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International Journal of Cancer

Selective osteopontin knockdown exerts anti-tumoral activity in a human glioblastoma model

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Osteopontin (OPN), a member of the SIBLING (Small Integrin-Binding LIgand N-linked Glycoprotein) family, is overexpressed in human glioblastoma. Higher levels of OPN expression correlate with increased tumor grade and enhanced migratory capacity of tumor cells. Based on these observations, we explored the possibility that knocking down OPN expression in glioblastoma cells could exert an anti-tumoral activity using an avian *in vivo* glioblastoma model that mimics closely human gliobastoma. Human U87-MG glioma cells transfected with specific anti-OPN small interfering RNAs (siRNAs) were grafted onto the chicken chorio-allantoic membrane (CAM). OPN-deficient U87-MG cells gave rise to tumors that were significantly smaller than tumors formed from untransfected cells (paired t-test, $p < 0.05$). Accordingly, the amount of proliferating cells in OPN-deficient tumors showed a six-fold reduction when compared to control tumors. However, OPN inhibition did not affect significantly tumor-associated angiogenesis. *In vitro*, OPN-silenced U87-MG and U373-MG cells showed decreased motility and migration. This is the first demonstration that OPN inhibition blocks glioma tumor growth, making this invasion-related protein an attractive target for glioma therapy.

Malignant gliomas, which include anaplastic gliomas and glioblastomas, are the most common type of primary brain tumor in human adults. A prominent characteristic of these tumors is their ability to infiltrate the normal brain tissue, making total resection of the tumor nearly impossible; thus tumor recurrence is a common event.[1] Therefore, identification of the major molecular players involved in glioma progression is essential for the development of future specific post-operative targeted therapies.

Osteopontin (OPN) is a member of the Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLINGs) family[2] and is expressed in normal mineralized tissues, the mammary gland and in several neoplastic tissues including central nervous system malignancies.[3] OPN plays a major role in many stages of cancer progression[4] and its expression significantly correlates with the tumor stage in various cancer types.[5] In gliomas, OPN expression has been correlated with the malignancy grade, suggesting that it may play a role in brain tumor progression.[6] This hypothesis was reinforced by another study aimed at the establishment of a molecular profile for human glioblastomas using serial analysis of gene expression (SAGE). In that study, C6 rat glioma cells were compared to primary cultured normal astrocytes. SAGE analysis allowed the identification of OPN as one of the most up-regulated genes in glioma cells.[7] More recently, Jang *et al.* demonstrated that OPN was expressed over 20-fold in tumoral brain tissue when compared to normal tissue, thus identifying OPN as a marker of glioma progression.[8] Furthermore, according to the same authors, OPN detection in glioma tumors could be correlated with the time when the lesions had become refractory to treatment.[9] Glioma cells express a variety of cell surface integrins enabling their interactions with extracellular matrix proteins, including OPN, and facilitating their invasion (for a review, see Reference 10).

Taken together these studies urged us to investigate whether glioma tumor development could be inhibited by specifically repressing OPN expression in tumor cells. RNA interference (RNAi) is widely accepted as the method of choice for rapidly assessing the biological significance of genes overexpressed in tumors and the subsequent validation of their protein products as potential targets in cancer. It has been demonstrated that U87-MG human glioma cells grafted onto the chorio-allantoic membrane (CAM) of the chicken embryo generate within a few days tumors whose growth and vascularization replicate significantly characteristics of the human glioblastoma.[11] Indeed, it was shown that CAM experimental glioma recapitulates hallmarks of the human

Key words: osteopontin, SIBLINGs, glioma, migration, proliferation
DOI: 10.1002/ijc.24751
History: Received 9 Mar 2009; Accepted 7 Jul 2009; Online 16 Jul 2009

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disease both at the transcriptional level and morphologically.[11] Therefore, we designed RNAi experiments to produce the specific silencing of OPN gene in human glioma cells and tested their behavior in the avian model.

In this loss-of-function study, we used western blot, ELISA and quantitative RT-PCR analysis to confirm the extent and stability of OPN knockdown. We demonstrated that OPN-silenced glioma cells are less able to generate tumors in the CAM model in correlation with a reduced proliferation rate, while the overall tumor angiogenesis was not affected. Both the motility and the migration potential of these cells were significantly reduced *in vitro*, indicating that OPN plays a major role in the promotion of glioma cell invasion. These results demonstrate for the first time that OPN suppression in glioma decreases tumor cell proliferation and migration, which are major features of glioma progression in humans.

Material and Methods

Cell lines

U87-MG human glioma cells were maintained in MEM with 10% FBS, 2 mM L-glutamine, 1% non essential amino acid (NEAA) and 1 mM sodium pyruvate. U373-MG human glioma cells were cultured in MEM with 5% FBS, 2 mM L-glutamine.

RNA interference

U87-MG and U373-MG cells were transfected with small interfering RNA (siRNA) at a concentration of 40 nM for 48 h using a calcium phosphate precipitation method as previously described.[12] siRNAs specific to OPN#1: 5′-CACAAGCAGUCCA GAUUAUUU, OPN#2[13] and VEGFA[14] were used to silence the corresponding target genes. GL3 luciferase siRNA and scrambled OPN#2 were used as negative controls. All siRNAs were purchased from Eurogentec (Seraing, Belgium).

OPN Enzyme-Linked Immunosorbent Assay (ELISA)

Sixteen hours after U87-MG and U373-MG cells transfection with the aforementioned siRNA, culture medium was replaced with serum-free medium. Forty-eight hours later, collected media were centrifuged and the supernatants were kept at −20°C until analysis. To evaluate OPN levels in experimental tumors, day 3 and 5 tumors (pools of 3 to 5 tumors/group) were homogenized in lysis buffer (CelLytic-MT, Sigma Aldrich, Bornem, Belgium) in the presence of a protease inhibitor cocktail (Roche Diagnostics) as previously described.[14] OPN protein concentration was measured using an ELISA kit (R&D Systems) according to the manufacturer's instructions and was normalized either to culture supernatant volume or to tissue weight. The assay was performed in triplicate wells and was repeated three times with the same results.

Western blot analysis

Proteins were separated on SDS-polyacrylamide gels and transferred to polyvinylidene-difluoride western-blotting membranes (Roche Inc.). Membranes were blocked with 5% non-fat dry milk for 1 hour at room temperature, and incubated with a polyclonal anti-human OPN antibody (R&D systems, Abingdon Oxon, UK). After washing, blots were incubated with peroxidase-conjugated secondary antibody for 1 hour. Blots were washed again and incubated in ECL detection reagent (Amersham Pharmacia Biotech, Buckinghamshire, England). β-actin was used as a control for equivalent protein loading.

RNA Extraction and Quantitative Real-Time RT-PCR Analysis

Total RNA was isolated from glioma cells using RNeasy columns (Qiagen, The Netherlands) according to the manufacturer's instructions. First-strand cDNA was synthesized using 1 μg of total RNA in 20 μl of reverse transcription (RT) reaction mixture containing 0.2 μg of pd(N)6 random hexamers (Amersham Biosciences), 2 mM of each deoxynucleotide triphosphate (Eurogentec), 1× first-strand buffer [50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2], 10 mM dithiothreitol and 100 units of SuperScriptTM II Reverse Transcriptase (Invitrogen). The reverse transcription was performed at 42°C for 50 min before a 15 min inactivation step at 70°C. Quantitative real-time PCR was performed in triplicate using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. OPN primers and TaqMan probe were designed using the primer design software Primer Express (Applied Biosystems): OPN forward 5′-TCACCTGTGC CATACCAGTTAA, OPN reverse 5′-TGAGATGGGTCAGGGT TTAGC, OPN probe FAM-5′-ACAAATACCCAGATGCTGT GGCCA-3′-TAMRA. The TaqMan glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control reagent kit (Applied Biosystems) was used for GAPDH detection. cDNA samples were mixed with 100 nM of each primer and TaqMan Universal PCR Master Mix containing 1× PCR buffer, 5.5 mM $MgCl_2$, 0.8 mM dNTPs mix, 100 nM probe and 1 unit of AmpliTaq Gold® thermostable DNA polymerase (Applied Biosystems) in a total volume of 25 μl. Real-time PCR was conducted using the following parameters: 94°C for 10 min, and 35 cycles at 94°C for 45 s, 57°C for 45 s and 72°C for 30 s, and 72°C for 2 min after the last cycle. Quantitative-real time PCR was performed for OPN and normalized to the amount of GAPDH mRNA from the same sample. Acquired data were analyzed by Sequence Detector software version 1.9 (Applied Biosystems).

Experimental glioma assay

On embryonic day 3, a window was cut in the eggshell after puncturing the air chamber and was sealed with Durapore

tape. On embryonic day 10, a plastic ring was placed on the CAM, and 5 million U87-MG cells in 15 μl of medium were deposited after gentle laceration of the CAM surface. Tumors were harvested 3, 5 or 7 days after implantation and were fixed with 4% paraformaldehyde for 30 min at room temperature. Microphotographs of each tumor were taken. Tumor volumes were estimated as described previously by Hagedorn *et al.*[11] using the formulation: $V = 4/3\ \pi\ r^3$, with $r = 1/2\ \sqrt{}\ (d1 \times d2)$ where r is the radius and d1 and d2, respectively the biggest and the smallest dimensions of the tumors. The CAM experiment was performed three times with the same results. Statistical comparison between the different tumor groups ($n \geq 6$ eggs per group) was performed using the Student's *t*-test.

Immunohistochemistry

Tumors were fixed at the indicated days with 4% paraformaldehyde and were embedded in paraffin. Proliferating cells were detected using mouse monoclonal anti-human Ki67 (Clone MIB-1, 1/100, Dakocytomation, Heverlee, Belgium). The signal was revealed using the vectastain ABC peroxidase kit (Vector laboratories, Inc, Burlingame, CA) and counterstained with hematoxylin. Non-immune horse serum was used as a negative control. Ki67-positive cells were counted in 5 fields of 4 different OPN#2 siRNA and control tumors. The experiment was performed twice. A statistical comparison was made using the Unpaired *t*-test.

Apoptosis assay

Tumor tissue was analyzed for apoptosis using the In Situ Cell Death Detection Kit (Roche Inc.). Tissue sections were washed and then permeabilized with proteinase K solution for 20 min at 37°C. TUNEL reaction mixture was added and incubated for 1 h at 37°C. Slides were washed and then incubated with an antibody conjugated with horseradish peroxidase for 30 min at 37°C. After substrate reaction, slides were visualized under light microscopy. Positive control consisted of a tissue section incubated with DNase solution (3 U/ml in 50 mM Tris-HCl pH7.5, 10 mM $MgCl_2$, 1 mg/ml BSA) for 10 min at room temperature to induce DNA strand breaks, prior to labeling procedures.

Scratch-induced migration assay

U87-MG and U373-MG glioblastoma cells were transfected with OPN#1 or OPN#2 siRNAs. No siRNA, siRNA against unrelated gene GL3 luciferase and scrambled OPN#2 were used as controls. Cells were grown to confluence on collagen coated 6-well plates as described elsewhere.[15] Eight areas were scratched per experimental condition. The region imaged at time zero was marked to enable the examination of the same population of migrating cells. Wound areas were measured at time zero and after 8 h. The data acquired from the 8 scratches were averaged to obtain the mean gap width at a given time and a percentage of wound closure was calculated. The assay was performed three times with similar results.

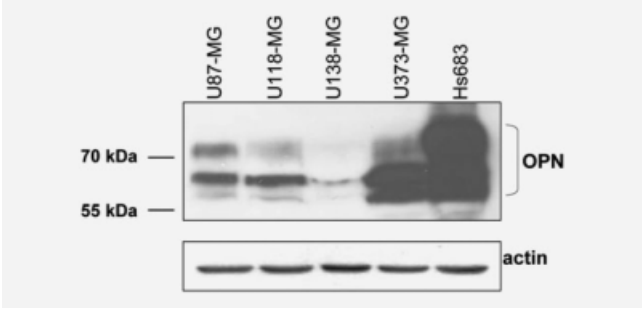

Figure 1. OPN expression in various human glioma cell lines. Western blot analysis of OPN expression was performed on total protein extracts from U87-MG, U118-MG, U138-MG, U373-MG and Hs683 glioma cell lines. The three bands, comprised between 55 and 70 kDa, corresponding to OPN were detectable in all 5 cell lines to different extents, with Hs683 showing the highest expression. Equal protein loading was assessed by anti-actin immunoblotting. The experiment was performed three times.

Motility assay

Cells (1.5×10^5) suspended in serum-free DMEM containing 0.1% bovine serum albumin (BSA) were seeded in triplicate into the upper part of a transwell filter (diameter 6.5 mm, pore size 8 μm; Costar, Cambridge, MA) and the lower compartment was filled with serum-free DMEM containing 1% BSA. After incubation for 24 hours at 37°C, non migrating cells of the upper surface of the filter were wiped with a cotton swab, and migrating cells in the lower surface of the filter were fixed and stained with the Diff-Quick kit (Medion Diagnostics, Switzerland). Motility rate was determined by counting cells in 3 random fields per well, and the extent of motility was expressed as the average number of cells per field. Three wells per condition were counted and the test was performed three times with similar results.

Results

Human glioma cell lines express OPN whose production can be efficiently and durably blocked using specific siRNAs

Western blot analysis revealed that all the 5 human glioma cell lines examined expressed OPN to different extents, with Hs683 showing the highest amounts and U138-MG the lowest. Using a specific polyclonal antibody, OPN protein was detected as two or three bands with apparent molecular weights ranging from 55 to 70 kDa, depending on the cell line investigated (Fig. 1). These bands probably correspond to known OPN isoforms (*a, b* and *c*)[16] and potentially to different degrees of post-translational modification.[17,18] We next used RNAi to induce specific silencing of OPN in human glioma cells. We tested two siRNAs specific for human OPN (OPN#1 and OPN#2) for their efficacy to reduce OPN

expression in U87-MG and U373-MG glioma cells. Both siRNAs induced a strong reduction of OPN protein production. Cells cultured without siRNAs (No siRNA) or transfected with irrelevant GL3 luciferase (GL3) and scrambled OPN#2 (ScrOPN#2) siRNAs served as controls and did not affect OPN protein levels (Fig. 2*a*). OPN is known to be secreted and was indeed readily detectable in U87-MG and U373-MG cell culture medium. We confirmed that glioma cells transfected with OPN siRNAs secreted less OPN as assessed by western blot performed on conditioned media (Fig. 2*b*, upper panel) and ELISA test (Fig. 2*b*, lower panel). Thus, both OPN siRNAs proved to be efficient inhibitors of OPN expression in both cell lines. In order to assess whether OPN inhibition was effective throughout the duration of the CAM experiment, parallel transfected cells were analyzed for OPN mRNA and protein levels at 3, 5 and 7 days post-transfection. Transient transfection with OPN siRNA led to a strong and prolonged OPN reduction suitable for short-term *in vitro* and *in vivo* experiments. Indeed, the OPN mRNA level stayed below 20% of controls for the first five days and was still below 55% on day 7 (Fig. 3, left panel). Consistently, the OPN protein level was stably decreased during the first five days and began to rise only at day 7 (Fig. 3, right panel).

OPN inhibition decreases glioma tumor growth in vivo

Hagedorn *et al.*[11] have recently established a robust and highly reproducible glioma progression model where U87-MG human glioma cells that are grafted onto the vascularized chicken CAM develop into a tumor within a short period of time. This model allows fast and precise analysis of the main steps of human glioma progression and tumor-associated angiogenesis. Untransfected U87-MG cells and cells transfected with either OPN#1 siRNA, OPN#2 siRNA or control siRNAs were grafted onto the CAM as described in the Materials and method section. No siRNA and control siRNAs tumors showed similar development and tumor volume increased notably over time (Fig. 4*a*). Even though tumor size varied slightly during the first days in the control groups, all tumors were fully vascularized at day 5 and vascular morphology did not change further until day 7, when the experiments were ended. In contrast, the OPN#1 and OPN#2 siRNA tumors were smaller than the control tumors and did not grow any further (Fig. 4*a*). We evaluated general growth characteristics of glioma tumors by estimating tumor volume 7 days after grafting (Fig. 4*b*). The volume of the experimental gliomas was significantly reduced after OPN knockdown: up to 40% compared to control tumors ($p < 0.005$; Fig. 4*b*). As a supplementary experimental control, U87-MG cells transfected with a specific siRNA directed against VEGFA were also grafted onto the CAM. In a recent study, we demonstrated the specificity and the efficiency of this VEGFA siRNA.[14] Indeed, using the same CAM glioma model, the blockade of VEGFA expression in grafted U87-MG cells induced a significant decrease in tumoral growth and vascularization. In this study, OPN siRNA tumors presented with a small size that was equivalent to VEGFA siRNA tumors (Fig. 4*b*). We next evaluated OPN protein levels in glioma tumors grown on the CAM. Tumor lysates analyzed by ELISA showed a significant basal expression of OPN *in vivo* (Fig. 4*c*). As expected, OPN#1 and OPN#2 siRNA tumors

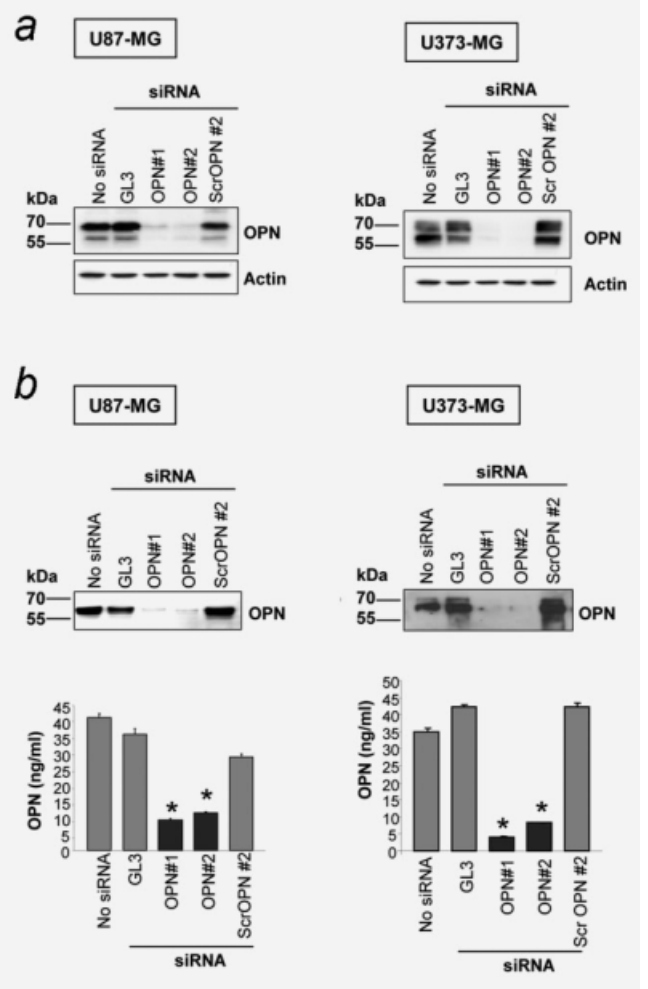

Figure 2. Inhibition of OPN secretion and expression on human glioma cells by RNA interference. (*a*) Two different small interfering RNAs (siRNAs) targeting OPN (OPN#1 and OPN#2) were tested in U87-MG and U373-MG glioma cells. After 48 h of transfection, both siRNAs strongly decreased OPN protein production compared to untransfected cells (No siRNA), cells transfected with an siRNA against GL3 luciferase unrelated gene (GL3) and scrambled OPN#2 (ScrOPN#2) used as controls. Equal protein loading was assessed by subsequent anti-actin immunoblotting. (*b*) OPN secretion was analyzed using western blot (upper panel) and ELISA techniques (lower panel). Conditioned culture medium of U87-MG and U373-MG cells demonstrated the secretion of OPN by glioma cells and confirmed the strong reduction of OPN protein levels after treatment with both siRNAs. The assays were repeated three times with the same results and a representative assay is shown. For the ELISA test, the mean ± SD of triplicates is represented; * indicates $p < 0.05$ versus No siRNA condition.

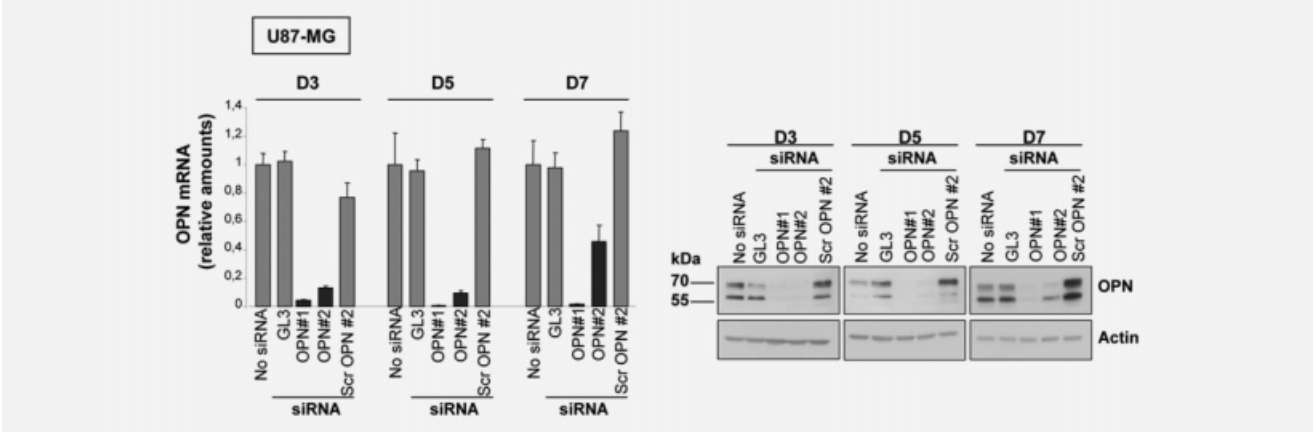

Figure 3. Inhibition of OPN expression by RNA interference throughout the duration of the CAM experiment. Total RNA and protein extracts from U87-MG cells transfected with controls, OPN#1 and OPN#2 siRNAs were analyzed by Real-time quantitative RT-PCR (left panel) and western blot (right panel) analysis. OPN mRNA and protein levels were determined 3, 5 and 7 days post-transfection and showed a significant reduction up to 5 days. Histograms show the relative level of OPN mRNA as compared with the No siRNA condition arbitrarily set to 1. The experiment was performed three times with similar results. Data are shown as the mean values ± SD of three replicates from a representative experiment.

contained less than 50% OPN when compared to control tumors ($p < 0.001$) (Fig. 4*c*).

OPN inhibition reduces glioma cell proliferation but does not affect apoptosis or angiogenesis in vivo

We next determined the proliferation index in OPN#2 siRNA tumors. In these tumors, the amount of proliferating cells showed a six-fold reduction when compared to control tumors (Fig. 5*a*). Since OPN has been implicated in the prevention of tumor cell apoptosis, we next performed a TUNEL assay on experimental glioma tumor sections. Only rare apoptotic tumor cells were observed in both siOPN#2 and the control (No siRNA, GL3 and ScrOPN#2) tumor sections, while the positive control consisting of DNase-treated tumor slides showed a high proportion of strongly stained nuclei (Fig. 5*b*). Several studies indicate that OPN plays an important role in tumor growth through the enhancement of angiogenesis *in vivo*. Therefore, we questioned whether the blockade of OPN expression in U87-MG cells influenced the vascularization of experimental glioma tumors. We did not observe any significant difference between OPN-expressing and non-expressing tumors. In contrast, control tumors derived from cells depleted in VEGFA displayed a visible decrease in their vascularization (Fig. 5*c*). These results indicate that OPN suppression in glioma cells influenced tumor proliferation without interfering with the angiogenesis and apoptosis processes *in vivo*.

OPN inhibition reduces glioma cell motility and migration in vitro

Glioma tumor cells are characterized by their highly invasive and infiltrative capacity, we therefore investigated whether OPN had an impact on glioma cell motility *in vitro* using a modified Boyden chamber assay. OPN-silenced U87-MG and U373-MG cells showed a 3-fold decreased motility when compared to their control counterpart (Fig. 6*a*). To further investigate the impact of OPN inhibition on glioma cell movement, we explored U87-MG and U373-MG cells migration using the scratch–wound assay. As shown in Figure 6*b*, the closure of the wound was significantly reduced in OPN-silenced cells. Eight hours after wounding, only one third of the scratched area was covered by migrating siOPN#1 and siOPN#2 U87-MG cells, whereas more than two thirds of the wound surface were covered by No siRNA, GL3 or ScrOPN#2 siRNAs-transfected cells (Fig. 6*c*, left panel). For siOPN#1 and siOPN#2 U373-MG cells, about 20% of the scratched area was covered by migrating cells, whereas about 53% of the wound surface was covered by control cells (Fig. 6*c*, right panel). The addition of mitomycin during the assay did not affect the rate of closure of the scratch (data not shown), indicating that the differences in closure rates were due to cell motility and not proliferation.

Discussion

Osteopontin (OPN) is a secreted protein that is overexpressed in most human cancers and has been associated with poor prognosis in cancer patients (for a review, see Reference 4). It is considered as a multifunctional protein that plays a central role in malignancy, notably through its diverse interactions with cell surface receptors (integrins, CD44), secreted matrix metalloproteinases (MMP2, MMP3), growth factor receptor pathways (EGFR) and through its activation of various key kinases (PI3K, MAPK) (for a review, see References 19 and 20). OPN is expressed in human glioma cells, with high-grade tumors expressing the highest levels.[6] The anti-tumorigenic and anti-metastatic effects of OPN knockdown have

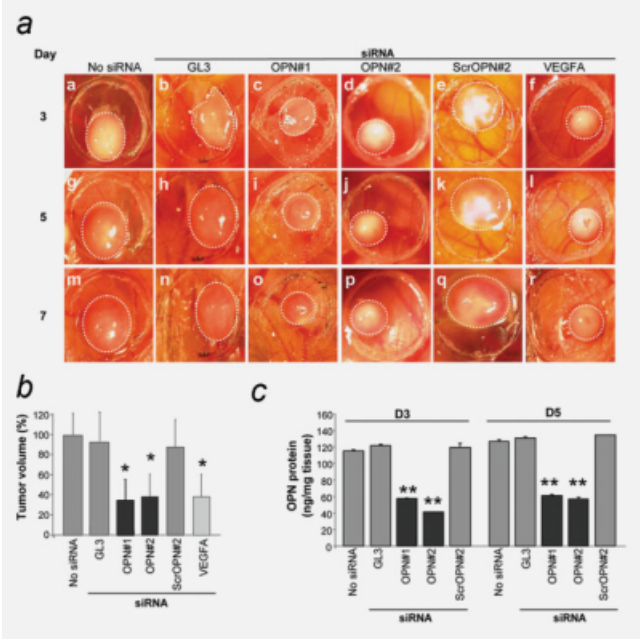

Figure 4. Experimental glioma tumors derived from OPN knockdown-U87-MG cells grafted onto the CAM. (*a*) Development of OPN#1, OPN#2 and control siRNAs tumors onto the chicken CAM was documented by biomicroscopy at 3, 5 and 7 days after U87-MG cell grafting. One representative tumor for each condition is shown. Glioma tumors derived from untreated cells (No siRNA) **(a, g, m)** resembled those derived from cells transfected with either control GL3 siRNA **(b, h, n)** or with ScrOPN#2 **(e, k, q)**. In contrast, OPN#1 (**c, i, o**), OPN#2 siRNA tumors (**d, j, p**) and VEGFA siRNA tumors (**f, l, r**) were smaller than the control tumors. (*b*) Seven days after grafting, tumor volume was calculated for each condition as indicated in the Materials and method section. OPN knockdown significantly decreased tumor volume compared to the No siRNA control (* $p < 0.005$). The experiment was performed three times with similar results. Data are shown as the mean values ± SD of three replicates from a representative experiment. (*c*) OPN protein levels of tumors grown on CAM were determined by ELISA on experimental glioma tumor lysates. OPN#1 and OPN#2 siRNA tumors contained significantly less OPN than control tumors (** $p < 0.001$ versus control siRNAs). After 3 and 5 days (D3 and D5), OPN#1 and OPN#2 siRNA tumors contained less OPN per mg of tumor tissue when compared to controls. The experiment was performed three times with similar results. Data are shown as the mean values ± SD of three replicates from a representative experiment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

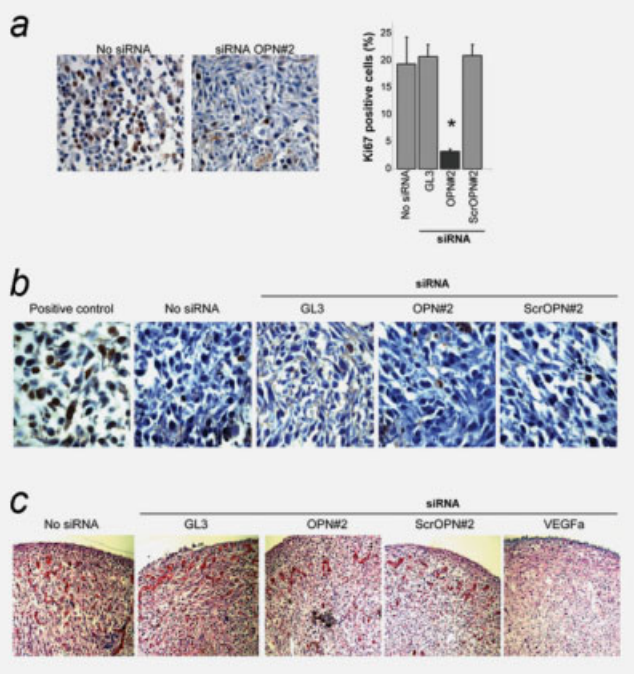

Figure 5. Effect of OPN inhibition on cell proliferation, apoptosis and angiogenesis in experimental glioma tumors. (*a*) Glioma tumors were embedded in paraffin and tissue sections were stained for the proliferation marker Ki67. Cell proliferation was significantly reduced in OPN#2 siRNA glioma compared to no siRNA, GL3 and ScrOPN#2 siRNAs as determined by the proportion of Ki67 positive nuclei (left panel). Quantification of Ki67 positive cells for each condition ($n = 4$) is presented in the right panel. The experiment was performed three times with the same results. Values represent mean ± SD (* $p < 0.005$). (*b*) TUNEL assay was performed on glioma tumor sections derived from U87-MG cells transfected with No siRNA, GL3, ScrOPN#2 and OPN#2 siRNAs. A representative staining for each condition is shown. The positive control is a tumor section in which DNA breakdown was induced by incubation with DNase solution prior to TUNEL assay. (*c*) H&E histology staining was performed on day 7 glioma tumor sections and microphotographs were taken right under the tumoral surface for each experimental condition. A representative staining for each condition is shown. Note the presence of numerous irregular and dilated capillaries in No siRNA, GL3 and ScrOPN#2 siRNA and in OPN#2 siRNA conditions, but not in VEGFA siRNA tumors used as a control of angiogenic inhibition. Magnifications (*a*) ×400, (*b*) ×630 and (*c*) ×100. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

been recently reported in breast, hepatocarcinoma and colon adenocarcinoma cells.[21–25] However, the importance of the blockade of endogenous OPN expression in glioma cells has not yet been addressed. In this study, a loss-of-function strategy allowed us to demonstrate the contribution of OPN expression to glioma cell motility, proliferation *in vitro* and tumorigenicity *in vivo*. Our data show that several human glioma cell lines express OPN and that the specific blockade of its expression in U87-MG cells induces a significant repression of glioma cell growth when grafted onto the CAM. This study is the first to reveal that endogenous OPN contributes to glioma tumor growth both *in vivo* and *in vitro*. In a microarray study comparing the gene expression pattern of N-ethyl-N-nitrosourea (ENU)-induced tumors in rat brain with those of contralateral and normal brains, OPN was

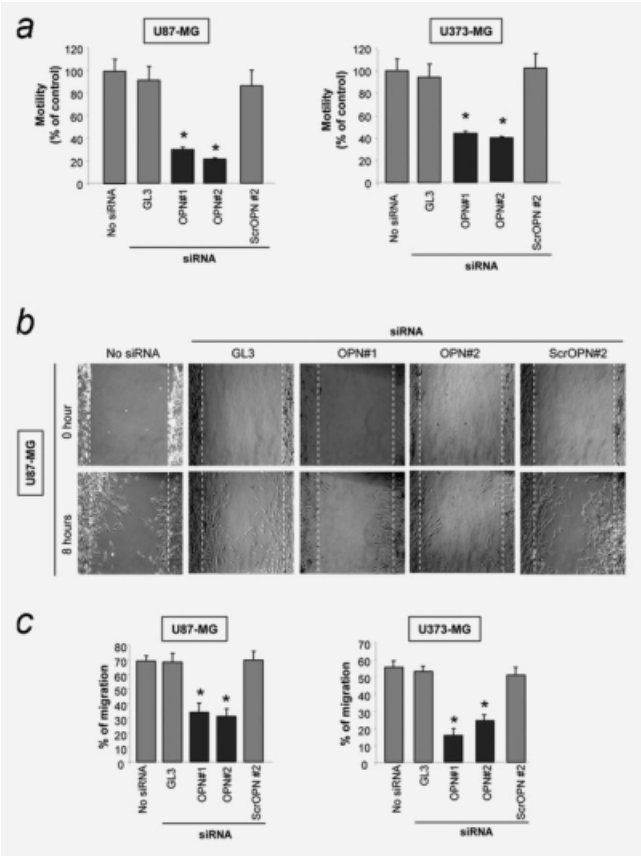

Figure 6. Effect of OPN inhibition on motility of human glioma cells *in vitro*. (*a*) The motility of U87-MG and U373-MG cells transfected with OPN#1, OPN#2 or control siRNAs (no siRNA, GL3 and ScrOPN#2) was analyzed using modified Boyden chamber assay. Cells were plated on the top of the membrane, serum-free medium was added in the bottom chamber and cells were incubated for 24 h. Columns represent the percentage of migrated OPN#1 and OPN#2 siRNA glioma cells relatively to control cells. OPN-depleted cells demonstrated a decreased motility when compared with control cells expressing OPN. The results of a representative experiment are shown ($n = 3$). The mean ± SD of triplicates is represented; * indicates $p < 0.0001$ *versus* controls. (*b*) Confluent monolayers of untransfected (No siRNA) U87-MG and GL3, ScrOPN#2, OPN#1 and OPN#2 siRNAs transfected U87-MG cells were wounded 48 hours after transfection. Phase-contrast microscopy photomicrographs were taken directly and 8 hours after wounding and representative fields are shown for each condition (magnification ×200). (*c*) Quantitative analysis of the wounded areas was performed as described in the Materials and method section and demonstrated that OPN inhibition significantly induced a decrease in U87-MG and U373-MG cell migration compared to the control (*$p < 0.0001$). The assay was repeated three times with the same results. Values are mean ± SD of 8 wounds for each condition from a representative experiment.

identified as the most up-regulated gene in glioma (21-fold). In the same study, the authors showed that OPN overexpression in ENU-induced tumor cells results in increased proliferation.[8] In contrast, in their recent study, Selkirk *et al.* demonstrated that OPN overexpression in rat F98 and human U87-MG high-grade glioma cells reduced tumor burden and promoted survival.[26] These authors also mention a differential effect of intermediate and high levels of OPN, pointing therefore to concentration-dependent cell responses, with OPN limiting the malignant character of glioma tumors only when threshold concentrations are reached and exceeded. Consistently, while OPN has been shown to be pro-angiogenic,[27] it has been proved to exert an opposite effect when overexpressed in endothelial cells.[28] Indeed, endothelial cells overexpressing OPN were shown to demonstrate a reduced motility in Boyden chambers and a reduced capacity to repair a wounded endothelial cell monolayer in an *in vitro* re-endothelization model after mechanical injury.[28] These authors suggest that the presence of high amounts of OPN may impair downstream cellular signaling through integrin ligation. This phenomenon, called integrin receptor "freezing", has also been reported in other studies[29,30] and may explain the opposite effects reported when OPN is overexpressed in cancer cells.

Here, we have answered the key question, especially from a therapeutic standpoint, of whether OPN expression is necessary for tumor progression. We showed that OPN silencing impedes the development of a tumor in the CAM assay pointing to a functional role of OPN in this process. Moreover, we observed that while OPN-deficient cells grafted onto the CAM were able to initiate the development of a tumor, they failed to sustain tumor growth throughout the duration of the CAM model experiment when compared with OPN-expressing tumor cells. Again, this observation is in total accordance with previous data describing the time-course of OPN expression in an ENU-induced glioma model, which allows the observation of the transition from a preneoplastic state to a fully developed tumor.[8] In this glioma progression model, early precursor lesions did not express OPN and its appearance coincided rather with the acquisition of a full glioma tumor phenotype. In human glioma, OPN expression correlated with aggressiveness as assessed by the malignancy grade.[6] Importantly, we demonstrated that OPN is secreted by glioma tumor cell lines, indicating that OPN contributes to the glioma extracellular matrix (ECM). In light of the abundant literature on the molecular nature of glioma ECM, as recently reviewed by Bellail *et al.*[31] and Lefranc *et al.*,[1] it is likely that glioma cells secrete specific proteins to create a permissive environment that favors their own proliferation and invasion. Accordingly, we observed that OPN-deficient glioma cells showed reduced motility and migration, suggesting that glioma cells that are unable to secrete OPN exhibit a lower invasive potential. In this regard, hyaluronic acid (HA), a proteoglycan abundantly found in the ECM of the human brain, has been shown to enhance the motility of glioma cells

through the induction of OPN expression in U87-MG cells via a PI3-K/Akt pathway.[32] Thus, the endogenous OPN expression levels of glioma cells can be influenced by ECM components present in the normal brain. Moreover, it has been demonstrated that the exogenous addition of human recombinant OPN to U87-MG cells increases their motility.[32] Taken together, these data and ours are consistent with a role of OPN as an autocrine factor that promotes glioma cell proliferation, motility and migration. The multifunctional role of OPN in cancer progression and metastasis is notably exerted through its interaction with integrins and CD44 cell surface receptors. Such receptors are known to be expressed at the surface of glioma cells and to contribute to the characteristic invasive phenotype of malignant gliomas.[10,33] OPN engagement, along with its cell surface receptors, leads to the activation of proteases including matrix metalloproteases (MMPs) that degrade the ECM, thus allowing tumor cells to migrate and infiltrate the surrounding tissue. In breast cancer cells, the silencing of OPN gene expression down-regulates the expression of oncogenic molecules such as uPA, MMP-2 and MMP-9 resulting in the inhibition of *in vitro* cell motility and *in vivo* tumorigenicity in mice.[21] Whether this is indeed the case for the invasion of brain parenchyma by glioma cells awaits further confirmation. Nevertheless, strong correlations between increased MMP levels and potential for high tumor cell invasiveness in human glioma have been already reported by several investigators (for a review, see Reference 34).

Recently, much has been focused on the biological role of OPN in the promotion of tumor-associated angiogenesis.[27,35] Endogenous VEGF has been previously shown to be crucial for the angiogenicity and tumorigenicity of U87-MG cells.[36] Furthermore, U87-MG cells deposited on the CAM have been shown to form highly vascularized tumors in a VEGF-dependent manner.[11] Accordingly, we observed that OPN suppression did not affect significantly tumor vascularization, while the inhibition of endogenous VEGF showed a marked reduction of tumor vessels. This observation suggests that, in the presence of VEGF, OPN is not essential for the formation of new capillary networks, at least in this model. Rather, OPN probably exerts a cooperative effect with VEGF on angiogenesis, notably by promoting the migration of endothelial cells induced by VEGF in an αvβ3 integrin-dependent manner.[37] Immunohistochemical detection of OPN in glioblastoma has been recently associated with microvascular density of the tumor and the authors propose that OPN could interact with VEGF in these tumors. However, VEGF levels were not assessed in that study.[35]

RNA interference approaches for the treatment of malignant gliomas have been recently reviewed by Mathulapa *et al.*[38] including the post-surgical delivery of siRNA in the resected-tumor cavity either via direct infusion[39] or *via* regulated delivery with implantable pumps.[40] We showed in a recent study that the *in vivo* delivery of siRNAs (directed against galectin-1) could be performed efficiently in the case of glioblastoma by means of Omaya reservoirs, thus minimizing systemic exposure and potential hepatotoxicity.[41] Thus, OPN gene silencing through RNAi could represent a promising new therapeutic tool for the cure of malignant gliomas and eventually for the prevention of their recurrence.

Acknowledgements

M. Le Mercier holds a "Télévie Grant", F. Lefranc is a Clinical Research Fellow, R. Kiss is a Director of Research and A. Bellahcène is a Senior Research Associate, all from the National Fund for Scientific Research (NFSR). This study was supported by grants from the NFSR and the University of Liège, Belgium. The authors thank P. Heneaux, V. Hennequière and N. Maloujahmoum for their expert technical assistance.

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