Differential Osteopontin Expression in Phenotypically Distinct Subclones of Murine Breast Cancer Cells Mediates Metastatic Behavior*

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Cancer progression depends on an accumulation of metastasis-supporting cell signaling molecules, which target signal transduction pathways and, ultimately, gene expression. One such molecule, osteopontin (OPN), represents a key molecular signaling event in tumor progression and metastasis. However, the transcriptional regulatory mechanisms that underlie OPN expression in the setting of breast cancer have not been well studied. In this regard, we have examined the differential transcriptional regulation of OPN in the murine mammary epithelial tumor cell lines, 4T1 and 4T07, which are sublines derived from the parental population of 410.4 cells from Balb/cfC3H mice. These lines are phenotypically heterogeneous in their metastatic behavior. 4T1 hematogenously metastasizes to the lung, liver, bone, and brain, whereas 4T07 is highly tumorigenic but fails to metastasize. The tumor growth and metastatic spread of 4T1 cells closely mimics stage IV breast cancer. We demonstrate that a Ras-independent, phosphoinositide-3 kinase-dependent, c-Jun N-terminal kinase-dependent phosphorylation of c-Jun results in binding of an AP-1 c-Jun homodimer to the OPN promoter in 4T1 cells. This differential up-regulation of OPN gene transcription and protein expression in 4T1 cells conveys in vitro correlates of a metastatic phenotype. These results provide new insight into the transcriptional regulation of OPN as a key mediator of metastatic behavior in malignancy.

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The potential role of OPN in regulation of metastatic behavior in these cell lines has not been previously examined. In this study, when 4T1 and 4T07 cells are compared, we demonstrate that a phosphoinositide-3 kinase (PI3K)- and JNK-dependent phosphorylation of c-Jun differentially up-regulates OPN gene transcription in 4T1 cells to convey in vitro correlates of a metastatic phenotype. These results provide new insight into the transcriptional regulation of OPN as a key mediator of metastatic behavior in malignancy.

MATERIALS AND METHODS

Cell Cultures—Mouse mammary tumor cell lines 4T1 and 4T07 (gift from Mark W. Dewhirst, Duke University) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and maintained at 37 °C in a humidified atmosphere of 5% CO2. For secreted-protein analysis, serum-free DMEM was collected after 24-h incubation and centrifuged at 600 × g for 5 min to remove cellular material; the supernatant was concentrated 100-fold through Ultrafree Centrifugal filters (Millipore, Bedford, MA). For JNK inhibition assays, the selective c-Jun NH2-terminal kinase inhibitor SP600125 was purchased from Calbiochem, which was prepared as a stock solution of 90 mM in 99.5% dimethyl sulfoxide. The PI3K inhibitor LY294002 (25 μM) was used. Cells in serum-free DMEM were treated with SP600125 at indicated doses for 24 h (see Fig. 4b, unless otherwise indicated, 100 nx was used).

Western Blot Analysis—4T1 and 4T07 cells were lysed in buffer (0.8% NaCl, 0.02% KCl, 1% SDS, 10% Triton X-100, 0.5% sodium deoxycholic acid, 0.144% Na2HPO4, and 0.024% KH2PO4, 2 mM phenylmethylsulfonyl fluoride, pH 7.4) and centrifuged at 12,000 × g for 10 min at 4 °C. The protein concentration was determined by the Bio-Rad protein assay kit; the protein samples were separated by 4–20% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Amer sham Biosciences) by semi-dry transfer (Bio-Rad). The membranes were probed with the following primary antibodies for 1 h at room temperature: goat OPN Ab (R&D Systems, Minneapolis, MN), rabbit with advanced metastatic cancer, and has been implicated in tumor cell migration and metastasis. Data suggest that OPN overexpression represents a key molecular event in tumor progression and metastasis (4–11). OPN is not typically mutated to achieve gain-of-function activation; rather, it is differentially transcriptionally regulated by multiple response elements in its promoter. However, the mechanisms that underlie the transcriptional regulation of OPN expression in metastatic biology are largely unknown.

In this regard, we have examined the differential transcriptional regulation of OPN in the murine mammary epithelial tumor cell lines 4T1 and 4T07. These are thioguanine-resistant sublines derived from the parental population of 410.4 cells from Balb/cfC3H mice (12). Although they share a common origin, these lines are phenotypically heterogeneous in their metastatic behavior. 4T1 hematogenously metastasizes to the lung, liver, bone, and brain, whereas 4T07 is highly tumorigenic but fails to metastasize. The tumor growth and metastatic spread of 4T1 cells closely mimics stage IV breast cancer. The potential role of OPN in regulation of metastatic behavior in these cell lines has not been previously examined. In this study, when 4T1 and 4T07 cells are compared, we demonstrate that a Ras-independent, phosphoinositide-3 kinase-dependent, c-Jun N-terminal kinase-dependent phosphorylation of c-Jun results in binding of an AP-1 c-Jun homodimer to the OPN promoter in 4T1 cells. This differential up-regulation of OPN gene transcription and protein expression in 4T1 cells conveys in vitro correlates of a metastatic phenotype. These results provide new insight into the transcriptional regulation of OPN as a key mediator of metastatic behavior in malignancy.
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c-Jun polyclonal Ab, goat c-Jun-Po4 (ser63/73) polyclonal Ab, rabbit p53 Polyclonal Ab, and goat JNK1/2-Po4 (Santa Cruz Biotechnology, Santa Cruz, CA) Ab. These antibodies were detected using the appropriate horseradish peroxidase-conju-
gated secondary antibody. The reactive proteins were visualized by means of the ECL kit (Amsham Bioscience). Relative protein expres-
sion was analyzed by laser densitometry and normalized to a β-actin standard.

Northern Blot Analysis—Total RNA was isolated using a TRIZol kit according to the manufacturer’s instructions (Invitrogen). RNA (10 μg) was separated by electrophoresis through denaturing 1.2% agarose-conju-
gated secondary antibody. The reactive proteins were visualized

Cell Migration and Invasion Assay—The migration and invasion assay were carried out in a Boyden Chamber system (Corning Glass
ware, Corning, NY). The 512 × 79 cells were seeded on the upper 12-well plates and incubated for 20 min at 30 °C. The microtiter plates were read at 80 °C.

Statistical Analysis—All data are presented as mean ± S.E. of three

RESULTS

Osteopontin Expression in 4T1 and 4T07 cells—We first ex-

Aminobutyric acid (GABA) is a neurotransmitter that regulates diverse cellular functions, including excitation and inhibition. In this study, the authors measured the expression of GABA in 4T1 and 4T07 cells using a competitive binding assay. The results showed that the expression of GABA was significantly higher in 4T1 cells than in 4T07 cells. This finding suggests that the GABAergic system may play a role in the regulation of cell proliferation and migration in these breast cancer cell lines.

Immunoblot analysis of cell lysate and culture media for cellu-

The results of this study indicate that the expression of GABA is significantly higher in 4T1 cells than in 4T07 cells. This finding suggests that the GABAergic system may play a role in the regulation of cell proliferation and migration in these breast cancer cell lines. Further studies are needed to elucidate the mechanisms underlying the observed differences in GABA expression between 4T1 and 4T07 cells.
actinomycin D (100 μg/ml), which was defined as time 0. Northern blots were then performed at 1, 2, 4, and 8 h. Expression of mRNA was normalized to that of the housekeeping gene, β-actin, and that of OPN at time 0 and then plotted semilogarithmically as a function of time to determine mRNA half-life. Half-life of OPN mRNA did not differ between the two cell lines. In 4T1 cells, OPN mRNA half-life was 5.0 ± 0.9 h, whereas that of 4T07 cells was 5.8 ± 0.7 h (p = not significant). These data indicate that OPN protein and mRNA are differentially expressed between 4T1 and 4T07 breast cancer cell lines.

**OPN Promoter Studies**—A 2.1-kb segment of the OPN promoter (OPN-Full), including the transcription start site, from 4T1 and 4T07 cells was amplified and submitted for automated sequencing in the Duke Sequencing Core Facility. The derived sequences were identical between the two cell lines. To further analyze differential OPN promoter activity, deletion-luciferase reporter constructs were transiently transfected into 4T1 and 4T07 cells. Serial deletion constructs demonstrated a significant 8-fold increase in luciferase activity between nt −69 and nt −258 in 4T1 cells. In contrast, luciferase activity in 4T07 cells was relatively stable among the various constructs. Sequences upstream of nt −258 to nt −2.1 kb did not further significantly increase OPN promoter activity in either 4T1 or 4T07 cells (data not shown). Using further serial deletion constructs between nt −69 and nt −258, the area of increased 4T1-associated OPN promoter activity was further localized to the length of the OPN promoter from nt −69 to nt −107 (Fig. 2a). Luciferase activity was noted to increase by more than 6-fold in 4T1 cells in the transition between the OPN −68 and OPN −107 promoter constructs.

The 38-bp segment between nt −69 and nt −107 was submitted to the TRANSFAC 6.0 data base to determine potential transcription factor binding sites. This analysis indicated the presence of two SP1 and one Yi, Oct 1, REB1, MyoD, D1, and AP-1 binding sites. To determine whether nuclear protein binds to any portion of this segment of OPN promoter, gel shift assays were performed using oligonucleotide sequences that corresponded to overlapping portions of this segment: nt −124 to nt −97 (Probe 1), nt −100 to nt −80 (Probe 2), and nt −84 to nt −64 (Probe 3) (Fig. 2b). Nuclear protein was extracted from 4T1 and 4T07 cells. With Probe 3, a single band was found to shift in the presence of 4T1 nuclear protein; in the presence of a 20-fold molar excess of cold Probe 3, this band was no longer present. No binding was seen with 4T07 nuclear protein. A consensus AP-1 binding sequence, TGACACA, is present in Probe 3 between nt −69 and nt −75. To determine whether AP-1 binds at this location in Probe 3 of the OPN promoter, supershift assays were performed with antibody to c-Jun. In this setting, the gel shift band was found to completely shift with c-Jun Ab, suggesting the presence of a c-Jun homodimer in 4T1 nuclear protein. Additional super shift studies were performed with antibody to c-Fos, JunB, and JunD; no band shift was noted (data not shown).

To further confirm potential AP-1 c-Jun homodimer binding performed and normalized to that of the β-actin standard. Blots are representative of three experiments. Data are expressed as mean ± S.E. (†, p < 0.01 versus 4T07).

**FIG. 1.** a, immunoblot analysis of OPN protein expression in 4T1 and 4T07 murine breast cancer cells. Protein levels were determined in cell lysates and concentrated culture media. Densitometric analysis was performed and normalized to that of β-actin standards. Blots are representative of three experiments. Data are expressed as mean ± S.E. (†, p < 0.01 versus 4T07).
at this site, DNA affinity chromatography was performed with the biotin-streptavidin technique. The sequence of the biotin-labeled oligonucleotide was: 5'-GTG GCA AAA ACC TCA UTGA CAC AUTC ACT CCA CCT-3', corresponding to positions nt -90 to nt -58 of the mouse OPN promoter. The putative corresponding transcription factor protein was then purified and isolated from nuclear extract isolated from 4T1 cells. When the eluate was analyzed by Western blot analysis, one major band was identified; this was excised and subjected to automated protein sequencing (Fig. 2c). Analysis of two separate tryptic digests yielded identical matches with only c-Jun. As a control, nuclear extract from 4T07 cells was also subjected to affinity chromatography with the biotin-labeled oligonucleotides. No band was found on Western blotting.
These data indicate that a c-Jun homodimer of AP-1 binds to the OPN promoter in 4T1 cells.

**Mutation Analysis of the OPN Promoter**—To further characterize this AP-1 site, mutation analysis was combined with transient transfection and gel shift assays. A point mutant of the AP-1 binding site was constructed in the full-length OPN promoter-reporter construct (M-OPN-Full) and OPN – 107 (M-OPN – 107). Using two-step PCR, the AP-1 binding site TGGCACA was mutated to TUCUAUTAUUA and confirmed by DNA sequencing. Transient transfection analysis was performed (Fig. 3a). Both M-OPN-107 and M-OPN-Full demonstrated significant depression of OPN promoter activity in 4T1 cells to levels equivalent to that of the OPN-69 construct. In contrast, transfection of mutant constructs into 4T07 cells did not alter OPN promoter activity.

Gelshift assays using 4T1 and 4T07 nuclear extracts were then performed using a labeled oligonucleotide with a mutated AP-1 site; 5’-AAA ACC TCA UTGA CAC AUTC AC-3’ was mutated to 5’-AAA ACC TCA UTCA TAT AUTC AC-3’ (Fig. 3b). In 4T1 cells, the typical AP-1 band was noted with the wild-type probe; in contrast, no binding was noted with the mutant probe. In addition, 20× excess unlabeled mutant probe did not compete with labeled wild-type probe for binding. No specific binding was found using nuclear protein from 4T07 cells. These mutation studies suggest that this AP-1 binding site is necessary for OPN promoter activity in 4T1 cells.

**Expression of c-Jun and JNK in 4T1 and 4T07 Cells**—Cell and nuclear protein from 4T1 and 4T07 cells were assayed to determine relative c-Jun protein levels (Fig. 4a). No differences in total or nuclear c-Jun protein levels were found between the two cell types. Because JNK activity is a well characterized activation mechanism for c-Jun, immunoblot analysis was also performed to determine c-Jun phosphorylated at Ser-63 and nuclear protein levels of constitutively phosphorylated c-Jun were noted to be significantly higher in 4T1 cells. β-Actin protein levels did not differ between 4T1 and 4T07 cells (data not shown). A specific pharmacologic inhibitor of JNK activity, SP600125, and an AS oligonucleotide inhibitor of JNK 1/2 (5’-TTT CAC TGA TCA ATA TAG TCC CTT-3’) were used in further studies. A dose-response relationship was determined for SP600125, and 100 nM SP600125 was found to ablate phosphorylation of c-Jun in 4T1 cells (Fig. 4b). No alteration in c-Jun and β-actin protein expression and no change in cell viability, as determined by trypan blue exclusion, were noted in the presence of this inhibitor. Transient transfection studies of the OPN promoter, gel shift assays, and immunoblots of OPN protein were then performed with SP600125 and/or AS-JNK (Fig. 4, c-e). Inhibition of JNK activity with SP600125 and/or treatment with AS-JNK 1/2 resulted in levels of OPN promoter activation in OPN-107 and OPN-Full that were not statistically different from that of OPN – 69. In a parallel fashion, using gel shift assays, AP-1 binding in 4T1 cells at nt – 69 to nt – 75 of the OPN promoter was ablated in the presence of SP600125 and/or treatment with AS-JNK 1/2. Finally, immunoblot analysis demonstrates dramatically diminished OPN protein levels in 4T1 cell lysates in the presence of SP600125 and/or treatment with AS-JNK 1/2. These results demonstrate that inhibition of JNK 1/2 activity significantly decreases AP-1 DNA binding, OPN promoter activity, and OPN protein expression in 4T1 cells.

Total and phosphorylated JNK 1/2 protein levels in the nuclear fraction and whole-cell lysates were measured in the 4T1 and 4T07 cell lines (Fig. 5). Monoclonal antibody to phosphorylated Thr-183 and Thr-185 on JNK-1/2 was used. Total cell lysate levels of JNK-1 and JNK-2 were similar between the two cell types; however, there was a significant difference in phosphorylated JNK-1 and JNK-2 between the two cell lines. This indicates that JNK-1/2 is constitutively phosphorylated in 4T1 cells.

**Ras-, PI3K-, and JNK-dependent OPN Expression in 4T1 and 4T07 Cells**—Because enhanced Ras activity can be a potential mechanism for constitutive activation of JNK 1/2, a dominant-negative form of Ras protein (pCMV-RasN17; BD Biosciences Clontech) that contains a serine-to-asparagine mutation at residue 17 was transfected into 4T1. Expression of this mutant Ras variant will “knock out” endogenous Ras ex-
pression in mammalian cells. Western blot analysis demonstrates dramatically enhanced Ras protein expression in 4T1 cells after transfection (Fig. 6a). In the presence of dominant-negative Ras protein, 4T1 levels of phosphorylated c-Jun, phosphorylated JNK1 and JNK2, and total OPN protein were then determined. Inhibition of Ras activity by dominant-negative
Ras protein did not alter levels of phosphorylated c-Jun, phosphorylated JNK, or total OPN, suggesting that alternative pathways for JNK activation are functioning in 4T1 cells to augment OPN transcription and protein expression.

PI3K is an additional signal transduction pathway that may activate JNK. To determine the role of PI3K, we used Δp85 (Gift from Dr. Julian Downward, Imperial Cancer Research Fund, London, UK), a dominant-negative form of the regulatory subunit of PI3K that is unable to bind the catalytic p110 subunit. Δp85 is thought to inhibit endogenous PI3K activation by sequestering upstream stimulatory proteins that bind to p85. When 4T1 cells are transfected with Δp85, levels of phosphorylated JNK-1 and JNK-2 protein are significantly decreased in association with decreased levels of phosphorylated c-Jun (Fig. 6b). OPN protein expression in the presence of PI3K inhibition is minimally detectable. These data suggest that 4T1 cells possess constitutively active PI3K, which ultimately increases OPN protein expression. To corroborate the corroborative role of PI3K in OPN expression in 4T1 cells, transient transfection studies were performed in 4T1, 4T1/Δp85, 4T1 + LY294002, a pharmacologic inhibitor of PI3K activity, using the OPN-107 and OPN-Full constructs (Fig. 6c). In the presence of Δp85 and LY294002, OPN promoter activation is significantly decreased by 6–8-fold in both the OPN-107 promoter and the full-length OPN promoter constructs. In association with the previous Western blot data in Fig. 6b, these results suggest that PI3K-dependent JNK- and c-Jun activation constitutively up-regulate OPN promoter activity to increase OPN protein expression.

**FIG. 5.** Immunoblot analysis of JNK-1/2 and phosphorylated JNK-1/2 protein expression in 4T1 and 4T07 murine breast cancer cells. Total and phosphorylated JNK-1/2 protein levels in the nuclear fraction and whole-cell lysates were measured in the 4T1 and 4T07 cell lines. Monoclonal antibody to phosphorylated Thr-183 and Thr-185 on JNK-1/2 was used. Blot is representative of three experiments.

**FIG. 6. a,** effect of DN-Ras transfection on OPN signal transduction pathway in 4T1 cells. Determination of nuclear expression of c-Jun PO4 used antibody directed at c-Jun phosphorylated at Ser-63 and Ser-73. Phosphorylated JNK-1/2 protein levels in the nuclear fraction were measured using monoclonal antibody to phosphorylated Thr-183 and Thr-185. Ras, β-actin, and OPN protein expression was determined in 4T1 cell lysates. Blot is representative of three experiments. **b,** effect of PI3K Δp85 transfection on OPN signal transduction pathway in 4T1 cells. Determination of nuclear expression of c-Jun PO4 used antibody directed at c-Jun phosphorylated at Ser-63 and Ser-73. Phosphorylated JNK-1/2 protein levels in the nuclear fraction were measured using monoclonal antibody to phosphorylated Thr-183 and Thr-185. PI3K Δp85, β-actin, and OPN protein expression was determined in 4T1 cell lysates. Blot is representative of three experiments. c, effect of PI3K inhibition on OPN promoter deletion constructs in 4T1 murine breast cancer cells. The lengths of the osteopontin promoter fragments tested were OPN-69 (−69 to +79), OPN-107 (−107 to +79), and OPN-Full. LY294002 (LY; 25 μM), a pharmacologic inhibitor of PI3K, or Δp85, a dominant-negative form of the regulatory subunit of PI3K that is unable to bind the catalytic p110 subunit, was transiently transfected in selected instances. Firefly luciferase activity was normalized for transfection efficiency using Renilla reniformis luciferase activity (pRL-SV40). The data are presented as mean -fold luminescence for three independent experiments performed in triplicate. (*, p < 0.01 versus OPN −69).
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Fig. 7. Adhesion, migration, and invasion characteristics of 4T1 and 4T07 murine breast cancer cells. Adhesion, migration and invasion assays were performed as described under “Materials and Methods.” In selected instances, SP600125 or AS-JNK 1/2 was added to inhibit JNK activation. AS-OPN was also used to inhibit OPN expression. Data are expressed relative to that of 4T1 cells; values are denoted as mean ± S.E. of three experiments. (*, p < 0.01 versus 4T1 + SP600125 and 4T1 + AS-JNK 1/2; #, p < 0.01 versus 4T1 + AS-OPN).

OPN is a secreted glycoprotein that is rich in aspartate and sialic acid residues and contains functional domains for calcium-binding, phosphorylation, glycosylation, and extra-cellular matrix adhesion (14). OPN seems to mediate cell-matrix interactions and cellular signaling through binding with integrin, primarily αVβ3, and CD44 receptors. OPN is expressed in multiple species, including humans and rodents (15). Cells that express OPN include osteoclasts, osteoblasts, kidney, breast and skin epithelial cells, nerve cells, vascular smooth muscle cells, and endothelial cells (14, 16–19). Activated immune cells such as T cells, natural killer cells, macrophages, and Kupffer cells also express OPN. The secreted OPN protein is widely distributed in plasma, urine, milk, and bile (20–22). Constitutive expression of OPN exists in several cell types, but induced expression has been detected in T lymphocytes, epidermal cells, bone cells, macrophages, and tumor cells in remodeling processes such as inflammation, ischemia-reperfusion, bone resorption, and tumor progression (14, 18, 19). A variety of stimuli, including phorbol 12-myristate 13-acetate, 1,25-dihydroxyvitamin D, basic fibroblast growth factor, tumor necrosis factor-α, interleukin-1, interferon-γ, and lipopolysaccharide, seemed to up-regulate OPN expression (14, 18, 19, 23). OPN has multiple molecular functions that mediate cell adhesion, chemotaxis, macrophage-directed interleukin-10 suppression, stress-dependent angiogenesis, prevention of apoptosis, and anchorage-independent growth of tumor cells (14, 18, 19, 23). A substantial body of data has recently linked OPN with the regulation of metastatic spread by tumor cells. However, the molecular mechanisms that define the role of OPN expression in tumor metastasis are incompletely understood.

With regard to breast cancer, OPN expression has been correlated with breast cancer metastasis. Fedarko and colleagues (9) demonstrated significantly increased serum levels of OPN in 20 breast cancer patients compared with those of normal control subjects. When breast cancer primary tumors were compared with bone metastases, elevated cellular OPN expression, as detected by immunohistochemistry, was noted to be significantly higher with bone metastasis (24). In addition, using expression microarray analysis, Korkola and colleagues (25) found that OPN is differentially expressed between lobular and ductal breast carcinomas. In contradistinction to the bulk of the correlative clinical data, Coppola et al. (7) recently surveyed immunohistochemical expression of OPN in a variety of human tumors. These investigators found little or no OPN expression in 23 of 26 breast cancers, although increased expression of OPN was noted in a number of other cancer types. Although speculative, these divergent pieces of data suggest a differential role for OPN in metastasis in contrast to tumorigenesis. As a result, OPN is the subject of a great deal of investigative interest as the result of its presumed primary role in mediating metastasis.

A number of studies have addressed the potential mechanisms by which exogenous OPN stimulates cell motility in breast cancer. However, little is known of the regulation of OPN expression. Several studies have addressed transcriptional regulation of OPN in the context of breast malignancy. When small 1-kb fragments of genomic DNA obtained from human malignant breast cancer lines are transfected into a benign rat mammary cell line, OPN gene expression is up-regulated with subsequent metastatic behavior when implanted into syngeneic rats (26). Subsequent work indicated that the endogenous inhibitory Tcf-4 is sequestered and as a result, OPN transcription is increased. Work by this same group (27) has also shown that Ets, PEA3, β-catenin-Lef-1, and c-Jun synergize to increase OPN transcription in a rat mammary cell line. It is interesting that although c-Jun was found to be essential for OPN transcription, these authors identified an AP-1 binding site at nt ~1872 and did not examine the nt ~107 region, which is integral to our observations. Finally, Liu and colleagues (28) investigated the transcriptional activation of the OPN promoter in the human metastatic cancer cell line A2058. They found that the region between ~170 and ~127 in the human OPN promoter acts as an enhancer element. This region was found to contain overlapped AML-1 and CCAAT/enhancer-binding protein binding site motifs. Functional analysis showed that the CCAAT/enhancer-binding protein α was more potent than the complex of AML-1 and its cofactor CCAAT-binding transcription factor β to up-regulate the OPN promoter. The authors conclude that AML-1 and CCAAT/enhancer-binding protein α play an important role in the up-regulation of the OPN gene in metastatic tumor cells. When our results are examined against this background, the relevant role of c-Jun corroborates the findings of El-Tanani and coworkers (27). Indeed, other activators, co-activators, or repressors may contribute to OPN expressionional programming.

Although OPN transcription has received some attention, even less is known of the upstream signal transduction pathways that regulate endogenous OPN expression in the setting of malignancy. Denhardt and coworkers (29) have studied the potential role of Ras in OPN regulation. Ras signaling in fibroblasts and epithelial cells stimulates binding of the Ras-response factor to a Ras-activated enhancer in the OPN promoter to enhance OPN transcription. In a further study, Wu and Denhardt (30) used 3T3 cells derived from wild-type and OPN-deficient mice and transformed by transfection with oncogenic Ras to assess the role of OPN in transformation in vitro and in tumorigenesis in vivo. Ras-transformed cell lines from both wild-type and OPN-deficient mice could form colonies in soft agar, indicating that this process can occur in the absence of
OPN. However, the ability of the OPN-deficient cell lines to form colonies was reduced compared with wild-type cell lines. Their results indicate that maximal transformation by Ras requires OPN expression, and implicate increased OPN expression as an important effector of the transforming activity of the Ras oncogene. However, in 4T1 cells, our results suggest that OPN expression is not dependent upon Ras activity.

The contribution of PI3K to a metastatic phenotype in breast cancer cells has been studied. Verbeek and colleagues (31) have demonstrated a critical role for PI3K in the migration characteristics of the ZR75–1 human breast cancer cell line. In a similar fashion, Tan and colleagues (32) found that heregulin-β1 activation of PI3K enhances breast cancer cell aggregation in MCF-7 and SKBR3 breast cancer cells. Finally, van Golen et al. (33) examined the role of PI3K in the SUM149 inflammatory breast cancer cell line and demonstrated a critical role for PI3K in anchorage-independent growth and survival. Very little is known with respect to the participation of PI3K in the expression of OPN. Zhang and colleagues (34) used benign breast epithelial cell lines to demonstrate that PI3K-Akt kinase activity can regulate accelerated cell division and increase OPN expression. Otherwise, in HepG2 cells, inhibition of the upstream activator of Akt, PI3K, using wortmannin significantly inhibited epidermal growth factor induced OPN expression (35). The underlying transcriptional regulatory mechanisms have not been previously addressed in the context of OPN-associated metastatic phenotypes.

In this study, we demonstrate that 4T1 breast cancer cells overexpress OPN compare with a sister subclone line, 4T07 cells. Enhanced expression of OPN is associated with significantly enhanced properties of adhesion, migration, and invasion. On the other hand, inhibition of OPN expression in these 4T1 cells ablates this more aggressive metastatic phenotype. Elevated OPN production is transcriptionally mediated via a Ras-independent, PI3K-dependent, and JNK-dependent pathway that activates c-Jun with a subsequent increase in binding of an AP-1 c-Jun homodimer to a region of the OPN promoter. The mechanism by which PI3K is constitutively activated is presently unknown, but the potential contribution of growth factors and/or phosphatase and tensin homolog is the subject of ongoing studies. Investigators have previously demonstrated a correlation between decreased PTEN expression in breast cancer. Garcia and colleagues (36) found that 40% of breast cancers show a decrease or absence of PTEN, with hypermethylation of the PTEN promoter in 48%. Likewise, Chung and coworkers (37) also found that loss of PTEN expression was common in the setting of breast cancer, and this loss correlates with tumor progression and lymph node metastasis. These data suggest that PTEN/PI3K and their relationship with OPN may come to be a fruitful area of investigation.
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