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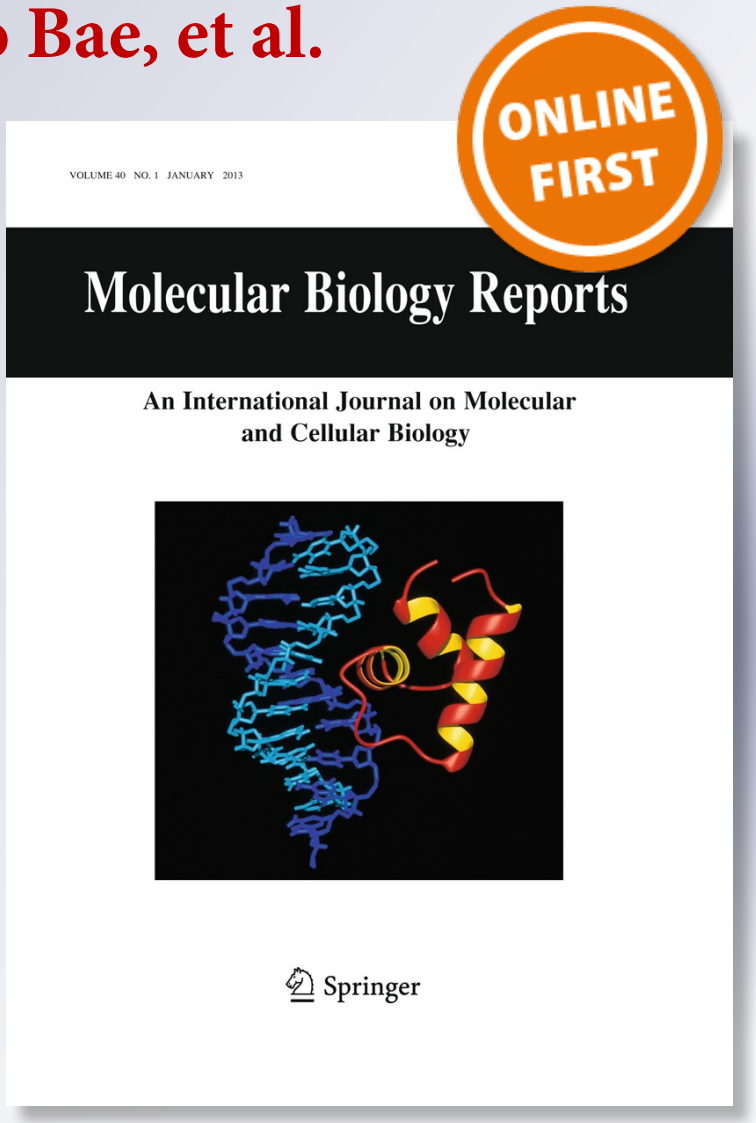

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Regulatory role of osteopontin in malignant transformation of endometrial cancer

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Abstract Osteopontin (OPN) involves in the tumor-promoting or metastasis in human endometrial cancer. Depletion of OPN gene expression in endometrial cancer cells was significantly decreased in cell viability and the cells undergo apoptotic cell death. The status of OPN in THESC, RL95, Hec1A and Ishikawa cell lines were analyzed by RT-PCR and western blot. After OPN-siRNA transfection, mRNA and protein expression levels of OPN were determined in Hec1A and Ishikawa cells. Cell proliferation and cell cycle distribution were observed by MTT and flow cytometry analysis. DNA fragmentation assay was used to measure cell apoptosis. Cell migration was assessed by wound healing assay. Depletion of OPN gene expression in endometrial cancer cell lines (Hec1A and Ishikawa cells) reproducibly changed their ability of proliferation. Concomitant changes were seen in the expression of OPN binding cell surface receptors, cell cycle-regulatory genes, cell invasion and colony formation nature of the tumor cells. Decreased colonizing potential in the absence of OPN was reversed in the presence of recombinant OPN. Inhibition of anchorage-independent growth was observed in the presence of metabolic inhibitors of the PI3K, Src and integrin signaling cascades, which was ameliorated in the presence of exogenously added OPN. Our result showed the role of OPN in endometrial cancer, in particular on the malignancy-promoting aspects of OPN that may pave way for new approaches to the clinical management of endometrial cancer.

Keywords Endometrial cancer · Osteopontin · siRNA · Apoptosis

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Introduction

Endometrial carcinoma is one of the most common malignant diseases of female reproductive tract affecting 2–3 % of women, and its incidence is on the increase in North America and Europe [1, 2]. A major problem in the therapeutic management of endometrial cancer is the growth and metastases in distant organs, but the genes orchestrating the process are yet to be identified for the rational design of new treatment.

Osteopontin (OPN) is an arginine-glycine-aspartic acid-containing acidic glycoprotein component of the extracellular matrix and contains functional domains for calcium binding, phosphorylation, glycosylation, and extracellular matrix adhesion [3]. OPN is expressed in multiple cell types including osteoclasts, oesteoblasts, epithelial cells of several organs and endothelial cells [4]. OPN has been

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reported to have multifunction as regulation of cell adhesion, chemotaxis, and anchorage-independent growth of tumor cells [5, 6]. OPN is postulated to bind to integrin receptors at the cell surface of human endometrium to mediate cellular adhesion and migration during embryo implantation [7]. There are conflicting reports about the expression of OPN in endometrial cancer [8, 9]. Additionally, the molecular mechanisms that define the role of OPN in tumor progression and metastasis in endometrial cancer are incompletely understood. We hypothesized that expression of OPN can independently predict the potential aggressiveness of endometrial cancer. The aim of the present study was to demonstrate whether OPN would serve as a tumor-promoting gene in endometrial cancer cells and be involved in tumor growth and maintenance of genomic stability.

Materials and methods

Cell culture and transfection

Human endometrial stromal cell (THESC) and endometrial cancer cells (RL95, Hec1A and Ishikawa cells) were purchased from ATCC. Cells were cultured in Dulbecco's Modified Eagle Medium, DMEM/F-12, supplemented with 10 % FBS and 1 % penicillin/streptomycin antibiotic mixture and grown to confluence. All the cell culture media and supplements were obtained from GibcoBRL (NY, USA). Transfection of siRNA was performed using ON-TARGET*plus* (Dharmacon, CO, USA) kit, according to the manufacturer's instruction. SMARTpool sequenced siRNA targeting OPN (Accession no. NM_000582) or nonspecific control pool (siRNA negative control) was diluted to a working stock concentration of 5 μM in RNase-free water. Transient transfection was done using the Lipofectamine 2000 reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Control experiments consisted of transfection with the non-targeting siRNA scrambled control or non-transfected (NT) cells. After 48 h of post transfection, cells were harvested and used for protein and RNA analysis.

Quantitative and semi-quantitative reverse transcription-PCR

Total RNA was isolated from endometrial cancer cell lines and OPN siRNA transfected Ishikawa. Five microgram of total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, WI, USA) according to the manufacturer's manual. The cDNAs of OPN and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified using sequence-specific primers (OPN sense primer, 5′-GGATCCCTCACTAC CATGAG-3′; OPN antisense primer, 5′-AAGCTTGACCTCA GAAGATGCACT-3′; GAPDH sense primer, 5′-GTATG ACAACGAATTTGGCTA CAG-3′; GAPDH antisense primer, 5′-AGCACAGGGTACTTTATTGATGGT-3′). The quantitative realtime PCR amplifications (40 cycles of 95 °C for 30 s and 60 °C for 60 s) were performed using the ABI 7900 (Applied Biosystems). For each target gene the average Ct values calculated from triplicate PCR reactions were normalized to the average Ct values for GAPDH. These normalized values were then used to calculate a value expressing the extent of OPN knock-down relative to the control according to the formula $2^{-(\text{mean}\Delta\Delta\text{Ct})}$.

Cell viability assay

To measure viability of OPN siRNA transfected Hec1A and Ishikawa cells, MTS colorimetric assay (Promega, WI, USA) was performed. Cells seeded in 96 well plates (2×10^3 cells/well) were transfected with OPN siRNA at various concentrations and incubated for 48 h. Then MTS reagent was added and incubated for 4 h at 37 °C. The intensity of the purple color was measured at 495 nm. Cell viability values were calculated relative to the control cells (100 %) and expressed as mean ± SD of three independent experiments.

Determination of cell cycle distribution

To determine cell distribution of the OPN siRNA treated Ishikawa cell, FACS analysis was performed. After 48 h incubation of siRNA, cells were harvested, washed with cold PBS, and were fixed in ice-cold 70 % ethanol at 4 °C for 24 h. Then cells were treated with 0.1 % RNaseA for 30 min at 37 °C and were stained with propidium iodide. DNA fluorescence was measured by flow cytometer (FACSCalibur™, Becton–Dickinson, NJ, USA). The percentage of cells in each cell cycle phase was determined using the ModFit LT™ software (Becton–Dickinson) based on the DNA histogram.

DNA fragmentation assessment

To evaluate apoptosis in OPN knock-down cells DNA fragmentation assay was performed. Ishikawa cells were harvested and incubated in DNA isolation buffer (50 mM Tris–HCl [pH 8.0], 10 mM EDTA, 0.5 % SDS and 0.5 mg/ml proteinase K) at 43 °C overnight. The cell lysates were treated with RNase A and extracted with phenol/chloroform/isoamyl alcohol. DNA was precipitated with 3 M Sodium acetate and 100 % ice-cold ethanol then DNA pellet was dissolved in TE buffer (10 mM Tris–HCl [pH 8.0] and 1 mM EDTA). Each DNA sample (10 μg/lane) was electrophoresed in a 2 % agarose gel.

Western blotting

The expressions of OPN, cell cycle regulatory proteins and apoptotic in endometrial cell lines were measured by Western blot analysis. After cells were lyzed and supernatant were collected, and the protein concentrations were measured using Bio-Rad Protein Assay Reagent (Bio-Rad, CA, USA). Fifty microgram of proteins were separated by 8-15 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidine difluoride (PVDF) membrane (Millipore, MA, USA). Then membrane was incubated with primary antibodies of p53, p21, p27, Rb, BAX, Bcl2, caspase-3, caspase-8, caspase-9, cdk2, cdk4 and β-tubulin (Cell Signaling Technology, MA, USA), cyclin A, cyclin E, cyclin D1, PARP, Fas (Santa Cruz, CA, USA), and OPN (R&D Systems, MN, USA). After following incubation with horseradish peroxidase conjugated secondary antibodies (Amersham Lifescience, Buckinghamshire, England), bands on the membranes were visualized by an enhanced chemiluminescence (ECL) system (Amersham Lifescience).

Cell migration assay

Ishikawa cells plated at 2.5×10^5 per 100-mm-diameter dish treated with control or OPN siRNA. After 2 days, cultured cells were disrupted by scratching with a pipette aid equipped with a 1000-μl tip. Repopulation of the cell-free area was examined under an inverted microscope after 48 h.

Anchorage-independent growth/Soft agar growth assay

Cells (5×10^5 per 60-mm-diameter dish) were plated with a top layer of 0.3 % agar (Difco, Detroit, MI) and a bottom layer of 0.6 % agar (both in 20 % FCS containing growth media). Every other day, 0.3 ml of medium was supplemented and the plates were examined microscopically for growth. After 3 weeks, number of cell clusters/microscopic field was counted.

Statistical analysis

The data are presented as mean ± SD. Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test for a post hoc comparison by SPSS 17.0 (SPSS Inc., USA). The p values of 0.05 or less was considered to be of statistical significance.

Results

Differential expression of OPN in endometrial carcinoma cell lines

To elucidate the function of OPN in endometrial cancer cell lines, we analyzed expression level of protein and mRNA of OPN in several immortalized endometrial stromal cell as well as endometrial cancer cell line. When compared to endometrial stromal cells and an endometrial cancer cell line RL95, androgen-independent cell line Hec1A and androgen-dependent cell line Ishikawa showed significantly high expression of OPN both at the mRNA (Fig. 1a) and protein level (Fig. 1b). Therefore, we used these two cell lines for further analysis.

OPN attenuates endometrial cancer cell proliferation

To examine whether OPN is involved in growth control, silencing of osteopontin using targeted siRNA sequences was carried out in Hec1A and Ishikawa cells. Reduction of OPN level in endometrial cancer cells was significantly associated with decrease in cell viability as a dose dependent manner (Fig. 2a, b). There was 51 % reduction in viability in Hec1A cell and 63 % in Ishikawa cell at 200 nM OPN siRNA.

To investigate the mechanism of the OPN level upon the endometrial cancer cell viability, we analyzed cell cycle distribution after incubation of OPN siRNA. Knock-down of OPN significantly disturbed cell cycle (Fig. 3a). The ratio of sub-G_0/G_1 was dramatically increased according to deprivation of OPN level compared with controls (Fig. 3b). This increased of sub-G_0/G_1 population can be attributed to apoptosis. DNA fragmentation assay showed extensive DNA laddering after deprivation of OPN (Fig. 3c).

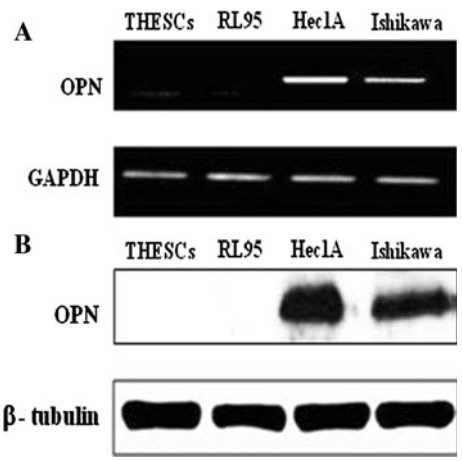

Fig. 1 Expression of OPN mRNA (**a**) and protein (**b**) in endometrial stromal cell (THESC) and endometrial cancer cell lines (RL95, Hec1A and Ishikawa cells)

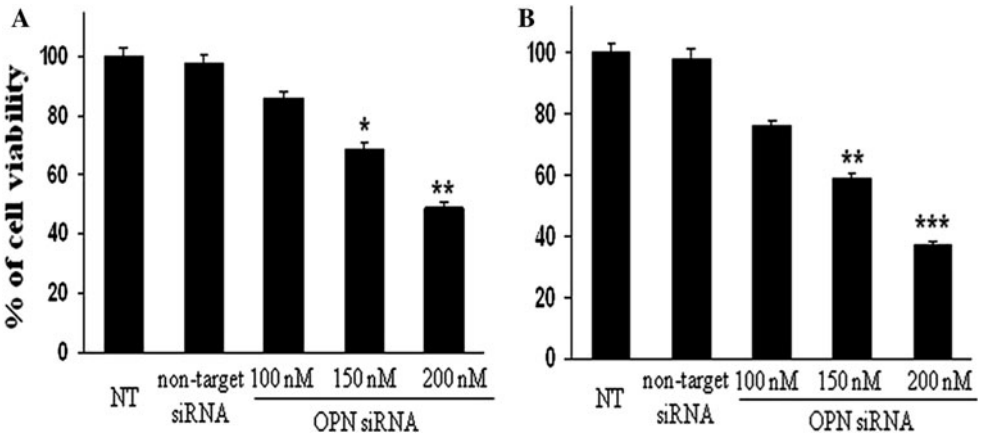

Fig. 2 Hec1A (**a**) and Ishikawa (**b**) cell viability after knock-down of OPN was measured using MTS assay. Data represents the mean ± SD of three independent experiments (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$)

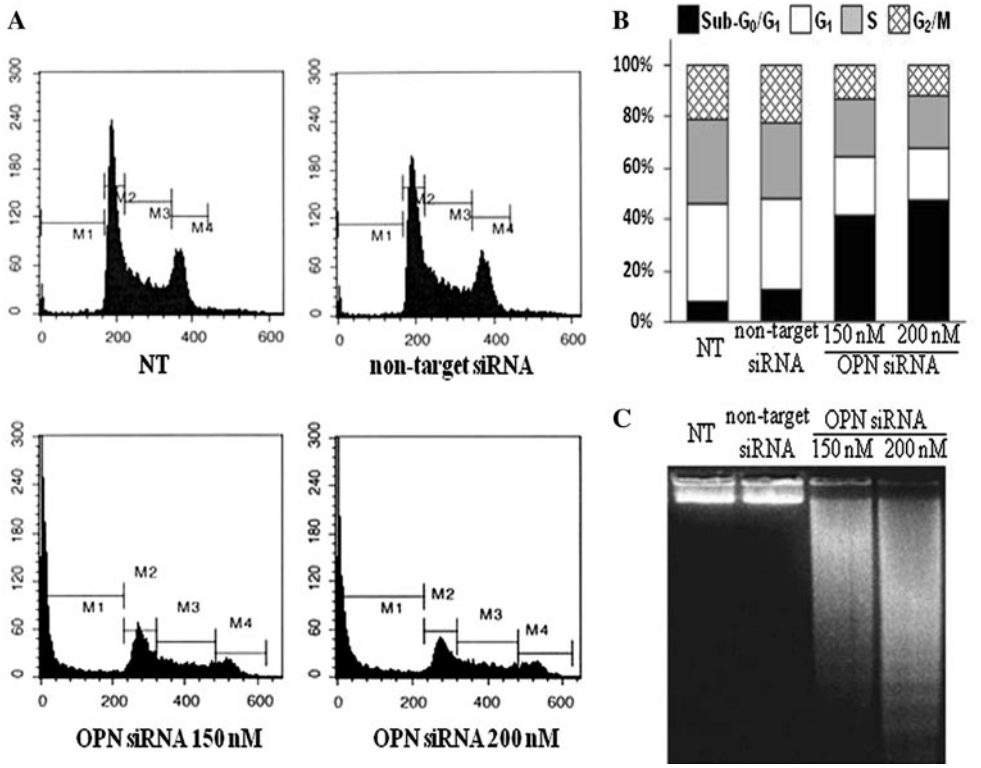

Fig. 3 **a** Effect of OPN silencing on Ishikawa cell cycle profile after incubation with indicated dose of OPN siRNA for 48 h. **b** The proportion of sub-G_0/G_1, G_1, S and G_2/M phases are measured as a percentage (%) of total cells. **c** Effect of OPN siRNA on DNA fragmentation in Ishikawa cells

As Ishikawa cells are androgen-dependent, we analyzed the expression of ER α and β in the absence of OPN. The ER β expression was not regulated by OPN (data not shown), whereas the ER α showed direct regulation with OPN expression. Expression levels of key OPN-binding receptors were decreased in the presence of OPN siRNA. This was accompanied by decreased expression of MMP-9 expression, which regulates cell survival and motility of the cells (Fig. 4).

OPN regulates cell cycle and apoptosis related proteins

As the deficiency of OPN affects cell cycle progression and increases apoptotic cell death. We analyzed the expression of cell cycle regulatory proteins. Expression of tumor suppressor genes p53, p21 and p27 were up regulated with concomitant down regulation of cyclin-dependent kinases and related cyclins after OPN knock-down (Fig. 5a). Death receptor signaling mediated through increased Fas ligand expression combined with cleavage of caspases was observed when OPN was knocked down in Ishikawa cells (Fig. 5b). In the absence of OPN there was significant cleaved PARP formation indicative of active apoptosis pathway.

Role of OPN in metastasis in endometrial cancer

To study the role of OPN in controlling the metastatic potential of endometrial cancer cells, cell migration assays

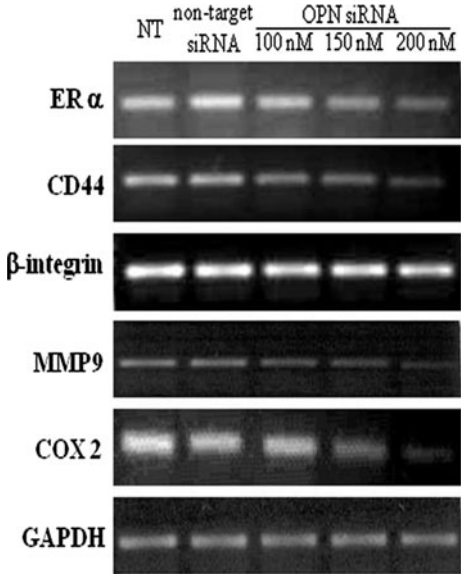

Fig. 4 Down regulation of expression levels of OPN receptors, MMP-9 and COX2 mRNA after silencing OPN

were carried out in the presence or absence of OPN. In the absence of OPN, cell migration dramatically reduced which was recovered after exogenous introduction of recombinant OPN (Fig. 6). This implicates that OPN play a crucial role in the invasive property of the endometrial cancer cells.

Anchorage-independent growth is a very salient feature of oncogenic cells. With anchorage-independent assay we observed that colony formation was increased in the presence of recombinant OPN and this event was nullified when OPN neutralizing antibody was added along with recombinant OPN (Fig. 7a). For further examination of underlying mechanism of OPN in colony formation, we carried out anchorage-independent growth of Ishikawa cells in the presence of specific inhibitors controlling different mechanisms. There was significant reduction in the formation of colonies in the presence of metabolic inhibitors which blocked cell growth signaling through the integrin, Src and PI3 kinase pathways (Fig. 7b). Inhibition brought by the inhibitors was reversed or minimized when recombinant OPN was exogenously added along with the inhibitors. Data from viability assay showed that the inhibition by these inhibitors did not cause cell death or cytotoxicity (Fig. 7c).

Discussion

OPN has been shown to promote the proliferation of cultured rat vascular smooth muscle cells and human coronary artery smooth muscle cells [10], whereas another study indicated that OPN exerts a negative regulatory effect on cell proliferation [11]. Our results indicate that OPN seems to modulate cell proliferation and potentially the survival

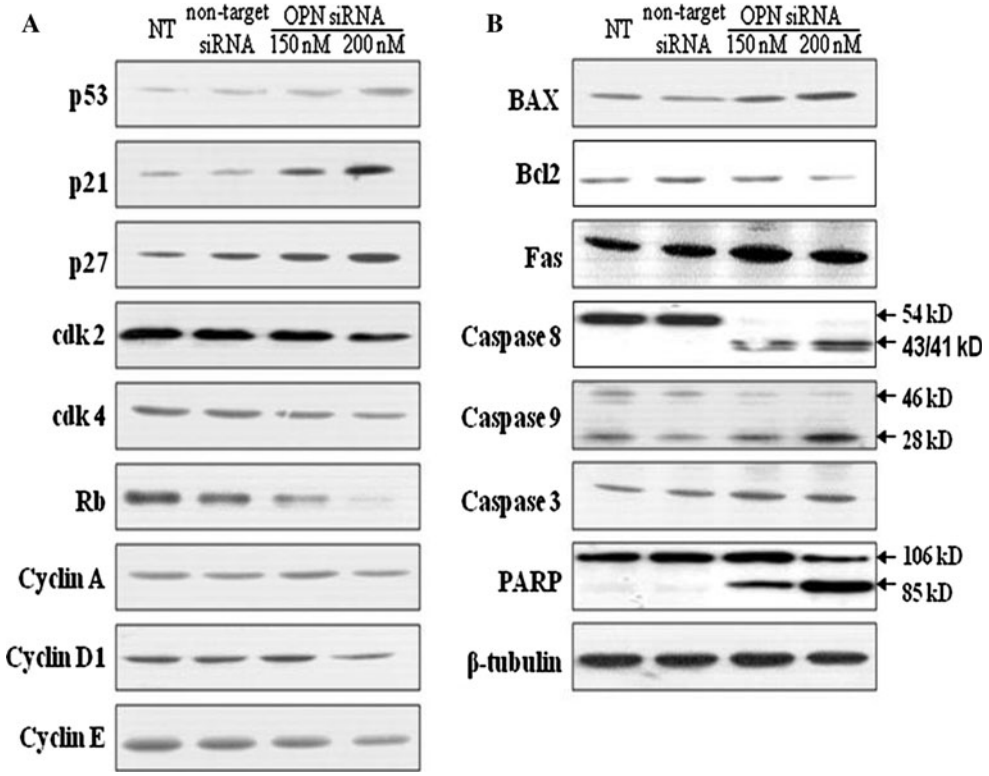

Fig. 5 Effect of OPN knockdown on cell cycle (**a**) and apoptotic (**b**) regulatory proteins. Protein analysis was performed after 48 h incubation with siRNA and β-tubulin was used as an internal control

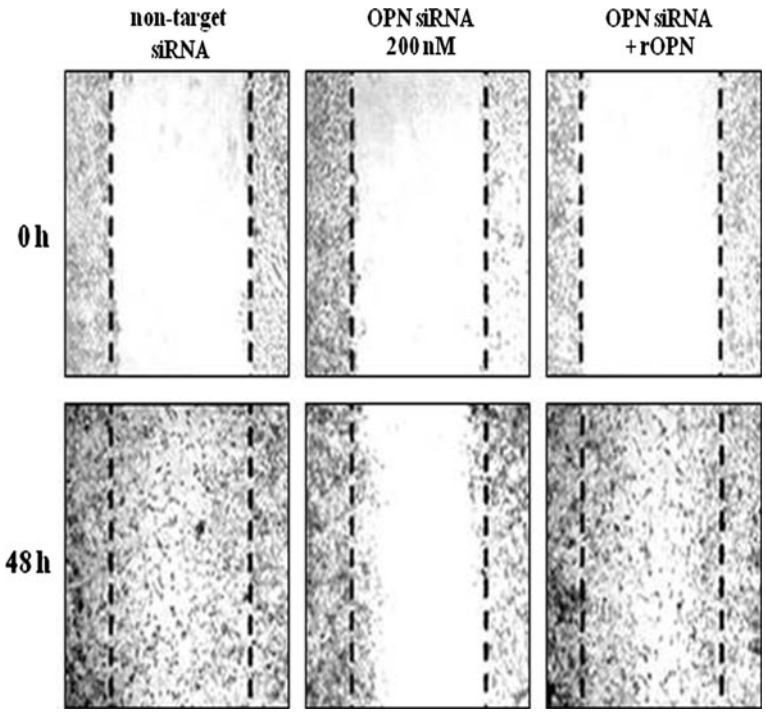

Fig. 6 Effect of OPN on cell migration activity in Ishikawa cells. ×50 magnification. Cells incubated with control or OPN siRNA for 48 h scratched then incubated further 48 h

of endometrial cancer cells. According to cell cycle distribution and DNA fragmentation assay, apoptotic sub-G_0/G_1 population was increased as the OPN level was decreased while the proportion of other cell cycles was unchanged when re-calculated without sub-G_0/G_1 population. As the expression of cell cycle regulatory proteins (p53, p21 and p27) were up regulated with concomitant down regulation of cyclin-dependent kinases and related cyclins in the absence of OPN, blockade of cell cycle progression forced endometrial cancer cell to apoptotic cell death. OPN deficiency-induced apoptosis was further confirmed with the changes of apoptotic protein level. In the absence of OPN there was significant cleaved PARP formation and caspase cascade which are typical indicatives of active apoptosis pathway. Although we did not prove whether presence of OPN prevents apoptosis or absence of OPN renders cells deficient in cell cycle progression which consequently induce apoptosis in endometrial cancer cells, apoptosis prevention of OPN in endothelial cell demonstrated in which OPN binding to αvβ3 increases NF-κB activity through *Ras* and Src [12].

As OPN plays an important role in cell adhesion that confers invasiveness and metastatic capacity to tumor cells [13], we assessed the role of OPN in controlling the metastatic potential of endometrial cancer cells. Cell migration assay showed that the absence of OPN reduced cell migration which was recovered when exogenously recombinant hOPN was added to the cells. As anchorage-independent growth is a very salient feature of oncogenic cells, our finding that recombinant OPN increased colony formation also implicates that OPN can increase invasion and metastasis in endometrial cancer cell.

As earlier report by Apparao [7] indicated that E2 and progesterone play an important role in regulating OPN expression in endometrium, we analyzed the expression level of ERα, which was down regulated when OPN expression was knocked-down. Thus, impaired ERα signaling may partly explain the loss of tumorigenicity in the absence of OPN in endometrial cancer. Furthermore, deficiency of OPN in endometrial cancer also deteriorated integrin and CD44 which can cause constitutive activation of signaling pathways leading to increased growth of tumor cells [6, 14].

An important question is how OPN might be involved in facilitating tumor cell invasion. OPN binds with several integrins and CD44 variants in both RGD sequence-dependent and sequence-independent manner [15]. To examine the signaling pathway downstream of OPN-CD44 interaction, we examined the involvement of Src kinase and PI3K pathways. Our data showed that PP1, a Src family kinase inhibitor, and the inhibitors specific to PI3K (Wortmanin) and integrin (RGD peptide) blocked OPN-mediated survival and significant reduction in the formation of colonies in the presence of metabolic inhibitors that blocked cell growth signaling through the integrin, Src and PI3K pathways. These results indicate that OPN contributes to several steps in the process of endometrial carcinogenesis and metastasis.

Results from our comparative comprehensive analysis in a series of cancer cell lines and OPN silencing studies

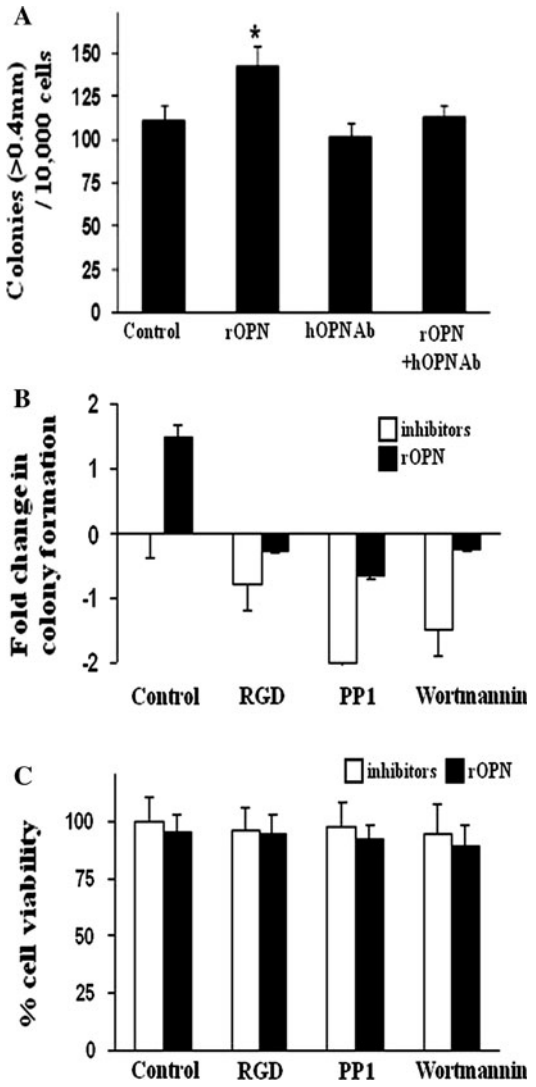

Fig. 7 **a** Colony formation of Ishikawa cells treated with exogenously added rOPN (10 μg/ml), human OPN specific antibody (hOPN Ab) and combination of both. **b** Statistical comparison of fold change in colony formation of Ishikawa cell in the presence of RGD peptide (integrin signaling inhibitor, 10 μg/ml), PP1 (Src-specific kinase inhibitor, 10 μM), or Wortmannin (PI3K inhibitor, 1 μM). **c** Changes in cell viability after incubation with inhibitors and rOPN. $*p < 0.05$

reveal that this cell cycle regulatory protein acts as a checkpoint in endometrial cancer growth and development.

The signaling pathways and transcription factors discussed in this study that regulated OPN expression, may explain, in part, the mechanism of OPN regulation. Since OPN can mediate cell adhesion, cell–cell interaction, invasion and colony formation in tumor cells in experimental systems, this molecule is a key candidate for a tumor prognostic marker and a potential target for therapeutic intervention in control of metastasis.

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