

# Endogenous Osteonectin/SPARC/BM-40 Expression Inhibits MDA-MB-231 Breast Cancer Cell Metastasis

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## Abstract

**Skeletal metastases occur with high incidence in patients with breast cancer and cause long-term skeletal morbidity. Osteonectin (SPARC, BM-40) is a bone matrix factor that is an *in vitro* chemoattractant for breast and prostate cancer cells. Increased expression of osteonectin is found in malignant breast tumors. We infected MDA-231 breast cancer cells with an adenovirus expressing osteonectin to examine the role of osteonectin expression in breast cancer cells and its effect on metastasis, in particular to bone. Expression of osteonectin did not affect MDA-231 cell proliferation, apoptosis, migration, cell aggregation, or protease cleavage of collagen IV. However, *in vitro* invasion of these osteonectin-infected cells through Matrigel and colony formation on Matrigel was decreased. Interestingly, high osteonectin expression in MDA-231 cells inhibited metastasis in a dose-dependent manner to many different organs including bone. The reduction in metastasis may be due to decreased platelet-tumor cell aggregation, because exogenous osteonectin inhibited platelet aggregation *in vitro* and the high osteonectin expression in MDA-231 cells reduced tumor cell-induced thrombocytopenia *in vivo* compared with control-infected cells. These studies suggest that high endogenous expression of osteonectin in breast cancer cells may reduce metastasis via reduced invasive activity and reduced tumor cell-platelet aggregation.** (Cancer Res 2005; 65(16): 7370-7)

## Introduction

Osteonectin [secreted protein acidic and rich in cysteine (SPARC), basement membrane protein-40 (BM-40)] is a secreted, phosphorylated, calcium-binding glycoprotein that was first isolated from bone where it comprises 15% of the noncollagenous protein (1). Osteonectin-null mice have osteopenia, cataracts, decreased skin tensile strength, increased adipose number, and accelerated wound closure (for review, see ref. 2). Although a cellular receptor for osteonectin has yet to be identified, it is known that this matricellular protein interacts with various matrix molecules, including collagen I, vitronectin, and thrombospondin (3–5). Osteonectin plays a role in the regulation of cell adhesion,

proliferation, migration, and tissue remodeling and is expressed during development and processes requiring extracellular matrix turnover such as wound healing and tumor progression (3, 4, 6–8).

Elevated osteonectin levels occur in a multitude of malignant tumors, including breast, brain, esophageal, and prostate carcinomas, as well as gliomas and melanomas (for reviews, see refs. 2, 9), suggesting that increased expression is associated with malignancy. The precise role that osteonectin plays in tumor growth and progression remains unknown. The promoting or inhibiting effects of osteonectin in different cancers seem dependent upon the cell type, the concentration, and the presence of full-length or proteolytic fragments of osteonectin (9). Although osteonectin promotes melanoma and squamous cell tumor growth (10, 11) and glioma invasion (12), there are reports that decreased osteonectin expression is associated with increased tumorigenicity and metastasis of human ovarian carcinoma cells (13) as well as transformed fibroblasts (14, 15). In neuroblastomas, expression of osteonectin is inversely correlated with malignant progression, and treatment of these tumors with osteonectin results in impaired tumor growth *in vivo* (16). Additionally, Lewis lung carcinoma, T-cell lymphoma, and pancreatic tumors all grew larger and more rapidly in osteonectin-null mice than tumors grown in control wild-type mice (17). These results suggest that osteonectin has multiple roles in tumor growth and progression.

Bellahcene and Castronovo (18) suggested that the increased expression of osteonectin in malignant breast tumors might play a role in the preferred homing of breast cancer cells to bone. Breast cancer is one of the most frequently diagnosed cancers in women and ranks as the second leading cause of cancer death among women. Approximately 60% to 70% of breast cancer patients who have died or are dying have bone metastases (19). Therefore, identifying the mechanisms of tumor metastasis to bone is critical to therapeutic approaches. Osteonectin is a factor in bone extract that promotes breast and prostate cancer cell invasion to bone *in vitro* (20). Additionally, bone extracts from osteonectin-null mice show reduced chemoattractant activity for prostate cancer cells (21). Here, we determine the effect of endogenous expression of osteonectin on invasion and metastasis of breast cancer cells to bone, using the osteonectin-negative MDA-231 breast carcinoma cell line. We find that induced expression of osteonectin in MDA-231 cells does not affect cell proliferation, apoptosis, cell aggregation, or migration but does inhibit tumor cell invasion *in vitro*. In addition, we find that metastases, including metastases to the bone, are inhibited by high cellular expression of osteonectin. We also found that exogenous osteonectin inhibits platelet aggregation *in vitro* and that high osteonectin expression

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in MDA-231 cells reduces tumor cell-induced thrombocytopenia *in vivo* compared with control infected cells. These results suggest that osteonectin inhibits MDA-231 breast cancer metastasis by decreasing invasion and tumor cell-platelet interaction.

## Materials and Methods

**Cell culture.** Human MDA-231, MDA-435, and HS578T breast carcinoma cell lines were maintained in DMEM/F-12 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). The MDA-231 and MDA-435 cell lines were transfected to constitutively express green fluorescent protein (GFP; ref. 22). The 293 cells were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin.

**Construction of osteonectin adenovirus.** The human osteonectin-coding region was subcloned from the *XhoI/BamHI* sites of pBluescript-hON2 (a gift from Drs. Marian Young and Larry Fisher, NIDCR, MD) and inserted in the sense orientation into the *SalI/BamHI* sites of the pACCMVpLpA shuttle vector to construct AdCMV-osteonectin. This resulted in the plasmid pACCMV-hON2, containing the cytomegalovirus (CMV) promoter/enhancer, hON2 cDNA, and a polyadenylate sequence. AdCMV-OSN was generated by homologous recombination of pACCMV-hON2 with pJM17 in 293 cells (23). The replication-deficient adenovirus Addl312 (a gift from Dr. T. Shenk, Princeton University, NJ; ref. 24) was used as a control virus. Both viruses were propagated in 293 cells, single viral plaques were isolated, amplified, purified on CsCl gradients, and titered by plaque assays.

A series of infections using various dilutions of AdCMV-OSN were conducted to determine the optimal multiplicity of infection (MOI) in which expression of osteonectin occurred with low cytotoxicity. The advantage of using adenovirus is that over 95% of the cells are infected and express osteonectin, making subcloning unnecessary; therefore, we were able to test a heterogeneous population of tumor cells expressing osteonectin.

**SDS-PAGE and immunoblot analysis.** MDA-231 breast carcinoma cells were infected for 1 hour in serum-free medium, grown on plastic in serum-containing medium, and then serum starved 18 hours before collecting the cell-conditioned medium. The cells were lysed in mPER buffer (Pierce, Rockford, IL), and the conditioned media were concentrated. Samples (cell lysate or media), normalized based on protein determinations (Bio-Rad detergent-compatible protein assay, Hercules, CA), and known amounts of purified bovine bone osteonectin (Hematologic Technologies, Inc., Essex Junction, VT), serially diluted (5, 10, 20, and 40 ng), were subjected to SDS-PAGE using 10% (w/v) gels (Bio-Rad). The protein in these gels was transferred to nitrocellulose and then immunoblotted using 1 µg/mL of mouse anti-human osteonectin monoclonal antibody (AON-5031, Hematologic Technologies) or 0.85 µg/mL of mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (6C5, Research Diagnostics, Inc., Flanders, NJ) in 5% milk-PBS with 0.05% Tween 20 (T-PBS). Membranes were probed with horseradish peroxidase-labeled goat anti-mouse IgG (Pierce) in 5% milk-T-PBS, and reactive proteins were detected using SuperSignal West Dura Extended Duration Substrate (Pierce). Chemiluminescence was detected using a Fuji LAS-1000 luminescent image analyzer "intelligent dark box" (Fujifilm Medical Systems USA, Inc., Stamford, CT) using exposure times at subsaturation levels. The immunoblots were quantified using Fujifilm Science Lab 98 Image Gauge software V3.3 (Fuji Photo Film Co., Ltd., Stamford, CT; ref. 25). A standard curve, within the linear range for quantification, was generated from known amounts of osteonectin. The amount of osteonectin in cell lysate and secreted after 1 day of infection was normalized to cell number. Each experiment was repeated at least thrice.

**Proliferation assays.** MDA-231 breast carcinoma cells were grown on 96-well tissue culture plates coated with either 50 µL per well of Matrigel (basement membrane extract, 11.5 mg/mL, Trevigen, Inc., Gaithersburg, MD) or neutralized Vitrogen-100 bovine collagen I (2.9 mg/mL, Cohesion, Palo Alto, CA; ref. 26) or uncoated. The cells were seeded immediately after

infection or 10 days postinfection in quadruplicate at a density of  $3.0 \times 10^3$  cells per well and grown for 4 days in serum-free media or in media containing 10% FBS. Growth was assessed 2 hours after addition of the CellTiter 96 AQ<sub>1</sub> One Solution Reagent according to the manufacturer's instructions (Promega, Madison, WI). Each experiment was repeated at least thrice. Data were imported into GraphPad Prism software V3.0a (San Diego, CA) for statistical analysis. A one-way ANOVA was done to determine the statistical differences among sample means. The conservative Bonferroni's multiple comparison post-test was combined with the ANOVA to compare differences between the mean values for each MOI of Addl312 and AdCMV-OSN.

**Matrigel colony assay.** MDA-231 cells were infected with either AdCMV-OSN or Addl312 or were not infected (control) and grown on Matrigel-coated (200 µL at 11.5 mg/mL) 14-mm microwell cover glass (MatTex Co., Ashland, MA) for 4 days, and then colonies were imaged using fluorescent optics on a Zeiss Axiovert 100 (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Five fields per sample were captured using an AxioCam digital camera and analyzed with Axiovision software V3.1 (Carl Zeiss MicroImaging). The size of the colonies was assessed using MetaMorph software V4.6r9 (Universal Imaging, Downingtown, PA). This experiment was repeated thrice. The colony size of AdCMV-OSN-infected MDA-231 cells was normalized to Addl312-infected cells. Statistical analysis was done using Prism software, ANOVA, and Bonferroni's post-test as described above.

**Apoptosis assays.** Cells were infected and grown as described above in the Matrigel colony assay. After 4 days, the cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 20 minutes, and the terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay was done following the manufacturer's protocol (Roche, Indianapolis, IN). Positive-stained cells were viewed with a LSM 510 Zeiss confocal microscope (Carl Zeiss MicroImaging).

**Aggregation assay.** MDA-231 cells were infected with either Addl312 or AdCMV-OSN as described above and grown overnight before collection with trypsin. The hanging drop assay for aggregation was done as described previously (27).

**Cell motility.** Cell motility was measured as described (28) by time-lapse video microscopy. MDA-231 cells were infected as described above. At least six cells were tracked per sample and each sample was run in duplicate. Each experiment was repeated at least thrice. Statistical analysis was done using Prism software, ANOVA, and Bonferroni's post-test as described above.

**Scratch "wound" migration assay.** Confluent monolayers (in triplicate) of MDA-231 cells infected for 24 hours with either AdCMV-OSN or Addl312 or not infected (control) were "wounded" using the narrow end of a pipette tip (for 0.1-1.0 mL). The wounds were photographed daily (0-4 days) in the same area. Three random measurements per wound per time point were measured using MetaMorph software. The experiment was repeated thrice. Statistical analysis was done using Prism software and linear regression analysis.

***In vitro* invasion assay.** *In vitro* invasion assays were done as described (29) with the following modifications. The upper chamber of FluorBlok 24-multiwell inserts (8 µm pore size, BD Biosciences, San Jose, CA) were coated with 50 µL of Matrigel (0.1 mg/mL) and dried overnight at 25°C. Twenty-four hours post-infection, MDA-231 cells were collected with Versene (Invitrogen), and  $2 \times 10^5$  cells in serum-free media containing 0.1% bovine serum albumin (BSA) were added to the upper chambers. Serum-free medium containing 0.1% BSA was added to the lower chambers. After 24 hours, the cells were stained with calcein AM (5 µg/mL, Invitrogen) for 30 minutes at 37°C and then fluorescence was measured in a Wallac 1420 Victor<sup>2</sup> multilabel plate reader (Perkin-Elmer, Shelton, CT). Each sample was measured in triplicate, and each experiment was repeated thrice. Data were analyzed using Prism, and statistical analysis was done as described above with ANOVA and Bonferroni's post-test.

**Protease activity assay.** DQ-collagen IV (Invitrogen) is a quenched fluorescent substrate. Proteolytic activity is detected by the presence of fluorescein (30). DQ-collagen IV (25 µg/mL) was mixed with Matrigel, and the microwell cover glasses were coated as described above. MDA-231 cells were infected and seeded onto the DQ-Collagen IV-Matrigel as described above. After 72 hours, protease activity was assessed by viewing the fluorescence with an LSM 510 Zeiss confocal microscope.

**Intracardiac injections.** MDA-231 cells were infected as described above and grown overnight before collection with Versene (Invitrogen). The cells ( $2 \times 10^5$  cells per mouse in 200  $\mu$ L of PBS) were injected into the left cardiac ventricle of 4-week-old athymic female nude mice (31, 32). At least nine mice per group were injected and the experiment was repeated at least twice. At  $\sim 5$  weeks, mice were sacrificed when they began to show adverse signs of disease, including weight loss and paralysis. Each mouse was dissected and the lung, heart, liver, kidneys, pancreas, spleen, and all bones, including the skull, ribs, humerus, ulna, radius, femur, tibia, and spine were examined for fluorescent tumors. The visible tumors were counted using a Zeiss Stemi SV11 Apo dissection microscope equipped with a GFP filter set. To examine tumors that may have been present within the liver, we made macroscopic sections in the liver with a scalpel. ANOVA and Bonferroni's post-test were used for statistical analysis as described above on data pooled from at least two experiments.

**Immunohistochemistry.** After imaging GFP tumors, the bone tumors were fixed in 4% paraformaldehyde, paraffin embedded, sectioned (5  $\mu$ m), and stained with H&E or anti-osteonectin antibodies. Tissue sections were deparaffinized, dehydrated with a graded series of ethanol, washed with H<sub>2</sub>O and then PBS, and finally treated with 1:500 dilution of rabbit anti-human (LF37) or mouse (LF23) osteonectin IgG (gifts from Dr. Larry Fisher; ref. 33) in 10% normal donkey serum (NDS) at room temperature overnight. Slides were then washed and incubated for 1 hour with a 1:100 dilution of CY3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, Inc., West Grove, PA) and a 1:250,000 dilution of SYBR Green (Invitrogen) in 5% NDS. Nonimmune serum was used as a control. Positive staining was viewed using a LSM 510 Zeiss confocal microscope.

**Platelet aggregation assay.** Human platelets were collected either as primary apheresis products (collected directly) or secondary to a leukapheresis product (prepared by manual centrifugation of the leukapheresis product) from healthy donors. All collections were done at the NIH Division of Transfusion Medicine with Institutional Review Board approval. Platelet-poor plasma (PPP) was obtained by centrifugation at  $2,000 \times g$  for 10 minutes at room temperature. Platelet-rich plasma (PRP) was adjusted to  $2 \times 10^8$  platelets/mL with PPP. PRP was preincubated with either 0, 9.5, 19, or 38  $\mu$ g/mL of bovine bone osteonectin (Hematological Technologies) for 20 minutes. Platelet aggregation was measured in a PAP-4 Platelet Aggregation Profiler (BIODATA Corp., Horsham, PA) at 37°C with constant stirring at 1,000 rpm. The aggregometer was calibrated with 200  $\mu$ L of PPP to express 100% transmission and with 200  $\mu$ L of PRP to express 0% optical transmission. ADP (20  $\mu$ mol/L) and epinephrine (300  $\mu$ mol/L) were added to each sample within 1 minute. Aggregation was measured for 10 minutes. Each assay was repeated thrice using platelets from three different patients. The difference in final aggregation compared with the nontreated sample was averaged. ANOVA and Bonferroni's post-test were used for statistical analysis as described above.

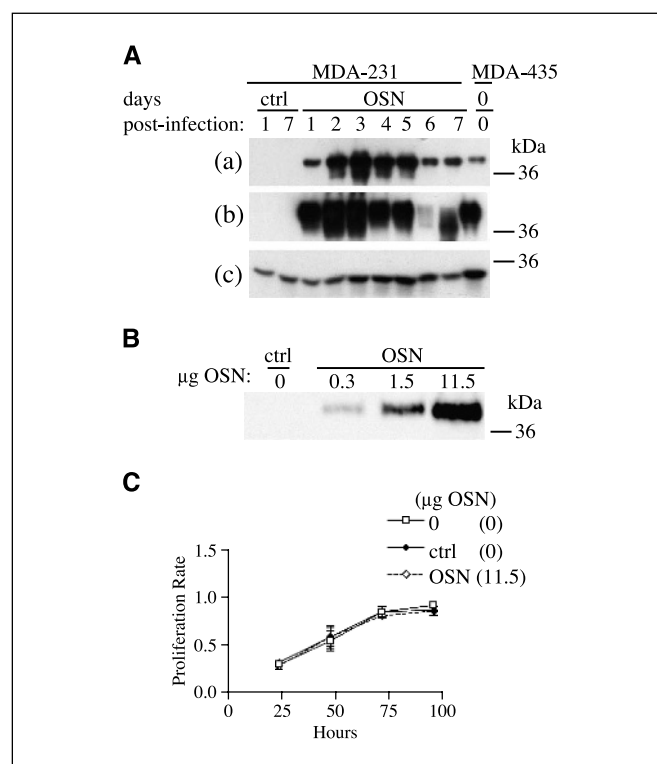
**Tumor cell-induced thrombocytopenia.** MDA-231 cells were infected with either Add1312 or AdCMV-OSN for 24 hours before intracardiac injection as described above. Sixty minutes after intracardiac injection of PBS or tumor cells, 0.4 mL of blood was collected in 3.8% sodium citrate buffer (1:9 ratio of whole blood) from each mouse. Platelet and RBC counts were determined using an ABX Pentra 60C+ Hematology Analyzer (ABX Diagnostics, Irvine, CA). The platelet count was normalized to RBC count. ANOVA and Bonferroni's post-test were used for statistical analysis as described above.

## Results

**Expression of osteonectin in MDA-231 breast cancer cells infected with AdCMV-OSN.** Many breast cancer cell lines express high levels of osteonectin (34, 35), and osteonectin expression is elevated in high-grade breast tumors (18, 36). We examined different levels of osteonectin expression in MDA-231 breast cancer cells for three reasons: (a) These cells metastasize to the bone in intracardiac models (32) and do not express detectable levels of osteonectin protein (34, 35, 37). (b) MDA-435

cells, which express high levels of osteonectin (34), metastasize to bone (38) but have melanoma and breast markers (39). Because the cell type may be important in the effect of osteonectin on tumor growth and metastasis, MDA-435 cells were thus not suitable for this study. (c) Other breast cancer cell lines, whether weakly invasive or highly invasive, do not metastasize well to bone.

AdCMV-OSN as well as control vector Add1312 were infected into MDA-231 breast carcinoma cells to examine the biological effects of osteonectin. We did not detect osteonectin protein in either the cell lysate or conditioned media from MDA-231 cells infected with control vector Add1312 (Fig. 1A). Osteonectin protein was present 1 day after infection with AdCMV-OSN in both the cell lysate and conditioned media (Fig. 1A). High expression of osteonectin protein is detected in the first 2 to 4 days post-infection, after which the protein levels decrease. Osteonectin was still present at 11 days, although at a reduced expression, as expected, and was no longer detectable at 14 days (data not shown). Osteonectin was immunolocalized in 95% of the cells 1 day post-infection with AdCMV-OSN and 100% of the cells expressed GFP (data not shown). After increasing amounts of AdCMV-OSN were infected into the MDA-231 cells, we quantitated the total amount of osteonectin expressed. One day post-infection,  $\sim 20\%$  of the protein was cell associated, and 80% of the protein was secreted



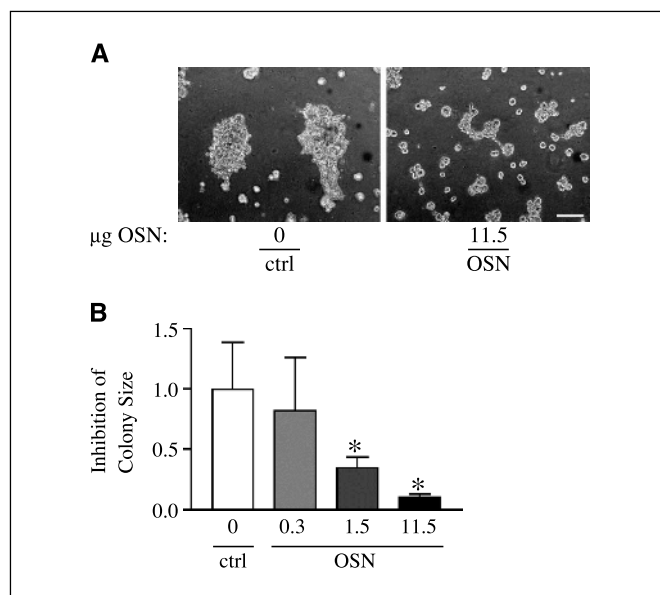
**Figure 1.** Expression of osteonectin protein in MDA-231 breast cancer cells after infection with AdCMV-OSN. This expression does not affect cell proliferation. MDA-231 cells were infected with either Add1312 (*ctrl*) or AdCMV-OSN (*OSN*) or not infected (*0*). A and B, samples were analyzed by immunoblotting. A, osteonectin in (a) cell lysate and (b) conditioned media; (c) GAPDH in cell lysates. B, osteonectin in conditioned medium from cells infected with adenovirus for 1 day. The amount of osteonectin secreted/ $10^6$  cells/24 h was quantitated. C, growth of MDA-231 cells cultured on plastic in the presence of 10% FBS. Points, means (where  $n = 4$  from one representative experiment of three with similar results); bars,  $\pm$ SE. The highest dose is shown as a representative of all doses used.

from the cell with amounts ranging from 0.3 to 11.5  $\mu\text{g}$  of osteonectin secreted/ $10^6$  cells/24 hours (Fig. 1B). The highest concentration of osteonectin protein expressed (11.5  $\mu\text{g}$  of osteonectin secreted/ $10^6$  cells/24 hours) in the MDA-231 cells 1 day post-infection was similar to that expressed by MDA-435 (Fig. 1A and B) and by HS578T (data not shown) breast cancer cell lines.

**Proliferation of MDA-231 cells is not affected by osteonectin.** Because osteonectin inhibits proliferation in some cell types (6, 13, 37), we examined whether osteonectin affected the proliferation of MDA-231 cells. Expression of osteonectin at all levels did not affect cell proliferation when the cells were grown in serum-free media (data not shown) or serum media on either plastic (Fig. 1C), collagen I, or Matrigel (data not shown) for the first 4 days after infection. Proliferation rates 10 to 14 days post-infection were also unaffected by osteonectin expression (data not shown).

**Invasive colony formation by MDA-231 cells is inhibited by expression of osteonectin.** Matrigel colony assays have been used to determine the invasiveness of cancer cell lines (29). Either AdCMV-OSN or Addl312 (control) was infected into MDA-231 cells, and the cells were grown on Matrigel. After 4 days, mean colony size was quantitated. Colony size and stellate colony formation of MDA-231 cells expressing high levels of osteonectin (1.5 and 11.5  $\mu\text{g}$  secreted/ $10^6$  cells/24 hours) were inhibited (Fig. 2A and B). The difference in colony size was not due to a difference in proliferation rates (Fig. 1C), apoptosis, or aggregation of the colonies (Table 1). Stellate morphology on Matrigel is indicative of malignancy involving both increased migration and invasion (29), suggesting that the cells expressing osteonectin may be less invasive and/or migratory.

**Expression of osteonectin does not affect *in vitro* cell migration by MDA-231 cells but does decrease cell invasion.** Exogenous osteonectin is a chemotactic factor that increases cell migration and invasion of renal (40), prostate, and breast (20)



**Figure 2.** Osteonectin inhibits stellate colony formation by MDA-231 cells plated in the Matrigel outgrowth assay. MDA-231 cells were infected with either Addl312 (*ctrl*) or AdCMV-OSN (*OSN*) and plated on Matrigel. *A*, images of live cell colonies after growth on Matrigel for 4 days. Bar, 100  $\mu\text{m}$ . *B*, colony size of cells infected with AdCMV-OSN was normalized to that of Addl312 infected cells. Representative of three experiments with similar results. *Columns*, mean ( $n > 300$ ); *bars*,  $\pm$ SE. \*,  $P < 0.05$ , significant difference from control as determined by one-way ANOVA with Bonferroni's post-test.

**Table 1.** Effect of osteonectin on MDA-231 activities

Assay	Effect of osteonectin
Proliferation	None
Colony size on Matrigel	Inhibition
Apoptosis (TUNEL staining)	None
Aggregation	None
Migration (scratch and time-lapse)	None
Invasion (Boyden chamber through Matrigel)	Inhibition
Protease Activity (DQ-Collagen IV)	None
Tumor cell-platelet aggregation*	Inhibition
Reduction of platelet number (platelet count <i>in vivo</i> )	Inhibition
Metastases, <i>in vivo</i> (intracardiac)	Inhibition

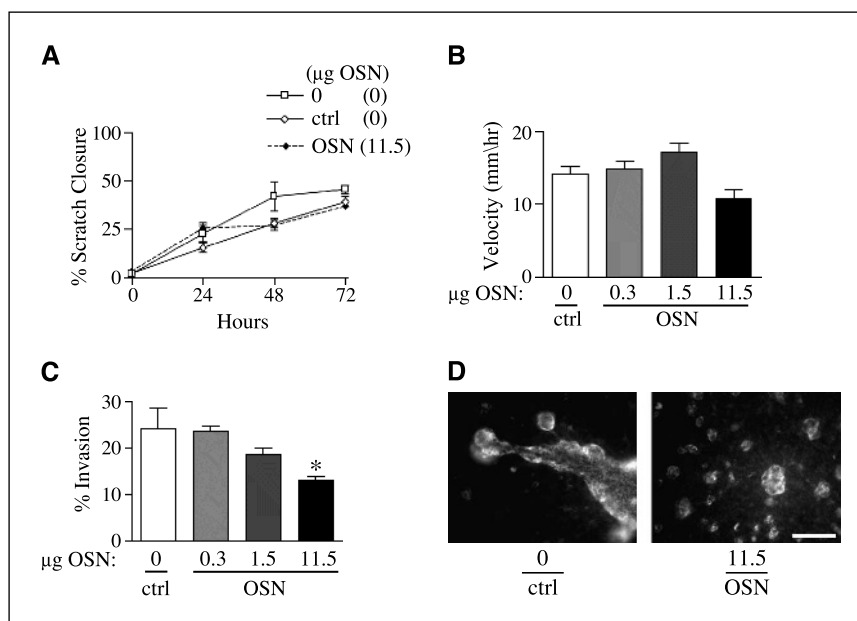
NOTE: These assays, measured as indicated in parentheses, were done *in vitro* unless otherwise noted. An effect of osteonectin expression is noted only if it was significantly different from the control-infected cells.

\*Osteonectin was added exogenously. In all other experiments, results are given for endogenous expression of osteonectin at the highest level secreted (11.5  $\mu\text{g}/10^6$  cells/24 h).

carcinoma cells. In addition, endogenous expression of osteonectin alters the migration of glioma cell lines, with either increased or decreased migration observed depending on the type of extracellular matrix proteins present (41). Therefore, we did two different migration assays: scratch wounding of confluent monolayers and video microscopy of single cells, to measure the migration of the MDA-231 breast tumor cells infected with either AdCMV-OSN or Addl312 (control) or not infected. Scratch assays were done over 4 days with all doses of osteonectin expression (Fig. 3A). Additionally, we tested the migration of the cells across plastic, recording the motility of single cell migration using time-lapse video microscopy (Fig. 3B). In both assays, expression of all levels of osteonectin in MDA-231 breast cancer cells did not significantly affect cell migration. Although osteonectin did not alter migration, the reduction of colony formation by osteonectin in Matrigel suggested that osteonectin may affect invasion.

Significantly less MDA-231 cells expressing high levels of osteonectin (11.5  $\mu\text{g}$  secreted/ $10^6$  cells/24 hours) invaded through Matrigel (13% of the cells expressing high levels of osteonectin invaded compared with 24% of the control cells; Fig. 3C). Such a decline in invasion through Matrigel is consistent with the reduced colony size and stellate appearance observed on Matrigel (Fig. 2A and B). We next examined whether or not the decrease in invasion was a result of decreased cleavage of collagen IV by observing the release of a quenched fluorescence substrate when collagen IV is cleaved. Sameni et al. (30) previously showed that breast cancer cell invasion correlates with the cleavage of fluorescently quenched-collagen IV by proteases. There was no decrease in the cleavage of collagen IV after 72 hours by MDA-231 cells expressing high amounts of osteonectin as seen by high fluorescence around all colonies; however, the smaller colony size of these cells is still observed (Fig. 3D).

**Metastases are decreased in animals injected with MDA-231 cells expressing osteonectin.** We next wanted to determine if endogenous expression of osteonectin in MDA-231 breast cancer



**Figure 3.** Expression of osteonectin decreased MDA-231 breast cancer cell invasion through Matrigel but did not affect migration. MDA-231 cells were infected with either Add1312 (*ctrl*) or AdCMV-OSN (*OSN*) or were not infected (*0*) for 24 hours before each experiment. **A**, confluent cell layers were wounded and the width of the wound was measured. The starting size of each wound was designated as 0% closure ( $n = 3$ ). The highest dose is shown as a representative of all doses used. **B**, cell motility of subconfluent cells grown on plastic was measured. At least six cells were tracked per experiment. **C**, invasion of cells through Matrigel-coated membranes was measured over a period of 4 hours. Percent invasion of these cells towards serum-free media was plotted. \*,  $P < 0.05$ , statistical analysis using one-way ANOVA with Bonferroni's as a post-test found significant differences, in invasion of cells expressing high levels of osteonectin compared with controls. **Columns**, means from one representative experiment of three with similar results; **bars**,  $\pm$ SE. **D**, cleavage of collagen IV is not affected by osteonectin expression in MDA-231 cells. Images of live cell colonies after growth on Matrigel for 3 days. The fluorescent (white) area represents cleavage of collagen IV. Bar, 100  $\mu$ m. **A** and **B**, statistical analyses done using linear regression analysis and one-way ANOVA, respectively, showed no significant differences between the rate of wound closure and cell migration in the presence or absence of osteonectin.

cells affected their metastasis to bone and to other organs. MDA-231 cells were infected for 24 hours with increasing levels of Add1312 (control) or AdCMV-OSN or were not infected (none) and then were injected into the left ventricle of the mouse heart. After 5 to 6 weeks, a significant increase in metastasis to only the kidneys was observed in mice injected with MDA-231 cells expressing low levels of osteonectin protein (0.3  $\mu$ g osteonectin secreted/ $10^6$  cells/24 hours, Table 2). There was no difference in the number of metastases in noninfected versus control infected cells (data not shown). Interestingly, mice injected with MDA-231 cells expressing the highest level of osteonectin protein (11.5  $\mu$ g osteonectin secreted/ $10^6$  cells/24 hours) had decreased number of metastases in every organ compared with the control infected cells. A significant reduction in the number of overall metastases (metastases to all organs, including bone) and of bone metastases was found (Table 2). In addition, a significant decreased incidence ( $P < 0.05$ ) of overall metastases (77% compared with 96% of control) and bone metastases (41% compared with 78% of control) was observed. A significant reduction was also found in the number of metastases in the ribs of mice injected with cells expressing 0.3 or 1.5  $\mu$ g osteonectin secreted/ $10^6$  cells/24 hours but total bone and overall metastasis were not significantly reduced (Table 2). Unexpectedly, we found that osteonectin was expressed in the bone metastases (detected by GFP and H&E staining) that arose from cells infected with AdCMV-OSN and the control virus Add1312 (data not shown) suggesting that once the tumor cells get to the bone osteonectin expression is induced. Thus, our data show that high levels of endogenous osteonectin expression, in tumor cells that reach the

blood circulation, reduce breast tumor cell invasion and metastases *in vivo* but has no effect on tumor cell migration, proliferation, or apoptosis *in vitro*.

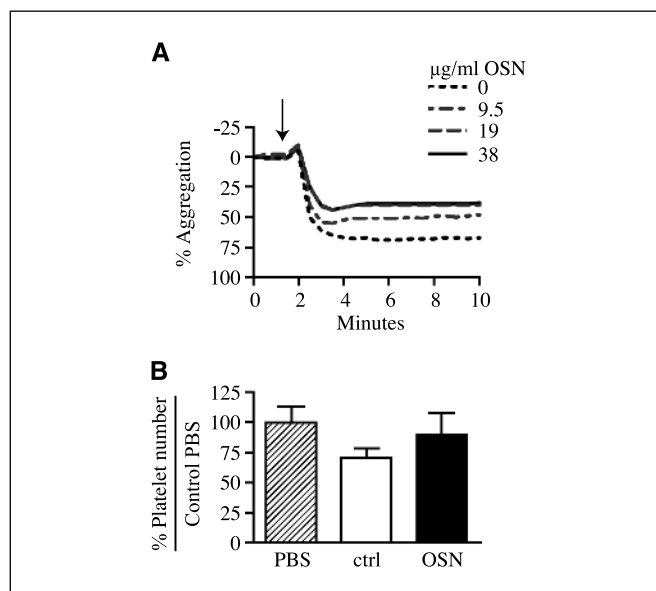
**Thrombocytopenia is reduced in mice injected with MDA-231 cells expressing osteonectin.** Osteonectin is secreted from platelets and binds to thrombospondin (5). Antibodies against osteonectin can inhibit the binding of thrombospondin to the platelet cell surface and platelet aggregation, suggesting that osteonectin is essential in platelet aggregation (42). Because platelet aggregation is important for tumor metastasis, especially to bone (43–45), we examined whether the MDA-231 breast cancer cells expressing osteonectin had an effect on platelet aggregation. Preincubation of human platelets with bone osteonectin significantly inhibited agonist-induced aggregation (Fig. 4A). Experimental metastasis studies have shown that once tumor cells are injected into the circulation, platelet count is reduced within 5 to 60 minutes, resulting in thrombocytopenia in these mice (44). PBS or MDA-231 cells infected for 24 hours with Add1312 (control) or AdCMV-OSN (secreting 11.5  $\mu$ g osteonectin/ $10^6$  cells/24 hours) were intracardiac injected. Mice injected with MDA-231 cells infected with Add1312 (control) had a 37% reduced platelet count, whereas injection of MDA-231 cells infected with AdCMV-OSN only dropped the platelet count 16% compared with mice injected with PBS (Fig. 4B). These *in vitro* and *in vivo* studies suggest that high expression of osteonectin in MDA-231 breast cancer cells inhibits tumor cell-platelet interactions, which combined with the reduced invasion, contributes to the decreased metastasis of these cells.

## Discussion

Osteonectin is an important regulator of cell growth and malignancy with complex biological effects that are cell and tumor type specific. Because breast cancer has a high propensity to metastasize to bone and because osteonectin is a bone-derived chemotactic factor elevated in breast carcinoma cells *in vivo* (18), we examined the effects of endogenous osteonectin expression on MDA-231 breast carcinoma cell growth, invasion, and metastasis. *In vitro*, proliferation, apoptosis, aggregation, and migration were not affected by osteonectin expression. However, expression of osteonectin inhibited MDA-231 tumor cell invasion and exogenous osteonectin inhibited platelet aggregation *in vitro*. High expression of osteonectin by MDA-231 breast cancer cells inhibited tumor cell-induced thrombocytopenia and overall metastasis, including metastasis to bone.

A recent study found that high osteonectin RNA is significantly correlated with breast cancer patient poor overall survival (46). On the other hand, Kim et al. (47) found no correlation between immunopositive osteonectin tumors and the 5-year survival rate of breast cancer patients. Other studies have shown increased osteonectin expression in breast cancer cells and tumors, but patient outcome in these studies has not been evaluated (18, 34, 48). Interestingly, osteonectin is significantly higher in primary breast tumors of patients who do not have bone marrow micrometastases (49). In addition, patients presenting with myeloma who have high plasma osteonectin levels are less likely to have osteolytic lesions (50). These data support our findings where high endogenous levels of osteonectin result in decreased metastasis of breast cancer cells.

Osteonectin regulates the proliferation of certain cells, including bovine aortic endothelial and ovarian carcinoma cells (6, 13), whereas proliferation in prostate cancer and melanoma cells is



**Figure 4.** Exogenous osteonectin inhibits platelet aggregation, and expression of osteonectin in MDA-231 cells decreases tumor cell-induced thrombocytopenia. **A**, bone osteonectin significantly inhibited final platelet aggregation that was induced when ADP and epinephrine were added (arrow). The inhibition of final aggregation (10-minute end point) was significant ( $P < 0.001$ ) for all doses of osteonectin treatment compared with the control (0, no addition of bone osteonectin). Significant differences of final platelet aggregation were determined by one-way ANOVA with Bonferroni's post-test. **B**, PBS or MDA-231 cells infected with either Add312 (ctrl) or AdCMV-OSN (OSN) were injected into the left ventricle of the mouse heart 24 hours after infection of the cells. Sixty minutes after the intracardiac injection, blood was drawn ( $n = 12$  for each group) and platelets were counted. The average platelet number from mice injected with PBS was set at 100%, and the average platelet number from mice injected with ctrl and osteonectin-infected cells was normalized to PBS.

unchanged (20, 51). Dhanesuan et al. (37) also induced osteonectin expression in MDA-231 breast carcinoma cells and found a small inhibition of tumor cell proliferation. Although using the same cells, we could not show a reduction in cell proliferation or apoptosis when the cells were grown on plastic, collagen I, or Matrigel. The level of osteonectin that is induced in MDA-231 cells by doxycyclin is not reported and may be low relative to our levels of expression (37). We expressed levels that are comparable to those endogenously made by other breast cancer cells.

High levels of osteonectin expression (11.5  $\mu\text{g}$  secreted/ $10^6$  cells/24 hours) inhibited the size of colonies formed by MDA-231 cells on Matrigel *in vitro*. A reduction in the stellate colony morphology by osteonectin expression suggested a potential decrease in cell migration and/or invasion (29). Whereas migration was unaffected, a decrease in Matrigel invasion was observed in osteonectin-expressing cells. Previous studies showed, however, that osteonectin is a chemotactic factor increasing breast and prostate cancer cell invasion (20) and matrix metalloproteinase-2 (MMP-2) activity (20, 35). MMP-2 cleaves collagen IV and this is a component in Matrigel. We found though that osteonectin expression in MDA-231 cells had no effect on the cleavage of collagen IV. As a chemoattractant, osteonectin has a gradient effect on breast cancer cells. In our studies, however, MDA-231 cells that secrete osteonectin constantly have a high concentration of osteonectin around the cells, and it is interesting to speculate that a saturation of the osteonectin receptors may be responsible for inhibiting invasion. Osteonectin may also have intracellular functions and/or induce expression of genes

**Table 2.** Effects of osteonectin expression on MDA-231 metastasis of cells infected with either Add312 (ctrl) or AdCMV-OSN (OSN)

Organ	Ctrl (0) <sup>†</sup>	OSN (0.3) <sup>†</sup>	OSN (1.5) <sup>†</sup>	OSN (11.5) <sup>†</sup>
Pancreas	4.0 (1.8)	2.7 (1.3)	0.4 (0.2)	1.5 (1.0)
Liver	2.7 (0.9)	2.4 (0.9)	1.1 (0.4)	0.4 (0.2)
Lung	12.7 (3.0)	15.5 (4.4)	9.6 (2.9)	4.6 (3.3)
Heart	2.2 (0.6)	2.3 (0.5)	1.9 (0.7)	0.5 (0.1)
Kidney	3.5 (0.9)	11.1* (3.1)	5.6 (2.1)	1.2 (0.5)
Ribs	3.1 (1.0)	0.2* (0.1)	0.2* (0.1)	1.0 (0.5)
Spine	1.9 (0.6)	0.5 (0.2)	0.6 (0.2)	1.0 (0.4)
Mandible	0.6 (0.1)	0.2 (0.1)	0.7 (0.2)	0.2 (0.1)
Skull	1.8 (0.6)	1.8 (0.6)	1.7 (0.4)	0.4 (0.2)
Hind limbs	1.5 (0.3)	1.0 (0.4)	1.7 (0.5)	0.5 (0.2)
Fore limbs	0.7 (0.3)	0.4 (0.2)	0.9 (0.2)	0.2 (0.1)
Overall	34.8 (6.2)	40.6 (6.5)	28.7 (4.1)	10.8* (3.9)
metastases				
Bone	9.6 (2.3)	4.0 (1.2)	5.9 (1.2)	3.4* (1.1)
metastases				

NOTE: Values shown are mean tumor number/mouse  $\pm$  SE. Ctrl,  $n = 27$ ; OSN (0.3),  $n = 20$ ; OSN (1.5),  $n = 19$ ; OSN (11.5),  $n = 27$ . \* $P < 0.05$  compared with control using one-way ANOVA with Bonferroni's multiple comparison post-test. <sup>†</sup>Micrograms of osteonectin secreted/ $10^6$  cells/24 h.

regulating cell invasion. There is evidence that osteonectin requires overexpression of other genes to affect migration and invasion in other cell types. Increased osteonectin expression in MCF-7 breast carcinoma cells overexpressing c-Jun results in increased migration and invasion (52). In these cells, the overexpression of both c-Jun and osteonectin were necessary for increased cell migration and invasion, suggesting that osteonectin alone is not sufficient for induction of migration or invasion.

It is unclear why osteonectin expression is associated with increased tumor growth and metastasis of some malignancies (glioma and melanoma; refs. 10, 12) and decreased tumor growth and metastasis with others (ovarian, neuroblastoma, and our data with MDA-231 cells; refs. 13, 16). The protease profiles of tumors vary and it has been suggested that proteolysis may release cryptic active sites in osteonectin. For example, *in vivo*, angiogenic activity and increased protease production have been mapped to individual domains of osteonectin (35, 53). Differential proteolytic cleavage of osteonectin by tumor-specific proteases may contribute to the distinct functions attributed to osteonectin in tumors. We did not see any evidence of cleavage of osteonectin *in vitro*. In addition, receptor expression, availability, and affinity may be altered in distinct tumor microenvironments. Tissue and cell variations might also explain conflicting results, but there also is added complexity from both tumor- and stroma-derived osteonectin. Although there was a decreased vascular area in Lewis lung carcinoma and T-cell lymphoma tumors grown in osteonectin-null mice, which agrees with osteonectin's ability to inhibit endothelial cell proliferation, the s.c. and metastatic tumors unexpectedly grew larger and more rapidly than tumors grown in control mice (17). There was no change in either cell proliferation or apoptosis of these tumors grown in osteonectin-null mice; however, there was a decrease in the production of collagen and an alteration in the organization of the collagen capsule was observed in pancreatic tumors grown in osteonectin-null mice (54). These results indicate that osteonectin is important in the organization of the extracellular matrix and that stromal-derived osteonectin may be important for tumor growth.

We found that only the high expression of osteonectin in MDA-231 breast carcinoma cells inhibited metastasis. Many tumorigenic and metastatic breast cancer cell lines (e.g., MDA-435, BT549, BT20, and Hs578T) express high levels of osteonectin (34, 35), suggesting osteonectin is not sufficient to inhibit metastasis. These cells may have additional mutations or expressed genes that allow them to overcome the inhibitory effects of osteonectin. Interestingly, only MDA-435 metastasizes well in the intracardiac model. MDA-435 cells contain both breast and melanoma markers (39), and because osteonectin increases metastasis in melanoma cells, it may have a different effect in MDA-435 cells.

Alternatively, the timing of osteonectin expression may have an important role in tumor metastasis. In our model system, osteonectin is only transiently expressed, suggesting that the inhibition of metastasis by osteonectin most likely occurs in the initial steps of this experimental metastasis model (e.g., survival in circulation, arrest, extravasation, or initial growth). The reduction of tumor cell-induced thrombocytopenia by high osteonectin expression in tumor cells suggests that tumor cell-platelet interactions are inhibited and that there is a reduction in the ability of the tumor cells to leave the circulation. Reduction of tumor cell-induced thrombocytopenia inhibited metastasis in other experimental metastasis models (44). Tumor cells in platelet emboli may escape immune surveillance, and tumor-platelet emboli may facilitate adhesion of tumor cells to the endothelium. Additionally, the interaction of platelets with tumor cell emboli prolongs tumor cell survival in the circulation (55) and may help mechanically lodge tumor cells in the microvasculature of organs, allowing extravasation and metastasis.

Platelet-derived osteonectin interacts with thrombospondin, and the binding of this complex to the platelet cell surface is important for platelet aggregation (5, 42); however, bone-derived osteonectin prevents the binding of thrombospondin to the platelet cell surface (42). Bone osteonectin has a smaller apparent molecular weight than platelet osteonectin, which is due to variable N-glycosylation (20, 56). This altered glycosylation affects the ability of platelet osteonectin to bind collagens I, III, and V (56) and, likely, the ability of bone osteonectin to bind with thrombospondin to the platelet cell surface (42). The osteonectin that is secreted from MDA-231 cells has the same apparent molecular weight as bone osteonectin (data not shown), suggesting that the osteonectin secreted from the MDA-231 cells may inhibit platelet aggregation by preventing binding of thrombospondin to the platelet cell surface.

Our data show that osteonectin can inhibit breast cancer cell invasion, platelet aggregation, and metastasis *in vivo*. The effect of osteonectin on tumor-platelet interactions has not previously been examined. Furthermore, metastasis to bone, a major clinical problem in breast cancer, is significantly decreased by osteonectin expression. Understanding the mechanism of action of osteonectin in metastasis may define new therapeutic or diagnostic approaches in cancer.

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