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Short communication

# THE INHIBITION OF *in vivo* TUMORIGENESIS OF OSTEOSARCOMA (OS)-732 CELLS BY ANTISENSE HUMAN OSTEOPONTIN RNA

SI-JIN LIU[1,6]*, DAO-QIANG ZHANG[2], XIU-MEI SUI[2], LIN ZHANG[3], ZI-WEI CAI[4], LI-QIU SUN[4], YA-JUN LIU[5], YAN XUE[5] and GUO-FA HU[1]
[1]Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100080, China, [2]Wendeng Central Hospital, Weifang Medical College, Weihai, Shandong Province 264400, China, [3]Research Institute of Sports Medicine, Soochow University, Suzhou, Jiangsu Province 215021, China [4]Department of Pathophysiology, Medical College of Shantou University, Shantou 515031, China, [5]Beijing Jishuitan Hospital, Peking University, Beijing 100035, China, [6]Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA, 02142, USA

**Abstract:** Osteopontin (OPN) is a secreted, non-collagenous, sialic acid-rich protein which functions by mediating cell-matrix interactions and cellular signaling via binding with integrins and CD44 receptors. An increasing number of studies have shown that OPN plays an important role in controlling cancer progression and metastasis. OPN was found to be expressed in many human cancer types, and in some cases, its over-expression was shown to be directly associated with poor patient prognosis. *In vitro* cancer cell line and animal model studies have clearly indicated that OPN can function in regulating the cell signaling that ultimately controls the oncogenic potential of various cancers. Previous studies in our laboratory demonstrated that OPN is highly expressed in human osteosarcoma (OS) cell line OS-732. In this study, we successfully reduced the tumorigenecity of OS-732 cells xenotransplanted into nude mice, using the antisense human OPN (hOPN) RNA expression vector.

**Key words:** Osteopontin, Osteosarcoma, Antisense RNA

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* Author for correspondence. Present address: Harvard-MIT Division of Health Sciences and Technology, MIT, 45 Carleton Str, E25-537, Cambridge, MA, 02142, USA. E-mail: sjliu@mit.edu, tel: 617-253-1446, fax: 617-253-3459

Abbreviations used: hOPN – human OPN; OPN – osteopontin; OS – osteosarcoma

## INTRODUCTION

Osteopontin (OPN) is a secreted, non-collagenous, sialic acid-rich, chemokine-like phosphorylated glycoprotein. It has diverse roles in both physiological and pathological conditions, including mineralization, accelerated blood vessel formation, enhanced cell survival, and acute and chronic inflammation [reviewed in 1, 2]. It regulates a series of signaling cascades and augments the expression of several oncogenic molecules by interacting with integrins and CD44 receptors [3].

Numerous studies have demonstrated that OPN plays an important role in the tumorigenesis and metastasis of various cancers [4, 5]. OPN is highly expressed in many cancer types, such as osteosarcoma, lung cancer, ovarian cancer, breast cancer, prostate cancer, glioblastoma, squamous cell carcinoma and melanoma [2, 6, 7]. Abundant secretion of OPN acts as a marker for these cancers, and high expression of OPN in primary cancer is associated with early metastasis and a poor prognosis [8]. Over-expression of OPN in benign cells may induce neoplastic-like cell behavior including increased attachment and invasion *in vitro*, and the ability to metastasize *in vivo* [9]. Conversely, the inhibition of OPN expression by antisense cDNA perturbs cell growth and cancer-forming capacity [10-12]. Cancer cells from OPN$^{-/-}$ mice show impaired colony formation in soft agar and slower cancer growth *in vivo* when compared with those from wild type mice [13]. Thus, targeting OPN might be an appropriate therapeutic strategy for the treatment of cancer.

Osteosarcoma (OS) is the most common type of primary bone cancer and is one of the most devastating, being the second most common cause of cancer-related death in the pediatric population [14]. OS is classified as a malignant mesenchymal neoplasm in which cancer directly produces defective osteoid (immature bone). In the past 30 years, combination chemotherapy together with surgical resection has been the major treatment for OS, with achievable 5-year rates for patients with non-metastasis [15]. However, the survival rate of OS patients with metastasis has not been improved significantly, and the prognosis for those patients who develop recurrent diseases following the surgical resection remains poor. The current treatment options are not desirable for these patients. Moreover, the chemotherapeutic agents used in OS treatment are highly toxic, and a portion of the cancer cells are resistant to these drugs. Therefore, new biological therapies are urgently needed as an adjunct to the conventional treatment options to improve morbidity and overall survival. To explore new biological therapies, in this study, we tried to reduce the *in vivo* tumorigenecity of OS-732 cells in nude mice, using antisense human OPN (hOPN) RNA.

## MATERIALS AND METHODS

### Plasmid preparation, cell culture and cell transfection

The portion of the hOPN cDNA that codes mature peptide (792 bp) was inserted into the pEGFP-N1 expression vector using Xho I and BamH I restriction sites.

The reading frame of hOPN is in the reverse direction to that of EGFP, so the antisense RNA of hOPN will be transcribed by the CMV promoter (Fig. 1). Human OS cell line OS-732 was kept in our laboratory under the same cell culture conditions as described previously [16]. Cells were grown in RPMI 1640 media (Hyclone) with 10% fetal bovine serum at 37ºC, 5% $CO_2$. The pEGFP-N1 vector expressing antisense hOPN RNA (pEGFP-N1-anti-hOPN) and the empty pEGFP-N1 vector were transfected into *in vitro* cultured OS-732 cells using LipofectAmine™ 2000 Reagent (Invitrogen). The transfection was carried out according to the protocol provided by Invitrogen. After selection with G418 (Invitrogen) for 2 weeks, we obtained stable transfectants integrated with either pEGFP-N1-anti-hOPN or pEGFP-N1 vector.

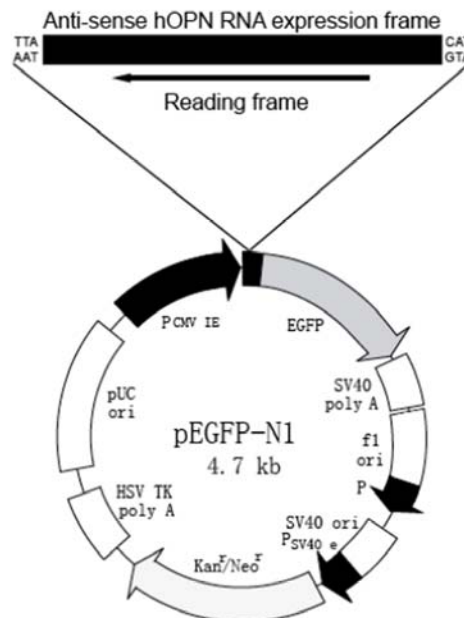


Fig. 1. The illustration of the antisense hOPN RNA expression vector. One 792-bp fragment of hOPN cDNA was reversely inserted into the pEGFP-N1 vector. The direction of its reading frame is opposite to that of EGFP.

**Transplantation and measurements of tumor volume and weight**

4-week old female mice (strain: Balb/c) were bred in specific pathogen-free conditions. The same number ($5.0 \times 10^6$, in 0.2 ml PBS) of OS-732 cells (control), OS-732 transfectant cells expressing antisense hOPN RNA (antisense hOPN) and OS-732 transfectant cells expressing EGFP (EGFP-control) were subcutaneously transplanted into the right axillae of nude mice. Starting on day 3 after transplantation, the length and width of the tumor were measured every 3 days, and the tumor volume was calculated according to the formula: $V = 1/2LW^2$. The mice were killed 27 days after transplantation and the tumor tissue in every mouse was weighed.

**Southern and Northern blot analyses**

Genomic DNA was isolated from OS-732 cells using a DNA isolation kit (Qiagen). An equal amount of genomic DNA (15 µg) was digested by Nhe I and Xba I. Upon digestion, the pEGFP-N1-anti-hOPN vector yielded a 1,500-bp fragment and the pEGFP-N1 vector yielded an 820-bp fragment. The digested DNA was fractioned on a 0.8% agarose gel, and then transferred onto a nitrocellulose membrane (Bio-Rad). Hybridization was carried out as previously described [17]. Total RNA was isolated from OS-732 cells using an RNA isolation kit (Promega). An equal amount of total RNA (25 µg) was separated by formaldehyde-denatured agarose gel electrophoresis and then transferred onto a nitrocellulose membrane. Northern blot analyses were performed as previously described [18]. GAPDH mRNA was used as a loading control. The EGFP cDNA (720 bp) and GAPDH cDNA (about 500 bp) were amplified via PCR, and the PCR products were used to yield $\alpha$-$^{32}$P-labeled fragments using the random primer method (Primer-a-Gene Labeling System kit from Promega). The $\alpha$-$^{32}$P-labeled EGFP cDNA fragments were used in Southern and Northern blot hybridization, and the $\alpha$-$^{32}$P-labeled GAPDH cDNA fragments were used in Northern blot hybridization.

**Statistical analysis**

Statistical analyses of the tumor volumes and weights for the various groups were performed using the two-independent-sample test (non-parametric test) with a P value of $< 0.05$ taken as statistically significant.

## RESULTS

**The expression of antisense hOPN RNA in OS-732 cells**

We obtained stable cell transfectants after selection culture with G418. For further experiments, we selected two transfectants which were stably integrated with either pEGFP-N1-anti-hOPN or pEGFP-N1 vectors, as verified by Southern blot analyses (Fig. 2A). Northern blot analyses showed that the antisense hOPN RNA complex and EGFP RNA were expressed in OS-732 cells (Fig. 2B).

**Tumor growth curve**

The average latent period (the time from transplantation to the appearance of a visible tumor) is a good parameter for evaluating tumor-forming ability. The average latent periods were 7 days for mice injected with OS-732 cells (control), and 9 days for mice injected with OS-732 transfectant cells expressing EGFP (EGFP-control). However, the average latent period was significantly longer (14 days) in mice injected with OS-732 transfectant cells expressing antisense hOPN RNA (the antisense hOPN group) compared with the control and EGFP groups, $P < 0.05$ (Fig. 3A). The tumor volume curves showed that the tumor volume in the antisense hOPN group was greatly reduced compared with the

other two groups during the time period between day 14 and 27. The representative growth curves for one mouse per group are shown in Fig. 3B.

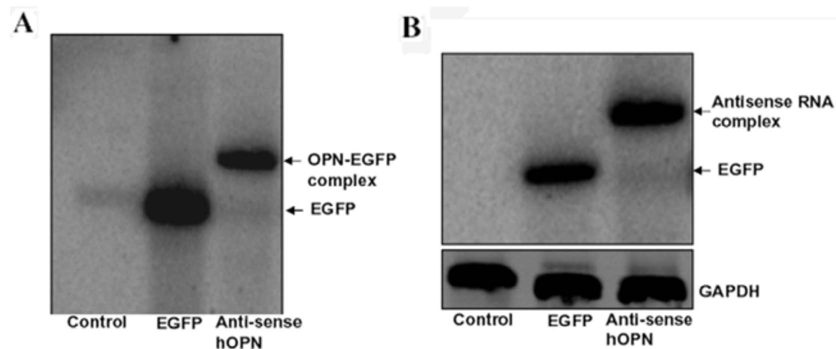


Fig. 2. The Southern and Northern blot analyses of transfectant cells. A – Southern blot analyses. The result showed a 792-bp band in the EGFP group and a 1,500-bp band in the antisense hOPN group. B – Northern blot analyses. The results showed EGFP mRNA was expressed in the EGFP group and the antisense RNA complex was expressed in the antisense hOPN group. The mRNA expression of GAPDH was used as the loading control.

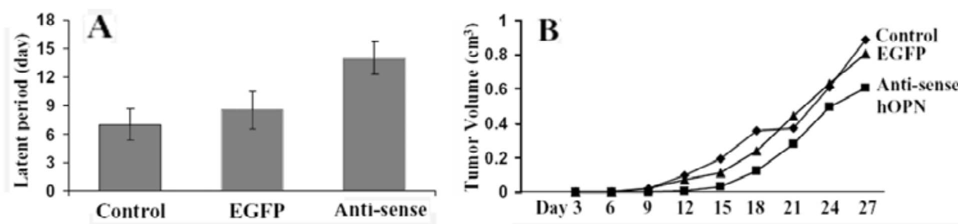


Fig. 3. Tumor growth was reduced in the antisense hOPN group. A – The average latent period for the tumor in all three groups. The average latent period in the antisense hOPN group was significantly delayed compared with the control groups, $P < 0.05$. The results are presented as the means ± SEM ($n = 3$). B – The representative tumor growth curves for all three groups.

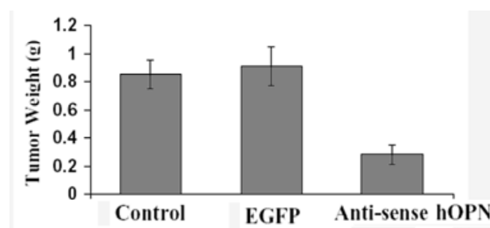


Fig. 4. The final tumor weights in all three groups. The final tumor weight in the antisense hOPN group was significantly diminished compared with the control groups, $P < 0.05$. The results are presented as the means ± SEM ($n = 3$).

**The final tumor weights**

The final tumor weights in the control group and EGFP group were 0.85 g and 0.91 g, respectively. However, the final tumor weight in the antisense hOPN group was 0.28 g, which is significantly lower ($P < 0.05$, Fig. 4).

## DISCUSSION

The suppression of cancer growth and progression is one of the major challenges facing modern medicine. The efficacy of traditional anti-cancer agents is hampered by their high toxicity to normal tissues, due to their lack of specificity for malignant cells. Therefore, new biological agents are needed to specifically target key regulators that are deregulated in malignant cells, with minimal toxicity to the patients. Antisense therapeutics act at the mRNA level and inhibit oncogene proteins with high specificity and efficiency. Thusfar, antisense nucleotides directed against signaling molecules, cytokine receptors and transcription factors have been applied in experimental models and have shown potential as therapeutics [19-21]. Several anitsense nucleotide-based drugs have been developed for the therapy of lung cancer, and some have recently reached clinical trials [22].

Inhibitory effects on cell growth and tumorigencity have been obtained using antisense RNA to silence cancer-associated genes. Bcl-2 [23] and telomerase [24], and IGF (insulin growth factor) [25, 26] and vascular endothelial growth factor (VEGF) [27, 28], were successfully silenced in cultured cells, resulting in the inhibition of *in vitro* cell growth and *in vivo* tumorigenicity. As in our study, EGFR was also successfully silenced by *in vivo* delivery of antisense RNA into subcutaneous xenografts into nude mice, leading to the inhibition of tumor growth [29].

Plasmid or viral vectors can be designed to produce antisense RNA which is complementary to a certain portion of mRNA. The inhibition of translation into protein occurs either by direct physical blocking of the ribosomal translation-machinery, or by inducing the degradation of mRNA by RNase [30]. Anitsense RNA, when transcribed into cells by plasmid or viral vectors, should be more efficient at long-term gene silencing than chemically synthesized antisense oligonucleotides that would have to be delivered exogenously [31].

Our laboratory previously demonstrated that OPN is highly expressed in human osteosarcoma (OS) cell line OS-732 [16]. Moreover, we found that OPN acts to facilitate OS-732 [16] and HEK 293 cell [32] proliferation and transmigration. Here, we used a plasmid vector to express antisense hOPN RNA, and this vector was demonstrated to be efficiently expressed in OS-732 cells (Fig. 2). The antisense hOPN RNA blocked endogenous hOPN mRNA translation in OS-732 cells. Consequently, the average latent period of subcutaneous xenografts in nude mice was significantly delayed in mice xenotransplanted with OS-732 transfectant cells expressing antisense hOPN RNA, compared with the two control groups (Fig. 3). The final tumor weight was also significantly less than

that in the two control groups (Fig. 4). These results were consistent with previous reports that antisense OPN cDNA could reduce cell growth and tumor-forming capacity [10-12].

The antisense nucleotide technique has long been used in studies of cancer treatment, and it has presented great advantages over other techniques. It offers one approach to target genes involved in cancer progression, especially those that are not amenable to small-molecule or antibody inhibition [33-36]. Blocking OPN mRNA translation using antisense RNA might be useful in developing novel molecular therapy for the treatment of various cancers.

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