani M, De Silva Rudland S, Barraclough R, Winstanley JH, Howitt R, West CR: Prognostic significance of the metastasis-associated protein osteopontin in human breast cancer. Cancer Res 2002, 62:3417–3427
33. Sodek J, Ganss B, McKee MD: Osteopontin. Crit Rev Oral Biol Med 2000, 11:279–303
34. Furger KA, Menon RK, Tuck AB, Bramwelll VH, Chambers AF: The functional and clinical roles of osteopontin in cancer and metastasis. Curr Mol Med 2001, 1:621–632
35. Xuan JW, Hota C, Shigeyama Y, D'Errico JA, Somerman MJ, Chambers AF: Site-directed mutagenesis of the arginine-glycine-aspartic acid sequence in osteopontin destroys cell adhesion and migration functions. J Cell Biochem 1995, 57:680–690
36. Tuck AB, Arsenault DM, O'Malley FP, Hota C, Ling MC, Wilson SM, Chambers AF: Osteopontin induces increased invasiveness and plasminogen activator expression of human mammary epithelial cells. Oncogene 1999, 18:4237–4246
37. Tuck AB, Hota C, Chambers AF: Osteopontin (OPN)-induced increase in human mammary epithelial cell invasiveness is urokinase (uPA)-dependent. Breast Cancer Res Treat 2001, 70:197–204
38. Tuck AB, Elliott BE, Hota C, Tremblay E, Chambers AF: Osteopontin-induced, integrin-dependent migration of human mammary epithelial cells involves activation of the hepatocyte growth factor receptor (Met). J Cell Biochem 2000, 78:465–475
39. Shijubo N, Uede T, Kon S, Nagata M, Abe S: Vascular endothelial growth factor and osteopontin in tumor biology. Crit Rev Oncog 2000, 11:135–146
40. Tuck AB, Hota C, Wilson SM, Chambers AF: Osteopontin-induced migration of human mammary epithelial cells involves activation of EGF receptor and multiple signal transduction pathways. Oncogene 2003, 22:1198–1205
41. Furger KA, Allan AL, Wilson SM, Hota C, Vantyghem SA, Postenka CO, Al-Katib W, Chambers AF, Tuck AB: Beta(3) integrin expression increases breast carcinoma cell responsiveness to the malignancy-enhancing effects of osteopontin. Mol Cancer Res 2003, 1:810–819
42. Adwan H, Bauerle T, Najajreh Y, Elazer V, Golomb G, Berger MR: Decreased levels of osteopontin and bone sialoprotein II are correlated with reduced proliferation, colony formation, and migration of GFP-MDA-MB-231 cells. Int J Oncol 2004, 24:1235–1244
43. Cook AC, Tuck AB, McCarthy S, Turner JG, Irby RB, Bloom GC, Yeatman TJ, Chambers AF: Osteopontin induces multiple changes in gene expression that reflect the six "hallmarks of cancer" in a model of breast cancer progression. Mol Carcinog 2005, 43:225–236
44. Rittling SR, Chambers AF: Role of osteopontin in tumour progression. Br J Cancer 2004, 90:1877–1881
45. Denhardt DT, Noda M, O'Regan AW, Pavlin D, Berman JS: Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. J Clin Invest 2001, 107:1055–1061
46. Vantyghem SA, Allan AL, Postenka CO, Al-Katib W, Keeney M, Tuck AB, Chambers AF: A model for lymph node metastasis of breast cancer: characterization of a metastatic variant of the MDA-MB-468 human breast cancer cell line. Clin Exp Metastasis 2005, 22:351–361
47. Xuan JW, Hota C, Chambers AF: Recombinant GST-human osteopontin fusion protein is functional in RGD-dependent cell adhesion. J Cell Biochem 1994, 54:247–255
48. Bautista DS, Xuan JW, Hota C, Chambers AF, Harris JF: Inhibition of Arg-Gly-Asp (RGD)-mediated cell adhesion to osteopontin by a monoclonal antibody against osteopontin. J Biol Chem 1994, 269:23280–23285
49. Kon S, Yokosaki Y, Maeda M, Segawa T, Horikoshi Y, Tsukagoshi H, Rashid MM, Morimoto J, Inobe M, Shijubo N, Chambers AF, Uede T: Mapping of functional epitopes of osteopontin by monoclonal antibodies raised against defined internal sequences. J Cell Biochem 2002, 84:420–432
50. Allred DC, Clark GM, Elledge R, Fuqua SA, Brown RW, Chamness GC, Osborne CK, McGuire WL: Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. J Natl Cancer Inst 1993, 85:200–206
51. Young MF, Kerr JM, Termine JD, Wewer UM, Wang MG, McBride OW, Fisher LW: cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). Genomics 1990, 7:491–502
52. Price JE, Polyzos A, Zhang RD, Daniels LM: Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. Cancer Res 1990, 50:717–721
53. Cailleau R, Olive M, Cruciger QV: Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. In Vitro 1978, 14:911–915
54. Vantyghem SA, Wilson SM, Postenka CO, Al-Katib W, Tuck AB, Chambers AF: Dietary genistein reduces metastasis in a postsurgical orthotopic breast cancer model. Cancer Res 2005, 65:3396–3403
55. Smith LL, Giachelli CM: Structural requirements for alpha 9 beta 1-mediated adhesion and migration to thrombin-cleaved osteopontin. Exp Cell Res 1998, 242:351–360
56. Liaw L, Skinner MP, Raines EW, Ross R, Cheresh DA, Schwartz SM, Giachelli CM: The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of alpha v beta 3 in smooth muscle cell migration to osteopontin in vitro. J Clin Invest 1995, 95:713–724
57. Hu DD, Lin EC, Kovach NL, Hoyer JR, Smith JW: A biochemical characterization of the binding of osteopontin to integrins alpha v beta 1 and alpha v beta 5. J Biol Chem 1995, 270:26232–26238
58. Halstead WS: The results of operations for the cure of cancer of the breast performed at Johns Hopkins Hospitals. Ann Surg 1894, 20:497–504
59. Brown P: Lymphatic system: unlocking the drains. Nature 2005, 436:456–458
60. Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, Jones M, Jackson DG: LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J Cell Biol 1999, 144:789–801
61. Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, Swartz M, Fukumura D, Jain RK, Alitalo K: Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science 1997, 276:1423–1425
62. Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA: Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci USA 1998, 95:548–553
63. Vlahakis NE, Young BA, Atakilit A, Sheppard D: The lymphangiogenic vascular endothelial growth factors VEGF-C and -D are ligands for the integrin alpha9beta1. J Biol Chem 2005, 280:4544–4552
64. Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K, Detmar M: Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med 2001, 7:192–198
65. He Y, Kozaki K, Karpanen T, Koshikawa K, Yla-Herttuala S, Takahashi T, Alitalo K: Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. J Natl Cancer Inst 2002, 94:819–825
66. Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG: VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med 2001, 7:186–191
67. Viale G, Maiorano E, Pruneri G, Mastropasqua MG, Valentini S, Galimberti V, Zurrida S, Maisonneuve P, Paganelli G, Mazzarol G: Predicting the risk for additional axillary metastases in patients with breast carcinoma and positive sentinel lymph node biopsy. Ann Surg 2005, 241:319–325
68. Joseph KA, El-Tamer M, Komenaka I, Troxel A, Ditkoff BA, Schnabel F: Predictors of nonsentinel node metastasis in patients with breast cancer after sentinel node metastasis. Arch Surg 2004, 139:648–651
69. Cserni G, Gregori D, Merletti F, Sapino A, Mano MP, Ponti A, Sandrucci S, Baltas B, Bussolati G: Meta-analysis of non-sentinel node metastases associated with micrometastatic sentinel nodes in breast cancer. Br J Surg 2004, 91:1245–1252
70. Torrenga H, Fabry H, van der Sijp JR, van Diest PJ, Pijpers R, Meijer S: Omitting axillary lymph node dissection in sentinel node negative breast cancer patients is safe: a long term follow-up analysis. J Surg Oncol 2004, 88:4–7, discussion 7–8
71. Van Zee KJ, Manasseh DM, Bevilacqua JL, Boolbol SK, Fey JV, Tan LK, Borgen PI, Cody HS, 3rd, Kattan MW: A nomogram for predicting the likelihood of additional nodal metastases in breast cancer pa-

tients with a positive sentinel node biopsy. Ann Surg Oncol 2003, 10:1140–1151
72. Denhardt DT, Guo X: Osteopontin: a protein with diverse functions. FASEB J 1993, 7:1475–1482
73. Ruco LP, Pomponi D, Pigott R, Stoppacciaro A, Monardo F, Uccini S, Boraschi D, Tagliabue A, Santoni A, Dejana E: Cytokine production (IL-1 alpha, IL-1 beta, and TNF alpha) and endothelial cell activation (ELAM-1 and HLA-DR) in reactive lymphadenitis, Hodgkin's disease, and in non-Hodgkin's lymphomas. An immunocytochemical study Am J Pathol 1990, 137:1163–1171
74. O'Regan AW, Nau GJ, Chupp GL, Berman JS: Osteopontin (Eta-1) in cell-mediated immunity: teaching an old dog new tricks. Immunol Today 2000, 21:475–478
75. Giancotti FG, Ruoslahti E: Integrin signaling. Science 1999, 285:1028–1032
76. Yokosaki Y, Matsuura N, Sasaki T, Murakami I, Schneider H, Higashiyama S, Saitoh Y, Yamakido M, Taooka Y, Sheppard D: The integrin alpha(9)beta(1) binds to a novel recognition sequence (SVVYGLR) in the thrombin-cleaved amino-terminal fragment of osteopontin. J Biol Chem 1999, 274:36328–36334
77. Katagiri YU, Sleeman J, Fujii H, Herrlich P, Hotta H, Tanaka K, Chikuma S, Yagita H, Okumura K, Murakami M, Saiki I, Chambers AF, Uede T: CD44 variants but not CD44s cooperate with beta1-containing integrins to permit cells to bind to osteopontin independently of arginine-glycine-aspartic acid, thereby stimulating cell motility and chemotaxis. Cancer Res 1999, 59: 219–226
78. Weber GF, Ashkar S, Glimcher MJ, Cantor H: Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science 1996, 271: 509–512