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Short communication

OVEREXPRESSION OF HUMAN OSTEOPONTIN INCREASES CELL PROLIFERATION AND MIGRATION IN HUMAN EMBRYO KIDNEY-293 CELLS

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Abstract: Malignant tumors are characterized by dysregulated cell growth and the metastasis of secondary tumors. Numerous studies have documented that osteopontin (OPN) plays a key role in regulating tumor progression and metastasis. Here, we show that the overexpression of OPN in human embryo kidney-293 cells significantly increases both the level of cell proliferation, by provoking the G_1/S transition, and the level of cell migration *in vitro*. These findings suggest that augmented OPN contributes to cell growth and motility. Inhibiting OPN or the pathway it stimulates may therefore represent a novel approach for the treatment of primary tumors and associated metastases.

Key words: Osteopontin, Overexpression, Proliferation, Metastasis

INTRODUCTION

The real threat to the lives of cancer patients is not the primary tumor, but the metastases to distant organs, accounting for 90% of cancer deaths. Once cancer cells form secondary tumors, the disease essentially enters an incurable phase. Cancer cells metastasize to distant organs with a ranked order of preference. Bone is one of the most frequent sites of metastasis for various cancers. The underlying mechanisms of metastasis have yet to be elucidated.

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Abbreviations used: ECM – extracellular matrix; hOPN – human OPN; IRES – internal ribosome entry site; OPN – osteopontin

Osteopontin (OPN) is a glycophosphoprotein containing the Arg-Gly-Asp (RGD) sequence, located in the extracellular (ECM) matrix. Since its first identification in bone, extensive studies have demonstrated that OPN is expressed by a wide spectrum of tissues and has multiple biological functions, such as regulating cell growth, apoptosis, adhesion and migration [1, 2]. Many studies have indicated that OPN is overexpressed in a variety of cancers including osteosarcoma, and breast and prostate cancers, especially those with high-grade/late-stage tumors and metastases [3-5]. Clinical studies reveal that overexpression of OPN corresponds to enhanced invasiveness and tumor metastasis, and is negatively correlated with overall survival in cancer patients [6-10]. OPN therefore has the potential as a diagnostic and/or prognostic marker of metastases, and as an indicator of overall clinical outcome for many cancers, including osteosarcoma, and breast, lung and brain cancers [11, 12].

As previously described, OPN acts as an autocrine growth factor to promote cell proliferation in a self-sufficient manner, and as a chemoattractant or a homing molecule to facilitate cancer metastasis to secondary sties [13, 14]. To test the hypothesis that overexpression of OPN increases cell growth and motility *in vitro*, we expressed the recombinant human OPN (hOPN) gene in human embryo kidney-293 (293) cells. Overexpressing hOPN resulted in increased cell cycle progression and migration in the 293 cells. The results of this study indicate the significance of OPN in tumor progression and metastasis, and its potential therapeutic value in cancer treatment.

MATERIALS AND METHODS

Cell culture

Human embryo kidney-293 cells were stored and cultured as described previously [15].

Construction of the hOPN expression vector and plasmid transfection

The portion of the hOPN cDNA encoding the whole protein was inserted into the pEGFP-N1 expression vector, and an internal ribosome entry site (IRES) was added between the hOPN cDNA and EGFP sequence. The recombinant expression vector is referred to as pEGFP-N1-hOPN. Cells were grown in RPMI 1640 media with 10% fetal bovine serum. The pEGFP-N1-hOPN and empty pEGFP-N1 plasmid was transfected into the 293 cells using LipofectAmine^{TM} 2000 Reagent (Invitrogen) according to the manufacturer's protocol. Stable transfectants were obtained after 2 weeks of selection with G418 (Invitrogen).

Western blot analysis and quantification

Protein extracts were prepared from cells using Mammalian Protein Extraction Reagent (M-PER; Pierce) and 20 μg of each was used for separation by 4-12% SDS-PAGE, and processed for Western blot analyses following the standard protocol. The antibodies used were the anti-GAPDH antibody (1:1,000), or anti-hOPN antibody (1:500). Both antibodies were obtained from Santa Cruz

Biotechnology. The intensities of the autoradiograms in the Western blots were quantified using the Image J software (rsbweb.nih.gov/ij).

Cell proliferation assay

Cell proliferation was assessed using an MTT-based colorimetric assay kit (Roche). The 293 cells were serum starved for 24 h and then seeded at a concentration of 5×10^4 cells/well in 100 µl medium. The cell culture continued for 48 h at 37ºC and 5% CO_2, and then 10 µl MTT-labeling reagent was added to each well. The 96-well plates were incubated for another 4 h followed by the addition of 100 µl of the solubilization solution into each well. The plate was incubated overnight and read at 570 nm with an ELISA reader.

5-Bromodeoxyuridine (BrdU) incorporation assay

The 293 cells were serum starved for 24 h and thereafter seeded at a concentration of 1×10^6 cells in 100-mm plates. The cells were then cultured in 10% serum medium for 48 h and 10 µM 5-BrdU (Sigma) was added to the medium 1 h before collection. The cells were fixed in ice-cold 70% ethanol, denatured in 2 mol/l HCl, neutralized with 0.1 M sodium borate, and finally stained with APC-conjugated anti-BrdU monoclonal antibody (BD Biosciences) according to the manufacturer's protocol. The cells were examined via FACS analysis. The percentage of cells that had incorporated BrdU corresponds to the APC-positive population.

Cell cycle determination

The cell cycle status was assessed via FACS analysis using propidium iodide (PI). Cells were fixed with ice-cold 70% ethanol overnight. Then, the cells were centrifuged and the cell pellet was re-suspended in 400 µl of PBS with 50 µl of RNase A (10 mg/ml) and 10 µl of PI (2 mg/ml). The mixture was incubated in the dark at 37ºC for 30 min, and was then analyzed using a flow cytometer (Becton Dickinson). The different phases of the cell cycle were assessed by collecting the signal in channel FL2-A. The percentage of the cell population in a particular phase was determined by ModFit LT software.

Transwell migration assay

The 293 cells were cultured in 1% serum medium for 24 h, and then the cell motility was assessed using the transwell (Corning) migration assay following the standard protocol. The cells were plated in the upper chamber (1×10^4/insert) and allowed to migrate for 24 h with 10% serum medium in the bottom as a chemoattractant. The cells on the filter-side of the upper chamber were cleaned with cotton swabs and the filters were stained with Hematoxylin and Eosin (H&E) staining solution after a cold methanol fix. The cells were counted under a microscope.

qRT-PCR analyses

Total RNA was isolated from *in vitro* cultured cells using Trizol (Invitrogen) according to the manufacturer's protocol. Quantitative measurements of gene

expression were carried out with a DNA Engine Opticon 2 (Bio-Rad) equipped with Opticon Monitor 2 software. GAPDH was used as an internal control. The primer sequences for the genes in RT-PCR analysis were from the Harvard Primer Bank (pga.mgh.harvard.edu/primerbank).

Statistical analysis

The two-tailed Student's t-test was used to analyze the statistical significance of the experimental data. A P-value < 0.05 indicated statistical significance.

RESULTS AND DISCUSSION

To investigate the potential role of OPN in regulating cell proliferation and motility, we overexpressed hOPN in human embryo kidney-293 (293) cells. We chose this cell line because it stands as an ideal model for the study of gene function. The 293 cells are very easy to grow, and can be very readily transfected with ectopic genes. In addition, since the 293 cells were transformed by an adenovirus from normal human embryonic kidney cells, they display the characteristics of cancer cells.

After selection with G418, we obtained 8 stable transfectants integrated with the pEGFP-N1-hOPN vector and 10 integrated with the pEGFP vector. Bright green fluorescence was observed inside the cells for these transfectants under a fluorescent microscope (data not shown) and most of the cells (> 99%) were EGFP-positive when assessed using FACS analysis (Fig. 2B). As the pEGFP-N1-hOPN construct has IRES between the hOPN and EGFP sequences, it expresses EGFP and hOPN separately in stably transfected cells. As a secreted protein, the hOPN protein is secreted out of the cells, while the EGFP protein stays inside the cells.

To confirm the ectopic expression of hOPN in the pEGFP-N1-hOPN cells, we assessed the expression of hOPN in two monoclonal transfectants (named clone #1 and #2) and in the polyclonal pool of all the transfectants in each treatment group via qRT-PCR and Western blot analyses. The expression of hOPN was dramatically increased (> 10-fold) in pEGFP-N1-hOPN (hOPN) 293 cells compared to that in the vehicle-controlled (pEGFP) cells at the mRNA and protein levels in both clone #1 and #2 ($P < 0.001$, Fig. 1A and B). A similar result was obtained in the polyclonal pools between the control and hOPN cells (data not shown). This result indicates that the presence of the pEGFP-N1-hOPN vector leads to overexpressed hOPN in the 293 cells. Regarding the cell's proliferative and migratory properties, similar results were observed for clone #1, clone #2 and the polyclonal pool in each treatment group. The data from clone #1 for each group is given in this paper.

An MTT-based cell proliferation assay shows that the cell growth in pEGFP-N1-hOPN 293 cells is significantly increased (by 46%) compared to that for pEGFP cells ($P < 0.005$, Fig. 2), a result similar to that in a previous report [16]. We then assessed the onset of DNA synthesis affected by the overexpression of hOPN using the BrdU incorporation assay. We found that hOPN overexpression

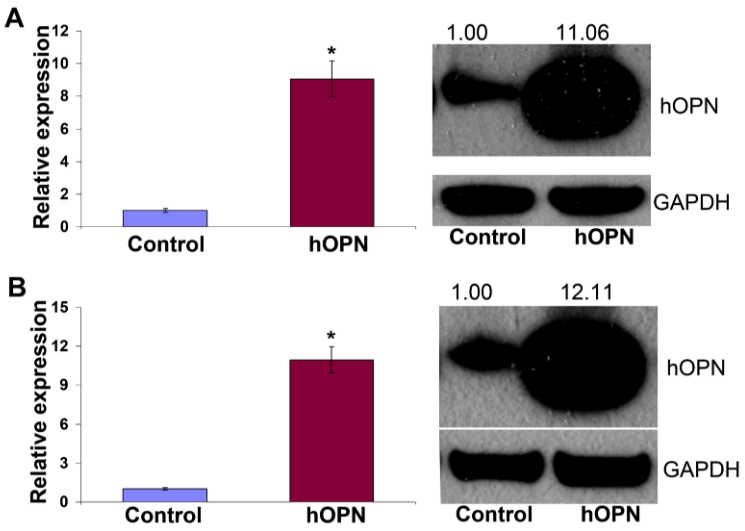

Fig. 1. Increased OPN expression in pEGFP-Nl-hOPN 293 cells from clone #1 (A) and clone #2 (B). The relative expression of hOPN in pEGFP-Nl-hOPN (hOPN) and pEGFP (control) cells was assessed via qRT-PCR analysis. GAPDH was used as the loading control. The results are presented as the means ± SEM (n = 3). An asterisk (*) indicates $P < 0.001$ compared with the control. Western blot analyses of hOPN in pEGFP-Nl-hOPN (hOPN) and pEGFP (control) cells were also performed. The ratios of hOPN to GAPDH are shown above the autoradiogram. The expression of hOPN in the control cells is defined as 1.

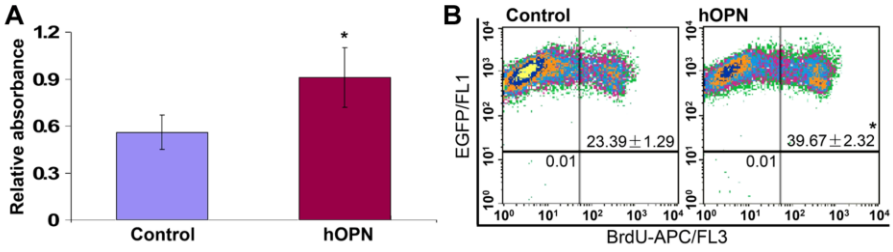

Fig. 2. Increased proliferation of the 293 cells upon overexpression of hOPN. A – An MTT-based cell proliferation assay was performed. The 293 cells were first serum starved for 24 h and seeded in a 96-well plate. The cells were then cultured for another 48 h and assessed for absorption at 570 nm. The relative absorption was calculated by defining the value in the control cells as 1. B – A 5-BrdU incorporation assay was performed. The 293 cells were serum starved for 24 h and then stimulated to grow with 10% serum medium followed by the FACS analysis. The cells in channel 1 (FL1) are EGFP-positive (the upper right + left), while the EGFP-negative cells are in the lower-left box (data shown in the box). The APC-positive (channel 3, FL3) population contains cells that have incorporated BrdU (the upper right). The percentage of APC-positive cells is shown in the box for each group. The results are presented as the means ± SEM (n = 4), and an asterisk (*) indicates $P < 0.005$, compared with the control. hOPN stands for pEGFP-Nl-hOPN cells, and control refers to pEGFP cells.

conferred a statistically significant advantage to the 293 cells in terms of the initiation of DNA synthesis upon serum deprivation/stimulation, as determined by the percentage of BrdU-incorporating cells (23% in control cells vs 40% in hOPN cells, $P < 0.001$, Fig. 2B). In order to investigate the mechanism responsible for increased cell proliferation with overexpression of hOPN, we examined the cell cycle status in pEGFP-N1-hOPN and pEGFP cells. As shown in Fig. 3, the percentage of cells in the G_1/G_0 phase decreases from 55.55% ± 1.34% in pEGFP cells to 44.54% ± 0.79% in pEGFP-N1-hOPN cells ($n = 4$, $P < 0.001$); and the percentage of cells in the S phase increases from 38.66% ± 2.09% to 48.90% ± 1.47% ($n = 4$, $P < 0.001$). These results suggest that cell cycle progression is stimulated by hOPN, which in fact facilitates the DNA synthesis and thereafter the transition of cells from the G_1 phase to the S phase.

Cancer cells often maintain aggressive growth in an autocrine manner independent of external signals. OPN, as a growth factor-like, calcified ECM-associated protein, is secreted by cancer cells and in turn enhances their proliferation by binding with its receptor αvβ3 and/or CD44 [13, 14], and then activating its downstream pathway, such as NF-kappa B-mediated pro-survival signaling [17].

Importantly, for the first time, we here demonstrated that overexpression of hOPN in the 293 cells significantly increases cell migration *in vitro*, as demonstrated by a transwell-based migration assay ($P < 0.001$, Fig. 4). This finding indicates that OPN is involved in the regulation of cell motility, although the mechanism has yet to be elucidated.

Although it is the main cause of cancer-related death, there is little in the way of metastasis-specific therapy. For example, osteosarcoma (OS) is the most common type of primary bone cancer, and the second most common cause of cancer-associated mortalities in children. The current therapy regimes for OS patients with metastasis are limited, and thus the overall clinical outcome of these patients remains poor. Metastasis involves a series of events: the escape of the cancer cells from the primary tumor, their spread via the circulatory system, and their eventual colonization of distant sites. Aggressive tumor growth and increased cell motility and invasiveness in a primary tumor contribute to the dissemination of cancer cells. In this study, we showed that overexprssion of hOPN significantly increases cell growth and migration. This study corresponds to the clinical finding of overexpression of OPN in primary tumors associated with a high pathological grade and metastases [11, 13]. Inhibition of OPN has been shown to suppress cell proliferation and motility *in vitro*, and malignancy and metastasis *in vivo* for various cancers [18-22].

The mechanism by which OPN facilitates tumor growth and particularly metastasis is still poorly understood. One possible mechanism is that OPN acts as a chemoattractant to induce cell migration via the binding between OPN and integrins [10]. For some cancers, OPN has been shown to be a homing molecule, enhancing cancer cell migration and then metastasis to the bone [23].

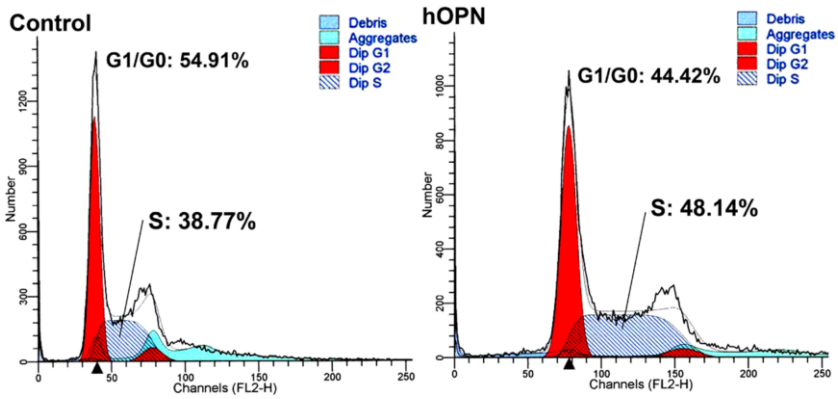

Fig. 3. The increased G_1/S transition in pEGFP-Nl-hOPN 293 cells. Cell cycle progression was determined using the ModFit LT software after collecting cells with a FACS machine. A representative profile of the cell cycle status in different phases for the pEGFP-Nl-hOPN (hOPN) and pEGFP (control) cells is presented. The percentages of cells in the G_1/G_0 and S phases are shown above the peaks.

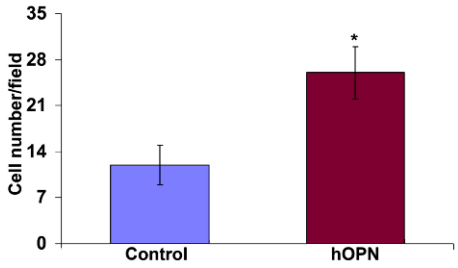

Fig. 4. hOPN increases cell migration in 293 cells. The bar graphs show the numbers of transmigrated cells in the chamber filters for pEGFP-Nl-hOPN (hOPN) and pEGFP (control) cells. pEGFP-Nl-hOPN (hOPN) and pEGFP (control) cells in 1% serum medium were added to the top of the transwell chamber, and 10% serum medium was used as the chemoattractant in the bottom of the well. At the end of the experiment, the cells on the top of the filter were scraped off with cotton swabs after fixing, and the cells that had migrated into the filter were counted under a microscope with H&E staining. Three images were randomly taken from three individual replicates under a microscope, and the cells in each image were counted. The results are presented as the means ± SEM (n = 9). An asterisk (*) indicates $P < 0.001$, compared with the control.

Furthermore, survival without adhesion is a prerequisite for metastatic cells to reach secondary sites during dissemination. OPN was recently found to contribute to cell survival in the absence of adhesion by increasing hyaluronic acid synthase [24].

This study shows that OPN is involved in the regulation of cell growth and motility. Inhibition of OPN expression or its downstream signaling appears to represent a novel therapeutic approach to tumor malignancy and metastasis.

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