Cancer Science
The official journal of the Japanese Cancer Association

Osteopontin is a new target molecule for ovarian clear cell carcinoma therapy

Motoki Matsuura,[1] Takahiro Suzuki and Tsuyoshi Saito

Department of Obstetrics and Gynecology, Sapporo Medical University, Sapporo, Japan

(Received February 20, 2010/Revised April 27, 2010/Accepted April 30, 2010/Accepted manuscript online May 12, 2010/Article first published online June 4, 2010)

Recent studies have demonstrated overexpression of osteopontin (OPN) in ovarian clear cell carcinoma. Here, we revealed the role of OPN in invasiveness in ovarian clear cell carcinoma. We used immunofluorescence analysis to detect OPN in a total of 160 patient-derived specimens. Ovarian clear cell carcinoma cell lines, RMG-1 and TOV-21G, were used to monitor changes in OPN and integrin levels, and cell invasiveness following treatment with OPN, simvastatin, and transfection with siRNA. Immunofluorescence analysis revealed statistically significant differences among the histological groups, and ovarian clear cell carcinoma expressed a strong OPN signal. The OPN receptors, alpha v and 5, and beta 1 and 3 integrins, were increased after treatment with OPN. Invasion assays indicated that OPN enhanced *in vitro* extracellular matrix invasion dose-dependently in ovarian clear cell carcinoma. Simvastatin significantly reduced expression of OPN and the integrins, and decreased ECM invasion. RNA interference also suppressed ECM invasion. These results suggest that down- or up-regulation of OPN is involved in carcinoma cell invasion. We thus conclude that OPN regulation could have a crucial role in ovarian clear cell carcinoma therapy. (*Cancer Sci* 2010; 101: 1828–1833)

Epithelial ovarian cancer is the most common malignant tumor arising from the ovary. This type of cancer has several different histological phenotypes. It is known to show resistance to platinum-based chemotherapy and to result in poor prognosis.[1–3] Chemotherapy with various cytotoxic drugs for ovarian clear cell carcinoma (OCCC) currently leads to limitations in therapy regimens. Therefore, a new potential molecular target for therapy needs to be developed. Hepatocyte nuclear factor-1 beta (HNF-1 beta) is currently recognized as an excellent marker for OCCC.[4] Recent studies have demonstrated the overexpression of osteopontin (OPN) in OCCC,[4,5] and *OPN* is the most direct target gene of HNF-1 beta.[6] HNF-1 beta is closely associated with OPN up-regulation in OCCC.[4]

Osteopontin (OPN) is expressed in a variety of normal and tumor tissues,[7,8] including bone,[9] breast,[10] prostate,[11] lung,[12] kidney,[13] stomach,[14] ovary,[9] and uterine endometrium,[15] and has been assessed as a potential diagnostic marker in malignancy.[5,16–18] A large number of hormones and cytokines – such as estrogen, progesterone,[15,19] vitamin D3, Rous sarcoma oncogene, and activator protein-1[9] – effect a change in OPN expression. Osteopontin (OPN) is known to be involved in a variety of physiological cellular functions such as tumorigenesis, metastasis, and angiogenesis.[9,19–21] In particular, OPN plays an important role in tumor growth, metastasis, and angiogenesis through alpha v, alpha 5, beta 1, beta 3, and beta 5 integrins, as well as CD44.[20,22–24]

Integrins are a family of glycosylated, heterodimeric transmembrane adhesion receptors that noncovalently bind alpha and beta subunits.[25] In a recent study, 18 alpha and eight beta subunits were identified, and they are known to form 24 different heterodimers. The combination of alpha and beta subunits determines the ligand-specificity of the integrins. Osteopontin (OPN) has been shown to bind integrins – in particular alpha v beta 3,[26] but also alpha v beta 1, alpha v beta 5,[22,27] and alpha 5 beta 1[28] – in malignancy, including ovarian cancer. It has been considered that OPN may contribute to tumor invasion and metastasis through integrin-mediated signaling.[23]

In the present study, we first examined several kinds of ovarian cancer cell lines and surgical specimens for OPN expression at both the mRNA and protein level. Subsequently, we revealed the role OPN plays in invasiveness in OCCC. From our results, we determined the effects of OPN in OCCC cell lines *in vitro* and investigated its tumor activity. Our data showed that OPN was referred to tumor growth and invasion.

Materials and Methods

Cell lines and cultures. The following ovarian cancer cell lines were used for the *in vitro* studies: clear cell carcinoma, RMG-1 (JCRB Cell Bank, Osaka, Japan), TOV-21G (ATCC, Manassas, VA, USA); serous adenocarcinoma, HTOA (Riken Bioresource Center, Ibaragi, Japan), MH and KFr (kindly provided by Dr Kikuchi, National Defense Medical College, Saitama, Japan); and mucinous adenocarcinoma, MCAS (JCRB Cell Bank). The cell lines were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Materials. For the *in vitro* studies, human recombinant OPN was obtained from Sigma-Aldrich (St Louis, MO, USA). Simvastatin was obtained from Cayman (Ann Arbor, MI, USA).

Biosamples. A total of 160 patient-derived specimens (nine healthy patients, 10 ovarian adenoma patients, 79 ovarian cancer patients, 20 corpus cancer patients, 42 cervical cancer patients) were obtained at Sapporo Medical University Hospital, Japan, between 2003 and 2008. For freshly-frozen sections, fresh specimens were embedded and snap-frozen in liquid nitrogen vapor, and stored at −80°C. All patients gave oral and written informed consent before surgical treatment.

Immunofluorescence analysis. For immunofluorescence staining of frozen sections, the OPN protein was detected by mouse antihuman monoclonal antibody (clone 1B20; IBL, Gunma, Japan) as the primary antibody.[21] Secondary antibodies included Alexa Fluor 488 and goat antimouse IgG (Molecular Probes, Eugene, OR, USA). The immunofluorescence study was performed as described previously.[29] Each section was given a score corresponding to the average of both staining intensity (no staining = 0, low staining = 1, medium staining = 2, strong staining = 3) and the percentage of positive cells (no-cells staining = 0, few-cells staining = 1, half-of-cells staining = 2, most-of-cells staining = 3). The score was evaluated using the average score of three observers who had no knowledge of any of the clinicopathological parameters.

[1]To whom correspondence should be addressed.
E-mail: mmatsuura@sapmed.ac.jp

doi: 10.1111/j.1349-7006.2010.01615.x

Quantitative real-time RT-PCR analysis. Total RNA was isolated from cell cultures using commercial spin columns (RNeasy; Qiagen, Tokyo, Japan) in accordance with the manufacturer's instructions, and cDNA synthesis was performed using the ThermoScript RT-PCR System (Invitrogen). Real-time quantitative RT-PCR was used to analyze the concentrations of OPN mRNA in cell lines using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The housekeeping gene was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Amplification of OPN was carried out using the following cycling profile: initial denaturation at 95°C for 10 min, 40 cycles of 15 s at 95°C, and 1 min at 60°C in a Chromo4system (Bio-Rad). To determine the relative level of OPN for each sample, the comparative C_T method was used as described previously.[5] Polymerase chain reaction (PCR) products were visualized on a 1.5% agarose gel containing 0.2 μg/μL ethidium bromide to confirm the specificity of the PCR reaction. A 100-bp ladder (New England BioLabs, Ipswich, MA, USA) was used as a size standard.

Invasion assay. Cell invasion through the extracellular matrix (ECM) was determined using the BD BioCoa Tumor Invasion System (BD Biosciences, San Jose, CA, USA) in accordance with the manufacturer's instructions. Briefly, a 24-multiwell insert plate that had been uniformly coated with BD Matrigel Matrix was used. Cell suspension (500 μL; 5×10^4 cells/mL) was added to the apical chambers, and the basal chambers were filled with 750 μL of chemoattractant with fibronectin (5 μg/mL). The cells were incubated for 22 h at 37°C in 5% CO_2 atmosphere. The invaded cells were stained with Giemsa and then counted using five randomly-selected visual fields.

RNA interference. SiRNA oligonucleotides with OPN which were pre-designed products (sense, 5′ -GGUCAAAAUCUAAGAAGUUtt-3′; antisense, 5′-AACUUCUUAGAUUUUGACCtc-3′), and negative control siRNA, were obtained by Ambion (Austin, TX, USA).

RMG-1 and TOV-21G cells were plated in 24-well plates at 30–50% confluence 24 h prior to transfection. Transfection of cells was carried out with Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using STATVIEW 5.0 (Hulinks, Tokyo, Japan). The statistical differences between the two groups were analyzed using the Mann–Whitney *U*-test (nonparametric data), and the differences among multiple groups were analyzed using the Kruskal–Wallis test. Values of $P < 0.05$ were considered significant.

Results

Strong OPN signals were observed in ovarian cancer tissues. Osteopontin (OPN) protein levels in frozen sections of 79 ovarian cancers, 20 corpus cancers, and 42 cervical cancers were assessed via immunohistochemical analysis (Table 1a). The relative mean immunostaining scores for OPN were 2.72 in ovarian cancer, 2.10 in corpus cancer, and 1.79 in cervical cancer. The OPN signals in ovarian cancer were strong compared with those in corpus and cervical cancer. To validate OPN expression in ovarian cancer, we analyzed the differences in immunostaining scores according to histological type. The immunostaining scores in ovarian serous, clear, endometrioid, and mucinous adenocarcinomas were 3.04 (95% CI: 2.47–3.62), 3.35 (95% CI: 2.66–4.03), 1.91 (95% CI: 1.15–2.69), and 2.67 (95% CI: 1.10–4.23), respectively. The differences among the histological groups were statistically significant ($P < 0.05$), and the clear cell carcinoma and serous adenocarcinoma types exhibited strong signals. Among the 71 invasive ovarian carcinomas, eight borderline ovarian tumors, 10 adenoma, and nine normal ovarian tissues, OPN-positive staining was observed in 80.3%, 66.7%, 30.0%, and 22.2%, respectively (Table 1b). The positive rates were significantly higher in malignant tissues than in normal and benign tissues ($P < 0.05$). Osteopontin (OPN) staining was observed in the cell membrane and cytoplasm. In particular, immunopositivity in serous and clear cell types was localized in the cytoplasmic region. There were no significant differences in immunostaining scores among different age groups and pathological stages (data not shown). Since OCCC showed the strongest signal of the various types of gynecological cancer tissues, we examined the relationship between OCCC and the role of OPN.

Table 1. Immunofluorescence analysis of osteopontin (OPN)

(a)

Cancer type	No. of patients	Scores		
		Mean (95% CI)	Range	OPN positive
Ovarian cancer				
Serous	23	3.04 (2.47–3.62)	0–6	95.7%
Clear	20	3.35 (2.66–4.03)	0–6	95.0%
Endometrioid	24	1.91 (1.15–2.69)	0–4	58.3%
Mucinous	12	2.67 (1.10–4.23)	0–6	66.6%
Endometrial cancer				
Endometrioid	20	2.10 (1.27–2.93)	0–4	65.0%
Cervical cancer				
SCC	23	1.91 (1.25–2.58)	0–6	69.6%
Adeno.	19	1.79 (1.03–2.55)	0–6	68.4%

(b)

Characteristics	Number	Mean scores (95% CI)	OPN positive
Healthy	9	0.89 (0–1.93)	22.2%
Adenoma	10	0.9 (0.11–1.69)	30.0%
Borderline	8	2.75 (0.98–4.52)	66.7%
Invasive	71	2.72 (2.30–3.13)	80.3%

(a) The relative mean osteopontin (OPN) scores of ovarian serous and clear cell carcinoma patients were significantly higher than those of the other ovarian histological types: corpus cancer and cervical cancer (*$P < 0.05$) . (b) Among the ovarian cancers, and benign and healthy tissues, the relative mean OPN scores were significantly higher in cancer groups than in benign and healthy tissues (*$P < 0.05$).
CI, confidence interval; SCC, squamous cell carcinoma.

Osteopontin (OPN) was strongly expressed in OCCC cell lines. To assess the expression of OPN mRNA in ovarian cancer, real-time RT-PCR was applied to six ovarian cancer cell lines. Relative OPN gene expression was calculated using the comparative C_T method (Fig. 1a). The reference was MH, which was considered to have a value of 1. The mean values of serous, clear, and mucinous adenocarcinoma cell lines were 107.6, 3917.9, and 2.0, respectively. High levels of OPN gene expression were therefore shown in OCCC cell lines ($P < 0.05$).

Immunofluorescence analysis was employed to examine OPN protein levels in the ovarian carcinoma cell lines (Fig. 1b,c). Osteopontin (OPN) expression was seen to have strong intensity in the cell surface membrane and also exhibited a granular pattern in the cytoplasm of OCCC cells. However, OPN was weakly-expressed in KFr (serous) and MCAS (mucinous) cells, in line with the results of real-time RT-PCR.

Osteopontin (OPN) enhanced integrin expression in ovarian clear cell carcinoma cell lines. Osteopontin (OPN) binds to a subset of integrin receptors, and thus we analyzed the type of integrin receptors in the clear cell carcinoma cells. To characterize the OPN/receptor interactions, two OCCC cell lines were treated with human recombinant OPN (0.2 μM) for 0–24 h in

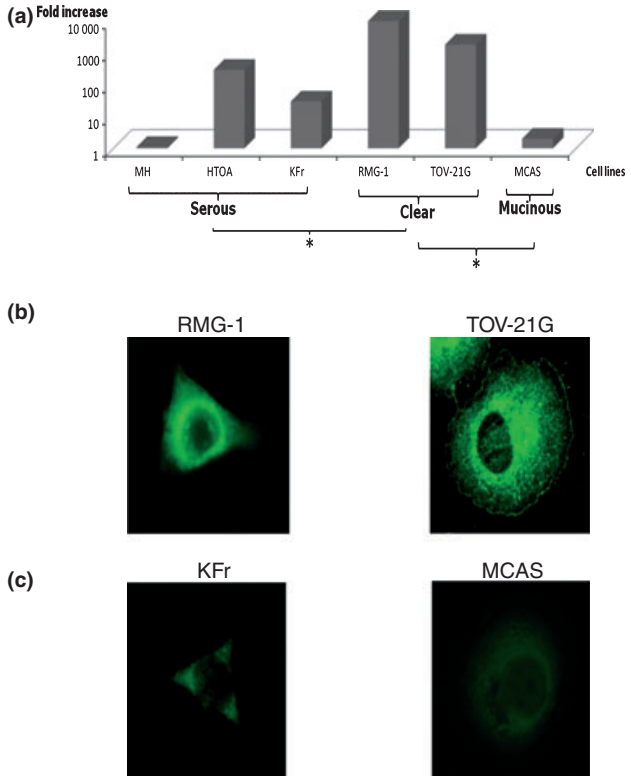

Fig. 1. Osteopontin (OPN) expression in ovarian cancer cell lines. (a) To compare the mRNA levels of OPN in ovarian clear cell carcinoma (OCCC) cell lines with those in other histological types, real-time RT-PCR was applied to six ovarian cancer cell lines. The mean values of clear, serous, and mucinous types were 3917.9, 107.6, and 2.0, respectively. Osteopontin (OPN) mRNA levels of OCCC were significantly higher than other histological types (*$P < 0.05$). (b,c) Immunofluorescence analysis was used to check the OPN protein levels of ovarian cancer cell lines. Osteopontin (OPN) was expressed with strong intensity in the cell surface membrane, and also exhibited a granular pattern in the cytoplasm in OCCC cell lines compared with serous and mucinous adenocarcinoma cell lines.

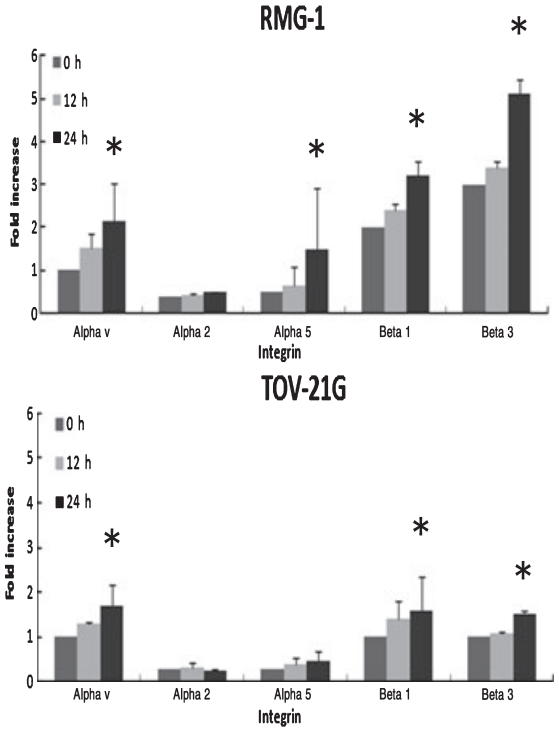

Fig. 2. Correlation between osteopontin (OPN) and integrins in ovarian clear cell carcinoma (OCCC) cell lines. To characterize OPN/receptor interactions, two OCCC cell lines were treated with human recombinant OPN (0.2 μM) for 0–24 h ($n = 3$). The levels of alpha v, 5, and beta 1, 3 integrin mRNA expression were shown to be significantly increased in this time-dependent analysis (*$P < 0.05$), although alpha 2 integrin mRNA expression was not increased significantly.

24-well plates, and the time-dependent changes in integrin gene expression were analyzed using quantitative real-time RT-PCR. The levels of alpha v, alpha 5, beta 1, and beta 3 integrin mRNA expressions were shown to be significantly increased in this time-dependent analysis, but the levels of alpha 2, which is not a specific receptor of OPN, were not increased significantly in both cell lines (Fig. 2). These results suggest that the combinations of alpha v beta 1, alpha v beta 3, and alpha 5 beta 1 subunits may be correlated with OPN in OCCC cells.

Osteopontin (OPN) enhanced *in vitro* ECM invasion. To evaluate the effect of OPN on ECM invasion, *in vitro* invasion assays were performed in two OCCC cell lines. Cell suspension (500 μL; 5×10^4 cells/mL) in serum-free medium was added to the apical chambers, and the basal chambers were filled with 750 μL of chemoattractant with 0–0.6 μM OPN and fibronectin (5 μg/mL). Figure 3 shows that cell invasiveness increased dose-dependently in both cell lines, and ECM invasion was significantly enhanced in RMG-1 (3-fold) and TOV-21G (3.1-fold), respectively, after treatment with 0.6 μM OPN.

Simvastatin reduced mRNA levels of OPN and integrins. To check the influence of simvastatin on OPN and integrin levels in OCCC, two OCCC cell lines were treated with 0–4 μM simvastatin for 48 h in 48-well plates (Fig. 4). Total RNA was isolated, and the changes in OPN and integrin mRNA levels were analyzed using real-time RT-PCR. The levels at 4 μM simvastatin were considered to be the reference values. Values shown are mean ± SD. The levels of OPN mRNA expression were shown to be significantly decreased (RMG-1: 1.3-fold, and TOV-21G: 1.2-fold) in this dose-dependent analysis (*$P < 0.05$). In addition, integrin mRNA levels were significantly decreased dose-dependently, except for beta 1 of TOV-21G, and alpha 2 of both cell lines (*$P < 0.05$). Each experiment was repeated three times with triplicate samples.

Simvastatin inhibited *in vitro* ECM invasion. We next investigated whether simvastatin inhibited the ECM invasion of OCCC cells by reducing OPN and integrins. To evaluate the effect of simvastatin on ECM invasion, *in vitro* invasion assays were performed in two OCCC cell lines (Fig. 5). Cells were pretreated with 4 μM simvastatin for 24 h in 24-well plates; 500 μL of cell suspension (5×10^4 cells/mL) in serum-free medium was then added to the apical chambers, and the basal chambers were filled with 750 μL of chemoattractant and fibronectin for 22 h (5 μg/mL). The reference was the control groups, which were considered to have a value of 1. Values shown are mean ± SD ($n = 3$). These data show that treatment with simvastatin inhibited ECM invasion in OCCC cell lines thus: RMG-1, 1.3-fold decrease; TOV-21G, 1.7-fold decrease (*$P < 0.05$).

RNA interference inhibited *in vitro* ECM invasion. We investigated whether pre-designed siRNA of OPN inhibited the ECM invasion of OCCC cells. Cells were treated with siRNA for 48 h, and 500 μL of cell suspension (5×10^4 cells/mL) in serum-free medium was added to the apical chambers, and the basal chambers were filled with 750 μL of chemoattractant and fibronectin for 22 h (5 μg/mL). Figure 6 shows that treatment with siRNA inhibited ECM invasion in OCCC cell lines thus:

doi: 10.1111/j.1349-7006.2010.01615.x

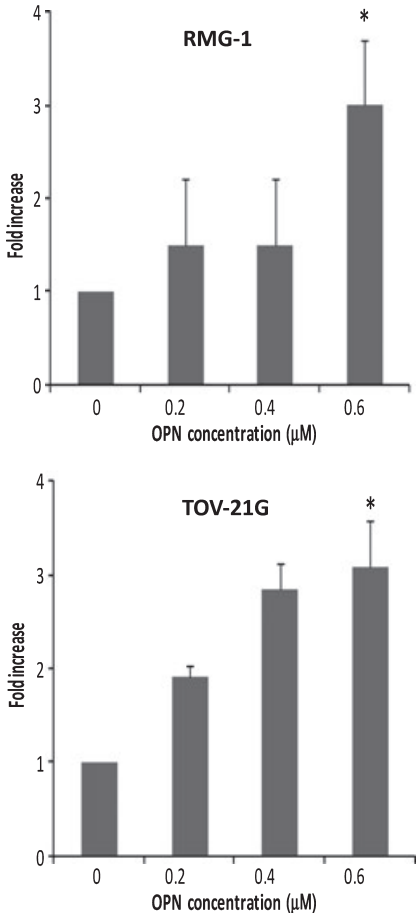

Fig. 3. Osteopontin (OPN) enhances extracellular matrix (ECM) invasion *in vitro*. To evaluate the effect of OPN on ECM invasion, *in vitro* invasion assays were performed in two ovarian clear cell carcinoma (OCCC) cell lines ($n = 3$). These data showed that OPN treatment caused a dose-dependent increase in ECM invasion in OCCC cell lines (*$P < 0.05$).

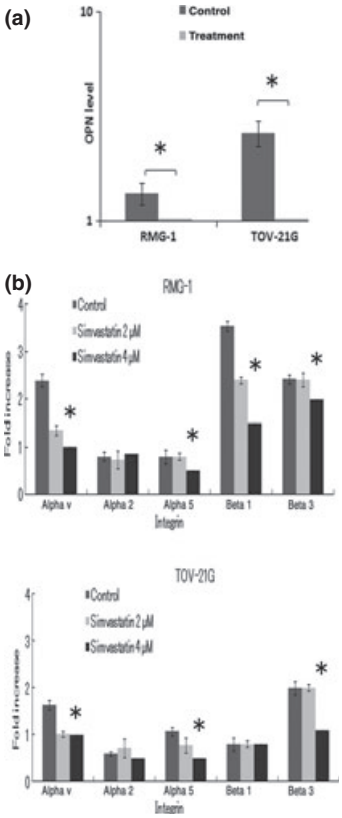

Fig. 4. Simvastatin influences the levels of osteopontin (OPN) and integrins. (a) To assess the influence of simvastatin on OPN levels, two ovarian clear cell carcinoma (OCCC) cell lines were treated with 0–4 μM simvastatin for 48 h. The levels of OPN mRNA expression were shown to be significantly decreased in this dose-dependent analysis (*$P < 0.05$). (b) Integrin mRNA levels were also analyzed in this experiment. After treatment with simvastatin, alpha v, 5, and beta 3 integrin mRNA levels were significantly and dose-dependently decreased (*$P < 0.05$), although alpha 2 integrin mRNA expression was not decreased.

RMG-1, 1.7-fold decrease; TOV-21G, 1.5-fold decrease, compared to negative control siRNA (*$P < 0.05$).

Discussion

Ovarian cancer is a major cause of morbidity and mortality among the gynecological malignancies.[30] While platinum plus taxan chemotherapy results in increased overall survival in patients with epithelial ovarian cancer,[31] OCCC is usually more resistant to chemotherapy than other types and has a poorer prognosis.[1,32] Ovarian clear cell carcinoma (OCCC) is increasing in East Asia compared with in Europe and the USA. Therefore, a new potential molecular target for therapy needs to be developed. Some studies have reported that OPN expression is increased in ovarian cancer,[4,5,16–18] and OPN correlates with other kinds of cancer progression and metastasis.[9,20,33–36]

To examine the clinical potential of OPN, we first used immunofluorescence analysis to check OPN protein expression in surgical specimens. Our study demonstrated that OPN protein was detected in ovarian cancer, corpus cancer, and cervical cancer. Ovarian cancer tissues expressed OPN protein more than did cervical and corpus cancer. Osteopontin (OPN) expression in OCCC and serous adenocarcinoma tissues was significantly higher than in other histological types, although OPN expression was significantly lower in benign tissues than in cancerous tissues. Our results suggest that different histological types have

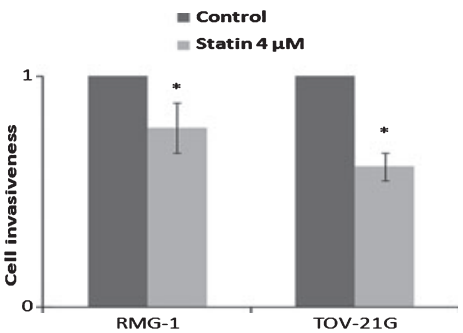

Fig. 5. Simvastatin inhibited extracellular matrix (ECM) invasion *in vitro*. To evaluate the effect of simvastatin on ECM invasion, *in vitro* invasion assays were performed in two ovarian clear cell carcinoma (OCCC) cell lines ($n = 3$). These data showed that treatment with simvastatin inhibited ECM invasion in OCCC cell lines thus: RMG-1, 1.3-fold decrease; TOV-21G, 1.7-fold decrease (*$P < 0.05$).

relatively different expression patterns. This might be due to the fact that OPN staining was observed in the cell membrane and cytoplasm. Recent studies[37–39] have revealed two OPN protein isoforms, the secreted isoform of OPN (sOPN) and the intracellular isoform of OPN (iOPN). Expression of the two isoforms

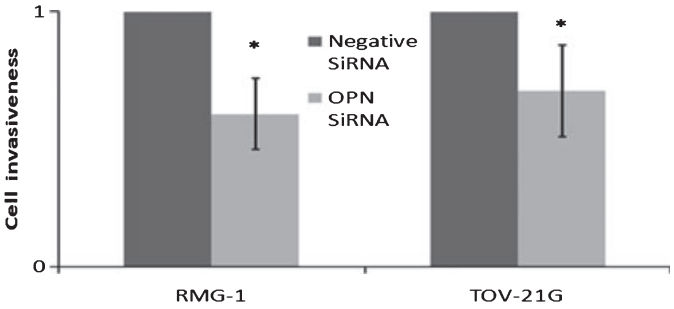

Fig. 6. RNAi showed the inhibition of extracellular matrix (ECM) invasion. Treatment with siRNA inhibited ECM invasion in two cell lines, compared to negative control siRNA (n = 3).

differs depending on the cell type. Most cancer cells contain high levels of iOPN, which is considered to be involved in cell survival. The expression levels and functional properties of iOPN in OCCC are still unclear. Osteopontin (OPN) staining in the cell membrane is due to sOPN, which binds to cell surface receptors such as integrins and CD44. In contrast, OPN staining in the cytoplasm indicates the subcellular localization of iOPN.

To further examine the differences in gene expression with histological subtypes of ovarian cancer, quantitative real-time RT-PCR was used to measure gene expression in the cells. Osteopontin (OPN) mRNA expression was elevated in OCCC cells, in line with the results of the immunofluorescence analysis. In addition, OPN protein was strongly expressed in OCCC cell lines, especially in the cell membrane and cytoplasm. This indicates that ovarian OCCC cells contain high levels of iOPN. The serous- and mucinous-type cancer cells expressed the OPN gene at low levels, yet the immunofluorescence data showed relatively high levels of OPN protein in the majority of the samples of serous and mucinous cancer tissues. From this, we deduced that OPN was actively synthesized in serous and mucinous-type cancer tissues.

Osteopontin (OPN) is an ECM protein containing an integrin-binding arginine-glycine-aspartate sequence (RGD)[7] and several pathways, such as that via Src to either epidermal growth factor receptor (EGFR)/mitogen activated protein kinase (MAPK) or PI3-KAkt: nuclear factor-kappa B (NF-κB),[36,40] is activated by the interaction between the RGD domain and, for the most part, the alpha v beta 3, alpha 5 beta 1 integrins.[24] This signal transduction results in the increased expression of a number of proteins, including matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA).[20] Matrix metalloproteinases (MMPs) and uPA are essential for the regulation of cell migration, ECM invasion, and metastasis in cancer cells.[24,35]

We also demonstrated that the expression of alpha v, alpha 5, beta 1, and beta 3 integrins was increased in OCCC, and human OPN affected the expression of alpha v, alpha 5, and beta 3 integrins. These results suggest that OPN correlated with alpha v beta 1, alpha v beta 3, and alpha 5 beta 1 integrins in OCCC.

Metastasis of cancer cells is the single characteristic that makes it more difficult to completely treat the disease. Increased invasiveness results in a higher metastatic capability of cancer cells. Cell adhesion molecules expressed in cancer cells are thought to play an important role in cancer cell-target organ adhesion and invasion. Although several families of cell adhesion molecules have been identified, cell–cell and cell-MMP interactions are mediated by integrins. Our results suggest that the combinations of alpha v beta 1 or 3 and alpha 5 beta 1 subunits correlated with OPN in OCCC cells. We then used an *in vitro* invasion assay to reveal whether OPN contributes to increased invasiveness. Our study demonstrated the dose-dependent effects of OPN on ECM invasion in OCCC cell lines.

One type of drug that may reduce OPN levels are the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins). It has been reported that statins reduce OPN expression in cultured rat aortic smooth muscle cells,[41] but it is unclear whether statins have an effect on OPN levels in malignant cells. Recent studies also suggest that statins, such as simvastatin, inhibit cancer cell growth and lead to apoptotic cell death through inhibition of the mevalonate pathway,[42–44] although another pathway may be involved in the induction of apoptosis, inhibition of cell proliferation, and cell invasiveness. Our data showed that simvastatin reduced not only the expression of OPN and integrins, but also cell invasiveness in clear cell carcinoma cells. As for serous cell carcinoma cells, we applied to add simvastatin, and OPN was reduced clearly (data not shown). But generally statins can decrease many kinds of tumors.[45] When administered as the sole agent in animal models, statins can decrease tumor load of acute myelogenous leukemia (AML), melanoma, hepatoma, pancreatic, lung, and neuroblastoma.[45,46] Although simvastatin could inhibit *in vitro* invasion of ovarian cancer cells, this does not prove that inhibition of invasion by simvastatin is due to the reduction of OPN expression. So we transfected two OCCC cells with OPN specific siRNA, and the transfected cells with OPN specific siRNA showed the reduction of cell invasiveness significantly. From these data, inhibition of OPN reduced cancer cell invasiveness. Mi *et al.* demonstrated that RNA aptamer-targeting of OPN has biological relevance for modifying tumor growth and metastasis in breast cancer cells,[47] while Zhao *et al.*[48] demonstrated that the RNA interference-mediated depletion of OPN may be a strategy for the treatment of hepatocellular carcinoma. Therefore, OPN can be considered a new molecular target for ovarian cancer therapy. These results suggest that simvastatin might be clinically effective against OCCC.

In summary, we have demonstrated that OPN expression is increased in OCCC, and that OPN induced ECM invasion *in vitro* by binding alpha v beta 1, alpha v beta 3, and alpha 5 beta 1 integrins in OCCC. Further, down-regulation of OPN by treatment with simvastatin, and transfection with OPN specific siRNA reduced cell invasiveness. Our results suggest that OPN regulation could play a key role in OCCC therapy through the control of integrins.

Disclosure Statement

The authors have no conflict of interest.

References

1 Sugiyama T, Kamura T, Kigawa J *et al.* Clinical characteristic of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy. *Cancer* 2000; **88**: 2584–9.

2 Tsuchiya A, Sakamoto M, Yasuda J *et al.* Expression profiling in ovarian clear cell carcinoma. Identification of hepatocyte nuclear factor-1 beta as a molecular marker and a possible molecular target therapy of ovarian clear cell carcinoma. *Am J Pathol* 2003; **163**: 2503–12.

3 Tammela J, Geisler JP, Eskew PN, Geisler HE. Clear cell carcinoma of the ovary: poor prognosis compared to serous carcinoma. *Eur J Gynaecol Oncol* 1998; **19**: 438–40.

4 Kato N, Motoyama T. Overexpression of Osteopontin in clear cell carcinoma of the ovary: close association with HNF-1 beta expression. *Histopathology* 2008; **52**: 682–8.

5 Kim JH, Skates SJ, Uede T *et al.* Osteopontin as a potential diagnostic biomarker for ovarian cancer. *JAMA* 2002; **13**: 1671–9.

doi: 10.1111/j.1349-7006.2010.01615.x

6 Senkel S, Lucas B, Klein-Hitpass L, Ryffel GU. Identification of target genes of the transcription factor HNF-1beta and HNF-1alfa in a human embryonic kidney cell line. *Biochim Biophys Acta* 2005; **20**: 179–90.
7 Sodek J, Ganss B, McKee MD. Osteopontin. *Crit Rev Oral Biol Med* 2000; **11**: 279–303.
8 Senger DR, Wirth DF, Hynes RO. Transformed mammalian cells secrete specific proteins and phosphoproteins. *Cell* 1979; **16**: 885–93.
9 El-Tanani MK, Campbell FC, Kurisetty V, Jin D, McCann M, Rudland P. The regulation and role of Osteopontin in malignant transformation and cancer. *Cytokine Growth Factor Rev* 2006; **17**: 463–74.
10 Tuck AB, Chambers AF, Allan AL. Osteopontin overexpression in breast cancer: knowledge gained and possible implications for clinical management. *J Cell Biochem* 2007; **102**: 859–68.
11 Jain S, Chakraborty G, Kundu GC. The crucial role of cyclooxygenase-2 in Osteopontin-induced protein kinase C alpha/c-Src/IkB kinase alpha/beta-dependent prostate tumor progression and angiogenesis. *Cancer Res* 2006; **66**: 38–48.
12 Chang YS, Kim HJ, Chang J, Ahn CM, Kim SK, Kim SK. Elevated circulating level of Osteopontin is associated with advanced disease state of non-small cell lung cancer. *Lung Cancer* 2007; **57**: 373–80.
13 Matusan K, Dordevic G, Stipic D, Mozetic V, Lucin K. Osteopontin expression correlates with prognostic variables and survival in clear cell renal cell carcinoma. *J Surg Oncol* 2006; **94**: 325–31.
14 Higashiyama M, Ito T, Tanaka E, Shimada Y. Prognostic significance of Osteopontin expression in human gastric carcinoma. *Ann Surg Oncol* 2007; **14**: 3419–27.
15 Apparao KBC, Murray MJ, Fritz MA *et al.* Osteopontin and its receptor alpha v beta 3 integrin are coexpressed in the human endometrium during the menstrual cycle but regulated differentially. *J Clin Endocrinol Metab* 2001; **86**: 4991–5000.
16 Nakae M, Iwamoto I, Fujino T *et al.* Preoperative plasma Osteopontin level as a biomarker complementary to carbohydrate antigen 125 in predicting ovarian cancer. *J Obstet Gynaecol Res* 2006; **32**: 309–14.
17 Schorge JO, Drake RD, Lee H *et al.* Osteopontin as an adjunct to CA125 in detecting recurrent ovarian cancer. *Clin Cancer Res* 2004; **10**: 3474–8.
18 Visintin I, Feng Z, Longton G *et al.* Diagnostic markers for early detection of ovarian cancer. *Clin Cancer Res* 2008; **14**: 1065–72.
19 Coppola D, Szabo M, Boulware D *et al.* Correlation of Osteopontin protein expression and pathological stage across a wide variety of tumor histologies. *Clin Cancer Res* 2004; **10**: 184–90.
20 Chakraborty G, Jain S, Behera R *et al.* The multifaceted roles of Osteopontin in cell signaling, tumor progression and angiogenesis. *Curr Mol Med* 2006; **6**: 819–30.
21 Kon S, Maeda M, Segawa T *et al.* Antibodies to different peptides in Osteopontin reveal complexities in the various secreted forms. *J Cell Biochem* 2000; **77**: 487–98.
22 Hu DD, Lin ECK, Kovach NL, Hoyer JR, Smith JW. A biochemical characterization of binding of Osteopontin to integrins alpha v beta 1 and alpha v beta 5. *J Biol Chem* 1995; **270**: 26232–8.
23 Liaw L, Skinner MP, Raines EW, Ross R, Cheresh DA, Schwartz SM. The adhesive and migratory effects of Osteopontin are mediated via distinct cell surface Integrins. *J Clin Invest* 1995; **95**: 713–24.
24 Rangaswami H, Bulbule A, Kundu GC. Osteopontin: role in cell signaling and cancer progression. *Trends Cell Biol* 2006; **16**: 79–87.
25 Van der Flier A, Sonnenberg A. Function and interactions on integrins. *Cell Tissue Res* 2001; **305**: 285–98.
26 Wai PY, Kuo PC. The role of Osteopontin in tumor metastasis. *J Surg Res* 2004; **121**: 228–41.
27 Davidson B, Goldberg I, Reich R *et al.* Alpha v- and beta 1- integrin subunits are commonly expressed in malignant effusion from ovarian carcinoma patients. *Gynecol Oncol* 2003; **90**: 248–57.
28 Sawada K, Mitra AK, Radjabi AR *et al.* Loss of E-Cadherin promotes ovarian cancer metastasis via alpha 5-Integrin, which is atherapeutic target. *Cancer Res* 2008; **68**: 2329–39.
29 Johnson GA, Burghardt RC, Spencer TE, Newton GR, Ott TL, Bazer FW. Ovine Osteopontin: Osteopontin and alpha v beta 3 integrin expression in the uterus and conceptus during the periimplantation period. *Biol Reprod* 1999; **61**: 892–9.
30 Jemal A, Murray T, Ward E *et al.* Cancer statistics, 2005. *CA Cancer J Clin* 2005; **55**: 10–30.
31 Bois AD, Luck HJ, Meier W *et al.* A randomized clinical trial of Cisplatin/Paclitaxel versus Carboplatin/Paclitaxel as first-line treatment of ovarian cancer. *J Natl Cancer Inst* 2003; **95**: 1320–9.
32 Enomoto T, Kuragaki C, Yamasaki M *et al.* Is clear cell carcinoma and micinous carcinoma of ovary sensitive to combination chemotherapy with paclitaxel and carboplatin. *Proc Am Soc Clin Oncol* 2003; **22**: 447 (abstract 1797).
33 Johnston NIF, Gunasekharan VK, Ravindranath A, O'Connel C, Johnston PG, EI-Tanani MK. Osteopontin as a target for cancer therapy. *Front Biosci* 2008; **13**: 4361–72.
34 Weber GF. The metastasis gene Osteopontin: a candidate target for cancer therapy. *Biochim Biophys Acta* 2001; **1552**: 61–85.
35 Philip S, Bulbule A, Kundu GC. Osteopontin stimulates tumor growth and activation of promatrix metalloproteinase-2 through nuclear factor-kB-mediated induction of membrane type 1 matrix metalloproteinase in murine melanoma cells. *J Biol Chem* 2001; **276**: 44926–35.
36 Song G, Cai QF, Mao YB, Ming YL, Bao SD, Ouyang GL. Osteopontin promotes ovarian cancer progression and cell survival and increase HIF-1 alpha expression through the PI3-K/Akt pathway. *Cancer Sci* 2008; **99**: 1901–7.
37 Cantor H, Shinohara ML. Regulation of T-helper-cell lineage development by osteopontin: the inside story. *Nat Rev Immunol* 2009; **9**: 137–41.
38 Zohar R, Suzuki N, Suzuki K *et al.* Intracellular Osteopontin is an integral component of the CD44-ERM complex involved in cell migration. *J Cell Physiol* 2000; **184**: 118–30.
39 Zohar R, Lee W, Arora P, Cheifetz S, McCulloch C, Sodek J. Single cell analysis of intracellular Osteopontin in osteogenic cultures of fetal rat calvarial cells. *J Cell Physiol* 1997; **170**: 88–100.
40 Standal T, Borset M, Sundan A. Role of Osteopontin in adhesion, migration, cell survival and remodeling. *Exp Oncol* 2004; **26**: 179–84.
41 Takemoto M, Kitahara M, Yokote K *et al.* NK-104, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, reduces osteopontin expression by rat aortic smooth muscle cells. *Br J Pharmacol* 2001; **133**: 83–8.
42 Hoque A, Chen H, Xu XC. Statin induces apoptosis and cell growth arrest in prostate cancer cells. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 88–94.
43 Fujiwara K, Tsubaki M, Yamazoe Y *et al.* Flivastatin induces apoptosis on human tongue carcinoma cell line HSC-3. *Yakugaku Zasshi* 2008; **128**: 153–8.
44 Koyuturk M, Ersoz M, Altiok N. Simvastatin induces proliferation inhibition and apoptosis in C6 glioma cells via c-jun N-terminal kinase. *Neurosci Lett* 2004; **370**: 212–7.
45 Clutterbuck RD, Millar BC, Powles RL *et al.* Inhibitory effect of simvastatin on the proliferation of human myeloid leukaemia cells in severe combined immunodeficient (SCID) mice. *Br J Haematol* 1998; **102**: 522–7.
46 Lishner M, Bar-Sef A, Elis A *et al.* Effect of simvastatin alone and in combination with cytosine arabinoside on the proliferation of myeloid leukemia cell lines. *J Investig Med* 2001; **49**: 319–24.
47 Mi Z, Guo H, Russell MB *et al.* RNA aptamer blockade of Osteopontin inhibits growth and metastasis of MDA-MB231 breast cancer cells. *Mol Ther* 2009; **17**: 153–61.
48 Zhao J, Dong L, Lu B *et al.* Down-regulation of Osteopontin suppresses growth and metastasis of hepatocellular carcinoma via induction of apoptosis. *Gastroenterology* 2008; **135**: 956–68.