

# Down-regulation of osteopontin inhibits metastasis of hepatocellular carcinoma cells via a mechanism involving MMP-2 and uPA

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**Abstract.** Osteopontin (OPN) has an important role in hepatocellular carcinoma (HCC) progression and metastasis. This study was to investigate the therapeutic potential of inhibition of OPN expression. A 2'-O-methoxyethylribose-modified phosphorothioate antisense oligonucleotides (ASO) was used to knock-down OPN expression in the human metastatic HCC cell line HCCLM6 and in nude mice orthotopically implanted with HCCLM6 showing highly spontaneous lung metastasis. Furthermore, we assessed the metastatic potential of HCCLM6 cells *in vitro* and *in vivo* after ASO treatment. Treatment of HCCLM6 cells with OPN ASO inhibited OPN mRNA expression in a dose- and time-dependent manner, whereas the control oligonucleotides had no effect. OPN ASO significantly suppressed migration and invasion of HCCLM6 cells *in vitro*. Specific suppression of OPN also inhibited matrix metalloproteinase 2 (MMP-2) and urokinase-type plasminogen activator (uPA) expression in HCCLM6 cells. In mice bearing orthotopic xenografts with HCCLM6, OPN inhibition following therapeutic treatment with OPN ASO significantly decreased lung metastases although tumor weight did not appear to be reduced. These

findings suggest that OPN-targeted therapy may be a promising strategy for the treatment of HCC metastases.

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common and aggressive malignancies, ranking the third in the world and the second in China in cancer-related deaths (1). Despite improvements in the available treatments such as surgical resection, liver transplantation and locoregional therapy, HCC prognosis remains poor due to the high rate of recurrence or metastasis after therapy (2). Thus, it is critical to identify the molecule controlling the invasive and metastatic potential of HCC, which would provide new targets for intervention.

Results from gene profiling analysis indicate that osteopontin (OPN) is identified as the lead gene overexpressed in metastatic HCC (3). OPN has recently been shown to be overexpressed in a variety of cancers including colon, breast, lung, prostate, and pancreatic cancer (4). OPN contributes to tumor progression, especially tumor metastasis through the promotion of cell proliferation, migration and extracellular matrix invasion *in vitro* (5,6). Specific inhibition of OPN signaling has also been shown to inhibit tumor progression and metastasis in other types of cancers (7). These findings suggest that OPN may be a novel drug target for therapeutic intervention in HCC progression and metastasis.

In our institute, human HCC cell line HCCLM6 with highly metastatic potential and the orthotopic model with spontaneous lung metastasis have been established previously (8), which is more reflective of HCC development and metastasis. Herein, we showed that inhibition of OPN using a specific 2'-O-methoxyethyl (2'-MOE)-modified antisense oligonucleotide (ASO) reduced cell migration and invasion of HCCLM6 cells *in vitro*. In addition, expression of matrix metalloproteinase 2 (MMP-2) and urokinase-type plasminogen activator (uPA) was reduced following OPN suppression. Moreover, the lung metastasis of HCCLM6 in nude mice treated with OPN ASO was effectively inhibited. Thus, the present results show that targeting OPN may have therapeutic potential in inhibiting HCC metastasis.

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*Abbreviations:* OPN, osteopontin; HCC, hepatocellular carcinoma; ASO, antisense oligonucleotides; MMP-2, matrix metalloproteinase 2; uPA, urokinase-type plasminogen activator; 2'-MOE, 2'-O-methoxyethyl; PI3K, phosphatidylinositol 3-kinase; NF- $\kappa$ B, nuclear factor  $\kappa$ B; MMPs, matrix metalloproteinases; MAPK, mitogen-activated protein kinases; AP-1, activator protein-1

*Key words:* hepatocellular carcinoma, osteopontin, metastasis, antisense oligonucleotides

## Materials and methods

**Cell culture and oligonucleotide treatment.** Human HCC cell line HCCLM6 established at the Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China, was cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) in 5% CO<sub>2</sub> at 37°C. Oligonucleotides were 20-mer in length with a phosphorothioate back-bone and five 2'-MOE-modified nucleotides at each end (underlined residues below). The 10 central bases were left unmodified to support RNase H-mediated cleavage of targeted mRNA. OPN ASO was designed to target the coding region of human OPN mRNA (GenBank NM\_000582; 5'-ACTTCGGTTGCTGGCAGGTC-3'). The control oligonucleotides had the same chemical design except for the randomized sequence. Oligonucleotides were synthesized as previously described. For cell treatment, oligonucleotides premixed with Lipofectamine 2000 (Invitrogen) at 1.25 µg/ml/100 nM ASO in serum-free DMEM were added to cells (37°C, 6 h). The oligonucleotide-lipid treatment was then replaced with culture medium and incubated for the indicated times.

**RNA isolation and reverse transcription-PCR.** Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and reverse transcribed into single-stranded cDNA. PCR was done on cDNA using oligo(dT) priming and amplified with the primer pairs for a 436-bp fragment of OPN (forward primer 5'-GGACTCCATTGACTCGAACG-3' and reverse primer 5'-TAATCTGGACTGCTTGTGGC-3') and a 366-bp fragment of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer 5'-ATCCCATCACCATCTTCCAG-3' and reverse primer 5'-GAGTCCTCCACGATACCAA-3'). Ten microliters of PCR product were analyzed on 2% agarose gels.

**Western blotting.** Cell lysate (50 µg) was separated by standard SDS-PAGE and then transferred to PVDF membranes. The membranes were washed, blocked, and incubated with the specific primary antihuman antibodies against OPN (1:500; Santa Cruz), or glyceraldehyde-3-phosphate dehydrogenase (1:5000; Santa Cruz), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The reactions were detected by enhanced chemiluminescence assay.

**Immunocytochemistry.** Cells cultured on cover slides in six-well plates (1.0x10<sup>5</sup>/well) were fixed in acetone and then stained with monoclonal mouse anti-human OPN (1:100; Santa Cruz) after a 3% hydrogen peroxide-methanol blocking solution. Following incubation with a horseradish peroxidase-conjugated secondary antibody (Dako), staining was developed using AEC chromogen substrate followed by counterstaining with hematoxylin.

**Cell proliferation assay.** The cellular proliferation of cells was measured by the MTT assay (Chemicon International, Inc.). Cells (1x10<sup>4</sup>) were plated in 96-well plates prior to treatment at the indicated concentrations. After incubation for 1-4 days, the cells were assayed at the indicated times following the manufacturer's protocol.

**Flow cytometry.** Cells were grown to subconfluence and treated with OPN ASO at the indicated concentration. The cells were trypsinized and fixed in 70% ethanol overnight and stained for 30 min with propidium iodide (0.1 µg/ml) followed by analysis.

**Matrigel invasion assay and migration assay.** The invasive ability of the transfected cells was determined by the Matrigel (BD Pharmingen) coated 24-well transwell chambers with upper and lower culture compartments separated by polycarbonate membranes with 8-µm pore (Costar, NY, USA). The bottom chamber was filled with DMEM containing 10% FBS as a chemoattractant. The transfected cells (1x10<sup>5</sup>) after treatment were seeded on the top chamber and incubated at 37°C with 5% CO<sub>2</sub>. After 40 h, the cells removed from the upper surface of the Matrigel by scrubbing with a cotton swab and cells that migrated to the underside of the membrane were stained with Giemsa (Sigma). Five high-power fields were counted and the mean number of cells per field was calculated. The migration assay was similar to the invasion assay, only without Matrigel. The experiments were carried out in triplicate.

**Enzyme-linked immunosorbent assay (ELISA).** Serum-free supernatants from 4x10<sup>5</sup> cells after treatment were measured for MMP-2, and uPA secretion by ELISA according to manufacturer's directions (MMP-2, MMP-9 ELISA kit from R&D Systems, Minneapolis, MN; uPA ELISA kit from American Diagnostica Inc.). Absorbance was measured at 450 nm and 570 nm using a microplate spectrophotometer. Each assay was conducted in triplicate.

**Gelatin zymography.** Gelatin zymography was performed as previously described. Briefly, 3x10<sup>5</sup> HCCLM6 cells were plated in six-well tissue culture plates and treated with oligonucleotides at the indicated time. Then, media were replaced with serum-free DMEM for 24 h and the supernatant was collected. The samples were resolved in 10% gelatin (Invitrogen) and the gel was developed according to the manufacturer's instructions. The enzyme activity was visualized by staining the gel with Coomassie brilliant blue R250.

**Therapeutic treatment of orthotopic HCCLM6 tumors in nude mice with OPN ASO.** Male 6- to 8-week-old BALB/c nu/nu mice were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China. Animals were maintained in accordance with the recommendations of the NIH Guidelines for Care and Use of Laboratory Animals. HCCLM6 cells were intrahepatically implanted into male BALB/c nude mice under sterile conditions as previously described. Mice were then randomly assigned into two groups (n=6/each group) and treated by intraperitoneal injection (i.p.) with 50 mg/kg oligonucleotide in saline solution. On day 35 after implantation (i.e., 3 days after the last dose), mice were sacrificed to examine tumor growth and metastases. Blood, liver tumor, and lung tissues were collected. Formalin-fixed liver tumors were examined for OPN protein levels using immunohistochemistry methods. Lung tissues were evaluated for metastases

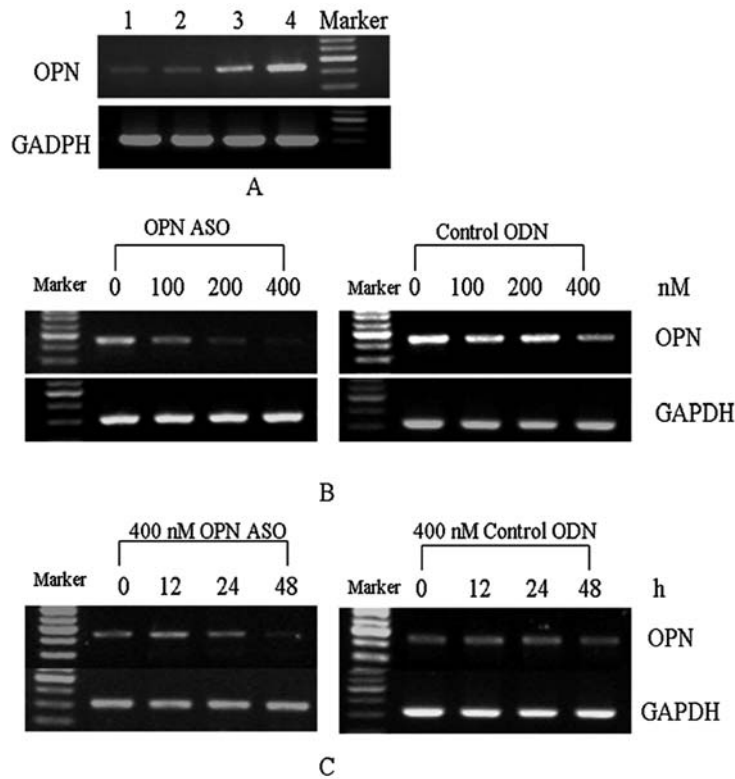


Figure 1. OPN mRNA expression was overexpressed in HCCLM6 cells and was inhibited by OPN ASO. (A) OPN mRNA expression of different cell lines was detected by RT-PCR. Lane 1, normal liver cell line L02; 2, non-metastatic HCC cell line SMMC-7721; 3, low metastatic HCC cell line MHCC97-L; 4, high metastatic HCC cell line HCCLM6. (B) OPN ASO inhibited OPN mRNA expression in a dose-dependent manner whereas control oligonucleotide (Control ODN) had no effect. (C) OPN ASO inhibited OPN mRNA expression at indicated concentrations (400 nM) in a time-dependent manner.

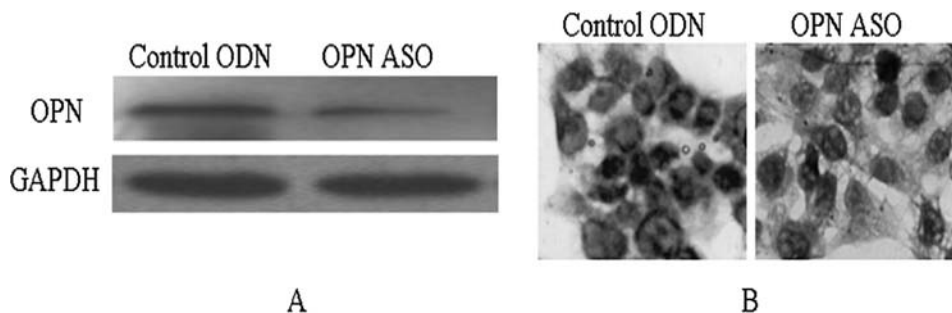


Figure 2. OPN protein expression was inhibited by OPN ASO in HCCLM6 cells. (A) OPN expression of HCCLM6 cells treated with OPN ASO (400 nM, 48 h) detected by Western blots. (B) Immunocytochemical staining of OPN in HCCLM6 cells treated with OPN ASO (400 nM, 48 h).

by sequential sections of paraffin-embedded tissue with H&E staining.

**Statistical analysis.** Statistical analyses were performed by the Statistical Package for the Social Sciences version 11.5 (SPSS, Inc., Chicago, IL). Data are expressed as the mean  $\pm$  SD, and analyzed using the two-tailed Student's t-test or the analysis of variance (ANOVA). The survival time of the animals was compared by the log-rank and Kaplan-Meier methods. The level of significance was set at  $P < 0.05$ .

## Results

**Knockdown of OPN expression in HCCLM6 cells by OPN ASO.** HCCLM6 cells overexpressed OPN protein compared

with low or non-metastatic HCC cell lines (Fig. 1A). Treatment of HCCLM6 cells with OPN ASO inhibited OPN mRNA expression detectable by RT-PCR in a dose- and time-dependent manner, whereas the same 2'-MOE-modified, sequence-randomized oligonucleotides had no effect (Fig. 1B and C), suggesting the presence of a sequence specific antisense activity. In agreement with RT-PCR results, OPN protein in HCCLM6 cells treated with OPN ASO was also reduced compared to control oligonucleotides (Fig. 2A). In addition, immunocytochemical staining revealed that OPN expression significantly decreased following OPN ASO treatment, but not by control oligonucleotides (Fig. 2B).

**Effects of OPN down-regulation by OPN ASO on cell proliferation, apoptosis, migration and invasion of HCCLM6**

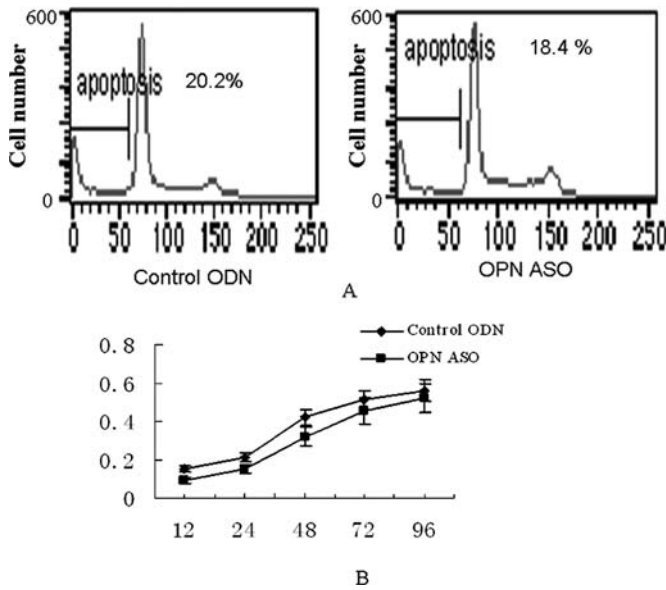


Figure 3. Effects of OPN suppression on the proliferation and apoptosis of HCCLM6 cells. HCCLM6 cells were treated with OPN ASO (400 nM, 48 h) or control oligonucleotide (Control ODN). (A) Flow cytometric analysis of apoptotic cells in HCCLM6 following propidium iodide staining. (B) The rate of proliferation in HCCLM6 cells treated with OPN ASO was not significantly different from that in Control ODN-treated cells.

*cells in vitro.* Effects of OPN knockdown by OPN ASO on the proliferation and apoptosis of HCCLM6 cells were measured by MTT assay and flow cytometry. Cell proliferation was not significantly inhibited (Fig. 3A) and apoptosis was not increased in the HCCLM6 cells administered with OPN ASO compared to those treated with control oligonucleotides (apoptotic rate: 18.4% vs. 20.2%, Fig. 3B). As migratory and invasive behaviors are the indicators of the metastatic potential, we examined HCCLM6 migration and invasion *in vitro* using the transwell assay after OPN was down-regulated by OPN ASO. The numbers of HCCLM6 cells treated with OPN ASO migrating toward the conditioned medium or invading

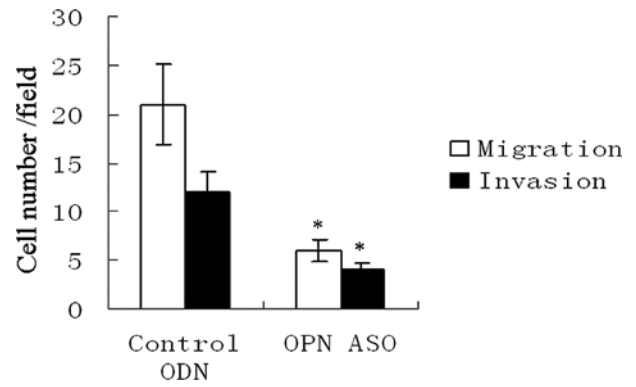


Figure 4. Inhibition of HCCLM6 migration and invasion *in vitro* by targeting OPN. HCCLM6 cells were treated with OPN ASO (400 nM, 48 h) or control oligonucleotide (Control ODN). Cell migratory and invasive ability was measured by transwell assay. Statistically significant difference of migration and invasion in OPN ASO-treated HCCLM6 cells and Control ODN-treated cells, \*P<0.05.

through the Matrigel were significantly fewer than those treated with control oligonucleotide (Migration assay: 6.80±2.49 vs. 20.60± 3.65, P<0.05; Invasion assay: 4.40±1.67 vs. 11.60± 3.36, Fig. 4), suggesting that the migration and invasion potential of HCCLM6 cells was decreased after inhibition of OPN.

*The decreased expression of MMP-2 and uPA following OPN ASO treatment.* Since OPN regulated HCCLM6 cells migration and invasion *in vitro*, we examined the expression of MMP-2 and uPA involved in the digestion of extracellular matrices, penetration of stroma, and establishment of metastasis. Following OPN ASO treatment, MMP-2 and uPA levels in culture supernatants of HCCLM6 cells were significantly reduced (MMP-2 level: 18.07±3.28 ng/ml vs. 40.08±7.74 ng/ml, P<0.05; uPA level: 1.69±0.32 ng/ml vs. 3.85±0.54 ng/ml, P<0.05, Fig. 5A). To furthermore determine if the reduced invasion of HCCLM6 treated by OPN ASO

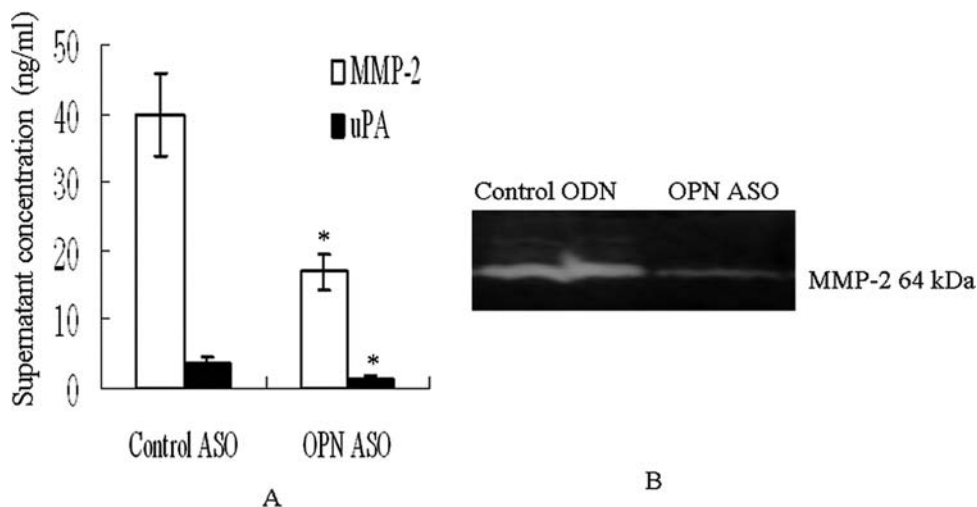


Figure 5. MMP-2 and uPA involved in the inhibitory effects of ASO OPN on the invasion and metastasis of HCCLM6 cells. (A) The levels of MMP-2 and uPA measured by ELISA analysis were significantly decreased in the medium of OPN ASO-treated HCCLM6 cells. (B) In the gelatin zymography, OPN ASO markedly decreased MMP-2 levels in HCCLM6 cells.

Table I. OPN ASO treatment reduces HCCLM6 cell lung metastasis *in vivo*.

Group	Tumor weight (g)	Lung metastasis <sup>a</sup>	
		Cases (%)	No. of metastases
OPN ASO	1.5±0.5	2/6 (33.3)	2.2±1.1
Control ODN	2.1±0.3	6/6 (100.0) <sup>b</sup>	6.8±2.1 <sup>b</sup>

<sup>a</sup>Lung metastasis is denoted as both the cases of positive lung metastases and the average number of metastases in lungs per positive case (mean ± SD). <sup>b</sup>P<0.05.

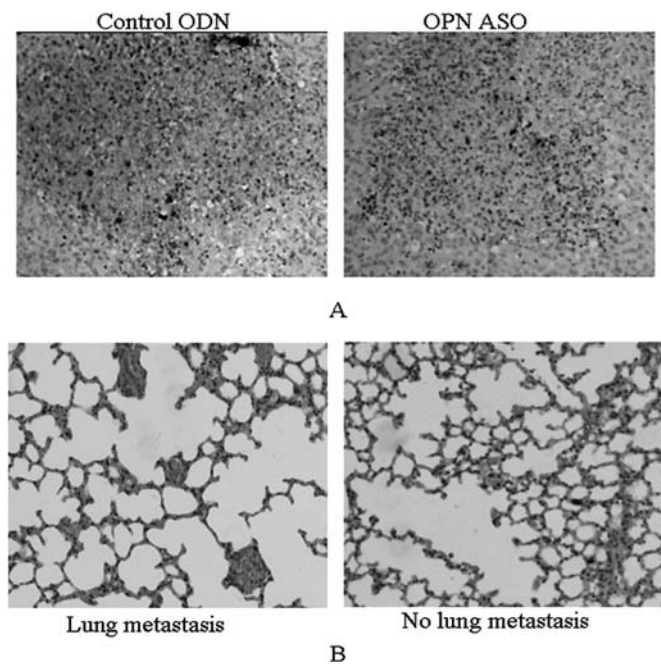


Figure 6. Inhibition of OPN expression in athymic mice bearing orthotopic HCCLM6 tumors following OPN ASO treatment. (A) OPN protein detected by immunohistochemistry was markedly reduced in intrahepatic tumor of OPN ASO-treated group (magnification, x50). (B) Representative lung tissue sections with metastatic lesions or no metastasis (hematoxylin and eosin; magnification, x50).

was due to reduced MMP-2, gelatin zymography was used to detect MMP-2 activity. The active form of MMP-2 was inhibited following OPN ASO treatment as indicated by the digestion of gelatin (Fig. 5B).

*Therapeutic treatment with OPN ASO inhibits lung metastasis of orthotopically transplanted HCCLM6 tumors in nude mice.* An advantage of the orthotopic HCCLM6 model with spontaneous lung metastasis mirrors clinical HCC progression and metastasis. We evaluated the therapeutic relevance of OPN ASO treatment in this model. Intraperitoneal injection of OPN ASO following orthotopic implantation inhibited OPN protein expression in HCC tumors (Fig. 6A). Tumor weight of OPN ASO-treated group was not significantly reduced compared with control oligonucleotide-treated group, but the incidence of lung metastasis in the animals treated with OPN ASO declined to 33.3% (2/6) from 100% (6/6) in control

oligonucleotide-treated animals (P=0.03, examined by Fisher's exact test) (Table I) (Fig. 6B). In addition, the number of lung metastatic lesions in OPN ASO treatment group was also greatly decreased compared to control oligonucleotide-treated group (Table I).

## Discussion

OPN expression has been linked to tumor progression and metastasis in a variety of human tumors including HCC (9). Many of the downstream signaling pathways and effectors including phosphatidylinositol 3-kinase (PI3K)/Akt, nuclear factor (NF)-κB, matrix metalloproteinases (MMPs) and uPA, are activated by OPN to facilitate tumor progression and targeting OPN signaling suppresses tumor progression in other types of cancers (10-13). As for HCC, OPN has been identified as the lead gene overexpressed in the metastatic HCC (3). Similar to other cancers, Sun *et al* (14) also found that OPN played an important role in HCC growth and metastasis through activating the mitogen-activated protein kinases (MAPK), NF-κB pathways and MMP-2. These findings indicate that OPN plays an important role in HCC progression and metastasis, and OPN might be a novel drug target for therapeutic intervention.

Human HCC cell line HCCLM6 features high lung metastatic potential and resembles typical cases of HCC in the clinic. OPN protein was overexpressed in HCCLM6 cells compared with non-metastatic HCC cell lines, suggesting OPN expression is associated with the highly metastatic potential. In the present study, suppression of OPN by 2'-MOE modified ASO inhibited malignant behaviors of HCCLM6 *in vitro* as well as in xenograft models. Cell migration and invasive capability of HCCLM6 cells following OPN knock-down by ASO were decreased and lung metastases were significantly reduced, supporting that the excessive expression of OPN is required for malignant phenotypes of HCC and OPN might be a new drug target for HCC progression and metastasis. Furthermore, the production of MMP-2 and uPA was reduced following the inhibition of OPN, suggesting MMP-2 and uPA might be the important mediators of OPN promoting HCC metastasis. This result is similar to that previously described (15). Mi *et al* (15) have demonstrated that OPN mediates metastatic behavior of murine mammary cancer through integrin-linked kinase-dependent activator protein-1 (AP-1) activation to up-regulate MMP-2 and uPA expression.

RNA interference is an effective method for a specific gene knockdown (16), ASO has been used for decades for clinical translations from basic research (17). Vitravene, an ASO drug, is already marketed and approximately 20 antisense drugs are in clinical development, thus, it appears that antisense drugs may prove of value in the disease treatment (18). Since most of HCC are associated with chronic liver disease (19), it is preferable to use a highly target-specific drug approach to treat HCC. In this study, the ASO-mediated specific suppression of OPN expression in HCC cells was shown by oligonucleotide concentration- and treatment time-dependency, as well as sequence-specificity. It has been shown that 2'-MOE ASO can accumulate in the liver following systemic administration in mice (20). The results of *in vivo* orthotopic HCC models showed that ASO displayed significant suppression of OPN expression in tumor tissue.

In summary, our results demonstrate that the ASO targeting OPN significantly suppressed HCC cells invasion *in vitro* and effectively inhibited lung metastasis in orthotopic HCC model *in vivo*, suggesting targeting OPN might have a therapeutic benefit for patients to prevent HCC metastasis.

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