Osteopontin Promotes the Invasive Growth of Melanoma Cells by Activating Integrin \( \alpha(v)\beta(3) \) and Down-Regulating Tetraspanin CD9

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Overexpression of osteopontin (OPN) is strongly associated with the invasiveness/metastasis of many cancers, including melanomas. However, the molecular mechanisms of OPN in these processes remain poorly understood. We found that forced expression of OPN in early vertical-growth-phase melanoma cells dramatically increased their migration/invasion and growth/survival in a three-dimensional collagen I gel. Neutralizing antibodies to OPN, integrin \( \alpha_v \beta_3 \), and integrin \( \alpha_v \beta_5 \), but not to CD44, negated the effects of OPN. Conversely, knocking down OPN in metastatic melanoma cells abrogated the invasive growth. OPN overexpression activated and OPN knockdown inactivated \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \) integrins, negligibly affecting their expression. We further found OPN expression to inversely correlate with tetraspanin CD9 expression. Early-stage melanoma cells displayed low OPN and high CD9 expression, and conversely, metastatic cells displayed high OPN and low CD9 expression. Overexpression of OPN in vertical-growth-phase melanoma cells induced down-regulation of CD9, and knockdown of OPN in metastatic melanoma cells up-regulated CD9. Reversion of these CD9 changes abolished the effects of OPN. Furthermore, knockdown of CD9 in early-stage melanoma cells stimulated their invasive capacity in three-dimensional collagen. Similarly, microarray analyses of benign nevi and primary melanomas from different stages revealed an inverse correlation between OPN and CD9. These data suggest that OPN promotes melanoma cell invasion by activating integrin \( \alpha_v \beta_3 \) and down-regulating CD9, a putative metastasis suppressor. (Am J Pathol 2014, 184: 842–858; http://dx.doi.org/10.1016/j.ajpath.2013.11.020)
metastasis, and poor prognosis in many other cancer types, such as breast, lung, ovarian, liver, colon, and other cancers. Besides the tumor cells, several tumor-associated stromal cells may also produce OPN and contribute to tumor progression. OPN is a multifunctional protein that can enhance proliferation, prevent apoptosis, contribute to angiogenesis, and promote invasion of various cancer cells. OPN can bind to several integrin receptors, including αvβ1, αvβ3, αvβ5, αvβ6, α4β1, α5β1, α9β1, and αβ3. The arginine-glycine-aspartate (RGD) motif of OPN is the major binding site for integrins, but the binding site for α6β1 is SVVYGLR. OPN is also able to bind CD44 receptors. Thus, the interaction of OPN with these cell-surface receptors can activate various intracellular signal transduction pathways and downstream molecules regulating cell behavior, such as AKT, NF-κB, and c-MET. However, little is still known about the downstream molecules involved in the regulation of the OPN-promoted invasion and metastasis.

In this study, we made use of previous studies on OPN in various cancers and microarray analyses of early vertical-growth-phase (VGP) melanoma cells and metastatic melanoma cells, and studied early-stage melanoma cells engineered to overexpress OPN, and metastatic melanoma cells knocked down in OPN expression, to further our understanding of the actions of OPN and identify molecules regulating the OPN-promoted invasion and growth of melanoma cells. We show that OPN is particularly important for the melanoma cell migration/invasion and growth in three-dimensional (3D) collagen I matrix, and that activation of integrin αvβ3 and down-regulation of tetraspanin CD9 are key events in the invasion process. We further show that knocking down CD9 in early VGP melanoma cells provides them with a high invasive capacity. Finally, we show that the expression levels of OPN and CD9 inversely correlate in clinical specimens of benign nevi and invasive melanomas.

Materials and Methods

Cell Culture

The VGP WM115 and G-361 melanoma cells (ATCC; LGC Promochem, Stockholm, Sweden) and the early VGP WM793 melanoma cells and metastatic WM239 melanoma cells (from a lymph node metastasis), kindly provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA), and their transfectants were grown in RPMI 1640 medium containing antibiotics (penicillin, streptomycin) and 10% fetal bovine serum (Invitrogen/Life Technologies, Carlsbad, CA) at 37°C in a 5% CO2 atmosphere.

Plasmids and Transfection

The human OPN cDNA-containing pcDNA3.1 vector [OP10/pcDNA3, kindly provided by Dr. Ann Chambers (University of Western Ontario, London, ON, Canada)], the human CD9 cDNA-containing pZeosV vector (pZeosV-CD9), kindly provided by Dr. Isao Tachibana (Osaka University, Osaka, Japan), empty pcDNA3.1 and pZeosV vectors (Invitrogen/Life Technologies), empty pLKO.1 vector, and the Expression TRC CD9 shRNA set (both from Open Biosystems/Thermo Fisher Scientific, Huntsville, AL) were transfected into WM793, G-361, and WM239 cells using Lipofectamine 2000 (Invitrogen/Life Technologies) according to the manufacturer’s instructions. Two days after transfection, the cells were subjected to selection with neomycin (for pcDNA3.1 vectors) or puromycin (for pLKO.1-based vectors), and pools of stable transfectants and individual clones (isolated by cylinder cloning) were used for experiments.

Generation of Cell Lines with a Tetracycline-Inducible System for Expression of Sense or Antisense OPN cDNA

The full-length human OPN cDNA was released from the pcDNA3.1 vector and subcloned into a blunt-ended inducible pLRT expression vector. The ligation products were analyzed by automated sequencing (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems/Life Technologies, Foster City, CA) to determine the fragment orientations. The pLRT-OPN sense and antisense plasmids were transfected into WM793 and WM239 cells, respectively, using Lipofectamine 2000 reagent, and stable transfectants were selected using blasticidin (5 μg/mL). Individual clones were picked up by cylinder cloning and tested for their inducibility of OPN expression (or antisense expression) by adding doxycycline (Sigma-Aldrich, St Louis, MO).

Lentiviral shRNA Transduction

WM239 cells were grown to 60% confluency and infected with shRNA lentiviral particles targeting OPN (sc-37252-SH, Santa Cruz Biotechnology, Dallas, TX), or with negative (scrambled) control shRNA particles (sc-108080) in the presence of 10 μg/mL Polybrene (hexadimethrine bromide). After 72 hours of transduction, the cells were collected for further experiments. Transductions were performed twice in duplicates in 24-well plates.

Cell Migration and Invasion Assays in 3D Matrices

The 3D collagen I gel invasion/migration assays were performed as described previously. Briefly, 1.5 to 2.0 × 10^4 cells in 100 μL of RPMI 1640 were seeded onto collagen I gels cast in 24-well plates (400 μL of collagen I/well, 2 μg/mL; BD Biosciences, Franklin Lakes, NJ) and allowed to adhere for 1 hour at 37°C. Excess RPMI 1640 was removed, and a second 300-μL collagen I gel layer was cast above the cells. Finally, 500 μL of RPMI 1640 containing 10% fetal bovine serum was added on top of the collagen I gel matrix. In the experiments with function-blocking antibodies, the neutralizing antibodies to OPN (R&D Systems, Minneapolis, MN), integrin β1 (6S6; EMD Millipore, Billerica, MA), integrin αvβ3 (LM609;
Millipore) or CD44 (IM7; BD Biosciences), or isotype control antibodies (10 µg/mL) were added into the collagen I gel and the growth medium. The growth medium was replenished every third day. The growth pattern and morphology of the cells was documented daily by phase-contrast microscopy and photography. The 3D Matrigel invasion assays were performed as previously described. The assays were repeated at least three times.

**Boyden Chamber/Transwell Migration Assays**

Migration of the cells was additionally analyzed using collagen I–coated Falcon cell-culture inserts (BD Biosciences), as described previously.

**Adhesion/Spreading Assays**

Cell-adhesion assays were performed on 96-well plates coated with plasma fibronectin, collagen I, collagen IV, and vitronectin, and with bovine serum albumin as a negative control.

**Cell Proliferation Analysis**

Cells were seeded in triplicate in 6-well plates or in 3D collagen I gel matrix in 24-well plates. At various time points, the cells cultured in two-dimensions (2D) were detached with trypsin, and the cells cultured in 3D collagen I gel were harvested by centrifugation at 300 × g for 5 minutes, after digestion of the gel with collagenase (0.2 mg/mL in PBS, for 90 minutes at 37°C; Sigma-Aldrich). Cells were counted by trypan blue exclusion.

**Annexin V Apoptosis Assays**

Cells were seeded on 24-well plates coated with 350 µL of collagen I gel in serum-free medium. After 36 hours, cellular apoptosis (phosphatidylserine externalization) was detected by annexin V staining using the Annexin V-FITC kit (BD Biosciences). Cell images were documented with a fluorescent microscope (Axiophot 2; Carl Zeiss, Oberkochen, Germany). The percentage of annexin V–positive cells was calculated from three randomly selected fields.

**Preparation of Secreted Proteins**

Secreted proteins from the cell-culture media were collected and concentrated using Amicon Ultracel-10 centrifugal filters (MWCO 10,000; Millipore), and equal amounts of proteins (10 to 25 µg) were subjected to Western blot analysis.

**Western Blot Analysis**

Western blot analyses of whole-cell protein lysates and secreted proteins were performed essentially as described previously, except that the analysis of CD9 was performed under nonreducing conditions. Proteins (10 to 20 µg) were run in SDS-PAGE (5% to 10%) and transferred onto a nitrocellulose membrane. The filters were incubated in blocking buffer overnight, and then incubated with mouse monoclonal antibodies (mAb) against OPN (Enzo Life Sciences, Farmingdale, NY), CD9 (MM2/57; EMD Millipore), and β-actin (Sigma-Aldrich) for 4 to 6 hours. The proteins were detected by enhanced chemiluminescence (Pierce antibodies; Thermo Fisher Scientific, Rockford, IL). Band densities were quantified using ImageJ version 1.44 (NIH, Bethesda, MD).

**Fluorescence-Activated Cell Sorting Analysis**

The cell-surface expression of tetraspanin CD9 and integrins αvβ3 and αvβ5 were evaluated by fluorescence-activated cell sorting (FACS) analysis. Cells were detached with 1 mmol/L EDTA in PBS and washed in blocking solution (PBS with either 0.5% bovine serum albumin or normal goat serum). The cells (1.0 × 10^5) were then incubated with 1.0 µg of mouse mAb to CD9 (ALB6; Beckman Coulter, Brea, CA), mouse mAb to αvβ3 (LM609), or mouse mAb to αvβ5 (P1F6; Santa Cruz Biotechnology) for 1 hour on ice, followed by sequential incubation with biotinylated secondary antibody and streptavidin-phycocerythrin, or Alexa Fluor 488–conjugated secondary antibody (Molecular Probes/Life Technologies). Finally, the expression levels of CD9 and integrins αvβ3 and αvβ5 were analyzed using a flow cytometer (FACScan, FACSAria II, or BD Accuri C6; BD Biosciences).

The amounts of active integrin αvβ3 were measured with a monovalent ligand-mimetic WOW-1 antibody (Fab fragment) (generously provided by Dr. Sanford J. Shattil, University of California, San Diego, CA), using Alexa Fluor 488–conjugated goat anti-mouse IgG (H+L) F(ab)2 (Molecular Probes/Life Technologies) as the secondary antibody.

**Immunofluorescence Staining of Actin**

Cells were cultured on top of thick collagen I matrix coated onto glass coverslips, and fixed with 3.5% paraformaldehyde. F-actin was stained with Alexa Fluor 594–conjugated phalloidin (Molecular Probes/Life Technologies). Fluorescence was visualized by using a Zeiss Axioscope 2 microscope and Axioscope2 photo module (Carl Zeiss), equipped with a Hamamatsu CCD digital camera (Hamamatsu Photonics, Hamamatsu City, Japan).

**Immunohistochemical Analysis of Cells Grown in 3D Collagen I Gels**

For immunohistochemical analysis of the cells grown within the thick 3D collagen I matrices, the gels were frozen in liquid nitrogen or fixed with formalin, embedded in paraffin, and cut into 5-µm sections. The sections were then examined by a routine immunohistochemical process as described. After deparaffinization, rehydration, heat-induced epitope
retrieval, and endogenous peroxidase blockage, the slides were incubated with rabbit anti-Ki67 mAb (SP6; Abcam, Cambridge, UK).

RT-PCR Analysis

Total RNAs were extracted from cells with the RNeasy kit (Qiagen, Crawley, UK). RNA quality/quantity was determined using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). One microgram of total RNA was reverse transcribed to cDNA. The primers of the different candidate genes and PCR conditions are listed in Table 1 and Kapyla et al.51 The PCR products were run on 2% agarose gels. After electrophoresis, the gels were stained with GelStar nucleic acid gel stain (Cambrex Bio Science Rockland, Rockland, ME). The bands were visualized and documented with Alpha Imager HP and Alpha Easer Software version 5.01 (Alpha Innotech Corporation, San Leandro, CA).

Patient Samples

Fresh biopsies from benign nevi (n = 12) and primary melanomas (n = 20) from healthy volunteers and melanoma patients were obtained by surgical resection at Helsinki University Central Hospital, Helsinki, Finland. The study was approved by the Ethics Committee of the Helsinki University Central Hospital, and informed consent was obtained from the patients.

Table 1  Primer Sequences and PCR Conditions

<table>
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<tr>
<th>Gene name</th>
<th>Primer Polarity</th>
<th>Sequence</th>
<th>Annealing (°C)</th>
<th>Cycles</th>
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<td>Osteopontin</td>
<td>Forward</td>
<td>5′-CCAAGTAAAGTCCAACGAAAAG-3′</td>
<td>55</td>
<td>20–28</td>
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<tr>
<td>CD9</td>
<td>Forward</td>
<td>5′-ACTGTTCCTCCTGTTGTA-3′</td>
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<td>35</td>
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<td>Integrin α1</td>
<td>Forward</td>
<td>5′-CACTGCGGCTGACAGCTGAG-3′</td>
<td>55</td>
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<tr>
<td>Integrin α2</td>
<td>Forward</td>
<td>5′-GGTGCCTGACAGCTGAG-3′</td>
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<tr>
<td>Integrin α10</td>
<td>Forward</td>
<td>5′-GGTGCCTGACAGCTGAG-3′</td>
<td>55</td>
<td>35</td>
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<tr>
<td>Integrin α11</td>
<td>Forward</td>
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<td>35</td>
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<tr>
<td>Integrin αν</td>
<td>Forward</td>
<td>5′-GGTGCCTGACAGCTGAG-3′</td>
<td>55</td>
<td>35</td>
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<tr>
<td>Integrin β1</td>
<td>Forward</td>
<td>5′-GGTGCCTGACAGCTGAG-3′</td>
<td>55</td>
<td>35</td>
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<tr>
<td>Integrin β3</td>
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<td>5′-GGTGCCTGACAGCTGAG-3′</td>
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<td>Integrin β5</td>
<td>Forward</td>
<td>5′-GGTGCCTGACAGCTGAG-3′</td>
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<td>β-actin</td>
<td>Forward</td>
<td>5′-GGTGCCTGACAGCTGAG-3′</td>
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<td>GAPDH</td>
<td>Forward</td>
<td>5′-GGTGCCTGACAGCTGAG-3′</td>
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Microarray Analysis

Microarray analyses with the Affymetrix Human Genome U133 Plus 2.0 arrays were performed according to the manufacturer’s protocols (Affymetrix, Santa Clara, CA), as previously described.10,48 Briefly, total RNAs were extracted using the RNeasy kit, and reverse-transcribed to cDNAs. After in vitro transcription, the biotinylated cRNAs were fragmented and hybridized to the chips. Washing and staining (with antibody amplification) was performed on an Affymetrix Fluidics 450 station, and the signals were scanned. The data were then processed by RMAExpress version 1.0.5, released May 22, 2010 (http://rmaexpress.bmbolstad.com).

Statistical Analysis

Data analyses were performed using a two-tailed Welch’s t-test. P < 0.05 was considered statistically significant.

Results

Ectopic Overexpression of OPN in WM793 Melanoma Cells Confers the Cells with the Ability to Migrate/Invade in 3D Collagen I

To examine the functional roles of OPN in melanoma cells, an early VGP melanoma cell line WM793 with low OPN expression was transfected with a constitutive expression vector for OPN or an empty control plasmid. After selection
of stable transfectants, the initial pools of transfectants harboring the control or OPN expression plasmids, and two clonal isolates of the latter (OPN5 and OPN8) with different levels of OPN protein expression (Figure 1, A and B), were subjected to more detailed analyses. As shown by Western blot analyses of the concentrated conditioned media and cell lysates (Figure 1B), the amount of secreted OPN was markedly increased in the OPN transfectants, whereas the corresponding intracellular levels of OPN were only slightly increased, if at all. These findings support the notion that OPN mainly conducts its functions as an extracellular, so-called matricellular protein.52

The OPN-overexpressing WM793 cells did not show any noticeable morphological change, and retained an epithelioid morphology, when grown on 2D tissue culture dishes in the presence of serum (Supplemental Figure S1A). However, the OPN transfectants showed an enhanced growth rate as compared with the control cells (Supplemental Figure S1B).

We then analyzed the behavior of the cells when embedded in 3D collagen I gels, to mimic the cellular growth and migration/invasion conditions of melanoma cells in the collagen-rich dermis. After 24 hours of culture, the control cells appeared to be in poor condition, displaying...
a round/roundish or oval morphology. By contrast, the OPN-overexpressing cells showed an elongated, spindle-shaped, or dendritic morphology typical of a migratory/invasive phenotype, and the cells contacted each other, forming a lattice (Figure 1, C and D). We further stained actin in the cells grown on top of thick 3D collagen I gels, and found the OPN-overexpressing cells to form invadopodia-like projections that extended into the 3D collagen I matrix (Figure 1 E). In addition, the OPN-overexpressing WM793 cells were found to show increased migratory activity in Boyden chamber/Transwell assays, using collagen I-coated membranes (Supplemental Figure S2, A and B).

To confirm that the invasive phenotype of the OPN transfectants in 3D collagen I gels was specifically associated with the production of OPN, and not due to any incidental transfection-related genetic alterations, a neutralizing antibody against human OPN was added to the assay system. Notably, the acquisition of the invasive phenotype by the OPN-overexpressing cells was effectively blocked in the presence of the OPN-neutralizing antibodies (Figure 2, A and B, and Supplemental Figure S3, A—D).

Because OPN is known to act as an attachment factor,28 we studied whether the differential behavior of the control and OPN-overexpressing cells could be due to the secreted OPN promoting cell adhesion. However, although exogenous OPN

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**Figure 2** The migratory/invasive activity of the OPN-transfected WM793 cells is inhibited by neutralizing antibodies to OPN and integrin αvβ3, in the absence of any increases in the mRNA expression of the integrin αvβ3 and αvβ5 receptor subunits. A: The migratory/invasive capability of the OPN-transfected WM793 cells (OPNp) in 3D collagen I in the presence of 10 μg/mL isotype control antibodies (IgG) and neutralizing antibodies to osteopontin (OPN Ab). B: The percentage of cells with a spread/elongated morphology after incubation of the cells with the control and OPN antibodies (A). C: The migratory/invasive capability of the OPN-transfected WM793 cells in 3D collagen I in the presence of 10 μg/mL isotype control antibodies (IgG) and neutralizing antibodies to integrin αvβ3 (αvβ3 Ab) for 36 hours. D: The percentage of cells with a spread/elongated morphology after incubation of the cells with the control and integrin αvβ3 antibodies. E: RT-PCR analysis of the integrin subunits ITGAV, ITGB3, and ITGB5. Actin (ACTB) was used as a control. Data are expressed as means ± SD of three replicates (B and D). *P = 0.244, Ctrl versus IgG; *P = 0.001, Ctrl versus OPN Ab (B); *P < 0.001, Ctrl versus integrin αvβ3 antibodies (D). Original magnification, ×100 (A and C). Ctrl, control.
was found to clearly support the attachment of the parental WM793 cells (Supplemental Figure S4A), the OPN-overexpression—promoted increase in invasion in 3D collagen I gels could not be explained by OPN acting as an attachment factor, at least to any larger extent, for the following reasons. First, the parental WM793 cells with low OPN expression can attach very well to collagen I alone (Supplemental Figure S4B). Second, the OPN-overexpressing cells did not spread within the 3D collagen I gels when incubated in the presence of neutralizing antibodies to integrin $\beta_1$ (Supplemental Figure S5, A and B), which block the cellular attachment to collagen I through the collagen receptors $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$. In this context, it is also noteworthy that the overexpression of OPN did not increase the expression of collagen receptors (Supplemental Figure S5C), which could have contributed to the different behavior of the parental WM793 cells and the OPN overexpressers in the collagen I gels.

We then studied the possible role played by integrin $\alpha_v\beta_3$, a major receptor for OPN, because it is known to be up-regulated in VGP and metastatic melanomas, it can rescue melanoma cells from apoptosis in 3D collagen, and its forced expression can convert radial-growth melanoma cells to VGP. We found that the addition of function-blocking/neutralizing antibodies to $\alpha_v\beta_3$ (LM609) to the 3D collagen I cultures of the OPN-overexpressing WM793 cells resulted in about 60% inhibition of cell spreading or survival (Figure 2, C and D, and Supplemental Figure S6, A–D). In this context, it is notable that the overexpression of OPN did not cause any increase in the mRNA expression levels of $\alpha_v\beta_3$ integrin subunits, or those of integrin $\alpha_v\beta_5$, another receptor for OPN. Rather, the integrin $\beta_3$ mRNA levels appeared to be slightly decreased (Figure 2E). Likewise, analysis of the cell-surface protein expression of integrin $\alpha_v\beta_3$ by flow cytometry with the LM609 antibody (which recognizes both the active and inactive conformations of $\alpha_v\beta_3$) suggested a small decrease in $\alpha_v\beta_3$ in the OPN-overexpressing cells as compared with the control cells, although all of the cells were positive for integrin $\alpha_v\beta_3$ expression (Figure 3A). We then assessed the amounts of active $\alpha_v\beta_3$ integrin on the cells with the monovalent ligand-mimetic antibody WOW-1, which selectively binds to conformationally active $\alpha_v\beta_3$. In these experiments, the OPN-overexpressing WM793 cells were found to display markedly (4.9-fold) higher amounts of active $\alpha_v\beta_3$ than the control cells (Figure 3B), indicating that OPN activates $\alpha_v\beta_3$ integrin. OPN overexpression was also found to slightly increase the cell-surface expression of integrin $\alpha_v\beta_5$, as determined with the P1F6 antibody (Figure 3C).

Because OPN is also known to bind the CD44 surface receptors and thereby promote the migration/invasion of many cancer cells, we tested whether the same could also be true for the OPN-overexpressing WM793 melanoma cells. However, administration of the CD44 function—blocking antibodies did not interfere with the

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**Figure 3** The overexpression of OPN does not increase the total levels of $\alpha_v\beta_1$ integrin on the cell surface, but increases the amounts of active $\alpha_v\beta_1$ integrin. FACS analysis of the expression of integrin $\alpha_v\beta_1$ (detected with LM609 antibody) (A), active integrin $\alpha_v\beta_1$ (detected with WOW-1 antibody) (B), and integrin $\alpha_v\beta_5$ (detected with P1F6 antibody) (C) on the cell surface of the transfectants. For experimental details, see Materials and Methods. Black lines indicate unlabeled cells; green lines, isotype controls; blue lines, empty vector-transfected WM793 cells (CTRL+), probed with specific antibodies; and red lines, OPN-transfected WM793 cells (OPN+), probed with specific antibodies. The M1 marker denotes the proportion of the cells considered to be positively stained for the integrins.
invasion/migration of these cells in 3D collagen I gels (Supplemental Figure S7, A—F).

**OPN Overexpression Promotes the Survival and Proliferation of WM793 Cells in 3D Collagen I Matrix**

Because OPN is known to contribute to antiapoptotic signaling of melanocytes in collagen gels, we analyzed the survival of the control and OPN-overexpressing WM793 cells when grown on thick collagen I gels. As shown in Supplemental Figure S8, A and B, the overexpression of OPN protected the WM793 cells from apoptosis, as assessed by annexin V staining.

Next, we analyzed the growth/proliferation of the cells within the 3D collagen I matrix by microscopy and cell counting (after digestion of the matrix with collagenase). All of the OPN transfectants showed a continuously increased growth rate/proliferation during the follow-up period for 1 to 2 weeks, whereas the control cells could not proliferate (except very marginally in the beginning) and appeared to die during prolonged culture (Figure 4, A and B, and data not shown). We further analyzed the cells grown inside the collagen gel for 2 weeks for the expression of the proliferation marker Ki-67 by immunohistochemistry in situ, after paraformaldehyde fixation, paraffin embedding, and vertical sectioning of the gels. As expected, only relatively few living control cells were detected in the sections at this stage anymore, whereas the OPN transfectants were detected in abundance. Notably, the expression of Ki-67 was up-regulated in the OPN transfectants, and the number of the Ki-67—positive cells as well as the intensity of the staining appeared to correlate with the OPN expression level (Figure 4, C and D).

We further analyzed the invasive growth ability of the OPN-overexpressing cells embedded in thick 3D Matrigel (a reconstituted basement membrane matrix), where the invasion requires proteolytic activity. As shown in Supplemental Figure S9, OPN overexpression was also found to promote the invasive growth of WM793 cells in 3D Matrigel, but in this matrix, the control cells also showed some invasive growth potential, consistent with our recent study. Thus, OPN appears to be more critical for the migration/invasion and growth of WM793 cells in 3D collagen I matrix than in Matrigel.

**Overexpression of OPN Down-Regulates CD9 Expression**

To identify molecules involved in the OPN-promoted invasive growth of melanoma cells in 3D collagen I gels, we first examined our genome-wide microarray data on early VGP WM793 melanoma cells that have a low OPN expression level, and metastatic WM239 melanoma cells that have high OPN expression, as well as public microarray databases on other early-stage primary and metastatic melanoma cell lines. Interestingly, the expression of CD9, a member of the tetraspanin family of transmembrane proteins, was found to often correlate inversely with OPN in the early-stage primary (eg, WM3211, SbC12, WM1361A, WM1552C, and WM793) and metastatic (eg, D24, D28,
support of the idea of the expressions of OPN and CD9 correlating negatively, our microarray and RT-PCR analyses revealed very low expression of the OPN mRNA and high expression of the CD9 mRNA also in primary melanocytes (Figure 5A and data not shown).

To further confirm that OPN expression is associated with down-regulation of CD9, we performed RT-PCR and Western blot analyses of CD9 in the OPN-transfected WM793 cells. Both the CD9 mRNA (Supplemental Figure S10A) and CD9 protein levels (Figure 5B) were significantly decreased in the OPN transfectants as compared to the control cells. Similar results were obtained with another VGP melanoma cell line, G-361 (Supplemental Figure S10B).

In addition, we evaluated the cell-surface expression of CD9, and these analyses confirmed the reduced expression of CD9 on the plasma membrane of the OPN transfectants as well (Figure 5C). To obtain still more convincing evidence for the regulation of CD9 by OPN, we transfected the
WM793 cells with a tetracycline-inducible OPN expression vector system, enabling analysis of the effects of OPN in exactly the same genetic context. As shown in Supplemental Figure S11, the addition of increasing amounts of doxycycline resulted in a progressive increase in the expression of OPN, accompanied by a reciprocal, graded decrease in the expression of CD9.

To assess the physiological significance of the down-regulation of CD9 by OPN overexpression, we restored CD9 expression in the OPN-overexpressing WM793 cells by transfection with a CD9 expression vector. Restoration of the CD9 expression abolished the migratory/invasive activity of the OPN-overexpressing cells (Figure 6, A–C), indicating that the down-regulation of CD9 is essential for the OPN-promoted cell migration/invasion in 3D collagen I.

**Down-Regulation of OPN in Metastatic WM239 Melanoma Cells Increases CD9 Expression and Impairs Invasive Growth**

Next, we studied the effects of knocking down the high expression of OPN in the metastatic WM239 cells by using a lentiviral shRNA delivery system. Analysis of the pools of the control and OPN shRNA transfectants already without isolating clones revealed an efficient down-regulation of OPN, which was accompanied by an up-regulation of CD9 expression (Figure 7A and Supplemental Figure S12). In 2D culture

**Figure 7** Down-regulation of OPN in metastatic WM239 melanoma cells inhibits the invasive growth of the cells in 3D collagen I and the attachment of the cells to vitronectin. A: RT-PCR analysis of the OPN and CD9 RNA expression levels in WM239 cells transduced with lentiviral control shRNAs or shRNAs targeting OPN. B: Growth curves of the control shRNA- and OPN shRNA-expressing WM239 cells in 2D culture in plastic Petri dishes. C: Culture of the control shRNA- and OPN shRNA-expressing WM239 cells in 3D collagen I gel for 24 hours. D: The percentage of spread/elongated cells after culture of the control and OPN-knockdown cells in collagen I gel for 24 hours. E: Growth curves of the WM239 cells transduced with control shRNA or OPN shRNA in 3D collagen I gel. F: Attachment/spreading of the OPN-knockdown cells on plasma fibronectin (pFN), collagen I (COL-I), collagen IV (COL-IV), and vitronectin (VTN), with bovine serum albumin (BSA) as a negative control. G: RT-PCR analysis of the expression levels of integrins ITGAV, ITGB3, and ITGB5 in the control shRNA- and OPN shRNA-expressing WM239 cells. Actin (ACTB) was used as a control. Data are expressed as means ± SD of at least three replicates (B and C–F). P = 0.01, WM239 ctrl shRNA versus WM239 OPN shRNA at 6 days of culture (B); P = 0.005 (D); P = 0.007, WM239 ctrl shRNA versus WM239 OPN shRNA at 14 days of culture in 3D collagen I (E); P < 0.001 (VTN) (F). Original magnification, ×100 (C). ctrl, control.

**Figure 8** The expression levels of integrins αvβ3 and αvβ5 on the cell surface are not changed after the down-regulation of OPN in metastatic WM239 melanoma cells. FACS analysis of the expression of integrins αvβ3 (A) and αvβ5 (B) on the cell surface of the control shRNA- and OPN shRNA-expressing WM239 cells. Green lines indicate isotype controls; blue lines, control shRNA-expressing WM239 cells probed with specific antibodies; and red lines, OPN shRNA-expressing WM239 cells probed with specific antibodies. The M1 marker denotes cells positively stained for the integrins.
conditions, the knockdown of OPN (and subsequent increase in CD9) had no effect on the cell morphology but inhibited the cell growth (Figure 7B), fitting with the preceding findings on the OPN-overexpressing WM793 cells (Supplemental Figure S1). In 3D collagen I gels, in turn, the knockdown of OPN affected both the morphology (spreading) and the growth of the cells (Figure 7, C–E). The control shRNA–transfected cells showed similar growth patterns to the parental WM239 cells (Supplemental Figure S13, A–C). The knockdown of OPN by shRNA expression was further found to induce apoptosis in the WM239 cells (Supplemental Figure S13, D and E), similar to that reported for hepatocellular carcinoma cells. During prolonged culture of the shRNA transfectant pool, a selection against the high OPN shRNA expressers with gradual diminution of the growth inhibitory effect was observed, as expected (Figure 7E and data not shown). Similar results were also obtained with the WM239 cells transfected with a tetracycline-inducible antisense OPN expression system (Supplemental Figure S14, A–C). Further studies of the WM239 OPN-knockdown cells strengthened our prior conclusions on WM793 cells that OPN is not needed for the attachment of melanoma cells to collagen I (or to collagen IV.

Figure 9 Down-regulation of CD9 in VGP WM793 cells induces an invasive phenotype and enables the cell growth in 3D collagen I. A and B: Analysis of the protein expression levels of CD9 by Western blotting and FACS, respectively, in WM793 cells and WM793 cells expressing the control shRNA (pLKO) or shRNAs (sh1 to sh5) targeting CD9. Relative protein expression levels of OPN in the control shRNA- and CD9 shRNAs-transfected WM793 cells are shown below the Western blot lanes (A) and the M1 bars (B). In B, gray lines and dark areas represent isotype controls and cells stained with CD9 antibody, respectively. The M1 marker denotes CD9 positive cells. C: Culture of the WM793 cells expressing the control or CD9 shRNAs in 3D collagen I gel for 36 hours. D: The percentage of cells with spread/elongated morphology after culture of the control or CD9 shRNA-expressing cells in collagen I for 36 hours. E: Growth curves of the control and CD9 shRNA-expressing WM793 cells in 3D collagen I gels. Data are expressed as means ± SD of three replicates (D and E). P < 0.001, WM793-pLKO versus WM793-CD9sh1; P < 0.001, WM793-pLKO versus WM793-CD9sh2 (D). P = 0.006, WM793-pLKO versus WM793-CD9sh1; P = 0.006, WM793-pLKO versus WM793-CD9sh2 at 14 days of culture (E). Original magnification, ×100 (C).
or plasma fibronectin), but showed that it is involved in the cell adhesion to vitronectin (Figure 7F). Here, it should again be noted that the attachment and spreading of the OPN-knockdown cells on vitronectin was profoundly inhibited without any decreases in the mRNA or protein expression levels of the integrins αβ1 or αvβ3, the main cellular receptors for vitronectin (Figure 7G and Figure 8, A and B). We further studied the significance of CD9 up-regulation for the OPN knockdown—induced effects on the behavior of WM239 cells in 3D collagen I culture by knocking down CD9. The knockdown of CD9 expression by CD9 shRNA1 transduction was found to largely abolish the inhibitory effects of OPN shRNA expression (Supplemental Figure S15, A and B, and Figure 7, C and D, for comparison).

Knockdown of CD9 Stimulates the Invasive Growth of WM793 Melanoma Cells

Next, we studied the importance of CD9 in the regulation of the invasive growth of melanoma cells in 3D collagen I matrix, and asked whether the down-regulation of CD9 expression by itself could induce an invasive phenotype. For this, the early VGP WM793 cells were transfected with plasmids encoding five different shRNAs to CD9 or with control plasmids (pLKO.1 or the nontarget shRNA control vector). Western blot (Figure 9A) and FACS (Figure 9B) analyses confirmed a clear down-regulation of CD9 in four of the five shRNA transfectants, two of which were selected for more detailed studies. In this context, it should also be noted that the down-regulation of CD9 was not associated with any reciprocal changes in OPN expression (Supplemental Figure S16).

The invasion/migration assays of the cells in 3D collagen I matrix demonstrated that knocking down the expression of CD9 in WM793 cells resulted in a similar morphological change as that observed with the WM793 cells overexpressing OPN. The cells became spindle-shaped and larger in size, acquiring a mesenchymal cell—like appearance characteristic of an invasive phenotype (Figure 9, C and D). Similarly to the WM793 cells overexpressing OPN, the CD9-knockdown cells showed a continuously increased growth rate, whereas the control cells again, after 1 week of marginal growth, started to die in the 3D collagen I matrix (Figure 9E).

The Development of Invasive Primary Melanomas Is Associated with Up-Regulation of OPN and Concomitant Down-Regulation of CD9

To assess the possible physiological significance of the observed gene expression alterations, we examined our microarray analyses of benign nevi and primary melanomas (Soikkeli et al.10 and unpublished data) for the expression of OPN and CD9. First, we compared OPN and CD9 expression in normal nevi (n = 12) and a group of patients (n = 9) with thin melanomas (Breslow’s depth <2 mm). The expression levels of OPN were very low in normal nevi but showed a moderate, statistically significant (P = 0.020) increase in thin melanomas, whereas the expression levels of CD9 were high in normal nevi and showed a minor, nonsignificant (P = 0.61) decrease in the thin melanomas (Figure 10). Analysis of a larger number of patients will be needed to define the precise association of the decrease in CD9 expression with the development of early-stage invasive melanomas. However, both the OPN and CD9 expression levels showed highly significant changes in the thicker melanomas with increased invasiveness11 (n = 11, Breslow’s depth >2 mm) as compared to normal nevi (P = 0.0014 and P = 0.00019, respectively). The expression of OPN mRNA was increased about 1000-fold, and the expression of CD9 was decreased roughly twofold in the thick melanomas (Figure 10). Similar results were obtained when comparing our microarray data on normal nevi to those of thin (n = 4) and thick (n = 10) melanomas deposited in data banks (Supplemental Figure S17).

Discussion

The secreted phosphoprotein SPP1/OPN has been implicated in the regulation of invasion and metastasis in a variety of cancers, including melanoma. Notably, SPP1/OPN is one of the most highly up-regulated genes/proteins in primary VGP melanomas compared to in benign nevi,10,12,61 and in micrometastatic melanomas,10 and its analysis may be of clinical value in prognostication13 and as a serological marker for metastasis, particularly in conjunction with other markers.62–64 In the metastatic cascade, OPN has particularly been associated with sentinel lymph node metastasis.10,13,65

Figure 10 The development and progression of primary melanomas is associated with up-regulation of OPN and down-regulation of CD9 expression. Analysis of the OPN and CD9 expression levels in human benign nevi (n = 12), thin primary melanomas (n = 9, Breslow <2 mm; median thickness 1.2 mm; range, 0.6 to 1.7 mm), and thick primary melanomas (n = 11, Breslow ≥2 mm; median thickness 7.0 mm; range, 2.5 to 27 mm) by Affymetrix HG U133 plus 2.0 arrays. Error bars indicate SD.
However, the mechanisms of OPN action in these steps of melanoma progression remain largely elusive.

Here, we found that the ectopic overexpression of full-length OPN cDNA in WM793 melanoma cells resulted in a markedly increased secretion of OPN, whereas the intracellular content of OPN was only marginally increased. Thus, although the secreted and intracellular forms of OPN have been documented to have distinct cellular functions,60 our data suggest that secreted OPN plays the major role in promoting melanoma growth. This is further supported by our finding that OPN neutralizing antibodies could block the growth of the OPN-overexpressing WM793 cells. In this context, it may be noteworthy that a specific splice variant of OPN (OPN-c lacking exon 4) has been associated with the invasiveness of breast cancer cells.57,68 However, we did not find this isoform to be expressed in the WM239 melanoma cells to any appreciable extent (data not shown). The same has also been reported recently in other human cancers.69 Thus, the expression of the different isoforms of OPN appears to be cell-type specific.

We found that the overexpression of OPN confers the WM793 melanoma cells with the ability to survive, migrate/invade, and grow in 3D collagen I gel. Thus, OPN is also likely to be important for the advancement of melanomas in the dermal environment, where collagen I is the most abundant extracellular matrix component. We further found that the OPN-promoted migration/invasion and growth of melanoma cells in 3D collagen was dependent on the activity of integrins β1 and αvβ3. Differing somewhat from a previous publication,7 we did not find integrin αvβ3 to be able to substitute for integrin β1 in mediating cell attachment to collagen I during culture in 3D collagen. This may be due to the fact that we used acid-extracted collagen I (resulting in normal collagen cross-linking), whereas Montgomery et al7 used protease-treated collagen I for preparing the gel or, as suggested by the latter authors, the collagen I was denatured by the action of cellular proteases, enabling cell binding to the matrix through αvβ3. It is also apparent from our data that the OPN-promoted survival/growth of the OPN-transfected WM793 cells in collagen I cannot be explained by the secreted (and deposited) OPN substituting for integrin β1 in the cell attachment to collagen I. Hence, these data, together with our studies of WM239 cells knocked down in OPN expression by shRNAs, suggest that OPN mainly promotes the migration/invasion and growth/survival of melanoma cells in 3D collagen by acting as an autocrine cytokine-like growth factor that activates the integrin αvβ3 receptor (and/or the other integrin receptors binding OPN). However, OPN may be involved in the regulation of adhesion of melanoma cells to substrata other than collagen I. Indeed, we found OPN to be essential for the adhesion of WM239 melanoma cells to vitronectin.

Because the main receptors for vitronectin are the integrins αvβ1, αvβ3, and αvβ5, and we found neither the overexpression nor the down-regulation of OPN to cause any major changes in the mRNA levels of the integrin subunits αv, β1, β3, or β5 or in the cell-surface expression of the αvβ3 and αvβ5 integrins, it is highly likely that OPN regulates the activation state/conformation of the αv (β1, β3, and β5) integrins. Indeed, we found OPN expression to increase the levels of active αvβ3 (measured with the WOW-1 antibody), whereas the total αvβ3 levels (measured with the LM609 antibody) on the cell surface appeared to be slightly decreased (which is in line with the observed slight decrease in integrin β3 mRNA expression). However, we cannot totally exclude the possibility that in the case of the LM609 antibody, the decreased fluorescence intensity observed in the OPN-overexpressing cells could also be due to a decreased accessibility of the antibody to the target, eg, as a result of OPN inducing αvβ3 integrin clustering or complexing with other proteins (OPN itself as one possibility). The WOW-1 antibody in turn as a monovalent Fab fragment is less sensitive to these kinds of changes, and is thus expected to reliably report the amounts of active αvβ3 on the cells.

Because vitronectin is abundantly present in serum and deposited on the vessel walls, elastic fibers in dermis, and reticular fibers in the lymph node cortex,70–72 it remains of interest to study whether the OPN-mediated activation of αv (β1, β3, and β5) receptors and consequent binding of the melanoma cells to vitronectin could also play a role in melanoma cell migration/invasion in dermis and homing of the metastatic cells in the lymph nodes. Indeed, it has been shown previously by using frozen sections of human lymph nodes that αvβ3 may be involved in the adherence and metastasis of melanoma cells to the lymph nodes.73

Moreover, the secreted OPN by melanoma cells may also activate stromal cells expressing αvβ3, such as endothelial cells and macrophages, and thereby promote tumor angiogenesis and other metastatic processes. All these data together make integrin αvβ3 an attractive target for therapy. Notably, several αv-integrin antagonists are already in clinical trials in melanoma.74,75

OPN is also known to signal through CD44 variant receptors in various cancers,76,77,78 but we did not find the blocking of CD44 with neutralizing antibodies to affect the behavior of the WM793 OPN-overexpressing cells in 3D collagen I. However, these data by no means exclude the possibility that OPN could signal through CD44 in other melanoma cell lines.79 It is notable, however, that our gene expression analyses of several primary human melanocyte and melanoma cell cultures or melanoma cell lines have not revealed any major difference in CD44 expression, whereas the expression of integrin αvβ3, especially that of the β3 subunit, appears to be markedly increased in the melanoma cells (unpublished data). All together, these data support the idea that, in human melanoma cells, OPN is mainly signaling through the integrin receptors.

Interestingly, our microarray analyses of melanocytes, early VGP melanoma cells, and metastatic melanoma cells revealed OPN expression to correlate inversely with the expression of tetraspanin CD9. In addition, the forced overexpression of OPN in WM793 cells caused down-regulation of CD9, and conversely, the knockdown of OPN in WM239 cells resulted in up-regulation of CD9; and reversion of these changes in CD9 by expression of CD9 cDNA and CD9 shRNAs,
respectively, negated the effects of OPN on cell migration/invasion in 3D collagen I. Further, knocking down CD9 in WM793 cells was found to induce an invasive phenotype, similar to that caused by OPN overexpression. In addition, our studies of clinical tumor specimens showed that the development and progression of primary melanomas is also associated with up-regulation of OPN and concomitant down-regulation of CD9. These data strongly suggest that CD9 is a downstream mediator of the OPN action in the promotion of the melanoma cell migration/invasion and growth.

CD9 is known to interact with other tetraspanins and many other transmembrane proteins, including various integrins (αβ1, αβ3, αβ6, and αβ4), growth factor receptors, peptidases, and EWI proteins; for a comprehensive list of interacting proteins see PINA (Protein Interaction Network Analysis platform query, http://cbg.garvan.unsw.edu.au/pina/home.do, last accessed September 10, 2013). Thus, through this tetraspanin web, CD9 may participate in a variety of physiological and pathological processes. Abundant evidence indicates that CD9 primarily acts as a tumor metastasis suppressor, although opposite functions have also been reported in some cases. A possible explanation of the contradictory results may be that CD9 regulates only distinct steps in the metastatic cascade or that its effects are context/organ dependent. In any case, in experimental mouse models of melanoma, the ectopic expression of CD9 in melanoma cells and intratumoral injection of an adenoviral CD9 expression vector have been documented to inhibit the pulmonary metastasis of melanoma cells. Of more physiological relevance, in an orthotopic lung cancer model, the adenoviral transduction of CD9 has been found to specifically inhibit metastasis to the regional lymph nodes.

There is also strong evidence for the involvement of CD9 in various clinical cancers. The down-regulation or loss of CD9 expression has been found to correlate with the invasiveness and/or metastasis of several cancers, including breast, lung, prostate, and gastrointestinal cancers, and melanoma. Further, high expression of CD9 has been associated with good prognosis, and the down-regulation/loss of CD9 with poor prognosis. Of particular interest, our data suggest that the down-regulation of CD9 in the invasive melanomas (which, consistent with a previous immunohistochemical study, correlated with the increased thickness of the primary tumors) is consequent to the increased expression of OPN. It is of note that also in clinical tumors, the down-regulation of CD9 appears to be primarily associated with lymph node metastasis, similar to that observed with the OPN-overexpressing tumor cells. Like OPN, CD9 has also been found to regulate the adhesion, epithelial-mesenchymal transition, migration/invasion, and proliferation/survival of tumor cells. In addition, OPN and CD9 are known to interact with and activate several proteins in common, such as integrins, signaling proteins, and secreted proteases according to PINA query (http://cbg.garvan.unsw.edu.au/pina/home.do, last accessed September 10, 2013), that critically contribute to the ability of tumor cells to invade and metastasize, although OPN and CD9 also display differences in their interaction partners, eg, as to the binding of integrin αβ3.

Collectively, our data indicate that OPN regulates the activation state of α3 (β1, β3, and β6) integrins and down-regulates the candidate metastasis suppressor CD9 in melanoma cells. The overexpression of OPN and down-regulation of CD9 had very similar consequences on the tumor cell behavior (growth and invasion), fitting with the idea that CD9 is an important mediator of the OPN actions. However, both of these proteins are multifunctional and apparently also have different mechanisms of action, which is evident, for example, from the differences in their binding partners and gene expression patterns of the OPN- and CD9-overexpressing tumor cell lines. Therefore, it remains of interest to test whether a combinational therapeutic approach, ie, a concomitant inhibition of OPN expression and activation of CD9 expression, particularly in the early phase of metastasis, could result in increased efficacy (over that attainable by either intervention alone) in the prevention of metastatic melanomas and other solid cancers.

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**Supplemental Data**

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