Tumorigenesis and Neoplastic Progression

Osteopontin Gene Expression Determines Spontaneous Metastatic Performance of Orthotopic Human Breast Cancer Xenografts

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A major problem in the therapeutic management of cancer is the growth of metastases in distant organs, but the genes orchestrating the process need to be identified for the rational design of new treatment. Here, we provide decisive experimental evidence demonstrating the causal involvement of a specific gene, osteopontin (OPN), in the pathogenesis of metastasis by human breast cancer cells and implicating some of its probable partners. Stable long-term depletion, or up-regulation, of OPN gene expression in a matched, isogenic pair of human breast cancer cell lines of differing metastatic proficiency reproducibly changed their ability to colonize distant organs. OPN down-regulation was achieved by transduction of the metastatic line with a DNA construct encoding a small hairpin RNA in a vector labeled with red fluorescent protein and resulted in a marked reduction of metastatic load ($P < 0.01$). Up-regulation of OPN in the negligibly metastatic line, with a green fluorescent protein-marked retroviral vector containing OPN cDNA driven by a strong promoter, resulted in heavy colonization of the lungs and lymph nodes ($P < 0.005$). The reciprocal changes in behavior of these matched cell lines cross-corroborate each other. Concomitant changes were seen in the expression of other metastasis-related genes in both modulated lines. The data indicate that therapeutic targeting of tumor OPN molecules could reset metastatically relevant gene networks, resulting in clinical benefit. (*Am J Pathol* 2007, 171:682–692; DOI: 10.2353/ajpath.2007.070232)

clinical objective of finding drugs that can inhibit or prevent secondary tumor growth. One of the molecules most frequently associated with human tumor metastasis in recent years is osteopontin (OPN). OPN is a secreted phosphoglycoprotein that exists in several alternatively spliced and differentially glycosylated isoforms in many body fluids (including urine, blood, and milk) and tissue compartments.^{1–4} Its known functions to date include roles in lymphocyte² and macrophage³ activation and migration, intercellular signaling between gonadotrophs and other cells in the anterior pituitary,⁵ signal transduction across the cell membrane, calcium binding, mammary morphogenesis, and lactation,⁶ and wound healing.⁷ It also binds to RGD receptors on the cell and to CD44⁸ and presents growth factors and other biologically active matrix components to the cell surface. It contains a thrombin cleavage site,² and the resulting fragments are soluble and have different functions from the full-length molecule.^{2,3} In different cells and tissues, its production is regulated by preferential binding of separate transcription factors to specific parts of its promoter.⁹ Currently, there is minimal information on the splice variants and on varieties of post-transcriptionally phosphorylated and glycosylated isoforms occurring in different body locations.

OPN has been implicated in tumor growth and metastasis by a number of investigative avenues, including DNA transfection studies,^{10,11} gene expression analysis of human and animal tumors,¹² observations on experimentally induced tumors,^{13,14} and clinical investigations on breast cancer,^{15,16} ovarian cancer,¹⁷ mesothelioma,¹⁸ bladder cancer,¹⁹ laryngeal cancer,²⁰ and other types of malignancies. Such work has demonstrated that OPN is elevated in patients with several types of tumors and shown good correlation of the OPN levels in tumors and body fluids with prognosis. However, OPN is also elevated in lactating mothers⁴ and in patients with a number of non-neoplastic conditions such as chronic pancreati-

The major problem in the therapeutic management of cancer is posed by the growth of multiple secondary tumors, metastases, in many distant organs. However, the critical molecular mechanisms enabling tumors to metastasize remain poorly understood, handicapping the

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tis,²¹ pulmonary diseases²² (including tuberculosis), and various inflammatory disorders,^{23,24} somewhat limiting its value as a diagnostic and prognostic marker. These studies have established a distinct, although not exclusive, association of raised OPN levels with malignant neoplasia but do not discriminate between whether this is a cause or an effect of neoplastic progression. To resolve this question, we conducted an investigation using a matched pair of cell lines from the same human breast cancer, one of which has much greater metastatic proficiency and OPN production than the other.

Recently, some other studies have examined the effects of experimentally up- or down-regulating OPN expression on the pathological activities of tumor cell lines. The studies have used a number of different methods to manipulate OPN levels including antisense RNA,^{25,26} RNA interference,^{27,28} retroviral “knockin” transduction,²⁶ induced mutations,²⁶ and transgenic animals.²⁵ Most of the reports describe the results of such interventions on the behavior of the genetically engineered cells *in vitro* and reported altered migratory and “invasive” proficiencies. A few studies have examined the behavior of the altered tumor cells *in vivo*, but only three of them^{25,26,28} studied metastasis from an orthotopic site and none attempted to quantitate the metastatic burden in the animals or the OPN levels in their tumors and their blood.

The current work contributes new information conclusively demonstrating a direct deterministic relationship between OPN production, OPN blood levels, and degree of spontaneous metastasis from an orthotopic site, using knockdown [by OPN-specific small hairpin (sh)RNA] and knockin technology, on a well-characterized pair of isogenic clonal human breast cancer cell lines of opposing metastatic proficiency. Fluorescent protein markers incorporated in the vector constructs enhanced the purification of transduced tumor cells and the sensitivity of detection of metastases in distant organs.^{29,30} This work also provides information on concurrent changes induced in some other genes believed to be mechanistically relevant. The collective data lay a firm foundation for deeper investigation of the potential cancer therapeutic benefits of targeting specific parts of the OPN molecule and of how other genes, in the network linked with osteopontin, play a causal role in metastasis.

Materials and Methods

Cell Lines

Two human breast cancer cell lines, NM-2C5 and M-4A4LM3-2, derived by single-cell cloning from the MDA-MB-435 cell line,³¹ were used in this study. M-4A4LM3-2 (hereafter called 4M3 for brevity) is the third generation descendant of the M-4A4 metastatic line originally isolated by Bao et al³² from MDA-MB-435 and was obtained by cyclically culturing and orthotopically re-inoculating the cells of successive generations of lung metastases as originally described by Fidler.³³ The metastatic proficiencies (strong for the 4M3 cell line³⁰; negligible for the NM-2C5 cell line^{32,34}) have been described previously. These isogenic cell lines were cultured in

RPMI 1640 medium supplemented with 10% newborn calf serum (Life Technologies, Inc., Gaithersburg, MD) and penicillin and streptomycin at 37°C in a humidified atmosphere of 5% CO₂/95% air. Transductions and analyses were performed on cultures passaged no more than 10 times from frozen vials of our original stocks at the time of *in vivo* inoculation. Cultures were regularly tested to confirm stability of metastatic performance and freedom from *Mycoplasma* and common murine pathogens. The pathogen tests were performed by University of California San Diego Animal Care Program Diagnostic Laboratory.

Some previous studies have speculated, on the basis of microarray analysis, that MDA-MB-435 is a melanoma cell line, not a breast cancer cell line,³⁵ because it expresses some melanocyte-related genes. However, more recent work,^{30,36} including our own unpublished data, demonstrate that it also expresses breast-specific proteins and grows better in the mammary gland, from which it can metastasize but cannot do so from the subcutis.³⁷ We are confident that it is of mammary origin and that its expression of melanocyte-related genes is an example of inappropriate gene expression³⁸ or lineage infidelity³⁶ often seen in human tumors.

Lentiviral Vector Construction

The plasmid pLNmR, containing both fluorescence and antibiotic resistance markers for selection, was used to incorporate the oligonucleotides down-regulating the OPN gene expression. The aim of using fluorescent protein markers was to enhance the purification of transduced tumor cells and the sensitivity of detection of metastases in distant organs.^{29,30} It was constructed using the pLLRed plasmid as the starting vector. pLLRed was assembled as follows: a sequence encoding the red fluorescent protein was polymerase chain reaction (PCR)-amplified from the pIRES2-DsRed plasmid (Clontech Laboratories, Inc., Mountain View, CA) with the oligonucleotides 5'-GTCACCGGTGTCACAACCATGGCCTCCTCC-3' (Agi site is underlined) and 5'-CGAGAATTCCTACAGGAACAGGTGGTGG-3' (EcoRI site is underlined). The PCR fragment was then digested with EcoRI-Agi and inserted into pLL3.7 (Addgene, Cambridge, MA), previously cut by these two restriction enzymes. To make the pLNmR vector, a BspHI-BspHI fragment containing the Kanamycin resistance gene from pIRES2-DsRed plasmid was cloned between the two BspHI restriction sites in pLLRed. The resulting plasmid, pLNmR, was checked by restriction mapping and sequencing.

siRNA Sequences and Constructs

A 21-bp DNA sequence encoding a sh interference RNA sequence designed to target a selected section of the OPN transcript was cloned into the lentiviral expression vector described above. The cDNA sequence of OPN was obtained from GenBank (accession number NM_000582) and scanned for potential target sequences for RNA interference using the small interfering (si)RNA Selection Program available from the Whitehead siRNA Selection Web Server (available at <http://jura.wi.mit.edu/>)

bioc/siRNAext/). Three target sequences were tested in preliminary studies, and the most effective for depletion of OPN transcripts was 5'-ATGAATTAGATAGTGCATCT-TCT-3', which has no significant homology with other genes. For construction of the DNA encoding the corresponding shRNA, the following two oligonucleotides were synthesized: (5'-TGAATTAGATAGTGCATCTTCTT-TCAAGAGAAAGATGCACTATCTAATTCAATTTTTC-3' and 5-TCGAGAAAAAATGAATTAGATAGTGCATCTT TCTCTTGAAGAAGATGCACTATCTAATTCA-3'. This second sequence (63 oligos) has a 4-bp overhang to provide for ligation into a vector. The 21-nucleotide sense or antisense section corresponding to the chosen OPN target is in bold letters, and the stem-loop sequences for the hairpin are in italics. Double-stranded DNA oligos were generated by mixing 1 μ l of each oligo (60 pmol/ μ l) with 48 μ l of annealing buffer (100 mmol/L potassium acetate, 30 mmol/L HEPES-KOH, pH 7.4, and 2 mmol/L magnesium acetate) and incubating at 95°C for 4 minutes, 70°C for 10 minutes, decreasing to 4°C slowly (0.1°C/minute), and finally 4°C for 10 minutes. Double-stranded DNA was ligated between the *Xho*I and *Hpa*I sites of the linearized pLNM vector. The resulting pLNM-siOPN constructs were sequenced to confirm correct alignment, orientation, and composition before being transfected into the packaging cell line. The control plasmid contained no insert but was otherwise identical.

Production and Transduction of Lentiviral Vectors

To accomplish incorporation into lentiviral vectors, pLNM-siOPN, or pLNM plasmid DNA was transfected into 293FT cells using ViraPower Packaging Mix and Lipofectamine 2000 (Invitrogen, Carlsbad, CA). In brief, the DNA-Lipofectamine 2000 complexes were added to a suspension of 293FT cells (6×10^6 in 10 ml of serum-supplemented medium) and incubated overnight. The medium containing the DNA-Lipofectamine 2000 complexes was then replaced with complete culture medium containing sodium pyruvate, and the virus-containing supernatants were harvested after 72 hours. For transduction into 4M3 cells, 1-ml aliquots of supernatant with 100 μ l of polybrene were added to cultures and incubated for 16 hours.

Cloning and Selection of OPN Down-Regulated Cells

Flow cytometry was used for the initial selection of transduced (red fluorescent protein-positive) cells with the strongest fluorescent signal. Parallel tests confirmed that these had the most OPN knockdown. To ensure uniformity of OPN production in the cell population used for further experiments, a large panel of monoclonal tumor cell lines was derived by limiting dilution cloning from the cells selected by fluorescence-activated cell sorting. Each monoclonal line was tested by quantitative PCR to identify the one with the least OPN transcripts, and for the selected clone, the

level was confirmed three times in consecutive culture samples. Stability of reduction was confirmed by measurements of mRNA transcripts and protein expression in long-term cultures (3 months) and in tumors resulting from intramammary inoculation (see below).

Up-Regulation of OPN Production

NM-2C5 cells were transduced using standard methods with a retroviral vector (pLNCX2) containing the entire OPN cDNA sequence under the control of the cytomegalovirus promoter and cassettes encoding green fluorescent protein (GFP) and the neomycin resistance gene. After selection in G-418 (a neomycin analog), individual clones were selected and tested for elevated OPN expression, by quantitative PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA). The clone (2 OPN UR) with OPN levels nearest to native 4M3 cells was chosen for further studies.

Isolation of Total RNA

Fresh cell pellets or frozen solid primary tumors were homogenized in TRIzol reagent (Life Technologies) for RNA extraction. The RNA pellets were resuspended in RNase-free water, and the contaminating DNA was removed from the preparations with DNase I using the DNA-free kit (Ambion, Austin, TX). The yield of total RNA was measured using a spectrophotometer (Eppendorf, Westbury, NY) and the quality assessed by running the samples in a 1% agarose gel.

Quantitative PCR

mRNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase and oligo(dT) from the Retroscript cDNA synthesis system (Ambion). The amplification reactions were conducted in 96-well plates in 25- μ l reaction volumes containing 12.5 μ l of 2 $\times$ SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA), 50 nmol/L each of forward and reverse primers, and 1 μ l of the cDNA and monitored in an ABI Prism 7700 Sequence Detector System (PE Applied Biosystems). The thermal profile for the polymerase chain reaction was 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds (denaturation step) and 60°C for 1 minute (annealing and elongation steps). Each sample was performed in triplicate, and the expression of the OPN gene was normalized to glyceraldehyde-3-phosphate dehydrogenase expression measured on the same plate.

Western Blotting

To prepare serum-free conditioned medium, cells were washed six times with serum-free medium and resupplied with fresh serum-free medium. After 24 hours, the culture supernatant was harvested, spun at $1500 \times g$ to remove cellular debris, and concentrated approximately $\times 200$ using Biomax Ultrafree centrifugal filters (Millipore, Bedford, MA). Protein concentration was determined with

the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Denatured and reduced protein samples were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis glycine gel, and proteins were transferred onto polyvinylidene difluoride membrane using a semidry apparatus (Bio-Rad, Hercules, CA). Blots were blocked in Tris-buffered saline-Tween 20 (TBS-T) buffer containing 5% dried milk for 2 hours at room temperature, followed by incubation with primary antibody, monoclonal anti-human osteopontin, from Chemicon International (Temecula, CA) at 1:2000 dilution for an additional 1 hour. After washing with TBS-T four times, blots were incubated with secondary antibody, horseradish peroxidase-conjugated anti-rat, from GE Healthcare (Piscataway, NJ) at 1:20,000 dilution for 1 hour at room temperature, and the binding of the secondary antibody was detected by enhanced chemiluminescence (GE Healthcare).

ELISA

Quantitative determination of human OPN in cell culture supernatants and tissue homogenates was performed using a commercially available ELISA kit (Assay Designs Inc., Ann Arbor, MI) according to the manufacturer's instructions. All quantification was done in triplicate. The concentration of OPN in a sample was determined by interpolation from a standard curve.

Cell Proliferation Assay

Cellular proliferation *in vitro* was determined using a CellTiter 96 colorimetric assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate (MTS/PMS) assay; Promega, Madison, WI] based on a modified tetrazolium/formazan procedure³⁹ in which the amount of reaction product (formazan) is time-dependent and proportional to the number of viable cells. For each cell line, 1×10^3 cells were seeded on day 0, and the number of cells was counted in triplicate after 24 hours to assess plating efficiency. The counts were repeated at daily intervals for 7 days.

In Vitro Cellular Adhesion Assay

Cellular adhesion to plastic, and to albumin- or fibronectin-coated surfaces, was evaluated for each cell line in 96-well microtiter plates. The assay plates were incubated at 37°C in 95% air/5% CO₂ for 30 minutes. After washing, attached cells were counted using the MTS/PMS assay.

Invasion Assay

The capability of the parental and genetically modified tumor cell lines to invade Matrigel-coated polycarbonate filters with 8- μ pores was measured using commercially available Boyden Chamber kits (Chemicon, Temecula,

CA) essentially as described by Albini et al.⁴⁰ Conditioned medium, obtained by incubation of NIH3T3 cells in serum-free medium for 24 hours with Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin and 0.05 mg/ml ascorbic acid, was placed in the lower compartment of the Boyden chamber as a chemoattractant. For each assay, 1×10^5 tumor cells suspended in complete medium (200 μ l) were placed in the upper chamber. After incubation for 12 hours at 37°C, cells that had invaded through the Matrigel-coated membranes onto their lower surfaces were stained and counted in 10 fields under a microscope ($\times 400$).

Migration Assay

Migratory ability was evaluated by scratch-wound healing assays on plastic plates. The surfaces of confluent monolayers were wounded by an 18-gauge needle, and cellular motility was evaluated by measuring the width of the wound at the same location immediately and 6 hours after wounding. Each assay was conducted in triplicate. Migration in response to chemoattraction was measured by movement toward fibronectin in Boyden chamber assays using uncoated membranes with open 8- μ m pores.

In Vivo Studies

One million cells in 50 μ l of mixture of RPMI 1640 medium and extracellular matrix gel (Matrigel; Sigma Chemical Co., St. Louis, MO) were inoculated into the mammary fat pad of anesthetized severe combined immunodeficient mice. Experimental protocols were approved by the University of California San Diego Animal Care Program, and rules relating to tumor growth and burden and to euthanasia were rigorously followed. Single clones, with optimal properties, were selected for *in vivo* testing from several available for each of the experimental groups in this study, in keeping with national and institutional ethical mandates to minimize the numbers of living animals used. When it became evident that statistically highly significant and convincing results on tumor metastasis were obtained by the genetic manipulations in each experimental category, relative to each other and to vector-only controls, testing of further clones could not be justified under the aforementioned regulations and ethical considerations relating to animal experimentation. Animals were euthanized and necropsied at 2 to 3 months after inoculation when the primary tumors reached ~ 20 mm in diameter. Metastasis formation by primary tumors was assessed by counting fluorescent tumor deposits of at least 1-mm diameter using a Leica MZ dissecting microscope (Leica Microsystems, Bannockburn, IL) and confirmed by histological examination. Although fluorescence imaging using this microscope is sufficiently sensitive to detect single cells, only cell clusters larger than 1 mm are regarded as true, established, metastatic colonies, incorporating host vessels and cells. Tissue from primary tumors and metastases were snap-frozen and stored at -80°C until used for RNA or protein extraction.

Table 1. Metastatic Behavior of Parental Clones 4M3 and NM-2C5 Transduced with Viral Vectors Containing GFP Alone

	4M3	NM-2C5
Number of mice	15	15
Primary tumors	15	15
Number with pulmonary metastases	13	4
	(Range 3 to 650 surface deposits, >1 mm; median = 55)	(Range 1 to 6 deposits >1 mm; median = 2)
Number with lymph Node metastases	12	4
	(Multiple; ie, ++ to +++, in locoregional, pelvic, abdominal, and thoracic nodes)	(Solitary; ie, +, only in locoregional nodes. No thoracic, axillary, or cervical involvement)

Statistical Analysis

For molecular measurements, data were expressed as a mean $\pm$ SD of at least three independent triplicate experiments.

For comparisons of metastatic load in different groups of animals, the Wilcoxon rank sum test was used. Numbers of metastases counted relate to colonies on the lung surface and are discontinuous variables that may not be normally distributed, and nonparametric analysis is more appropriate than standard *t*-tests, which are parametric.

Results

Properties of Parent Cell Lines: 4M3 and NM2C5

The 4M3 and NM-2C5 cell lines used in this work differ considerably in metastatic proficiency (strong for 4M3, negligible for NM2C5; see Table 1) and human OPN expression. Figure 1, a and b, shows a 27-fold difference in OPN level by qPCR and 100-fold by ELISA between these *cell lines*. In the corresponding *tumors* the differences are 10-fold in the primary tumors and about 17-fold in the blood (Figure 3). We have confirmed that both these isogenic human breast cancer lines express all three known isoforms of osteopontin and that the A-form is most abundant. In the xenogeneic tumors produced by these lines in immunocompromised mice, there is also an up-regulation of *mouse* OPN expression (unmeasurable in virgin mammary glands but elevated approximately equally in 4M3 and NM-2C5 tumors, see below for details).

Properties of Transduced Lines in Vitro

Lines derived from 4M3 were named 4OPNVO (transduced with vector only) and 4OPsi (transduced with the OPN knockdown construct). Lines derived from NM2C5 were named 2C5GFP (vector alone) and 2OPUR (transduced with the OPN up-regulating construct).

The transduced lines showed excellent efficiency of incorporation and expression of the fluorescence-encoding constructs. After selection by fluorescence-activated cell sorting or drug in the culture medium, the cells showed ubiquitous but variable expression of the incorporated genes, judged by fluorescence (Figure 1, e–h). Single-cell cloning enabled identification and selection of

cell lines with uniform, optimally altered levels of expression. The altered expression of OPN was confirmed to be stable over 3 months *in vitro* for each line (Figure 1, a and b). The OPN-down-regulated line 4OPsi exhibited marked spindle-shaped morphology, relative to 4M3 cells (Figure 1, c–f), and the cells transduced with vector-

Figure 1. a: Human OPN transcript levels in control and test cell lines measured by qPCR. b: Human OPN secretion levels into medium by control and test cell lines measured by ELISA. c: Morphology of unmodified 4M3 cells. d: Morphology of transduced 4OPsi cells. Note increased tendency to spindle-shape. e: Transduced 4OPVO cells after flow sorting. Note all cells fluoresce, but intensity varies. f: Transduced 4OPsi cells after flow sorting. Note spindle shape and variation in fluorescence intensity although all cells are labeled. g: Transduced 2C5GFP (control) cells after G-418 selection. Note uniform intensity of fluorescence. h: Transduced 2OPUR cells after sorting. Fluorescence is uniform, and note that there are no obvious morphological differences from control cells.

Figure 2. **a:** Growth curves of control and genetically modified cells *in vitro*. **b:** Attachment of control and modified cells to fibronectin- and albumin-coated plastic. **c:** Motility of control and modified cell lines tested by scratch-wound healing assay. **d:** Chemotaxis of cell lines toward fibronectin and bovine serum albumin. **e:** Invasion of control and test cell lines through Matrigel matrices.

only (4OPVO), but the OPN up-regulated line 2OPUR did not show any significant morphological change relative to its own specific vector-only control, 2C5GFP (Figure 1, g and h). The down-regulation of all three isoforms of OPN in 4OPsi cells was so complete that transcripts were undetectable by qPCR and protein was unmeasurable by ELISA in concentrated culture medium (Figure 1, a and b). In contrast, up-regulation of OPN expression in 2OPUR cells to levels comparable with 4M3 cells was demonstrated by qPCR, Western blotting, and ELISA. Levels in control OPVO cells and 2C5GFP cells transduced with vector-only were not significantly altered from parental levels. Growth curves for all transduced lines *in vitro* differed only slightly from the untransduced cells (Figure 2, a and b). The changes were inversely related to the substantial effects seen on metastatic proficiencies and are therefore unlikely to be responsible for them.

Stability of the Changed OPN Expression Levels in Genetically Engineered Cell Lines and Corresponding Tumors

Measurement of the levels of OPN transcripts and protein produced by engineered and parent cell lines showed that the alterations induced by these manipulations were stable in over at least 3 months' growth of cell lines *in vitro* (Figure 1, a and b) and in primary tumors *in vivo* (see below).

Altered Attachment, Motility and Invasive Capabilities of OPN-Transduced Lines in Vitro

Attachment to bovine serum albumin-coated plastic dishes was not altered in any of the transduced lines, although attachment to fibronectin was mildly diminished in 4OPsi cells and elevated slightly in 2OPUR cells (Figure 2b). Motility as measured by a scratch assay and by migration

through porous membranes was increased in 2OPUR cells relative to their parent line but was not decreased by down-regulation of OPN in the 4OPsi cells (Figure 2c). However, alteration of OPN expression did have opposing effects on chemotactic responses to fibronectin by these isogenic cell lineages, being decreased when OPN was down-regulated in 4OPsi cells and vice versa in 2OPUR cells (Figure 2d). Invasion by 4OPsi cells through stromal matrices (Matrigel) in Boyden chambers was similarly decreased to the level of 2C5GFP cells, whereas experimental manipulation of OPN expression had the opposite effect in 2OPUR cells (Figure 2e).

OPN Production in Tumors

OPN production was greatly diminished in tumors generated by 4OPsi cells relative to those produced by the parental metastatic line 4M3 and by the vector-only control cells 4OPVO (Figure 3b). It should be noted that the constituent cells of the 4OPsi tumors are the progeny of several replicative cycles and that the suppression produced by the experimental strategy used in this work was therefore stable over many generations. However, it is interesting to note that although the extinction of OPN expression (Figure 1, a and b) was total in cells cultured *in vitro* for over 12 weeks, some tumors began expressing the human protein again at low levels (Figure 3b).

Tumorigenesis and Spontaneous Metastasis after Orthotopic Inoculation

Incidence of Tumor Takes after Intramammary Inoculation

Tumors appeared in all 15 inoculated animals in each of the study groups. Their growth rates (Figure 3a) were

Figure 3. **a:** Growth rates of tumors generated by intramammary inoculation of control and modified cell lines. **b:** Human OPN levels in tumors made by control and test cell lines (ELISA). **c:** Human OPN levels in blood of corresponding tumor-bearers measured by ELISA. **d:** Human uPA levels in tumors generated by control and modified cell lines (ELISA). **e:** Human MMP-2 levels in blood of animals bearing tumors generated by control and test cell lines (ELISA).

not remarkably different for any of the cell types studied. They corresponded approximately to those of tumors made by their ancestral cell lines,^{34,41} M4A4 and NM-2C5, from which they were derived by selection in animals.

Incidence and Degree of Metastatic Spread

The numbers of animals with metastases in the 4OPsi and 4OPVO groups were the same (15/15), but the degree of metastatic burden in the lungs and lymph nodes in 4OPsi animals was dramatically diminished ($P < 0.01$) relative to 4OPVO (Table 2 and Figure 4). Metastasis formation by 4OPsi tumors was not completely abrogated, but PCR on DNA extracted from the few metastases formed, using primers flanking the insertion site of the siRNA transcribing section into the vector, indicated that they were formed by cells that had eliminated the insert by rearrangement. This is supported by the re-emergence of lowered levels of OPN expression in some tumors made by OPN-deficient 4OPsi cells (Figure 3b).

In addition, the incidence of metastasis and the numbers of metastases in each animal inoculated with 2OPUR cells was substantially greater ($P < 0.005$) than the numbers seen in animals inoculated with control vector-only NM2C5GFP cells and NM-2C5 parental cells (Tables 1 and 2). Thus, the down-regulation of OPN in OPsi tumors and the increased production of this protein in OPUR tumors had major effects on metastasis of the corresponding tumor types. It should be mentioned that the evaluation of the metastatic load in each animal was enhanced by the fluorescent markers.

The Source of the Increased OPN in the Metastatic Tumors

Analysis of the source of OPN, from the tumor or the host, was accomplished by choosing primers and antibodies that were species-specific for human or murine gene products. The normal, adult, non-neoplastic virgin mammary gland does not produce any detectable amount (Figure 5a), but it was found that both human and murine OPN were greatly elevated in 4M3 tumors relative to their weakly metastatic NM2C5 counterparts (Figure 5b), human much more so than murine. Hence, the pres-

Table 2. Metastatic Load in Lungs and Lymph Nodes of Animals Inoculated with Genetically Engineered Cell Lines

Mouse no.	4OPVO		4OPsi		OPUR*	
	P	LN	P	LN	P	LN
1	10		9		0	++ Pel
2	61	+ Th	2		2	+ Sci
3	28		10	+ Sac, Ren	0	+ Ren
4	52	++ Th	8		5	+ Sac, Lum
5	21		8		5	
6	39	++ Th	57	+ Th, Ax, Mes	76	+ Lum, Ax
7	45	+ Th	115	++ Th, Ax	34	+ Ax, Lum, Sac, Mam
8	29	+ Pel	59	+ Th	58	+ Pel, Ren, Th
9	52	++ Ing, Lum	18	+ Ax	85	+ Lum, Ren
10	88	++ Th, Abd	419	+ Abd	245	+ Th, Sc
11	251	++ Th, Abd	26	+ Ren, Th	11	+ Th, Lum, Sac
12	750	++ Th, Cer, Rec	30		59	+ Ax, Ren, Cer
13	186	++ Th, Mes, Rec	6		28	+ Th, Lum, Sac
14	168	++ Th, Cer, Rec, Del, Lum	7		482	+ Th, Ren, Om, Cer
15	224	++ Th Cer, Ax	83	++ Th, Cer	—	—

P, numbers of surface deposits; LN, degree and distribution of lymph node involvement; Th, thoracic; Pel, pelvic; Ing, inguinal; Lum, lumbar; Abd, abdominal; Cer, cervical; Mes, mesenteric; Rec, rectal; Del, deltoid; Ax, axillary; Ren, Renal; Sci, Sciatic; Om, Omental; Sac, sacral; Mam, mammary.
 *One animal in this group died of unrelated causes before necropsy.

Figure 4. Necropsy results. **a** and **d**: Survey views of whole animal (**a**) and lungs (**d**) of 4OPVO animal show red fluorescent primary tumor and many metastases. **Arrows** in **a** show tumor deposits in lymph nodes. **b** and **e**: Survey views of animal inoculated with 4OPSi cell line show reduction in metastases in lymph nodes (**b**) and lungs (**e**). **White arrow** in **b** shows weakly fluorescing normal gall bladder. Primary tumor in mammary gland fluoresces red. **c**: Survey view of animal inoculated with 2OPUR cells shows GFP-labeled metastases in abdominal, thoracic, axillary, and cervical lymph nodes (**red arrows**). P, primary tumor. **f**: Increased metastases in lungs of 2OPUR animals compared with controls (see **j**). **g**: Closeup of **f** to show metastases and many intervening scattered cells in the lungs (x50). **h**: Lymph node from 2OPUR animal shows complete colonization of node and obstruction of adjacent lymph vessels (**arrows**) with tumor cells (x40). **i**: Survey view of the body of a control 2C5GFP animal shows an occasional positive lymph node and large green fluorescent primary tumor in mammary gland. **j**: Lungs of control 2C5GFP animal with occasional small metastases in lungs. Only deposits above 1 mm (See scale included) are metastases. **k**: Lungs of control 4M3-inoculated animal show the vigorous metastatic capability of the parental cell line.

Figure 5. **a:** RT-PCR products using murine-specific (m) and human-specific (h) OPN primers demonstrate the absence of mouse OPN in normal murine mammary glands. NM2C5 tumor was added as positive control. (NTC, no template negative control.) Mouse glyceraldehyde-3-phosphate dehydrogenase amplicons were used as loading control. **b:** Human and murine OPN in xenogeneic tumors analyzed by species-specific qPCR. The tumor cells induced the non-neoplastic gland to secrete murine OPN, but the majority of the secreted protein is human. **c:** ELISA analysis showing that genetically increased OPN secretion is accompanied by decreased MMP-8 secretion.

ence of human tumor cells induced mouse mammary tissue to produce OPN, and metastatic cells induced much more than their nonmetastatic counterparts, although the bulk of the elevated OPN made by metastatic tumors is of tumor cell rather than host cell origin.

Plasma Levels of Mouse and Human Osteopontin in Tumor-Bearing Animals

Human and murine OPN were both elevated in the plasma of tumor-bearing animals, and the elimination of human OPN by siRNA in 4OPsi cells was accompanied by a significant decrease in the plasma OPN levels (Figure 3c) in the corresponding group of animals. Conversely, blood OPN levels were higher in 2OPUR tumor-bearers. As expected, the majority of the plasma OPN was produced by the human tumor cells rather than the murine stroma.

Activities of Other Metastasis-Relevant Genes: Mechanistic Effects of OPN Modulation

Parallel studies on some other proteins that may be involved in the network affecting metastasis, conducted to investigate potential mechanistic pathways for the observed effects, showed that matrix metalloproteinase (MMP)-2 and uPA (Figure 3, d and e) were up-regulated

in 2OPUR cells/tumors and down-regulated in 4OPsi cells/tumors concomitantly with OPN in the various engineered cell lines. Moreover, genes for other proteins, such as MMP-8 and TSP-1, which we have previously linked with the nonmetastatic phenotype, were regulated in the opposite direction in the engineered cells. Thus we found that MMP-8 is down-regulated about fourfold in 2OPUR cells relative to parental NM-2C5 (Figure 5c), although not much up-regulated in LM-3 cells with diminished OPN (data not shown). Conversely, we found that in NM-2C5 cells transduced with an antisense RNA construct designed to down-regulate MMP-8,⁴² OPN production measured by qPCR and ELISA became elevated up to 30-fold. The down-regulation effect, in cells producing much OPN (ie, OPUR and unmodified LM-3 cells) was probably due to secreted OPN acting in an auto/paracrine mode through a cell surface receptor, because studies *in vitro*, using antibodies that block the $\alpha 5 \beta 3$ receptor, inhibited both native and recombinant OPN from exerting this effect. In another experiment, we noted dose-dependent down-regulation of thrombospondin 1 (*TSP1*) expression by NM-2C5 cells (which normally express high *TSP1* and low osteopontin³⁴), with increasing concentrations of recombinant human OPN to the medium, indicating multiple inter- and counter-regulatory networks in the production of matricellular proteins and proteases in metastasis.

Discussion

The results of this investigation provide compelling new evidence that OPN plays a decisive role in human breast cancer metastasis. The reciprocal behavioral changes induced in the isogenic metastatic and nonmetastatic cell lines that we used provide valuable cross-corroboration of the effects of altering OPN expression on the metastatic behavior of a tumor and demonstrate that this protein is causally involved in the metastatic process. This does not necessarily imply that it is the prime mover in this complex process, but it does provide reproducible hard evidence that OPN and its regulatory pathways are mechanistically involved and a strong platform for further dissection of the molecular causation of this biologically unique phenomenon.

The data we provide are convincingly consistent with previous clinical^{15,16} and experimental work^{10,11,13,14} showing a strong association between elevated OPN expression and tumor growth and progression. This new evidence also supports and extends previous interventional studies^{25–28} describing alterations in tumor behavior following manipulation of OPN gene expression in other model systems. Some of these studies used assays involving intravascular^{27,43} or subcutaneous inoculation as convenient surrogates for true orthotopic tumor metastasis, which takes longer to study. Others used a purely murine transgenic model incorporating a polyoma middle T gene²⁵ or a murine cell line engineered to silence OPN.⁴³ Another study using siRNA to suppress OPN expression in human MDA-MB-435 breast cancer cells²⁸ reported reduction in tumorigenicity after intramammary inoculation. The incidence of metastasis (numbers of animals found to have metastases at necropsy) was reported to be reduced in the experimental group relative to controls but, as this was due to the absence of primary tumors in some animals, the data do not provide information specifically and directly relevant to the involvement of OPN in metastasis. The differences between the target sequences used in the Shevde et al study²⁸ and in the current work could perhaps account for the preponderant effect being on tumorigenesis in that work and more clearly on metastasis in ours, since the OPN gene is known to produce a number of splice variants and post-translationally phosphorylated or glycosylated products, which could have different roles in tumorigenesis relative to metastasis. In our work, we know by the highly sensitive measurement methods of PCR and ELISA that all OPN transcripts and proteins measurable by the primers and antibodies that we used were substantially altered by the genetic manipulations in the manner described in Results, leading to the observed effects on metastasis, but this does not formally eliminate the possibility that the absence of significant effects on tumorigenesis in our work was because of the persistence of other unknown variants.

The current report progresses from previous studies by unequivocally showing that decreasing or increasing OPN expression in isogenic, cloned human breast cancer cell lines, with opposing metastatic proficiencies, reverses their metastatic phenotypes. Concomitantly, the

levels of OPN in the tumors and plasma increase and decrease in accordance with the conclusion that OPN plays a critical mechanistic role in the process. The data presented in Figure 2 suggest that this may occur through effects on migration, invasion, and operationally effective attachment of the tumor cells to substrates in the intercellular matrix of the primary and metastatic sites via appropriate binding sites on the molecule. Analysis of the source of the OPN clearly indicates that there is cross talk between the tumor cells and the supporting host stroma. The majority of the secreted OPN is produced by the human tumor cells, but neighboring (murine) host cells are also induced to up-regulate OPN secretion (see Montel et al^{44,45} for further discussion of tumor host interactions in metastatic gene regulation). In this context, it is useful to mention a previous study from this laboratory showing clearly that stable knockdown of MMP-8 production by genetic engineering increases the metastatic performance of NM-2C5 cells and that elevation of the level of production of this protease decreased the metastatic capability of isogenic M-4A4 human breast cancer cells,⁴² which constitute the ancestral lineage from which 4M3 was derived.

Taken in conjunction with the current work, it seems reasonable to conclude that these investigations have identified two important opposing components of the metastatic process, OPN and MMP-8. These projects have also demonstrated that additional genes, implicated by other investigators in the mechanism of metastasis, such as MMP-2 and uPA,⁴⁶ vary in coordinate manner when OPN and MMP-8 are genetically altered and thus provide a new opportunity to probe how this clinically important process is initiated and sustained by a dynamically interactive network of genes regulating each other. We postulate that hierarchical stimulatory and inhibitory relationships exist among its components and that functional integrity of some nodes of the network (eg, OPN) is absolutely critical for the balance of the system to tip toward metastatic tumor spread. Moreover, the data strongly suggest the possibility that blocking specific parts of variants (as yet unidentified) of the OPN molecule could be therapeutically useful in controlling metastasis of some cancers. The data do not establish that OPN and MMP-8 are implicated in all types of cancer metastasis but do provide useful clues for investigating molecules that are involved in metastasis of malignant tumors of other organs and the rational design of drugs to inhibit the process.

References

1. Rittling SR, Chambers AF: Role of osteopontin in tumour progression. *Br J Cancer* 2004, 90:1877–1881
2. Wai PY, Kuo PC: The role of osteopontin in tumor metastasis. *J Surg Res* 2004, 121:228–241
3. Weber GF: The metastasis gene osteopontin: a candidate target for cancer therapy. *Biochim Biophys Acta* 2001, 1552:61–85
4. Nagatomo T, Ohga S, Takada H, Nomura A, Hikino S, Imura M, Ohshima K, Hara T: Microarray analysis of human milk cells: persistent high expression of osteopontin during the lactation period. *Clin Exp Immunol* 2004, 138:47–53
5. Ehrchen J, Heuer H, Sigmund R, Schafer MK, Bauer K: Expression

- and regulation of osteopontin and connective tissue growth factor transcripts in rat anterior pituitary. *J Endocrinol* 2001, 169:87–96
6. Nemir M, Bhattacharyya D, Li X, Singh K, Mukherjee AB, Mukherjee BB: Targeted inhibition of osteopontin expression in the mammary gland causes abnormal morphogenesis and lactation deficiency. *J Biol Chem* 2000, 275:969–976
7. Kyriakides TR, Bornstein P: Matricellular proteins as modulators of wound healing and the foreign body response. *Thromb Haemost* 2003, 90:986–992
8. Khan SA, Cook AC, Kappil M, Gunthert U, Chambers AF, Tuck AB, Denhardt DT: Enhanced cell surface CD44 variant (v6, v9) expression by osteopontin in breast cancer epithelial cells facilitates tumor cell migration: novel post-transcriptional, post-translational regulation. *Clin Exp Metastasis* 2005, 22:663–673
9. Inman CK, Shore P: The osteoblast transcription factor Runx2 is expressed in mammary epithelial cells and mediates osteopontin expression. *J Biol Chem* 2003, 278:48684–48689
10. Oates AJ, Barraclough R, Rudland PS: The identification of osteopontin as a metastasis-related gene product in a rodent mammary tumour model. *Oncogene* 1996, 13:97–104
11. El-Tanani M, Barraclough R, Wilkinson MC, Rudland PS: Metastasis-inducing DNA regulates the expression of the osteopontin gene by binding the transcription factor Tcf-4. *Cancer Res* 2001, 61:5619–5629
12. 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
13. Hsieh YH, Juliana MM, Hicks PH, Feng G, Elmets C, Liaw L, Chang PL: Papilloma development is delayed in osteopontin-null mice: implicating an antiapoptosis role for osteopontin. *Cancer Res* 2006, 66:7119–7127
14. Jin H, Valverde P, Chen J: Cloning of hamster osteopontin and expression distribution in normal tissues and experimentally induced oral squamous-cell carcinoma. *Arch Oral Biol* 2006, 51:236–245
15. 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
16. Bramwell VH, Doig GS, Tuck AB, Wilson SM, Tonkin KS, Tomiak A, Perera F, Vandenberg TA, Chambers AF: Serial plasma osteopontin levels have prognostic value in metastatic breast cancer. *Clin Cancer Res* 2006, 12:3337–3343
17. Seidman JD: Osteopontin as a biomarker for ovarian cancer. *JAMA* 2002, 287:3209; author reply 3209–3210
18. Pass HI, Lott D, Lonardo F, Harbut M, Liu Z, Tang N, Carbone M, Webb C, Wali A: Asbestos exposure, pleural mesothelioma, and serum osteopontin levels. *N Engl J Med* 2005, 353:1564–1573
19. Ang C, Chambers AF, Tuck AB, Winquist E, Izawa JI: Plasma osteopontin levels are predictive of disease stage in patients with transitional cell carcinoma of the bladder. *BJU Int* 2005, 96:803–805
20. Celetti A, Testa D, Staibano S, Merolla F, Guarino V, Castellone MD, Iovine R, Mansueto G, Somma P, De Rosa G, Galli V, Melillo RM, Santoro M: Overexpression of the cytokine osteopontin identifies aggressive laryngeal squamous cell carcinomas and enhances carcinoma cell proliferation and invasiveness. *Clin Cancer Res* 2005, 11:8019–8027
21. Nakamura M, Oka M, Iizuka N, Kawauchi S, Gondo T, Ueno T, Tangoku A: Osteopontin expression in chronic pancreatitis. *Pancreas* 2002, 25:182–187
22. O'Regan A: The role of osteopontin in lung disease. *Cytokine Growth Factor Rev* 2003, 14:479–488
23. Masuda H, Takahashi Y, Asai S, Takayama T: Distinct gene expression of osteopontin in patients with ulcerative colitis. *J Surg Res* 2003, 111:85–90
24. 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
25. Jessen KA, Liu SY, Tepper CG, Karrim J, McGoldrick ET, Rosner A, Munn RJ, Young LJ, Borowsky AD, Cardiff RD, Gregg JP: Molecular analysis of metastasis in a polyomavirus middle T mouse model: the role of osteopontin. *Breast Cancer Res* 2004, 6:R157–R169
26. Allan AL, George R, Vantyghem SA, Lee MW, Hodgson NC, Engel CJ, Holliday RL, Girvan DP, Scott LA, Postenka CO, Al-Katib W, Stitt LW, Uede T, Chambers AF, Tuck AB: Role of the integrin-binding protein osteopontin in lymphatic metastasis of breast cancer. *Am J Pathol* 2006, 169:233–246
27. Ito T, Hashimoto Y, Tanaka E, Kan T, Tsunoda S, Sato F, Higashiyama M, Okumura T, Shimada Y: An inducible short-hairpin RNA vector against osteopontin reduces metastatic potential of human esophageal squamous cell carcinoma in vitro and in vivo. *Clin Cancer Res* 2006, 12:1308–1316
28. Shevde LA, Samant RS, Paik JC, Metge BJ, Chambers AF, Casey G, Frost AR, Welch DR: Osteopontin knockdown suppresses tumorigenicity of human metastatic breast carcinoma, MDA-MB-435. *Clin Exp Metastasis* 2006, 23:123–133
29. Hoffman RM: The multiple uses of fluorescent proteins to visualize cancer in vivo. *Nat Rev Cancer* 2005, 5:796–806
30. Montel V, Huang TY, Mose E, Pestonjamas K, Tarin D: Expression profiling of primary tumors and matched lymphatic and lung metastases in a xenogeneic breast cancer model. *Am J Pathol* 2005, 166:1565–1579
31. Cailleau R, Olive M, Cruciger QV: Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In Vitro* 1978, 14:911–915
32. Bao L, Pigott R, Matsumura Y, Baban D, Tarin D: Correlation of VLA-4 integrin expression with metastatic potential in various human tumour cell lines. *Differentiation* 1993, 52:239–246
33. Fidler IJ: Selection of successive tumour lines for metastasis. *Nat New Biol* 1973, 242:148–149
34. Urquidí V, Sloan D, Kawai K, Agarwal D, Woodman AC, Tarin D, Goodison S: Contrasting expression of thrombospondin-1 and osteopontin correlates with absence or presence of metastatic phenotype in an isogenic model of spontaneous human breast cancer metastasis. *Clin Cancer Res* 2002, 8:61–74
35. Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, Iyer V, Jeffrey SS, Van de Rijn M, Waltham M, Pergamenschikov A, Lee JC, Lashkari D, Shalon D, Myers TG, Weinstein JN, Botstein D, Brown PO: Systematic variation in gene expression patterns in human cancer cell lines. *Nat Genet* 2000, 24:227–235
36. Sellappan S, Grijalva R, Zhou X, Yang W, Eli MB, Mills GB, Yu D: Lineage infidelity of MDA-MB-435 cells: expression of melanocyte proteins in a breast cancer cell line. *Cancer Res* 2004, 64:3479–3485
37. Bao L, Matsumura Y, Baban D, Sun Y, Tarin D: Effects of inoculation site and Matrigel on growth and metastasis of human breast cancer cells. *Br J Cancer* 1994, 70:228–232
38. Tarin D: The fallacy of epithelial mesenchymal transition in neoplasia. *Cancer Res* 2005, 65:5996–6000
39. Buttkie TM, McCubrey JA, Owen TC: Use of an aqueous soluble tetrazolium/formazan assay to measure viability and proliferation of lymphokine-dependent cell lines. *J Immunol Methods* 1993, 157:233–240
40. Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM, McEwan RN: A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res* 1987, 47:3239–3245
41. Goodison S, Kawai K, Hihara J, Jiang P, Yang M, Urquidí V, Hoffman RM, Tarin D: Prolonged dormancy and site-specific growth potential of cancer cells spontaneously disseminated from nonmetastatic breast tumors as revealed by labeling with green fluorescent protein. *Clin Cancer Res* 2003, 9:3808–3814
42. Montel V, Kleeman J, Agarwal D, Spinella D, Kawai K, Tarin D: Altered metastatic behavior of human breast cancer cells after experimental manipulation of matrix metalloproteinase 8 gene expression. *Cancer Res* 2004, 64:1687–1694
43. Wai PY, Mi Z, Guo H, Sarraf-Yazdi S, Gao C, Wei J, Marroquin CE, Clary B, Kuo PC: Osteopontin silencing by small interfering RNA suppresses in vitro and in vivo CT26 murine colon adenocarcinoma metastasis. *Carcinogenesis* 2005, 26:741–751
44. Montel V, Pestonjamas K, Mose E, Tarin D: Tumor-host interactions contribute to the elevated expression level of alpha1-antichymotrypsin in metastatic breast tumor xenografts. *Differentiation* 2005, 73:88–98
45. Montel V, Mose ES, Tarin D: Tumor-stromal interactions reciprocally modulate gene expression patterns during carcinogenesis and metastasis. *Int J Cancer* 2006, 119:251–263
46. Mi Z, Guo H, Wai PY, Gao C, Kuo PC: Integrin-linked kinase regulates osteopontin-dependent MMP-2 and uPA expression to convey metastatic function in murine mammary epithelial cancer cells. *Carcinogenesis* 2006, 27:1134–1145