Osteopontin silencing by small interfering RNA suppresses in vitro and in vivo CT26 murine colon adenocarcinoma metastasis

Philip Y. Wai, Zhiyong Mi, Hongtao Guo, Shiva Sarraf-Yazdi, Chengjiang Gao, Junping Wei, Carlos E. Marroquin, Bryan Clary and Paul C. Ku

Abbreviations: EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OPN, osteopontin; pS-OPN, plasmid vector expressing siRNA against OPN; pS-MM, plasmid vector expressing mismatch siRNA; PCNA, proliferating cell nuclear antigen; RNAi, RNA interference; siRNA, small interfering RNA; sR1, CT26 cells transiently transfected with OPN siRNA to target 1; sR2, CT26 cells transiently transfected with OPN siRNA to target 2; uPA, urokinase plasminogen activator; VGEF, vascular endothelial growth factor.

Introduction

Colorectal cancer continues to be the second leading cause of cancer-related death in the United States (1, 2). The natural history of the disease predicts that many patients will experience metastasis to distant organs and fail locoregional therapy. The most common extra-colonic site of tumor growth is the liver, with up to one-third of colon cancer patients developing hepatic metastases (2, 3). The molecular mechanisms underlying tumor metastasis are not completely understood but recent evidence suggests that osteopontin (OPN) plays a significant role in regulating cancer cell metastasis.

OPN was originally characterized as an extra cellular matrix (ECM) protein secreted by transformed epithelial cells (4). While constitutive expression of OPN at low levels exists in several normal cell types, induced expression at high levels occurs during cellular remodeling processes and in tumor progression (5–9). The secreted phosphoprotein contains functional domains for calcium binding, phosphorylation and glycosylation, and serves dual functions by acting as a structural, scaffolding protein in the ECM and also as a cytokine whose signal is transduced by the α, β integrin and CD44 families of receptors (9–15). Cumulative evidence from investigators studying models of breast cancer and melanoma suggests that OPN can activate metastatic mechanisms. But in the context of colorectal cancer, the evidence has been less definitive. Certainly, gene-profiling studies have identified a correlation between advanced and metastatic colon tumors and abundant expression of OPN (16–17). However, the lack of mechanistic or functional data has led to an incomplete understanding of how OPN mediates colorectal metastasis. In order to address this issue, we have designed this study to establish a functional correlation between OPN and colon cancer metastasis.

In this study, we used RNA interference (RNAi) (18–19) to produce specific and long-term silencing of OPN in CT26 colon cancer cells. CT26 is an undifferentiated murine colon adenocarcinoma cell line syngeneic to the BALB/c mouse strain (20). CT26 exhibits aggressive in vivo tumorigenic activity, produces pulmonary and hepatic metastases in murine models (20, 21) and expresses OPN at high levels. We have designed our experiments as a loss-of-function study using western blot-analysis to confirm the extent and stability of OPN knockdown in our clones that stably express siRNA against OPN. We evaluate the metastatic phenotype of OPN-silenced CT26 clones using standard in vitro migration and invasion assays and demonstrate a functional correlation in vivo using a model for experimental hepatic metastases (22, 23). We also demonstrate that OPN suppression is associated with matrix-metalloproteinase-2 (MMP-2) downregulation and suggest that this represents a mechanism for the OPN-dependent invasiveness we observed. These results indicate that OPN mediates colorectal–hepatic metastasis by enhancing tumor cell invasion and migration through the ECM by regulating MMP-2 expression, independent of cellular proliferation.
Materials and methods

Cell culture

CT26 murine colon carcinoma cells, syngeneic to BALB/C mice, were grown as monolayer cultures in DMEM-10% FBS (Invitrogen, Carlsbad, CA) supplemented with 100 IU/ml Penicillin and 100 μg/ml Streptomycin. Cells were maintained in a 37°C incubator with 5% CO₂, humidified air. Cell lines that were stably transfected with plasmid vectors were maintained in DMEM-10% FBS supplemented with 100 IU/ml Penicillin, 100 μg/ml Streptomycin and 750 μg/ml Geneticin (Invitrogen).

siRNA sequences and constructs

Using GenBank™ sequence NM 009263 for murine OPN cDNA and computer analysis software developed by Ambion, Inc., we selected two candidate sequences in the OPN cDNA sequence for RNAi (Figure 1A). These 21-nt sequences showed no homology with other known mouse genes. The corresponding sense and antisense siRNA sequences for each target are also shown (Figure 1A). Synthetic, annealed, siRNA oligonucleotides were synthesized chemically and gel-electrophoresis purified (Ambion, Austin, TX) and used during transient transfection experiments. Murine mismatch or scrambled siRNA sequences (Ambion) possessing limited homology to mouse genes served as a negative control. For stable RNAi we designed a hairpin siRNA sequence that contains both sense and antisense siRNA sequences against OPN target 2 and flanking BamHI and HindIII sites (Figure 1E). This sequence was chemically synthesized and PAGE-purified (Sigma-Genosys, The Woodlands, TX) and cloned into pSilencer neo™, an expression vector containing an H1 RNA polymerase III promoter (24) and a neomycin antibiotic resistance gene (Ambion). A pSilencer neo™ vector that expresses mismatch hairpin siRNA with limited homology to mouse genes (Ambion) served as our negative control. Plasmids were amplified and purified using QIAfilter™ Plasmid Maxi Kit (QIAGEN, Valencia, CA).

Transfection of siRNA oligonucleotides and stable siRNA plasmids

Harvesting of CT26 cells using Trypsin was done 24 h prior to transfection, and plated at a density of 5 x 10⁵ cells/well in 6-well plates (Costar, Corning Inc., NY) in DMEM-10% FBS without antibiotics. Reconstituted, annealed siRNA was added to the cells and siRNA against target 2 and R2 were diluted in OPTIMEM I (Invitrogen) to a final concentration of 50 nM and transiently transfected into CT26 using Lipofectamine 2000 (Invitrogen). The medium was replaced with DMEM-10% FBS after 4 h. CT26 wild-type cells (WT), CT26 incubated with Lipofectamine 2000 alone in the absence of siRNA and CT26 incubated with Lipofectamine 2000 and mismatch siRNA (pS-MM) were used as controls. Cells were harvested 48 h after transfection and OPN protein levels were quantified by western blot-analysis in triplicate assays. In separate experiments, 2 μg of purified pSilencer neo™ expression vectors containing either the OPN siRNA insert (pS-OPN) or the negative-control mismatch-siRNA (pS-MM) were transfected into CT26 cells as described above. Antibiotic selection using Geneticin (Invitrogen) was initiated 24 h after transfection to generate stable clones. Western blotting for OPN was performed 72 h after transfection, to assess the selectivity of OPN knockdown amongst WT, CT26-pS-OPN and CT26+pS-MM cell-lines. Clonal cell lines that stably express OPN-siRNA and mismatch-siRNA were selected and cultured for up to 4 months.

Western blot-analysis

Total lysates were prepared and analyzed by SDS-PAGE as previously described (25). Briefly, 35 μg of protein/lane was resolved on 4-20% polyacrylamide gels (Gradipore Inc., Hawthorne, NY) and transferred to polyvinylidene (PVDF) membranes (Amersham Biosciences, Piscataway, NJ). The primary antibodies used were: anti-OPN (1:500; R and D Systems, Minneapolis, MN), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2000; Ambion, Austin, TX), anti-MMP-2 (1:500; Santa Cruz, CA) and anti-MMP-9 (1:500; Santa Cruz, CA) and anti-VEGF (1:500; Santa Cruz, CA). Secondary antibodies used were: donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA; 1:2000), donkey anti-mouse IgG-HRP (Santa Cruz Biotechnology; 1:2000) or goat anti-rabbit (Santa Cruz Biotechnology; 1:2000). Immunodetection was performed using HRP-based SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL). For quantification, the bands were scanned into AlphaImager 3400 (Alpha Innotech, San Leandro, CA) and normalized by dividing the measured density of protein bands by the density of GAPDH control bands from corresponding cell lysates.

In vitro migration and invasion assays

The motility and invasiveness of CT26 cells stably expressing pS-OPN were evaluated in 24-well transwell chambers with upper and lower culture compartments separated by polycarbonate membranes with 8-μm sized pores (Costar 3422, Corning Inc., NY). WT and CT26 stably expressing pS-MM were used as controls. Cells were grown to 60% confluence, harvested using Cell-stripper (Cellgro, Herndon, VA), washed with 1× PBS and resuspended in DMEM-0.1% BSA. Prior to plating cells into the transwells, DMEM-0.1% BSA was incubated in the top chamber of each transwell at 37°C for 1 h to saturate the specific binding sites. Then suspended cells were seeded into respective bottom chambers at 5 x 10⁴ cells/well for each cell line was calculated and plotted against time. Assays were performed in triplicate and data are expressed as mean ± SD.

Cellular proliferation assay

The in vitro growth rates of WT, pS-OPN clones and pS-MM clones were assessed at 24, 48 and 72 h. Cells were grown in monolayer culture to 60% confluence, harvested using Trypsin and plated at a density of 5 x 10⁴ cells/ml into separate wells of a 12-well plate (Costar, Corning Inc., NY). DMEM-10% FBS supplemented with 100 IU/ml Penicillin and 100 μg/ml Streptomycin was used as culture medium and changed every 24 h. For pS-OPN and pS-MM cells, 750 μg/ml Geneticin was added to the culture medium. Cells were harvested at 24, 48 and 72 h after the initial trypsinization, subjected to trypsin blue exclusion and viable cells were counted using a Bright-Line hemacytometer (Hausser Scientific, Horsham, PA). The total number of cells/well for each cell line was calculated and plotted against time. Assays were performed in triplicate and data are expressed as mean ± SD.

Animal studies

Female BALB/c mice, 6-7 weeks old (Charles River Laboratories, Wilmington, MA) were acclimated for 1 week at the Duke University Medical Center Vivarium while caged in groups of five. All animal studies were carried out according to the Institutional Animal Care and Use Committee of Duke University Medical Center. The in vivo metastatic phenotype of OPN-downregulated cells was evaluated using a hepatic metastasis model (22,23). In this model, intrasplenic injection of tumor cells produced hepatic metastases by circulating through the portal venous system. WT and pS-MM cells served as controls. Prior to injection, cells were resuspended in 1× PBS, trypsinized and reseeded in DMEM. Cell number and viability were determined by trypsin blue exclusion (>90% viability). A total of 33 mice were randomly assigned to three groups (11 mice/group) corresponding to groups WT, pS-OPN or pS-MM. After the mice were anesthetized, the left flanks were shaved and sterilized using iodinated solution. A small, left subcostal incision was made and the spleen was isolated and exteriorized. A suspension of 5 x 10⁴ cell/mouse pS-MM were injected into the spleens of the mice to produce liver metastases. Splenectomies were performed 5 min after intrasplenic injection. Hemostasis was obtained using surgical clips and incisions were closed with vicryl suture. Mice were observed daily, then killed by cervical dislocation after anesthesia on day 19; after that hepatocarcinomas were performed. The harvested livers were

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Fig. 1. Inhibition of OPN expression in CT26 cells by RNAi. (A) DNA sequences of targets in OPN-cDNA selected for RNA interference (Target 1: 5'-AAT CTC CTT GCG CCA CAG AAT-3'; Target 2: 5'-AAG TCA GCT GGA TGA ACC AAG-3'). Corresponding sense and antisense sequences for siRNA oligonucleotides are shown below each target sequence. (B) Western blot showing levels of OPN protein in cell lysates from WT (lane 1), sR- (lane 2), sR1 (lane 3), and sR2 (lane 4). GAPDH levels served as an internal control for loading. (C) Histogram showing spot densitometry for the bands in Figure 1B. Western blots were scanned into AlphaImager 3400. The measured density for OPN bands was divided by the measured density for GAPDH bands to normalize the samples. Results from WT (lane 1), sR- (lane 2), sR1 (lane 3), and sR2 (lane 4) are shown. * Indicates P<0.05 versus WT. (D) Western blot showing that RNAi mediated silencing of OPN by transient transfection is time-dependent. Using sR2, the extent of OPN suppression was measured at 24, 48 and 72 h (lanes 3, 6 and 9). OPN protein levels in WT (lanes 1, 4 and 7) and sR- (lanes 2, 5 and 8) were used as controls. Levels of GAPDH are shown and were used as an internal control for loading. (E) The DNA sequence of the OPN-siRNA insert subcloned into pSilencer neo™ is shown. BamH1 and HindIII restriction enzyme sequences flank the insert. The sense and antisense sequences are separated by a loop sequence (loop). An RNA pol III terminator sequence (poly-T) is positioned 3' to the antisense sequence to terminate transcription. (F) Western blot and spot densitometry showing the extent of OPN protein suppression in cells stably transfected with pS-OPN at 4 months after initial transfection. Results for WT (lane 1), pS-OPN-A1, pS-OPN-A2, pS-OPN-A3 and pS-OPN-A4 (lanes 2-5) and clonal cell lines stably transfected with pS-MM control plasmids—pS-MM-V6 and pS-MM-V7 (lanes 6,7), are shown. Levels of GAPDH in corresponding cell lines are also shown and served as an internal control for loading. Histograms showing spot densitometry for the bands (OPN/GAPDH) are shown below the western blot. The mean ± SD of triplicate assays for each cell line is shown. Compared with WT control: *, P<0.05; †, P = 0.44; ‡, P = 0.14.
analyzed by measuring liver weights and counting the number of surface nodules as indices of metastatic burden.

Immunohistochemistry

Staining for Hematoxylin and Eosin (H&E), proliferating cell nuclear antigen (PCNA), Ki-67, MMP-2, MMP-9, urokinase plasminogen activator (uPA), vascular endothelial growth factor (VEGF) and OPN were performed on sections of liver specimens selected from the animal studies. Parafin-embedded sections, 5 μm-thick, were mounted on glass slides, dried overnight, deparaffinized and treated with hydrogen peroxide to block endogenous peroxidase activity. Specimens were treated with microwaves for 5 min in citrate buffer to retrieve antigens. In addition to the primary antibodies listed in Materials and methods, Western blot-analysis, we also used anti-PCNA (Santa Cruz Biotechnologies; 1:100) and anti-Ki-67 (Santa Cruz Biotechnologies; 1:300). The secondary antibodies used were biotinylated anti-goat IgG (Vector, Burlingame, CA), biotinylated anti-rabbit IgG (Vector) and biotinylated anti-mouse IgG (Vector). Specimens were then treated with Vectastain (Vector), diaminobenzidine (Innovex Biosciences, Richmond, CA), counterstained with Hematoxylin, dehydrated and mounted with permanent mounting medium. Negative controls underwent the same procedure with the omission of the primary antibody and also with the corresponding isotype antibody. Immunolabeling was assessed by counting positively stained tumor cell nuclei (PCNA, Ki-67) or cytoplasm (OPN, MMP-2, MMP-9, uPA and VEGF) from areas with the highest rates of staining. Necrotic areas were avoided. The labeling index was defined as the fraction of the number of cells with well-defined positive staining divided by the total number of tumor cells within the field (100× magnification). Counting and analysis were performed on three different tumor tissue samples from each cell line. The average number of tumor nuclei per high power field was 735.2 ± 110.2 cells (n = 18).

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using SigmaStat Version 3 (Systat software, Point Richmond, CA). Individual comparisons were made with Student’s t-test. The criterion for significance was P < 0.05 for all comparisons.

Results

RNAi stably decreases OPN expression

CT26 murine colon cancer cells are highly invasive and normally constitutively express OPN at high levels. We specifically suppressed OPN expression in CT26 using RNAi. Initially, we selected two 21-nt targets within the OPN cDNA for RNAi (Figure 1A). Based on these targets, we designed double-stranded 21-nt siRNA constructs encoding sense and antisense siRNA (Figure 1A). Western blot-analysis of sR1 and sR2 demonstrated 2.0-fold (P = 0.041) and 2.5-fold (P = 0.006) decreased OPN protein expression, respectively, in comparison with control (Figure 1B and C). CT26 (WT), CT26 treated with Lipofectamine 2000 in the absence of siRNA (data not shown) and CT26 treated with Lipofectamine 2000 and mismatch/scrambled siRNA (sR–) served as controls and they demonstrated no significant difference in OPN protein levels. This RNAi mediated effect was specific, as GAPDH levels did not differ significantly among the treated cells and controls (Figure 1B). Using siRNA against target 2, we determined that transient-transfection mediated RNAi is time dependent as OPN silencing was maintained for up to 72 h (Figure 1D). In order to assess the phenotype of tumor cells in which OPN expression was inhibited over long-term, we directed our attention to generating stable, OPN-downregulated clonal cell lines. Based on the higher efficiency of sR2 compared with sR1, we selected target sequence 2 in the OPN-cDNA (5’-AAG TCA GCT GGA TGA AAG-3’) for use in designing our siRNA expression plasmids.

We designed and constructed plasmids that would express siRNA against OPN-cDNA target sequence 2, as described under Materials and methods. Sense and antisense siRNA sequences were arranged sequentially to facilitate the formation of hairpin siRNA upon transcription (Figure 1E). CT26 cells were transfected with purified pSilencer neo™ expression vectors containing either the OPN-siRNA insert (pS-OPN) or the mismatch siRNA insert (pS-MM). pSilencer neo™ contains a neomycin resistance gene and we initiated antibiotic selection 24 h after transfection. At 72 h after transfection, western blot-analysis of pS-OPN cells confirmed a 2.3-fold decrease in OPN expression compared with WT (data not shown). pS-MM cells showed similar levels of OPN protein compared with WT. At 4 months, western blot-analysis of our clones demonstrated decreased OPN protein expression of 1.6-fold (pS-OPN-A1), 2.6-fold (pS-OPN-A2) and 2.4-fold (pS-OPN-A3), compared with WT (P < 0.05). Our most significant clone, pS-OPN-A4, demonstrated 3.0-fold decrease in OPN expression (Figure 1F; P < 0.05). Clones pS-MM-V6 and pS-MM-V7 showed OPN protein levels similar to WT (Figure 1F; P = 0.41, P = 0.14). Protein levels of GAPDH, a housekeeping gene, were similar between different cell lines indicating that the RNAi-mediated knockdown of OPN was specific and did not result from a global decrease in gene expression (Figure 1F).

Downregulation of endogenous OPN protein expression inhibits colon cancer cell migration and invasion in vitro, independent of cellular proliferation

We evaluated whether the suppression of OPN expression would alter the in vitro metastatic phenotype of CT26 cells. We assessed the motility of pS-OPN clones across transwell polycarbonate membranes as described under Materials and methods. WT, pS-MM-V6 and pS-MM-V7 served as controls. Cell motility in pS-OPN-A1, pS-OPN-A2, pS-OPN-A3 and pS-OPN-A4 were decreased by 1.7-, 2.5-, 2.9- and 3.6-fold, respectively, compared with WT (Figure 2A; P < 0.05 for each cell line). No significant differences were detected between WT and pS-MM-V6 (P = 0.49) or WT and pS-MM-V7 (P = 0.20). Parallel experiments were performed and membranes used for the migration assays were fixed, stained with 0.2% crystal violet and mounted on glass microscope slides. Representative sections at 100× magnification are shown for WT, pS-OPN-A4 and pS-MM-V6 (Figure 2B).

Next, we evaluated whether downregulation of OPN expression would alter the ability of CT26 cells to invade into Matrigel™, a solubilized basement membrane preparation extracted from the EHS mouse sarcoma. The invasiveness of pS-OPN-A4 was assessed using Matrigel coated transwell-invasion chambers as described under Materials and methods. pS-OPN-A1, pS-OPN-A2, pS-OPN-A3 and pS-OPN-A4 demonstrated 2.0-, 2.9-, 3.2- and 4.1-fold decreased invasionness, respectively, compared with WT (Figure 3A; P < 0.05 for each clone). No significant difference in invasiveness was observed between WT and pS-MM-V6 (P = 0.44) or WT and pS-MM-V7 (P = 0.50). Parallel experiments were performed in triplicate and membranes used for the invasion assays were prepared, as described above and shown in Figure 3B.

To confirm that the data from the in vitro migration and invasion assays did not result from differences in cellular proliferation amongst our cell lines, we measured growth rates using an in vitro cellular proliferation assay as described under Materials and methods. No significant difference in the rate of proliferation was observed amongst cell lines at 24 h (data not shown). Similar rates of growth were observed at 48 h
Fig. 2. The impact of OPN silencing on in vitro CT26 cell motility. (A) The motility of WT, pS-OPN-A1–pS-OPN-A4, pS-MM-V6 and pS-MM-V7 cells were assessed by incubating cells in transwell chambers for 24 h using 10% FBS as a chemoattractant. Non-migrated cells were removed. Migrated cells were stained with 0.2% crystal violet, subjected to elution by acetic acid and quantified in a microplate reader (A590). ψ, by definition, WT cells were assigned a migration index of 1. The data represent the mean ± SD of triplicate assays. Compared with WT control: *, P < 0.05; †, P = 0.48; ‡, P = 0.19. (B) Light microscopy demonstrating representative fields of transwell membranes used in the in vitro migration assays comparing the motility of WT, pS-OPN-A4 or pS-MM-V6 cells. A 0.2% crystal violet was used for staining. The 8-μm pores of the transwell polycarbonate membranes are visible in the background. Magnification, 100×.

Fig. 3. The impact of OPN silencing on in vitro CT26 cell invasiveness. (A) The invasiveness of WT pS-OPN-A1–pS-OPN-A4, pS-MM-V6 and pS-MM-V7 cells were assessed by incubating cells in Matrigel-coated transwell chambers for 24 h using 10% FBS as a chemoattractant. ψ, by definition, WT cells were assigned an invasion index of 1. The data represent the mean ± SD of triplicate assays. Compared with WT control: *, P < 0.05; †, P = 0.44; ‡, P = 0.50. (B) Light microscopy demonstrating representative fields of transwell membranes used in the in vitro invasion assays. Membranes used for WT, pS-OPN-A4 and pS-MM-V6 cells are shown. 0.2% crystal violet was used for staining. The 8-μm pores of the transwell polycarbonate membranes are visible in the background. Magnification, 100×.
and 72 h. These data indicate that the expression of siRNA against OPN, the expression of the neomycin resistance gene and the use of our pSilencer neo™ vector have no significant effect on CT26 cellular proliferation over 72 h. Taken together with the results from our in vitro migration and invasion assays that were conducted over an incubation period of 24 h, these data suggest that the inhibition of OPN expression in pS-OPN cells resulted in decreased cell motility and invasiveness, independent of cellular proliferation in CT26 cells.

Suppression of OPN expression in CT26 cells decreases MMP-2 expression

We evaluated the protein expression of several metastatic markers that have been previously shown to be associated with OPN in models of breast cancer and melanoma. We evaluated our OPN-downregulated clones for alterations in expression of MMP-2, MMP-9, uPA and VEGF using western blot-analysis. Interestingly, MMP-2 expression was decreased in each of our OPN-downregulated clones (Figure 4). No significant changes in expression were detected for MMP-9, uPA or VEGF (Figure 4). The degree and extent of downregulation was conserved between OPN and MMP-2 with our most downregulated OPN clone, pS-OPN-A4, demonstrating the most significant attenuation of MMP-2 expression (3.0-fold versus WT; \( P < 0.05 \)).

Downregulation of endogenous OPN attenuates hepatic metastasis of CT26 colon adenocarcinoma in vivo

Using an in vivo model for hepatic metastasis, we assessed the metastatic phenotype of our most significantly downregulated clone, pS-OPN-A4, in comparison with control cell lines, WT and pS-MM-V6, as described under Materials and methods. After an incubation period of 16 days, some mice became morbid with grossly distended abdomens and lowered physical activity and five mice died over the period of days 16–18 (WT, 2 deaths; pS-MM-V6, 3 deaths; pS-OPN-A4 mice, no deaths). The mice were killed on day 19. Livers were harvested, their weights were measured and the number of surface tumor nodules were counted (Figure 5). Previous experiments determined that livers harvested from normal mice had weights measuring 1.06 ± 0.07 g (data not shown). Suppression of OPN expression significantly decreased metastatic burden with differences grossly discernible compared with controls (Figure 5). Livers colonized by pS-OPN-A4 cells demonstrated a mean 2.01-fold decrease in liver weight compared with WT cells (Figure 6A, 1.88 ± 1.34 versus 3.79 ± 1.49 g, \( P = 0.009 \)). Mean liver weights did not differ significantly between livers colonized by WT cells and pS-MM-V6 cells (Figure 6A, \( P = 0.94 \)). Eight of 9 livers (89%) in the WT group and 6 of 8 livers (75%) in the pS-MM-V6 group had ≥20 metastatic nodules on the liver surface (Figure 6B, D). In contrast, only 2 of 11 livers (18%) in the pS-OPN-A4 group had ≥20 metastatic nodules on the liver surface (Figure 6C). At the same time, 4 of 11 (36%) pS-OPN-A4 livers showed no evidence of hepatic metastases, whereas only 9 of 11 (81%) WT livers and none of the eight (0%) pS-MM-V6 livers demonstrated an absence of hepatic metastases (Figure 6). A total of 2 of 11 mice (18%) from the WT group and 3 of 11 mice (27%) from the pS-MM group died during the 19-day incubation period but no mice from the pS-OPN-A4 group died.

The in vivo status of the hepatic metastases derived from our animal studies was determined using immunohistochemical analysis for OPN, PCNA, Ki-67, MMP-2, MMP-9, uPA and VEGF. Livers from the animal studies were fixed in 10% formalin, embedded into paraffin, sectioned and stained for H&E, OPN, PCNA, Ki-67, MMP-2, MMP-9, uPA and VEGF (data not shown). The labeling index for PCNA was not significantly different between cell lines (WT, 0.29 ± 0.01; pS-OPN-A4, 0.28 ± 0.02; pS-MM-V6, 0.26 ± 0.037, \( P > 0.05 \)). Similar results were obtained by evaluating Ki-67 (WT, 0.11 ± 0.004; pS-OPN-A4, 0.11 ± 0.004; pS-MM-V6, 0.10 ± 0.018, \( P > 0.05 \)). Interestingly, OPN expression was also conserved in the hepatic metastases resulting from different cell lines (WT, 0.87 ± 0.02; pS-OPN-A4, 0.85 ± 0.03;
pS-MM-V6, 0.87 ± 0.02, P > 0.05), suggesting that pS-OPN-A4 metastases represent cells in which the siRNA effect was not preserved. Staining MMP-2, MMP-9, uPA and VEGF also confirmed equivalent protein expression between different liver tumors (data not shown). These data suggest that the RNAi effect was not conserved in the hepatic metastases derived from pS-OPN-A4 and that these pS-OPN-A4 metastatic foci were derived from a small subpopulation of pS-OPN-A4 that retained residual expression of OPN and MMP-2.

Discussion

Osteopontin is a ~298-amino acid, secreted phosphoprotein that binds the αvβ integrin and CD44 families of receptors. Recently, increased OPN expression has been correlated with

Fig. 5. The effect of OPN knockdown on the in vivo metastatic phenotype of CT26 colon cancer cells. Photographs of representative livers removed from mice injected intrasplenically with WT, pS-OPN-A4 or pS-MM-V6 cells. Scale, 4 cm.

Fig. 6. The impact of OPN silencing on in vivo tumor burden. (A) The graph shows the liver weights of mice injected intrasplenically with WT, pS-OPN-A4 or pS-MM-V6 cells after an incubation period of 19 days. Data are expressed as mean ± SD of triplicate assays. Compared with WT control: *, P = 0.009; †, P = 0.94. (B) The graph shows the proportion of livers from mice injected intrasplenically with WT cells (n = 9) that contained 0, 1–19 or ≥20 surface tumor nodules. Data are expressed as a percentage of WT livers. (C) The graph shows the proportion of livers from mice injected intrasplenically with pS-OPN-A4 cells (n = 11) that contained 0, 1–19 or ≥20 surface tumor nodules. Data are expressed as a percentage of pS-OPN-A4 livers. (D) The graph shows the proportion of livers from mice injected intrasplenically with pS-MM-V6 cells (n = 8) that contained 0, 1–19 or ≥20 surface tumor nodules. Data are expressed as a percentage of pS-MM-V6 livers.
tumor invasion, progression or metastasis in cancers of the breast (28–30), stomach (31), lung (32,33), prostate (34), liver (35,36) and colon (16,17). In models of breast cancer and melanoma, OPN has been shown to activate molecular mechanisms that regulate tumor cell migration and invasion. However, there are currently no studies that describe the precise, mechanistic role of OPN in models of colon carcinoma. Certainly, data from gene-profiling studies strongly support that increased OPN expression correlates with colorectal tumor progression. Results from a screen of 12,000 human genes have recently uncovered OPN as a lead marker of colon cancer progression (16) and established a significant association between the degree of OPN expression and advancing Astler Collier (AC) stage. Northern blot-analysis confirmed a ~10- to 20-fold increase in OPN expression in samples with liver metastases compared with normal mucosa (16) and a gradient of increasing OPN expression was observed when a precursor polyp (low OPN expression) was directly compared with its adjacent, associated invasive cancer (high OPN expression) (17). Despite these interesting observations, there are limited data in the literature to promote a functional understanding of how OPN mediates tumor metastasis in models of colon cancer.

In this study, we have shown that plasmid-based RNAi suppresses OPN expression in CT26 colon adenocarcinoma cells in a stable and long-term fashion. We confirmed a mean decrease in OPN protein expression of ~3-fold over 4 months. In vitro, this attenuation of OPN expression is associated with a 3.6-fold decrease in cell motility (P < 0.05 versus WT) and 4.1-fold decrease in cell invasiveness (P < 0.05 versus WT) in pS-OPN-A4. Importantly, we demonstrated that these phenotypic differences are independent of cellular proliferation, as the rate of growth is conserved between CT26 WT and pS-OPN clones. We screened our OPN-downregulated clones for specific target genes that have been previously associated with OPN regulation. In contrast to data from breast cancer studies by Kundu and colleagues (37,38), no significant difference in uPA or MMP-9 expression was observed in our OPN-downregulated clones. In addition, no change in VEGF expression was observed between CT26 WT and pS-OPN clones. These data are consistent with studies by Senger et al., which demonstrate that VEGF induces OPN and αβ, expression during endothelial cell migration (39). These data suggest that OPN-potentially operates downstream to VEGF signaling; and that in CT26 cells, downregulation of OPN does not significantly modulate VEGF expression. Western blot-analysis did, however, uncover a mean 3.0-fold decrease in MMP-2 expression in pS-OPN clones compared with control. Together, these data suggest that the set of OPN-mediated signaling pathways in previously described models of breast cancer is different from the assortment of pathways that are regulated in CT26 colon cancer.

The liver is the primary extra-colonic site for colon cancer metastasis and represents the most common location and clinical presentation for recurrent disease in patients who fail locoregional therapy. To address the biology underlying these later stages of colorectal metastasis, we directed our animal studies to investigate the role of OPN in regulating hepatic end-organ metastasis. Certain advantages and limitations are associated with both spontaneous and experimental models of tumor metastasis and no single system can model the exact biological environment that is encountered during human tumorigenesis and progression. We selected an intrasplenic-injection model of experimental hepatic metastasis over a cecal-injection model of spontaneous metastasis for multiple reasons. The model of intrasplenic hepatic metastasis has been well established and described (22,40–45), provides a consistent and reliable method of generating liver metastasis with high penetrance (22,41,43,44,46,47) and satisfies our experimental goal of characterizing the role of OPN in regulating end-organ metastasis. On the other hand, cecal-injection models can be variable with respect to the latency and penetrance of hepatic metastases (22,48,49), can result in mechanical disruption of cecal tissue during the implantation process which may permit escape of tumor cells into the circulatory system at the time of implantation (50) and may lead to intestinal obstruction which alters experimental morbidity (48). Using this murine model, we demonstrated that pS-OPN clones that exhibited a mean 3.0-fold decrease in OPN protein expression (P = 0.036 versus WT) also demonstrated a 2.01-fold (or 50.4%) decrease in tumor burden in vivo (P = 0.099 versus WT). These data suggest that OPN plays a functional role in the metastasis of CT26 colon cancer cells in vivo.

We analyzed these hepatic metastases derived from our OPN downregulated cell line using IHC for OPN, PCNA, Ki-67, uPA, MMP-2, MMP-9 and VEGF. PCNA plays an essential role in nucleic acid metabolism during DNA replication and repair (51). It functions as the accessory protein for DNA polymerase δ and is required for chromosomal DNA synthesis in S phase (52,53). Ki-67 antigen is expressed by cells in the G1, G2, S and M phases of the cell cycle and is commonly used as a marker of proliferative activity (54). Both antigens serve as markers for tumor proliferation and decreased survival in several carcinomas (52, 55–58). We have included both antigens in our study as PCNA is also expressed in cells undergoing DNA repair and can be non-specific for proliferative states (53). Our labeling indices for PCNA and Ki-67 indicated that in vivo proliferation was similar across cell lines and consistent with our in vitro proliferation assays. Interestingly, expression of other protein markers including OPN, were also similar in hepatic metastases derived from different cell types. We hypothesize that the hepatic metastases derived from pS-OPN cells resulted from the minority of pS-OPN that retained residual OPN expression either through incomplete penetration of the siRNA effect or pS-OPN cells that acquired molecular escape mechanisms to counter the expression of OPN-specific siRNA. Our in vitro data support this hypothesis as the degree of silencing shown by western blotting confirmed that OPN was not completely silenced with mean OPN expression decreased by ~3-fold. The hypothesis of residual expression is also consistent with the results of our functional assays, which show that suppression of OPN expression neither resulted in complete inhibition of cellular migration and invasion nor complete prevention of the development of in vivo hepatic metastases. Residual OPN expression in pS-OPN also accounts for the similar levels of MMP-2 expression between WT and pS-OPN-derived hepatic metastases in our in vivo IHC analysis. Importantly, in contrast to these in vivo IHC results, our in vitro western blots clearly demonstrated differential expression of OPN and MMP-2 in pS-OPN clones compared with WT, prior to intrasplenic injection. Together, these data suggest that the majority of pS-OPN-A4 cells have suppressed expression of OPN and MMP-2, are motility- and invasion-impaired and fail to invade and seed the liver. In turn, the majority of these OPN-downregulated cells are removed
by the circulatory or immune system in this in vivo model. At the same time, the minority of pS-OPN that have retained residual OPN and MMP-2 expression succeed in colonizing the liver.

Together, these in vitro and in vivo data represent the first report describing mechanistic evidence for OPN as a mediator of metastasis in a model for colon carcinoma using CT26 cells. Our data concerning MMP-2 represent the results of a simple screen to establish a basic mechanism of action for OPN. Certainly, successful seeding of tumor in end-organs such as the liver is the result of complex, cellular cascades that coordinate cellular processes including (but are not limited to) (i) tumor cell attachment to basement membrane through cell-surface adhesion molecules, (ii) proteolytic degradation of the ECM by tumor-derived proteinases and (iii) tumor cell migration through the ECM (26). A thorough examination of all possible OPN-downstream targets and mediators of OPN-dependent signaling pathways was beyond the scope of this study, but is the focus of ongoing research in our laboratory. Cumulative evidence in models of breast cancer and melanoma suggest that there are a number of OPN-downstream targets. Recent reports suggest that MMP-mediated remodeling of the ECM is one of several initiating events allowing cancer cells to invade the surrounding stroma (59,60). Philip et al. have recently shown that OPN upregulates pro-MMP-2 expression in a NF-κB-dependent fashion during ECM invasion by melanoma cells. OPN also increases cell invasiveness in human mammary carcinoma through stimulation of uPA (61). Das et al. have confirmed that OPN induction of uPA secretion is NF-κB-IkBα-IκK-mediated and dependent on PI3'-kinase/Akt activity (37,62). Migration of the tumor cell through the ECM is another fundamental metastatic process (63). OPN also induces epidermal growth factor receptor (EGFR) mRNA expression, EGFR tyrosine kinase activity, hepatocyte growth factor receptor (Met) mRNA and protein expression and increases Met kinase activity during OPN-mediated migration in human mammary cancer cells (64,65). Specific inhibition of EGFR and EGFR kinase results in reduced OPN-mediated migration (65). OPN-induced cell migration may also be mediated by signaling through the CD44 receptor. The molecular mechanisms governing these phenomena are not completely understood but some investigators suggest that OPN and CD44 interact with the ezrin, radixin and moesin (ERM) proteins and alter cytoskeletal dynamics through the cortical actin filaments (66). These data clearly suggest that there are a number of OPN-downstream targets. In the context of colorectal cancer, our laboratory is currently in the process of identifying relevant molecular targets in a systematic fashion using suppressive subtraction hybridization.

The regulation of OPN expression is another area that is not completely understood and warrants further investigation. Several investigators have demonstrated that OPN expression is induced by a variety of stimuli in tumor progression and controlled by complex regulatory pathways (7-9). Early investigations with the human OPN promoter uncovered multiple candidate elements that contain consensus sequences for known transcription factors. Potential regulatory sequences include TATA-like (−27 to −22 nt) and CCAAT-like (−73 to −68 nt) sequences, vitamin-D-responsive (VDR)-like motifs (−1892 to −1878 and −698 to −684 nt), GATA-1 (−851 to −847 nt), AP-1 (TGACACA, −78 to −72 nt), PEA3 (−1695 to −1690 and −1418 to −1413 nt) and Ets-1 (−47 to −39 nt) binding sequences and multiple TCF-1 recognition sequences (67). More recently, several transcription factors including AP-1, Myc, Oct-1, USF, v-Src, PEBP2αA/CBFα1, Smad, Hox, Tcf-4/β-catenin and TP53 have also been shown to regulate OPN expression in various cancer models (68,69).

To our knowledge, there are currently no studies that describe the molecular regulators of OPN expression in colon cancer. Ongoing investigations in our laboratory are currently directed toward uncovering the regulatory molecules that control OPN expression in colon cancer cells. Given the substantial evidence supporting a significant molecular function for OPN in tumor metastasis, focus on the molecular determinants that up-regulate OPN may lead to the identification of potential targets which can be modulated to inhibit the metastatic phenotype in colorectal cancer.

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