Lung cancer osteopontin isoforms exhibit angiogenic functional heterogeneity

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Objective: Osteopontin is a multifunctional phosphoprotein with an important but poorly understood role in non–small cell lung cancer pathogenesis. We hypothesize that osteopontin isoforms (*OPNa*, *OPNb*, and *OPNc*) have divergent roles in non–small-cell lung cancer angiogenesis and divergent impact on vascular endothelial growth factor secretion.

Methods: We examined mRNA expression using reverse transcriptase-polymerase chain reaction primers for 3 osteopontin isoforms in non–small-cell lung cancer and immortalized bronchial epithelial cell lines, and correlated expression with osteopontin secretion into media detected by enzyme-linked immunosorbent assay. Angiogenic properties conferred by osteopontin isoforms were evaluated by transfecting cDNA plasmids specific to each isoform and controls into non–small-cell lung cancer cell lines, H153 and H358 (low endogenous osteopontin) and A549 and H460 (high endogenous osteopontin), analyzing conditioned media on a bovine capillary endothelial platform, and measuring vascular endothelial growth factor levels by enzyme-linked immunosorbent assay.

Results: *OPNa* mRNA expression correlated with osteopontin secretion in cell lines ($r = 0.912$, $P = .0006$). *OPNa* overexpression significantly increased tubule length compared with controls, *OPNb* had a similar, but less pronounced effect, and *OPNc* significantly decreased tubule length compared with controls in each cell line. *OPNa* overexpression was associated with significant increases in vascular endothelial growth factor secretion, whereas *OPNb* had no effect and *OPNc* overexpression was associated with significant decreases in vascular endothelial growth factor compared with controls in each cell line.

Conclusion: We demonstrated divergent effects of osteopontin isoforms on non–small-cell lung cancer angiogenesis and vascular endothelial growth factor secretion. *OPNa* overexpression was associated with increased bovine capillary endothelial tubule length and vascular endothelial growth factor secretion, whereas *OPNc* was associated with decreases in both. These findings may lead to therapeutic strategies for selective isoform inhibition in non–small cell lung cancer. (J Thorac Cardiovasc Surg 2010;139:1587-93)

Osteopontin (OPN) is a multifunctional phosphoprotein with a significant role in the pathogenesis of non–small-cell lung cancer (NSCLC). In NSCLC, elevated OPN levels in tumors and serum are associated with poor patient prognosis and increased risk for recurrence.[1] NSCLC cell lines that natively express OPN have greater metastatic potential and invasive behavior,[2] but the molecular pathways for OPN tumorigenicity are incompletely understood. Three human mRNA splice variants of OPN have been identified. Osteopontin-a (*OPNa*) represents the full-length cDNA, osteopontin-b (*OPNb*) is defined by a deletion at exon-5, and osteopontin-c (*OPNc*) is defined by a deletion at exon-4. Expression and function of the individual isoforms in clinical NSCLC samples or tumor model systems have not been reported.

Increased angiogenesis is essential for growth and metastasis of solid tumors. In NSCLC, an important relationship exists between microvessel development and progression to invasive disease.[3-5] Tumor microvessel formation is dependent on adhesive interactions between tumor cells and the extracellular matrix, and is essential to establish conduits for nutrient delivery. Increased microvessel development has also been directly correlated with tumor maturation and is an independent risk factor for the development of metastatic disease.[5,6]

Vascular endothelial growth factor (VEGF) is a potent angiogenic and mitogenic protein that plays a central role in tumor neovascularization. In NSCLC, VEGF levels correlate directly with clinical outcome.[7,8] VEGF also has an established role in the progression of tumor invasion caused by receptor stimulated capillary in-growth and microvessel formation.[4,9] Binding of VEGF to endothelial cell surface receptors increases migration and proliferation. VEGF

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Abbreviations and Acronyms

BCE = bovine capillary endothelial
bp = base pair
DMEM = Dulbecco's Modified Eagle Medium
ELISA = enzyme-linked immunosorbent assay
NSCLC = non–small-cell lung cancer
OPN = osteopontin
RT-PCR = reverse transcriptase-polymerase chain reaction
VEGF = vascular endothelial growth factor

further supports the growth and stabilization of new blood vessels, which facilitates tumor expansion and formation of metastasis.[10] Interruption of this pathway via VEGF inhibition results in endothelial cell apoptosis and restricted tumor progression.[10]

VEGF and OPN are frequently expressed simultaneously during angiogenesis, both by tumor cells and by activated macrophages associated with ischemia and necrosis.[11] Coexpression of VEGF and OPN is associated with enhanced tumor microvessel development and is a marker for poor prognosis in early-stage NSCLC.[12] VEGF-mediated angiogenesis is significantly increased by concurrent OPN stimulation of endothelial cells.[10] The aim of this investigation is to evaluate the effect of individual OPN isoforms (*OPNa*, *OPNb*, and *OPNc*) on tumor-associated angiogenic properties of NSCLC and to determine whether concurrent VEGF expression and secretion is affected in an isoform-specific manner.

MATERIALS AND METHODS

Seven human lung cancer cell lines (A549, H358, H153, H157, H1299, H460, and Calu-3) were obtained from American Type Tissue Collection (Manassas, VA). Two human immortalized bronchial endothelial cell lines, Beas2 B and HKT3, were received as gifts (Dr John Minna, University Texas Southwestern, Dallas, Tex). Cell lines were maintained in culture in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Grand Island, NY) with 10% fetal bovine serum (Invitrogen) at 37°C, 5% CO_2.

Reverse transcriptase-polymerase chain reaction (RT-PCR) primers were designed on the basis of the 3 National Cancer for Biotechnology Information GeneBank sequences to be inclusive of the regions of exon-4 and exon-5 at the N-terminus, which are deleted from *OPNc* and *OPNb,* respectively. The resulting primers, 5′TTGCAGTGATTTGCTTTTGC-3′ (sense) and 5′GTCAATGGAGTCCTGGCTGT-3′ (antisense), amplify a 305-base pair (bp) product from *OPNa*, a 263-bp product from *OPNb,* and a 224-bp product from *OPNc* in a single reaction, which are separated by electrophoresis.

With the use of these primers, we examined mRNA expression of OPN by RT-PCR and correlated that with OPN secretion into media, detected by enzyme-linked immunosorbent assay (ELISA) (IBL, Gunma, Japan) in the series of 9 cell lines. Conditioned media was collected from cultures after 72 hours in serum-free conditions, and mRNA was isolated from the cell lines using the RNEasy mini kit (QIAGEN, Valencia, Calif). PCR amplification was carried out in a total volume of 20 μL using the standard Invitrogen PCR buffer system (Invitrogen, Carlsbad, Calif) and optimized concentrations of $MgCl_2$. The reactions were performed with denaturing for 15 seconds at 94°C, annealing for 30 seconds at 58°C, and elongation for 30 seconds at 70°C for a total of 40 cycles; the final extension was at 72°C for 5 minutes. RT-PCR products were analyzed by Tris-acetate EDTA agarose (2% w/v) gel electrophoresis. The amplification of peptidylprolyl isomerase A with primers 5′-TCTGAGCACTGGAGAGAAAGG-3′ (sense) and 5′GGAAAACATGGAACCC-3′ (antisense) served as controls for equal loading and integrity of all PCR reactions.

We evaluated the functional impact of each OPN isoform by transfecting cDNA plasmids specific to each isoform (Origene, Rockville, Md) into 4 NSCLC cell lines (H153, H358, A549, and H460). H153 and H358 cell lines have low endogenous OPN expression, whereas A549 and H460 have high endogenous OPN expression. Plasmids were derived from single-colony *Escherichia coli* culture and purified by silica chromatography. The protein coding sequence of each clone matched the published National Cancer for Biotechnology Information GeneBank NM references for each OPN isoform. NSCLC cell lines were transfected using Lipofectamine^{2000} (Invitrogen) with either OPN isoform plasmid construct or pCMV empty vector, which served as a control. A pooled population of cells was used for RT-PCR and functional analysis.

Angiogenic properties were measured using an in vitro bovine capillary endothelial (BCE) platform. BCE cells (Cell Applications Inc, San Diego, Calif) were maintained on gelatin-coated, 10-cm plastic dishes in 10 mL of endothelial growth media (Cell Applications, Inc), supplemented with 10% fetal bovine serum, 5 U/mL penicillin, and 5 mg/mL streptomycin at 37°C, 5% CO_2. Cells were subcultured for 9 to 13 passages before commencing experiments.

The BCE angiogenesis platform was performed as previously described.[13,14] In brief, BCE cells were trypsinized, washed in phosphate-buffered saline, and resuspended in conditioned media harvested from OPN isoform transfections and control cell lines at a concentration of 16,000 cells/100 mL. Diluted 1:100 Matrigel (Becton Dickinson, Bedford, Mass) was used to coat 96-well plates. Plates were incubated at room temperature for 1 hour and washed with phosphate-buffered saline. BCE cells in conditioned media were suspended on the Matrigel platform in triplicates. DMEM, DMEM + fibroblast growth factor (1 μg/100 mL) (R&D Systems, Minneapolis, Minn), and DMEM + VEGF (2 μg/100 mL) (R&D Systems) were used as controls. BCE cells were incubated with conditioned media on the Matrigel platform for 18 hours at 37°C, 5% CO_2, and then stained with 0.05% crystal violet. Tubule length was measured under 10× magnification using a 9-grid measuring platform. The sum of all tubules within an 889 μ^2 area was measured in triplicate and compared between experimental cell lines and controls. Tubules with a clear origin from an endothelial cell nucleus were included in the measurement. Tubule length is reported as a mean of total microns (μ) ± standard deviation.

We evaluated the impact of individual OPN isoforms on VEGF by measuring protein secretion into media from the OPN isoform transfected cell lines. Human VEGF ELISA Kits (R&D) were used to quantify VEGF secretion into media (picograms/milliliter). Changes in OPN protein secretion as a result of isoform overexpression were simultaneously evaluated by ELISA (described above).

To explore the relationship between VEGF secretion and OPN-induced mediators of endothelial cell proliferation, we used an Affymetrix Gene Chip (Santa Clara, Calif) U133 Plus2 platform to evaluate gene expression profiles in A549 (high endogenous OPN) with overexpression of *OPNa*, *OPNc*, and pCMV controls. mRNA was used from the same population of cells used in the BCE angiogenesis assays. Differences in VEGF expression identified in gene array were validated in all 4 cell lines by RT-PCR. VEGF primers, 5′CCTTGCTGCTCTACCTCCAC-3′(sense) and 5′ATCTGCATGGTGATGTTGGA-3′(antisense), amplify a 280-bp product. The RT-PCR protocol was performed as previously described.

A 2-sided Student *t* test was used to evaluate differences in BCE tubule length and VEGF secretion between cell lines transfected with the individual OPN isoforms and empty vector controls. Statistics were performed

using the Statistical Package for the Social Sciences version 11.5 (SPSS Inc, Chicago, Ill). Data are presented as mean ± standard deviation.

RESULTS

We noted a correlation between OPN mRNA expression and OPN secretion in the series of lung cancer and bronchial epithelial cell lines ($r = 0.9120$; $P = .0006$; confidence interval, 0.6286–0.9816). Five cell lines (A549, H460, H157, H1299, and Calu-3) had high endogenous OPN mRNA expression and OPN secretion detected in conditioned media. Two NSCLC cell lines (H153 and H358) and the 2 bronchial epithelial cell lines (Beas2 Bs and HKT3) had minimal endogenous OPN mRNA expression and no detectable OPN secretion into media. *OPNa,* the full-length isoform, was dominantly expressed in OPN-secreting cell lines. Concurrent *OPNb* expression was noted in the same cell lines, but to a lesser extent than *OPNa*. *OPNc* was not noted to be endogenously expressed in these cell lines (Figure 1).

Validation of plasmid expression in each cell line is demonstrated in Figure 2. Initial angiogenic experiments focused on overexpression of the individual OPN isoforms in H153, an NSCLC cell line with low endogenous OPN expression. Conditioned media harvested from H153 cells with forced overexpression of *OPNa* resulted in a significant increase in BCE tubule length (1599.23 μ ± 18.45) compared with empty vector controls (725.02 μ ± 12.75, $P < .0001$). *OPNb* overexpression had a similar effect on tubule length (1050.22 μ ± 13.22, $P < .0001$). Conversely, media from cells that overexpressed *OPNc* resulted in a significant decrease in tubule length compared with controls (523.05 μ ± 9.25, $P < .0001$) (Figure 3).

This divergent pattern of behavior with overexpression of the individual OPN isoforms was validated in 3 additional NSCLC cell lines: H358, with low endogenous OPN expression, and A549 and H460, both with high endogenous OPN expression. In all 4 cell lines, the pattern of effect on tubule length was consistent: Overexpression of *OPNa* significantly increased BCE tubule length compared with controls, overexpression of *OPNb* also significantly increased tubule length but to a lesser extent than *OPNa*, and overexpression of *OPNc* significantly decreased BCE tubule length compared with controls (Figure 3). Empty vector controls from A549 and H460 formed significantly longer tubules compared with controls with low endogenous OPN ($P <$ .0001), most clearly demonstrated in bar graft depiction of results from the BCE assay (Figure 4, *A*).

Overexpression of the individual OPN isoforms was also associated with a divergent effect on VEGF secretion. *OPNa* overexpression was associated with a significant increased in VEGF secretion compared with controls in all 4 cell lines. *OPNc* overexpression was associated with a significant decrease in VEGF secretion in all cell lines compared with controls. However, *OPNb* overexpression was not associated with significant alterations in VEGF secretion compared with controls in any of the cell lines (Figure 4, *B*).

Overexpression of the individual OPN isoforms was also associated with a divergent effect on OPN secretion. Overexpression of *OPNa* and *OPNb* was associated with small increases in the concentration of OPN protein in conditioned media, but *OPNc* overexpression was associated with a significant increase in OPN protein secretion in all 4 cell lines (Figure 4, *C*).

Significant differences in downstream patterns of regulation were noted on expression profiles of A549 overexpressing *OPNa* and *OPNc*. Relative expression of VEGF was increased 2-fold compared with controls with overexpression of *OPNa* and decreased 40% with overexpression of *OPNc*. This pattern of divergent VEGF expression was validated by RT-PCR in all 4 cell lines. We noted that control cell lines with high endogenous OPN (A549 and H460) had appreciably higher VEGF mRNA expression than control cell lines with low endogenous OPN expression (H153 and H358). In all cell lines, overexpression of *OPNa* was associated with increased VEGF mRNA expression, most appreciably in cell lines with low endogenous OPN, and

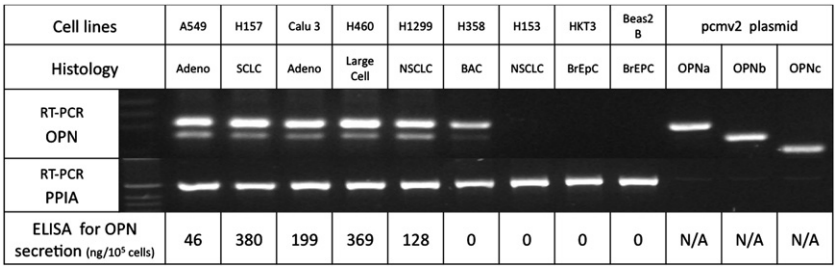


FIGURE 1. OPN mRNA expression in human lung cancer and immortalized bronchial epithelial cells. Primers amplify each of the 3 isoforms, which are then separated by electrophoresis on 2% agarose gel. *OPNa* produces the 305-bp dominant band seen at the top. *OPNb* amplifies a 263-bp fragment seen below the dominant band in A549, H157, Calu-3, H460, and H1299. *OPNc* amplifies a 224-bp fragment that migrates below the *OPNb* and is not seen in these cell lines. Amplification of pCMV2 plasmid specific to each isoform serves as positive controls, and amplification of peptidylprolyl isomerase A serves as control for equal loading. OPN expression correlates with OPN secretion as detected by ELISA after 72 hours in serum-free media. *BrEpC,* Immortalized bronchial epithelial cells; *adeno,* adenocarcinoma; *ELISA,* enzyme-linked immunosorbent assay; *SCLC,* small-cell lung cancer; *NSCLC,* non–small-cell lung cancer; *OPN,* osteopontin; *PPIA,* peptidylprolyl isomerase A; *RT-PCR,* reverse transcriptase-polymerase chain reaction; *N/A,* not available.

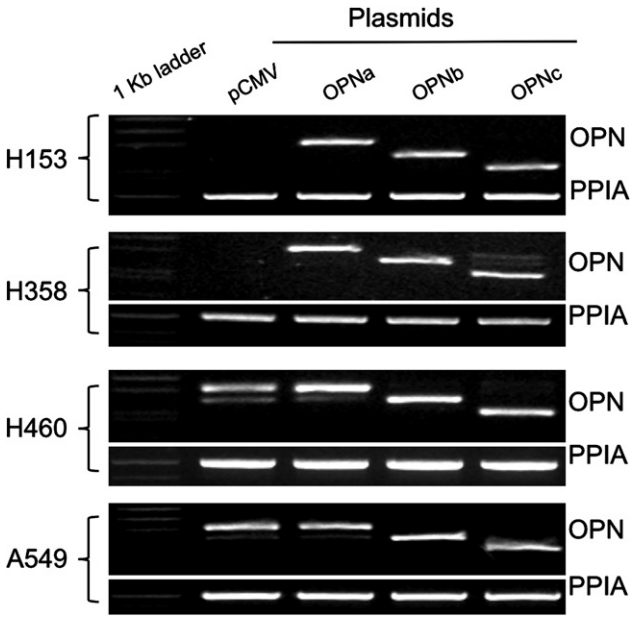


FIGURE 2. RT-PCR demonstrating forced expression of individual OPN isoforms in 4 NSCLC cell lines. Pooled populations of transfected cells were used in RT-PCR and angiogenesis assay. H153 and H358 have low native OPN expression. A549 and H60 have high native OPN expression. *OPN*, Osteopontin; *PPIA*, peptidylprolyl isomerase A.

OPNc was associated with decreased VEGF expression compared with controls, most appreciably in cell lines with high endogenous OPN. *OPNb* overexpression, which was not evaluated by gene array, seems to have a less consistent impact on VEGF expression (Figure 5).

DISCUSSION

OPN has been identified in a remarkable range of normal and pathologic contexts.[15] It is an important adhesive bone matrix protein and plays a key role in immune cell recruitment, wound healing, and tissue remodeling.[16,17] Overexpression of OPN is also recognized in pathologic conditions that involve inflammation, ischemia, and malignancy. The importance of OPN in tumor pathogenesis is highlighted in gene transfer experiments, in which transfection of OPN increases the malignant phenotype,[18] and OPN knock-out experiments with antisense oligonucleotides that decrease malignant potential.[19,20] OPN's principle functions are related to cell migration and adhesion, and are mediated by integrin binding to its central and well-conserved RGD (arginine-glycine-aspartate) domain. OPN is overexpressed by immunohistochemistry in human NSCLC tumors compared with surrounding normal lung paranchyma.[2] In vitro experiments demonstrate increased migration and invasion by NSCLC cell lines that overexpress OPN.[2] These data suggest an important role for OPN in determining the metastatic potential of NSCLC.

Increased angiogenesis is a key feature of malignant transformation that allows for tumor growth and facilitates tumor cell entry into the circulation.[21] VEGF is a crucial intermediate in this process, which acts via endothelial cell activation, facilitating mitogenic and vascular permeability-enhancing activities.[3,4,7,9] Inhibition of VEGF decreases tumor neovascularization and substantially inhibits primary tumor growth.[22] OPN has an established relationship with VEGF in the angiogenic development of early stage

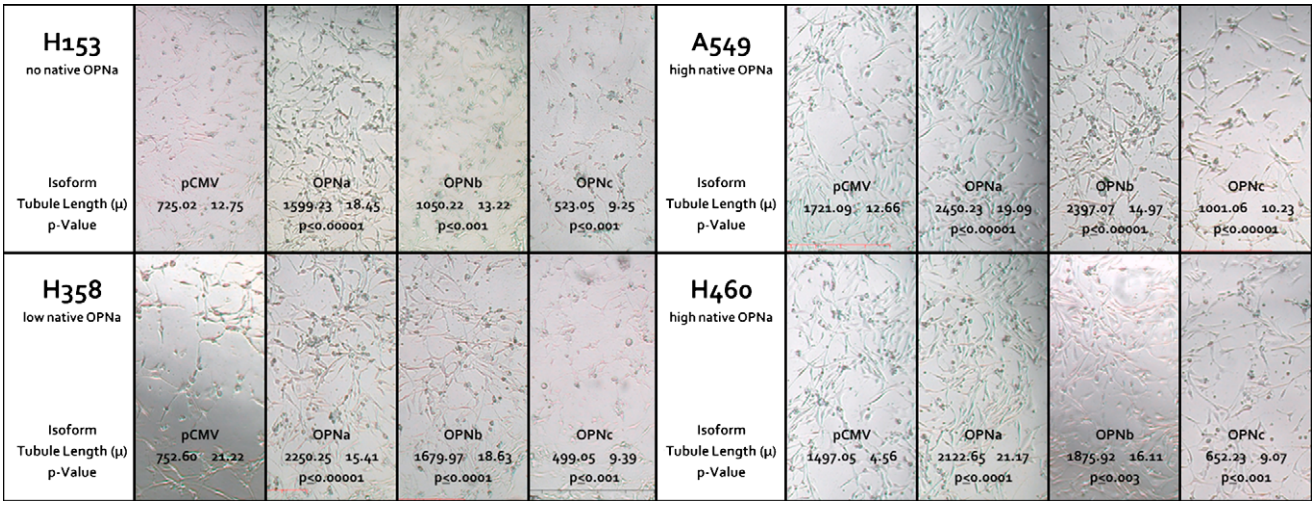
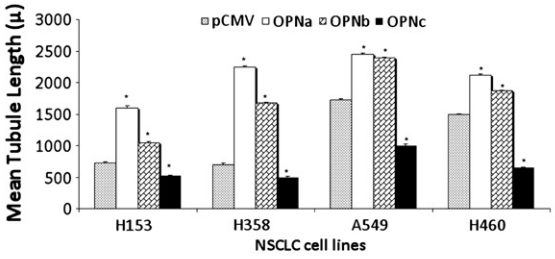
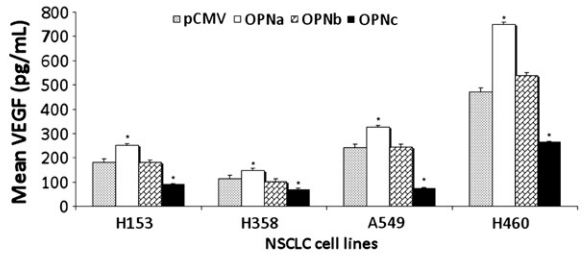


FIGURE 3. Representative sections showing tubule formation and mean tubule lengths for BCEs plated on Matrigel (Becton Dickinson, Bedford, MA) and cultured in conditioned media collected from NSCLC cell lines transfected with the individual OPN isoforms and pCMV empty vector controls. H153 and H358 have low native OPN expression, and A549 and H460 have high native OPN expression. *OPNa* overexpression in all cell lines resulted in significantly more tubule formation compared with controls. *OPNb* overexpression also significantly increased tubule length in all cell lines compared with controls, but to a lesser extent then *OPNa*. *OPNc* overexpression resulted in a significant decrease in tubule formation compared with controls in all 4 NSCLC cell lines. *OPN*, Osteopontin.



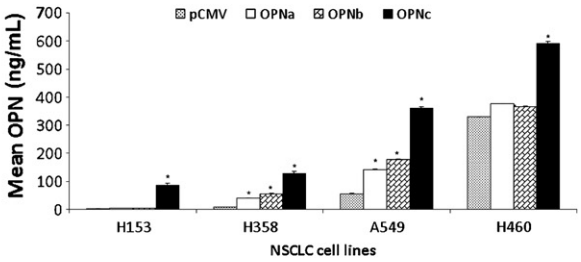
	H153		H358		A549		H460	
	BCE Tubule Length (u)	P-Value	BCE Tubule Length (u)	P-Value	BCE Tubule Length (u)	P-Value	BCE Tubule Length (u)	P-Value
pCMV	725.02 ± 12.75		752.6 ± 21.22		1721.09 ± 12.66		1497.05 ± 4.56	
OPNa	1599.23 ± 18.45	0.0001	2250.25 ± 15.41	0.0001	2450.23 ± 19.09	0.0001	2122.65 ± 21.17	0.0001
OPNb	1050.22 ± 13.22	0.001	1679.97 ± 18.63	0.0001	2397.07 ± 14.97	0.0001	1875.92 ± 16.11	0.003
OPNc	523.05 ± 9.25	0.001	499.05 ± 9.39	0.001	1001.06 ± 10.23	0.001	652.23 ± 9.07	0.001

A



	H153		H358		A549		H460	
	VEGF ELISA Media (pg/ml)	P-Value	VEGF ELISA Media (pg/ml)	P-Value	VEGF ELISA (pg/ml)	P-Value	VEGF ELISA Media (pg/ml)	P-Value
pCMV	190.75 ± 20.02		112.63 ± 38.22		241.97 ± 13.43		472.29 ± 10.05	
OPNa	250.54 ± 22.15	0.01	148.36 ± 17.02	0.03	325.58 ± 6.01	0.001	679.54 ± 13.09	0.001
OPNb	180.47 ± 19.72	1	102.11 ± 18.97	1	245.26 ± 7.32	1	539.31 ± 24.07	0.06
OPNc	91.25 ± 6.07	0.001	70.8 ± 16.51	0.001	73.88 ± 3.50	0.001	264.78 ± 5.75	0.001

B



	H153		H358		A549		H460	
	OPN ELISA Media (pg/ml)	P-Value	OPN ELISA Media (pg/ml)	P-Value	OPN ELISA (pg/ml)	P-Value	OPN ELISA Media (pg/ml)	P-Value
pCMV	1.22 ± .15		8.27 ± 3.35		55.52 ± 4.98		329.82 ± 22.09	
OPNa	4.05 ± .22	1	39.43 ± 5.02	0.03	142.72 ± 6.09	0.001	376.23 ± 18.67	1
OPNb	3.95 ± .19	1	55.85 ± 3.95	0.01	178.32 ± 8.22	0.001	366.23 ± 14.52	0.09
OPNc	87.23 ± 2.35	0.001	128.33 ± 15.05	0.001	360.75 ± 14.05	0.0001	592.75 ± 21.05	0.001

C

FIGURE 4. A, Bar graft depicting the effect of the overexpression of individual OPN isoforms compared with pCMV empty vector controls on mean BCE tubule length (μ) in 4 NSCLC cell lines. Asterisk denotes a significant difference between OPN isoform overexpression in cell lines and pCMV controls. Mean tubule length ± standard deviation and *P* value are listed below bar graft. H153 and H358 wild-type cells had significantly shorter BCE tubule lengths than A549 and H460, which have high native OPN expression. Tubule formation was significantly increased in all cell lines with forced overexpression of *OPNa* and *OPNb* compared with controls. Conversely, overexpression of *OPNc* resulted in significantly fewer tubules formed compared with controls in all cell lines. B, Bar graft depicting associated changes in VEGF secretion (picograms/milliliter) with overexpression of individual OPN isoforms compared with pCMV empty vector controls in 4 NSCLC cell lines. Asterisk denotes a significant difference between OPN isoform and pCMV controls. Mean VEGF concentrations ± standard deviation and *P* value are listed below bar graft. H153 and H358 have low native OPN expression, and A549 and H460 have high native OPN expression. Significant increases in VEGF secretion were associated with *OPNa* overexpression compared with controls. Conversely, *OPNc* overexpression was associated with significant decreases in VEGF secretion compared with controls in all cell lines. *OPNb* overexpression was associated with no significant change in VEGF secretion in any of the 4 cell lines. C, Bar graft depicting the effect of the overexpression of individual OPN isoforms compared with pCMV empty vector controls on OPN secretion (nanograms/milliliter) in 4 NSCLC cell lines. Asterisk denotes a significant difference between OPN isoform overexpression in cell lines and pCMV controls. Mean OPN concentrations ± standard deviation and *P* value are listed below bar graft. In H153, H358, and H460 overexpression of *OPNa* and *OPNb* had no significant impact on OPN protein concentrations in conditioned media. In A549, overexpression of *OPNa* and *OPNb* caused significant step-up in OPN protein secretion. Overexpression of *OPNc* caused a significant increase in OPN secretion in all cell lines. OPN protein is measured by ELISA, which is not isoform specific. *OPN,* Osteopontin; *NSCLC,* non–small-cell lung cancer; *BCE,* bovine capillary endothelial; *VEGF,* vascular endothelial growth factor; *ELISA,* enzyme-linked immunosorbent assay.

NSCLC,[10,12] and VEGF-mediated angiogenesis is enhanced by concurrent up-regulation of OPN.[10]

OPN and VEGF are known to act synergistically via $\alpha v\beta 3$ integrin on the endothelial cell surface. Exposure to VEGF increases $\alpha v\beta 3$ expression, allowing for increased endothelial cell migration in the presence of OPN.[11] The $\alpha v\beta 3$ integrin is up-regulated in NSCLC tumors compared with normal tissue, and antibody-mediated inhibition of this integrin results in endothelial cell apoptosis and restricted invasion.[23,24] This common pathway for OPN and VEGF could serve as an important target for the regulation of malignant behavior in NSCLC.[10]

We have demonstrated on an in vitro angiogenesis platform that overexpression of the individual OPN isoforms has a divergent impact on angiogenic properties in NSCLC. *OPNa* increases BCE tubule formation, whereas *OPNc* reduces tubule length. We have also demonstrated that *OPNa* overexpression is associated with increased VEGF secretion and expression, whereas *OPNc* is associated with decreased VEGF secretion and expression, particularly in

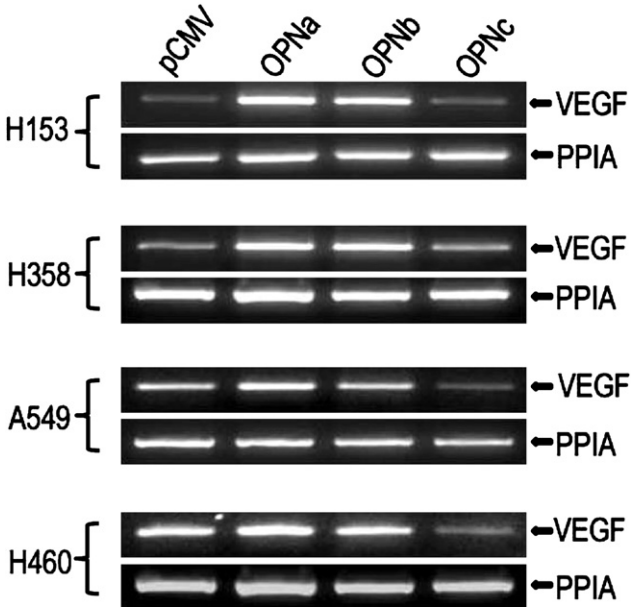


FIGURE 5. Gene-array analysis demonstrated *OPNa* overexpression was associated with a 2-fold increase in relative expression of VEGF, whereas OPNc overexpression was associated with a 40% decrease in VEGF expression compared with controls. RT-PCR validation of gene array with regard to divergent VEGF expression with overexpression of the OPN isoforms is shown. Overexpression of *OPNa* in all 4 cell lines resulted in elevated VEGF mRNA expression, whereas *OPNc* overexpression decreased VEGF expression in all cell lines compared with wild-type cells. Elevated VEGF mRNA expression was appreciated in control cell lines with high native OPN expression (A549 and H460) compared with cell lines with low native OPN expression (H153 and H358). *OPN,* Osteopontin; *VEGF,* vascular endothelial growth factor; *PPIA,* peptidylprolyl isomerase A.

cell lines with high endogenous OPN. These findings suggest that some of the angiogenic properties conferred by OPN may be due to modulation of VEGF production by tumor cells. Studies that initially described the BCE platform used in our experiments noted a relationship between VEGF activity and increased tubule formation.[25] Enhanced capillary tubule formation with simultaneous increases in VEGF secretion by NSCLC cells that overexpress *OPNa* in our experiments is consistent with these observations. We also noted that wild-type NSCLC cell lines with high endogenous OPN (A549 and H460) had higher endogenous VEGF secretion and greater angiogenic potential measured by BCE tubule formation compared with wild-type cell lines with low endogenous OPN (H153 and H358). However, *OPNb* overexpression increased BCE tubule length without associated changes in VEGF secretion or expression, indicating that additional pathways are likely important, and further demonstrating that each isoform has a unique effect in this complex process. Senger and colleagues[11] demonstrated OPN's ability to increase endothelial cell migration via binding to the $\alpha v\beta 3$ integrin. It is reasonable to assume that *OPNb* has the ability to activate endothelial receptors via binding to $\alpha v\beta 3$ integrin, which would result in increased tubule formation observed in these experiments, but it does so without a concomitant augmentation of VEGF secretion or expression by tumor cells. This ''2-hit'' effect on angiogenesis is likely exclusive to the full-length protein.

The relationship between overexpression of *OPNa* and increased tubule formation was not linear. This is best appreciated in H460 (high endogenous OPN), where overexpression of *OPNa* significantly increased tubule formation compared with controls, but to a smaller extent than in H358 (low endogenous OPN) (Figure 4). VEGF and OPN are known to act synergistically via $\alpha v\beta 3$ integrin to increase angiogenesis in NSCLC. A similar nonlinear increase in endothelial cell migration has been noted with exposure of increasing concentrations of the 2 mediators.[11] Cell lines with low endogenous OPN expression demonstrated a larger percentage increase in tubule length with overexpression of *OPNa*, but a smaller increase in VEGF secretion, compared with cell lines with high native OPN. This may indicate that the introduction of *OPNa* into a system without endogenous OPN may have a greater impact on endothelial cell activation than increasing expression in the presence of already high levels of *OPNa*.

The impact of *OPNc* overexpression, which decreased VEGF secretion and tubule length, appeared more linear. Cell lines with high endogenous OPN had relatively large decreases in tubule length and VEGF secretion compared with cell lines with low endogenous OPN in the presence of the c-isoform. *OPNc* seems to act as a dominant negative in this system, overriding the effects of the native OPN isoforms.

Our data suggest that OPN's effect on tumor angiogenesis is multifactorial and that each OPN isoform has a unique effect. *OPNa*, the full-length protein and dominant isoform expressed in NSCLC cell lines, increases angiogenesis by activating endothelial cells, likely via binding to the $\alpha v\beta 3$ integrin, with associated increases in VEGF secretion by the tumor cells. *OPNb,* which has a deletion at exon-5, seems to activate endothelial cells but does not increase VEGF secretion by tumor cells. Neither *OPNa* nor *OPNb* overexpression resulted in significant increases in OPN protein secretion, indicating that the effects in endothelial cell activation are not merely due to the presence of more OPN protein. *OPNc,* which lacks exon-4, has a dominant negative effect in the system, decreasing endothelial cell activation and VEGF secretion by the tumor cells. This supports our hypothesis that individual OPN isoforms have divergent roles with respect to tumor-associated angiogenesis and VEGF production in NSCLC. The proangiogenic properties of OPN function in an isoform-specific manner, and this phenomenon is most pronounced in the *OPNa* isoform. Structural similarities between *OPNa* and *OPNc*, which behave divergently, may make OPN ideal for isoform-specific targeted therapies aimed at reducing the angiogenic potential of NSCLC.

Appreciable weaknesses of this study include the inability to evaluate isoform-specific inhibitory effects of OPN on VEGF expression, secretion, and endothelial cell activation because of the lack of isoform-specific antibodies currently available. Our laboratory is currently working on production of such antibodies. Future experiments include silencing RNA in cell lines with high endogenous OPN to further define the role of OPN on tumor angiogenesis. The lack of quantitative PCR or gene array data from all transfected cell lines weakens our findings with respect to isoform-specific mRNA expression.

CONCLUSIONS

These in vitro data demonstrate that *OPNa* confers greater angiogenic properties with increased BCE tubule formation and an associated increase in secretion of VEGF in NSCLC. *OPNb,* which lacks exon-5, activates endothelial cells, but has no effect on VEGF secretion. *OPNc*, which lacks exon-4, has a dominant negative effect, decreasing tubule length and VEGF secretion. *OPNa* is the isoform preferentially up-regulated in NSCLC cell lines. An enhanced understanding of the specific behavior of these subunits may lead to therapeutic strategies that selectively inhibit OPN isoforms to alter the angiogenic and metastatic potential of NSCLC.

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