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ORIGINAL RESEARCH

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# *In vitro* effects of osteopontin on endometrial cancer cells and signaling pathways in epithelial-mesenchymal transition

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## Abstract

This study aims to demonstrate *in vitro* effects of osteopontin (OPN), an extracellular matrix molecule, on proliferation and migration in human umbilical vein endothelial cells (HUVEC) and Ishikawa human endometrial adenocarcinoma cells (Ishikawa) cultures. In addition, the effects of OPN on phosphatidylinositol 3 kinase (PI3K) and extracellular signal regulatory kinase 1/2 (ERK1/2), which are involved signaling pathways in cell cycle, were investigated. This research was carried out as a prospective cell culture study in Aydın Adnan Menderes University Biochemistry Laboratory. Two types of cell culture were used, HUVEC representing endothelial cells and Ishikawa representing tumour cells. The HUVEC and Ishikawa were extracted from liquid nitrogen and seeded in culture medium. With the increasing concentrations of recombinant human OPN (rhOPN) in both cell lines, proliferation and migration of cells and PI3K and ERK1/2 levels in the medium were investigated. Detection of cell proliferation in these two cell types with MTT test, and migration assay were performed. Proliferation was found to increase in both cell types with increasing levels of rhOPN ($p < 0.01$). In both cell cultures, rhOPN induced mesenchymal transformation, indicating migration at the highest (400 ng/mL) concentrations. Increasing levels of PI3K and ERK1/2 were measured with the increasing concentrations of rhOPN ($p < 0.01$ and $p < 0.05$, respectively). Proliferation, migration, and PI3K and ERK1/2 molecules are increased with rhOPN in both cell cultures studied. This suggests that OPN could be a candidate prognostic marker in endometrial cancer.

## Keywords

Endometrial cancer; Extracellular signal regulatory kinase 1/2; Human umbilical vein endothelial cells; Ishikawa cells; Osteopontin; Phosphatidylinositol 3 kinase

## 1. Introduction

Endometrial cancer is the most common malignancy of the female genital tract, affecting 2%–3% of women. The main perspective is the prevention of metastases and recurrences [1]. Many molecules are being studied to elucidate the mechanism of metastases and recurrences in cancer. One such molecule is osteopontin (OPN), an extracellular matrix (ECM) molecule involved in many physiological and pathological processes, including cell adhesion, angiogenesis, and tumour metastases. This molecule has been implicated in the progression of several cancers, including endometrial cancer [2]. In addition, cells with a high rate of metastatic change have higher levels of OPN than cells that do not form tumours [3].

However, there have been few studies in the literature investigating the effect of OPN on epithelial mesenchymal transition (EMT) in Ishikawa human endometrial adenocarcinoma cells (Ishikawa) [2, 4, 5]. Therefore, the aim of this study was to investigate the tumourigenic effects of recombinant human (rh) OPN in Ishikawa via two different signaling pathways. The effects of rhOPN at different concentrations were investigated in Ishikawa and human umbilical vein endothelial cells (HUVEC) cultures. Proliferation, migration and tube-like structures indicative of new blood vessel formation were demonstrated *in vitro*. In addition, the study aimed to determine the expression of PI3K and ERK1/2 molecules in the cell medium, which play an important role in the PI3K and ERK1/2 signaling pathways.

## 2. Material and methods

This was a prospective cell culture study and was conducted at Aydın Adnan Menderes University Biochemistry Laboratory in 2020. Ishikawa was used as the tumour cells material and HUVEC was used as the endothelial cells material. The effects of rhOPN on cell proliferation, migration and angiogenesis were demonstrated. PI3K and ERK1/2 expressions were determined by detecting PI3K and ERK1/2 molecules in the cell


  

medium. No rhOPN was introduced to the control medium in both Ishikawa and HUVEC cultures. The effects of rhOPN on angiogenesis were investigated in HUVEC.

## 2.1 Human umbilical vein endothelial cell supply and cultivation

HUVEC medium preparation and cell viability maintenance were performed as described by Grant and Auerbach *et al*. [6, 7].

1. 5 mL of Medium 199 (M199) was placed into the vial containing 100 mg endothelial cell growth supplement (ECGS) and dissolved.
2. 100 mL fetal calf serum, 5 mL glutamine, 5 mL penicillin/streptomycin amphotericin B, 0.5 mL gentamicin and 250 $\mu$L heparin were added to a 250 mL sterile bottle.
3. 5 mL of ECGS solution previously dissolved with M199 was added into this mixture.
4. The entire content was sterilized through a 0.22-micron filter and added into M199.
5. The sterile HUVEC medium was made ready for use by portioning it into 50 mL sterile conical tubes and placed at +4 °C.

## 2.2 Supply and cultivation of Ishikawa human endometrial adenocarcinoma cell lines

Ishikawa are well-differentiated human endometrial adenocarcinoma cells. The cells used in this study were obtained from the cell stocks owned of Prof. Dr. Çiğdem Yenisey, faculty member of the Department of Medical Biochemistry, and stored in liquid nitrogen at −196 °C in the laboratory of Adnan Menderes University Science and Technology Centre (BİLTEM).

The cells were grown in Eagle's Minimum Essential Medium + 2 mM glutamine + 1% Non-Essential Amino Acids + 5% Fetal Bovine Serum (FBS) + penicillin (100 U/mL) + streptomycin (100 $\mu$g/mL) and the examinations were performed when the cells reached 80–90% density.

## 2.3 Cell thawing

Cell thawing involved the steps of removing HUVEC and Ishikawa, which are primary endothelial cells previously frozen in a liquid nitrogen tank at −196 °C, from liquid nitrogen and seeding them in culture medium in accordance with appropriate procedures.

## 2.4 Cell counting and viability assay

Cells were automatically counted using the EVE™ Automated Cell Counter, the NanoEnTek Cell Counter, and the percentage of viable cells (unstained cells) was calculated.

## 2.5 Harvesting of tumor cells ambient medium

Approximately 125,000 cells were seeded in a 6-well plate, and the FBS-containing medium on top was discarded when it was occupied by approximately 80–90% full.

## 2.6 Detection of cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay

The absorbance of the color produced by Ishikawa was measured in the wells containing only growth medium (GM) as a control. In addition, a serum-free medium solution (FBS) was added to a well similar to the well containing 0 ng/mL rhOPN as described by Li *et al*. [4]. The presence of two controls in this study can be explained as follows: cells absolutely require serum while they are growing, and since serum contains all growth factors and proteins, a sample in a serum-free medium must also be included in order to evaluate the effect of a substance. All concentrations of rhOPN were prepared using a FBS-free medium (Recombinant Human Osteopontin Protein, Secreted Phosphoprotein 1 with a catalogue number of PKSH032000, Elabsciense, United States). The tests were repeated by using 8 samples and the same concentration of substances on 3 different days, and the average value of 24 measurements was considered to significantly represent the effect of each substance on cell viability.

## 2.7 Migration/invasion assay

The lowest and highest doses of rhOPN 400 ng/mL were used to assess migration in HUVEC. In this migration assay performed on HUVEC, the number of cells could only be assessed by images as there was no software in the camera system. For this reason, migration assessment at intermediate concentrations was not performed. The same assay was performed with Ishikawa to show the effects of rhOPN 400 ng/mL on migration at 48 hours.

Cells were seeded in a 6-well plate with an average of 125,000 cells. When the cells in the wells reached approximately 90% density, a line was drawn in the middle with the help of a sterile pipette tip. Different doses of rhOPN were then added and checked every 12 hours. The migration of the cells was visualized. The wells were photographed and the study ended in the 48th hour, when the best results were obtained.

In addition, the migration study was repeated using the insert. First, HUVEC was seeded in 200 $\mu$L of growth medium in the insert with 8 $\mu$m pores placed in a 24-well plate, and a further 200 $\mu$L of M199 + 1% bovine serum albumin (BSA) solution was added. Before the inserts were placed in the 24-well plate, 600 $\mu$L of the medium obtained from Ishikawa incubated with 100 and 400 rhOPN was added to the wells, and the inserts were placed in these wells. Although migration was observed after 6 hours, we removed the inserts after approximately 12 hours due to our working conditions. The removed inserts were first washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS), then incubated with freshly prepared 3.7% formaldehyde for 15 minutes, washed again with DPBS twice and stained with Giemsa dye for 5 minutes. The cells were then fixed with methanol for approximately 20 minutes at room temperature and washed again with DPBS twice. The cells that remained inside the inserts and did not pass through the pores were cleaned with a cotton swab and images were obtained from different areas.

## 2.8 *In vitro* angiogenesis (*in vitro* tube) assay

*In vitro* angiogenesis, formation of capillary vessels by endothelial cells (HUVEC) using a tumour medium, was performed as described by Grant *et al*. [7]. Angiogenesis was only studied in HUVEC in this study as the capillary endothelial cells begin to organize to form new vessels and a new basement membrane is secreted to protect the newly formed vasculature.

1. 50 $\mu$L of Matrigel was placed in the designated wells of 96-well plates
2. The plate was incubated in an oven with 5% $CO_2$ at 37 °C for 30 minutes.
3. At the end of the incubation, 200 $\mu$L of serum-free medium (1% BSA and 1% insulin-transferrin-selenium in endothelial basal medium solution) was added to the wells.
4. Immediately afterwards, approximately 30,000 HUVEC were seeded into the wells in 100 $\mu$L of M199 solution.
5. After incubation in an oven with 5% $CO_2$ at 37 °C for one hour, 100 $\mu$L of Ishikawa medium (with and without rhOPN) previously obtained from tumour cells was added to the wells.
6. After the plate was incubated in an oven with 5% $CO_2$ at 37 °C for 16 hours, the medium was carefully removed and images were obtained under an inverted microscope.

## 2.9 Detection of PI3K and ERK1/2 molecules in tumour cells medium

The levels of all markers in the tumour cell medium (conditioned medium, CM) were determined using commercially available Human Enzyme-Linked Immunosorbent Assay (ELISA) kits. The PI3K ELISA kit; (Catalogue No. E0896Hu, Product number. 85-6685-27, Bioassay Technology Laboratory, Shanghai, China). ERK1/2 ELISA kit; (Catalogue No. E4633Hu, Product number. 85-6713-87, Bioassay Technology Laboratory, Shanghai, China).

Samples were added to the wells of the antigen coated plate and incubated at 37 °C followed by the addition of antibody. After the washing steps, the substrate solution was added, and after the formation of blue colors, the reaction terminating reagent was added and the resulting yellow color was read at 450 nm in the microplate reader (ELx800, UV-Visible Multiscan Spectrophotometer, Diagnostic Automation Inc., Los Angeles, CA, USA). The concentrations in the samples were automatically calculated from the graph generated by the instrument using the standards included in the kit.

## 2.10 Statistical analysis

Statistical analyses were performed by using The Statistical Package for Social Sciences for Windows, Version 25 (Released 2017, IBM Corp., Armonk, NY, USA). Normality of the data for the numerical variables was checked using the residual values obtained from the One-Way Analysis of Variance. To this end, Kolmogorov-Smirnov test ($n > 50$) or Shapiro-Wilk test ($n < 50$) was used. The homogeneity of variance was tested using the Levene test. $F$ (when variances are homogeneous) or Welch (when variances are not homogeneous) statistics were used for multiple comparisons between OPN levels (ng/mL). Pairwise comparisons were performed using Tukey HSD (post-$F$-Test) or Dunnett T3 (post-Welch-Test). The significance level was set at $p = 0.05$ for all the hypothesis tests. As the two cell culture structures were different, no comparison was made between the groups.

# 3. Results

## 3.1 Cell proliferation assay

The ordered groups on the plates were K (GM) as a control group in which HUVEC or Ishikawa were grown in a growth medium containing normal FBS, K (CM) as another control group in which HUVEC or Ishikawa were again amplified, but without FBS or rhOPN, and other groups with rhOPN at 100, 200, 300 and 400 ng/mL. When the effects of rhOPN on HUVEC and Ishikawa were examined, almost every increase in the concentration of rhOPN resulted in a significant difference in the proliferation of the cell lines (Fig. 1).

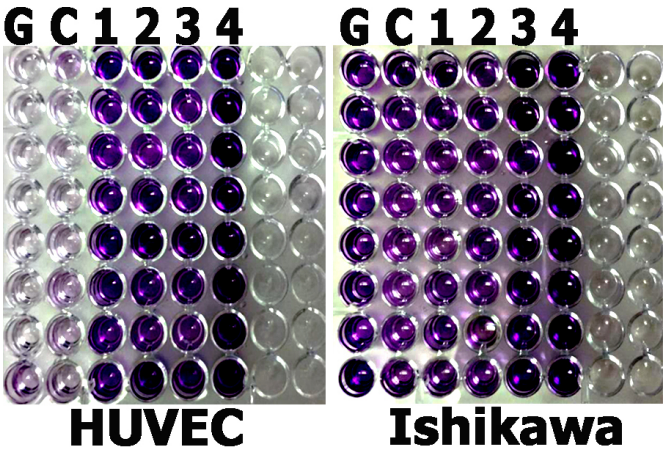


**FIGURE 1. Cell proliferation assay.** Demonstration of OPN-induced proliferation in HUVEC and Ishikawa.

We wanted to replicate this study in endothelial cells and investigate the effect of rhOPN on the transformation of primary epithelial cells, which have not undergone malignant transformation but are the most important cells in the formation of angiogenesis. To achieve this, we worked with HUVEC, a human umbilical cord vein cell and a cell line often used to model angiogenesis *in vitro*.

As shown in Table 1, when the effects of rhOPN on Ishikawa were examined, the difference between the mean values of the control and no rhOPN added groups and the groups given 200, 300 and 400 ng/mL rhOPN was found to be statistically significant. Again, the difference between the 100 ng/mL rhOPN added group and the 200, 300 and 400 ng/mL rhOPN added groups, the difference between the 200 ng/mL rhOPN added group and the 300 and 400 ng/mL rhOPN added groups, and the difference between the 300 ng/mL rhOPN added group and the 400 ng/mL rhOPN added group were significant. The difference between the rhOPN added groups was found to be statistically significant.

Besides, as presented in Table 1, when the effects of rhOPN on HUVEC were examined, the difference between the mean values of the control and no rhOPN added groups and the groups given 200, 300 and 400 ng/mL rhOPN was found to

**TABLE 1. Effects of increasing concentrations of rhOPN on HUVEC and Ishikawa.**

| rhOPN (ng/mL) | HUVEC in the 48th hour (Mean ± SD) | Ishikawa in the 48th hour (Mean ± SD) |
|---|---|---|
| Control (GM) | $0.059 \pm 0.008^{a}$ | $0.147 \pm 0.035^{g}$ |
| 0 | $0.063 \pm 0.011^{b}$ | $0.153 \pm 0.028^{h}$ |
| 100 | $0.635 \pm 0.103^{c}$ | $0.182 \pm 0.022^{i}$ |
| 200 | $0.675 \pm 0.115^{d}$ | $0.536 \pm 0.022^{j}$ |
| 300 | $1.358 \pm 0.216^{e}$ | $1.269 \pm 0.146^{k}$ |
| 400 | $2.536 \pm 0.622^{f}$ | $2.804 \pm 0.514^{l}$ |

*HUVEC: human umbilical vein endothelial cell; Ishikawa: Ishikawa human endometrial adenocarcinoma cell; rhOPN: recombinant human osteopontin; GM: growth medium; SD: standard deviation.*
*Statistical significance was set at $p < 0.001$ for comparisons of a with c, d, e, f; b with c, d, e and f; c with e and f; d with e; g with j, k, l; h with j, k, l; i with j, k, l; j with k, l; k with l.*
*Statistical significance was set at $p < 0.01$ for the comparison of e with f.*

be statistically significant. Furthermore, the difference between the group given 100 ng/mL rhOPN and the group given 300 ng/mL rhOPN, the difference between the group given 200 ng/mL rhOPN and the groups given 300 and 400 ng/mL rhOPN, and the difference between the group given 300 ng/mL rhOPN and the group given 400 ng/mL rhOPN were statistically significant. The difference between the rhOPN groups was found to be statistically significant (Table 1, Figs. 2,3).

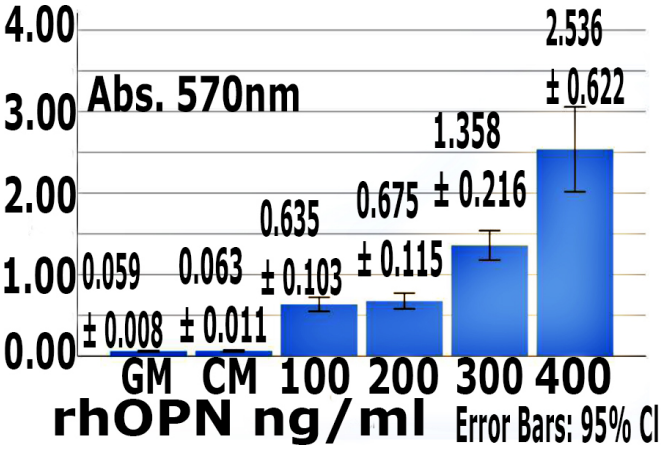


**FIGURE 2. Graphical representation of the effect of rhOPN on proliferation in HUVEC.** rhOPN: recombinant human osteopontin; GM: growth medium; CM: conditioned medium; CI: confidence interval.

## 3.2 Migration/invasion assay

Ishikawa migration cells closed the opening created with the pipette tip between the 0th and 48th hours. This is an expected situation for tumuor cells as they have migration capacity. When rhOPN 400 ng/mL was added to Ishikawa, the distance between them much more decreased at the end of the 48th hour. In other words, the migration of the cells increased. There was no migration in the cells containing their own medium but not containing rhOPN after 48 hours. However, when rhOPN 400 ng/mL was added, migration and mesenchymal transformation of the cells increased (Fig. 4A,B).

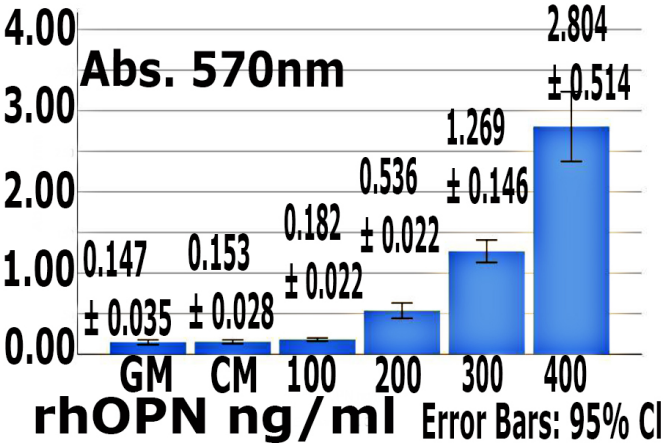


**FIGURE 3. Graphical representation of the effect of rhOPN on proliferation in Ishikawa.** rhOPN: recombinant human osteopontin; GM: growth medium; CM: conditioned medium; CI: confidence interval.

The images obtained from the inserts showed that the cells passed through the pores of the inserts and migrated. As there was no software in the camera system, evaluation could only be made with images. Even at the lowest concentration of rhOPN, 100 ng/mL, cells were detected in the images obtained from the inserts. It was therefore thought that rhOPN stimulated endothelial cell migration and induced angiogenesis, the formation of new blood vessels, thus contributing to metastasis (Fig. 4C).

## 3.3 *In vitro* tube formation and angiogenesis

HUVEC are endothelial cells that form tube-like structures resembling blood vessels on Matrigel (an authentic basal membrane) within 16–18 hours. This assay is called *in vitro* angiogenesis and is used in drug discovery and to study the anti-tumour effects of substances. The tube structures formed in the control well containing HUVECs' own medium were compared with those formed after the addition of rhOPN 400 ng/mL.

Both the frequency and the branching points of the tube

structures increased in HUVEC treated with rhOPN at the highest dose used in this study (Fig. 5).

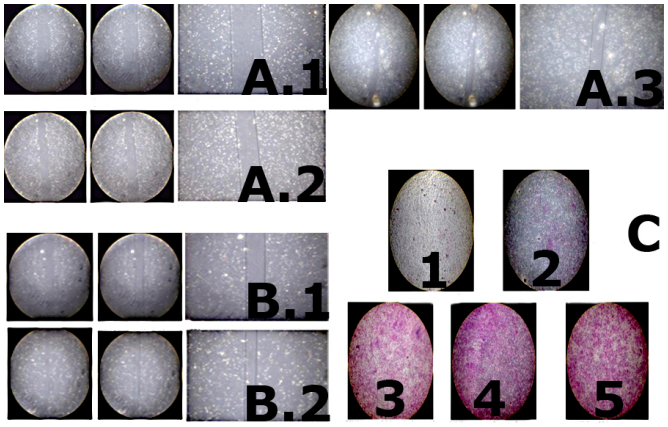


**FIGURE 4. Migration (invasion) assay.** The Effect of rhOPN 400 ng/mL on Ishikawa and HUVEC migration. (A) The Effect of rhOPN 400 ng/mL on Ishikawa migration, Line method. (A.1) Ishikawa Control 0th hour. (A.2) Ishikawa Control 48th hour. (A.3) Ishikawa + rhOPN 400 ng/mL 48th hour. (B) The Effect of rhOPN 400 ng/mL on HUVEC migration, Line method. (B.1) HUVEC Control 48th hour. (B.2) HUVEC + rhOPN 400 ng/mL 48th hour. (C) The Effect of rhOPN on HUVEC migration, Insert method. (C1) HUVEC Control. (C2) HUVEC + rhOPN 100 ng/mL. (C3) HUVEC + rhOPN 200 ng/mL. (C4) HUVEC + rhOPN 300 ng/mL. (C5) HUVEC + rhOPN 400 ng/mL.

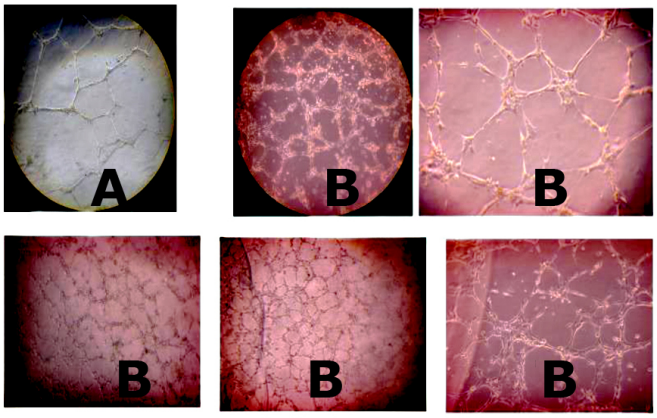


**FIGURE 5. *In vitro* tube formation (angiogenesis) after addition of rhOPN 400 ng/mL to HUVEC.** (A) HUVEC Control. (B) HUVEC + rhOPN 400 ng/mL. The tube structures formed in the images obtained from the control well, where HUVEC grew in their own medium, were compared with the tube structures formed after addition of 400 ng/mL rhOPN. The other 5 images showed an increase in the frequency of tube structures and branch points. This suggests that rhOPN, at the highest dose we used, increases the frequency of tube structures formed in HUVEC, *i.e.*, angiogenesis, which means that it may contribute to the formation of metastasis.

### 3.4 ELISA test of PI3K and ERK1/2 signal molecules

The results concerning the PI3K and ERK1/2 signaling molecules detected by ELISA in the medium obtained after treatment of HUVEC and Ishikawa with different concentrations of rhOPN are summarized in Tables 2 and 3.

As shown in Table 2, the difference between the mean values of the PI3K and ERK1/2 molecules obtained from the control medium (containing FBS) and the second control medium (containing FBS) and the wells with 200, 300 and 400 ng/mL rhOPN added was statistically significant. Besides, when the levels of the signaling molecule in the 100 ng/mL rhOPN well and the 200, 300 and 400 ng/mL rhOPN well were compared, the difference between them was statistically significant and as the dose increased so did the amount of PI3K molecule (Fig. 6, Table 2, Figs. 7,8).

As shown in Table 3, the difference between the PI3K and ERK1/2 levels obtained in the wells of the control medium (containing FBS) and the second control group which did not contain FBS and rhOPN, and the average values of the PI3K molecule in the medium obtained from the wells containing 200, 300 and 400 ng/mL rhOPN were statistically significant. The increase in PI3K molecule appeared to be dose dependent (Fig. 9, Table 3, Figs. 10,11).

Tables 2 and 3 show that the levels of PI3K and ERK1/2 molecules increased significantly at almost all increased doses of rhOPN.

## 4. Discussion

This study showed that rhOPN increased migration and proliferation in HUVEC and Ishikawa, and induced angiogenesis in HUVEC. In both endothelial and malignant cell cultures, rhOPN showed similar effects in terms of migration and proliferation. These effects were consistent with the increase in the levels of PI3K and ERK1/2 molecules in the medium of the cell cultures. HUVEC were incorporated into the study to investigate the effects of rhOPN on endothelial cells as well as on cancer cells of Ishikawa.

It was shown in a study of angiogenesis that new vessels arise from pre-existing capillaries, postcapillary venules or terminal venules, but that arteries, arterioles or veins are not angiogenic in nature [8]. Endothelial cells begin to organize to form new vessels, secreting basement membrane components to maintain the newly formed vasculature. Endothelial cells line up in a single row on a basement membrane and form a layer that covers the entire vascular system. These cells, which are polygonal or spindle-shaped, are surrounded by a second layer of pericytes and smooth muscle cells. This complex structure is located in the microenvironment called the extracellular matrix [8]. In the formation of angiogenesis, there are stages of activation, proliferation, invasion and migration of endothelial cells, maturation of endothelial cells and formation of the lumen [9]. Angiogenic molecules released from tumour cells and surrounding inflammatory cells bind to the receptors on the endothelial surface and initiate angiogenesis [10]. For this reason, angiogenesis was studied in HUVEC cells in the present study. The increase in the concentrations of PI3K

**TABLE 2. Effects of increasing concentrations of rhOPN on PI3K Signaling Molecule levels in HUVEC and Ishikawa.**

| rhOPN (ng/mL) | PI3K (ng/mL) Mean ± SD | |
|---|---|---|
| | HUVEC | Ishikawa |
| Control | $9.87 \pm 0.58^{a}$ | $11.67 \pm 2.29^{g}$ |
| 0 | $10.02 \pm 1.15^{b}$ | $11.61 \pm 2.15^{h}$ |
| 100 | $11.97 \pm 2.85^{c}$ | $14.90 \pm 2.47^{i}$ |
| 200 | $20.25 \pm 3.58^{d}$ | $21.75 \pm 3.63^{j}$ |
| 300 | $28.94 \pm 4.18^{e}$ | $22.87 \pm 3.14^{k}$ |
| 400 | $32.75 \pm 3.46^{f}$ | $30.82 \pm 3.91^{l}$ |

*HUVEC: human umbilical vein endothelial cell; Ishikawa: Ishikawa human endometrial adenocarcinoma cell; rhOPN: recombinant human osteopontin; PI3K: phosphatidylinositol 3 kinase; SD: standard deviation.*
*Statistical significance was set at $p < 0.001$ for comparisons of a with d, e, f; b with d, e, f; c with d, e, f; d with f; g with j, k, l; h with j, k, l; i with j, k, l; j with l; k with l.*
*Statistical significance was set at $p < 0.01$ for the comparison of d with e.*

**TABLE 3. Effects of increasing concentrations of rhOPN on ERK1/2 Signaling Molecule levels in HUVEC and Ishikawa.**

| rhOPN (ng/mL) | ERK1/2 (ng/mL) Mean ± SD | |
|---|---|---|
| | HUVEC | Ishikawa |
| Control | $912.80 \pm 110.90^{a}$ | $1128.80 \pm 72.92^{g}$ |
| 0 | $910.40 \pm 203.41^{b}$ | $1126.40 \pm 78.99^{h}$ |
| 100 | $2092.70 \pm 647.25^{c}$ | $1554.20 \pm 209.70^{i}$ |
| 200 | $2830.40 \pm 499.11^{d}$ | $2038.20 \pm 705.10^{j}$ |
| 300 | $2840.11 \pm 497.77^{e}$ | $2154.50 \pm 384.40^{k}$ |
| 400 | $4038.22 \pm 904.81^{f}$ | $2920.90 \pm 466.40^{l}$ |

*HUVEC: human umbilical vein endothelial cell; Ishikawa: Ishikawa human endometrial adenocarcinoma cell; rhOPN: recombinant human osteopontin; ERK1/2: extracellular signal regulatory kinase 1/2; SD: standard deviation.*
*Statistical significance was set at $p < 0.01$ for comparisons of a with c; b with c; c with f; g with i; h with i; i with k.*
*Statistical significance was set at $p < 0.001$ for comparisons of a with d, e, f; b with d, e, f; g with k, l; h with k, l; i with l.*
*Statistical significance was set at $p < 0.05$ for comparisons of d with f; d with e; g with j; h with j; k with l.*

and ERK1/2 molecules with increasing doses of rhOPN in the media of the cells was considered as an indicator of the activation of PI3K and ERK1/2 signaling pathways via CD44 and $\alpha v\beta 3$ receptors. The increase in these molecules in both cell culture series suggests that migration and angiogenesis are triggered. The values of the two groups were not compared because the cell characteristics were different.

Du *et al*. [2] isolated tumor-associated human endometrial epithelial cells (HEEC) from endometrial cancer cells and investigated the role of OPN in the development of endometrial cancer-related angiogenesis by inhibiting the OPN gene in HEEC and Ishikawa. They silenced OPN-producing genes in cell lines and observed that reduced OPN production in HEEC and Ishikawa resulted in a decrease in cell migration and *in vitro* tube formation. They suggested that OPN secreted from HEEC may be responsible for triggering angiogenesis by binding to $\alpha v\beta 3$ integrins found on cell surfaces. However, they showed that proliferation was not inhibited in cells in which the OPN genes were silenced (siOPN). They also investigated the effect of OPN on tumour growth in nude mice using Ishikawa. Although no significant changes were observed in the growth of cells *in vitro*, tumours grew much more slowly in the mice given siOPN-Ishikawa than in the mice given only Ishikawa, which is consistent with the results of the present study. However, Du *et al*. [2] reported that although OPN expression was inhibited, proliferation in HEEC and Ishikawa was not inhibited. In the current study, a significant increase in proliferation of both Ishikawa and HUVEC was noted with increasing concentrations of rhOPN. This might have been due to the difference in the study design.

An increasing number of studies have shown in recent years that OPN triggers the progression of various cancer types, including endometrial cancers, by regulating angiogenesis [2, 5, 11–13]. OPN binds to various integrin structures and CD44 molecules that induce migration in endothelial cells. It also increases the regulation of endothelial cell migration induced through VEGF signaling. Tissue factor, OPN and $\alpha v\beta 3$ integrins, which are vascular endothelial growth factor (VEGF)-induced proteins in endothelial cells, play an important role in angiogenesis *in vivo* [2, 3, 14]. It is emphasized in the literature that OPN has an important role in tumour growth by enhancing *in vivo* angiogenesis and that any abnormality in the expression

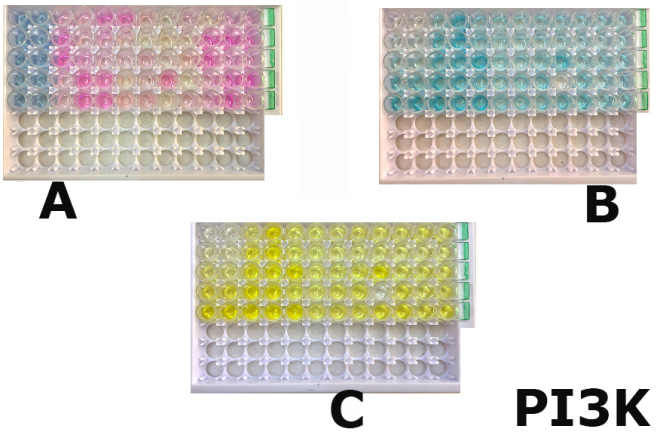


**FIGURE 6. Detection of PI3K molecule in tumour cells medium.** (PI3K: phosphatidylinositol 3 kinase). The levels of all markers in the tumour cell conditioned medium (CM) were determined using commercially available Human Enzyme-Linked Immunosorbent Assay (ELISA) kits. (The PI3K; Catalogue No. E0896Hu, Bioassay Technology Laboratory, Shanghai, China). (A) Medium added to antibody coated plates. (B) Substrate solution was added to the sample. (C) The yellow color formed when stop solution was added and measured at 450 nm. Samples were added to the wells of the antigen coated plate, incubated at 37 °C followed by the addition of antibody. After washing steps, the substrate solution was added and after the formation of blue colours, the reaction terminating reagent was added and the resulting yellow colour was read at 450 nm in the microplate reader (ELx800). The concentrations in the samples were automatically calculated from the graph generated by the instrument using the standards provided in the kit. Using the standards provided in the kit, graph was automatically generated on the ELISA reader and the *R*-values of the graph was *R*-square = 0.9997 for PI3K, demonstrating that the test is sensitive and reproducible.

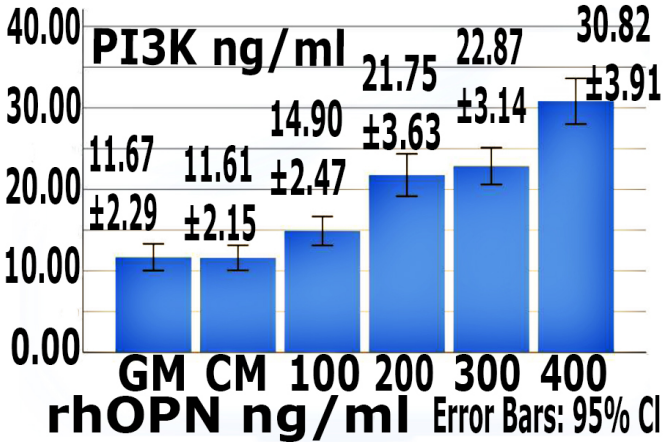


**FIGURE 7. Effects of increasing concentrations of rhOPN on PI3K Signal Molecule in Ishikawa.** rhOPN: recombinant human osteopontin; PI3K: phosphatidylinositol 3 kinase; GM: growth medium; CM: conditioned medium; CI: confidence interval.

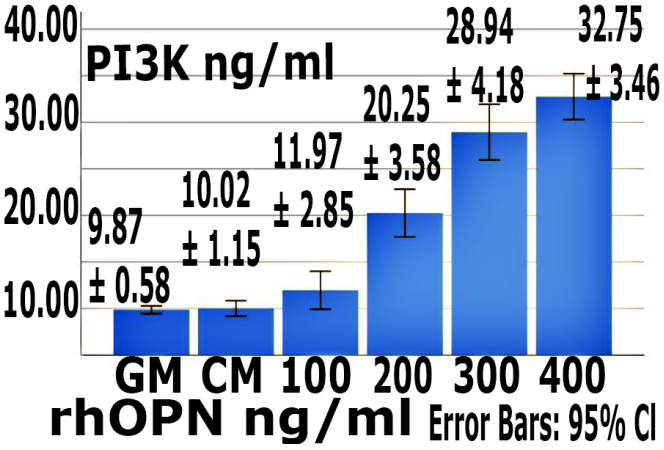


**FIGURE 8. Effects of increasing concentrations of rhOPN on PI3K Signal Molecule in HUVEC.** rhOPN: recombinant human osteopontin; PI3K: phosphatidylinositol 3 kinase; GM: growth medium; CM: conditioned medium; CI: confidence interval.

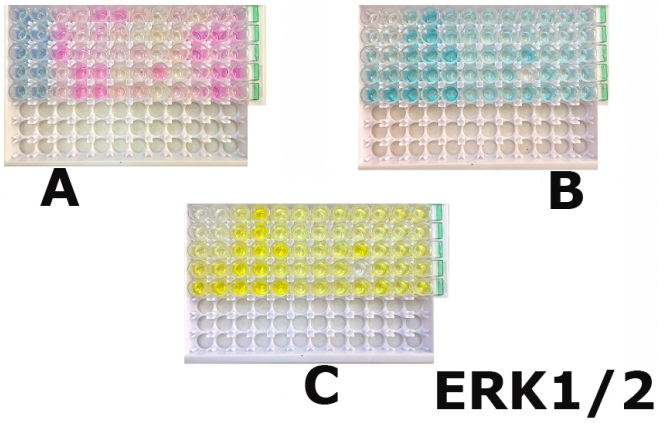


**FIGURE 9. Detection of ERK1/2 molecule in tumour cells medium.** (ERK1/2: extracellular signal regulatory kinase 1/2.). The levels of all markers in the tumour cell medium (conditioned medium, CM) were determined using commercially available Human Enzyme-Linked Immunosorbent Assay (ELISA) kits. (The ERK1/2; Catalogue No. E4633Hu, Bioassay Technology Laboratory, Shanghai, China). (A) Medium added to antibody coated plates. (B) Substrate solution was added to the sample. (C) The yellow color formed when stop solution was added and measured at 450 nm. Samples were added to the wells of the antigen coated plate and incubated at 37 °C followed by the addition of antibody. After the washing steps, the substrate solution was added and after the formation of blue colors, the reaction terminating reagent was added and the resulting yellow color was read at 450 nm in the microplate reader (ELx800). The concentrations in the samples were automatically calculated from the graph generated by the instrument using the standards included in the kit. Using the standards provided in the kit, graph was automatically generated on the ELISA reader and the *R*-values of the graph was *R*-square = 1.000 for ERK1/2, demonstrating that the test is sensitive and reproducible.

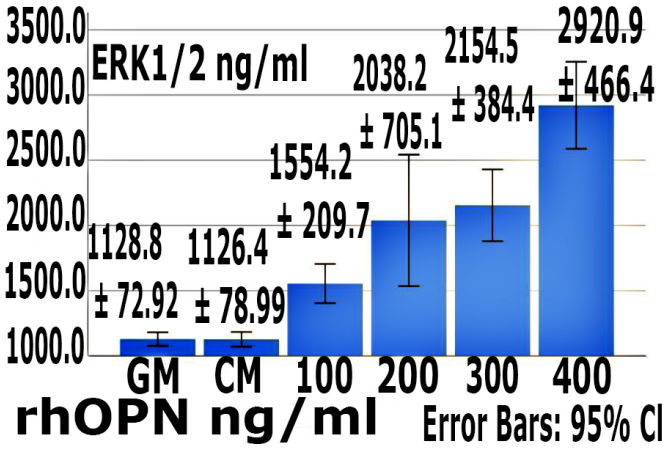


**FIGURE 10. Effects of increasing concentrations of rhOPN on ERK1/2 Signal Molecule in Ishikawa.** rhOPN: recombinant human osteopontin; ERK1/2: extracellular signal regulatory kinase 1/2; GM: growth medium; CM: conditioned medium; CI: confidence interval.

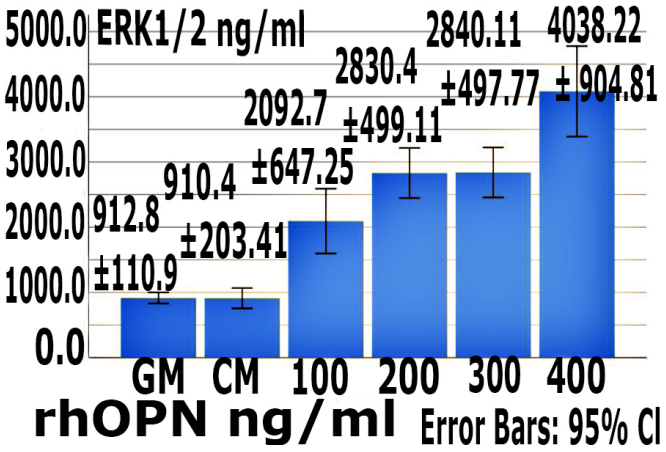


**FIGURE 11. Effects of increasing concentrations of rhOPN on ERK1/2 Signal Molecule in HUVEC.** rhOPN: recombinant human osteopontin; ERK1/2: extracellular signal regulatory kinase 1/2; GM: growth medium; CM: conditioned medium; CI: confidence interval.

of OPN or in the signaling pathway will play an important role in the development and progression of endometrial cancers [4, 15].

During EMT, epithelial cells lose their characteristic properties. They acquire mesenchymal features such as weakening of cell-cell adhesion, increased motility and spread of cells, development of resistance to apoptosis, and changes in cellular morphology. It can be suggested that EMT increases migration in tumour cells and gives more metastatic potential [16, 17]. Fedarko *et al.* [18] found that OPN levels increased in patients with breast, lung, and prostate cancer but not in patients with colon cancer. Li *et al.* [4] investigated the effects of rhOPN (0, 100, 200, 300 and 400 ng/mL) on migration and EMT in HEC-1A (Human Endometrial Carcinoma Cell) and the molecules in signaling pathways. The highest proliferation was observed at the 300 ng/mL level of OPN on the 4th day. As the rhOPN levels of HEC-1A increased so did the number of migrating cells. They reported that rhOPN increased the secretion of MMP-2 (Matrix metalloproteinase-2), an EMT related molecule, in HEC-1A. Li *et al.* [4] also measured metalloproteases. However, the present study did not aim to determine the levels of major metalloproteases. In the migration assay of the present study, the number of cells could only be assessed from images due to the lack of software in the camera system, which is a limitation of our study.

Carvalho *et al.* [19] found that rhOPN increases proliferation in a dose-dependent manner in human bone marrow mesenchymal stem cells. They also investigated the angiogenic potential of rhOPN and osteocalcin in HUVEC and reported a significant increase in the number of tube structures. By adding rhOPN 1 $\mu$g/mL to the HUVEC, they planted these cells on Matrigel and discovered that the tube structures and branching increased in the same way at 72 hours [19]. The present study showed that the number of tube structures formed by HUVEC and their branching points significantly increased between the 16th and 18th hours at the highest dose of rhOPN.

Cho *et al.* [11] investigated whether the OPN molecule can be a biomarker and its role in prognosis of endometrial cancer. They showed that the preoperative plasma OPN level measured by ELISA was very high compared to the control group. They also found that plasma OPN levels were associated with tumour stage, grade, myometrial infiltration, cytology and histological type [11]. Moreover, it was recommended in the literature that further studies should be conducted by adding HE4 and CA 72-4 (Cancer Antigen 72-4) OPN to the panel due to the low sensitivity of CA 125 in the detection of early-stage ovarian cancer [20, 21].

Rani *et al.* [22] found OPN levels to be higher in ovarian cancer and showed that it has better specificity than CA 125. They say that OPN can better distinguish benign and malignant ovarian masses compared to CA 125 [22].

In a study by Al-Maghrabi *et al.* [23], nonneoplastic endometrial tissues were shown to have higher OPN expression compared to cancerous endometrial tissues through the immunohistochemistry method. Besides, increased expression of OPN in endometrial carcinoma was found to be associated with a better survival outcome, but no relationship was demonstrated with other prognostic factors. The conflicting results were attributed to technical factors.

Shi *et al.* [24] suggested that OPN may be a target molecule associated with the prognosis of patients with lung cancer and recurrence/metastasis of lung cancer. They showed that OPN plays a decisive role in lung cancer cell motility, proliferation and EMT formation through activation of OPN-PI3K and OPN-MEK pathways. They found that PI3K and MEK inhibitors reduced the EMT and biological behavior process in lung cancer cells, possibly by altering vimentin-related cytoskeletons. They stated that OPN could be a metastasis-related or specific biomarker for lung cancer and a potential target for antimetastatic therapy [24]. These findings support the results of the present study.

In a study by Qin *et al.* [25], OPN expression was positively correlated with age, FIGO (The International Federation of Gynecology and Obstetrics) stage, tumour size, lymphovascular invasion and unfavourable prognosis. They also found that OPN plays a role in immunosuppression, hypoxia,

high glycolytic metabolism, apoptosis, angiogenesis, EMT and multiple signalling pathways (p53 pathway, PI3K/Akt pathway, IL6/STAT3 (Interleukin-6, Signal transducer and activator of transcription 3) signalling, mTORC1 (Mammalian target of rapamycin complex 1) signalling and KRAS (Kirsten Rat Sarcoma Viral Oncogene Homologue) signalling). In this study, it was shown that high OPN expression increased the immunosuppressive state in cervical cancer. Furthermore, high expression of OPN was associated with poor survival and therapeutic resistance in cervical cancer. They propose that OPN represents a potential therapeutic target and favourable prognostic factor in cervical cancer patients [25].

Poleboyina *et al*. [26] concluded that OPN is one of the most up-regulated genes in cervical cancer and Entrectinib is a promising potential OPN inhibitor to reduce cervical cancer progression.

Kariya *et al*. [27] explained that tumours are surrounded by environmental components called the tumour microenvironment (TME). Recent evidence suggests that the interaction between tumour cells and the TME modulates tumour formation, tumour cell invasion, metastasis, chemoresistance and immune response, leading to tumour development and aggressiveness. One of the components of TME is osteopontin. OPN is a matricellular protein secreted by tumour cells, endothelial cells, fibroblast cells and also by immune cells within the TME. It is increasingly recognised that OPN is a critical factor in tumour progression. OPN is expressed in many normal cells and plays important roles in physiological processes such as cell adhesion, migration, proliferation, survival, differentiation and immune modulation. anti-OPN drugs can target the OPN molecule not only in tumour tissues but also in normal tissues. To avoid these problems, it is necessary to develop tumour cell-specific OPN-targeting drugs [27].

EMT has a key role in cancer metastasis [17]. The OPN molecule stimulates the formation of EMT in cells through two different signaling pathways, and these pathways contain different molecules. The first one is the signaling pathway mediated by the $\alpha v\beta 3$ receptor, where the ERK1/2 molecule is expressed and increases motility, migration, and invasion in cells. The other one is the pathway in which proliferation and angiogenesis are stimulated in cells in which the CD44 receptor-mediated PI3K molecule is expressed [7]. There is a limited number of studies investigating the effect of OPN on EMT in Ishikawa. The current study is original research in which all relevant studies were evaluated in terms of the proliferation and migration of cells, which are the steps of angiogenesis formation, and the formation of tube-like structures *in vitro*. In addition, the two important signaling molecules in tumour progression, PI3K and ERK1/2, which are activated following the occurrence of OPN-receptor binding, were detected at the molecular level in HUVEC and Ishikawa medium.

The present study was conducted *in vitro* only and by applying different doses of rhOPN to HUVEC and Ishikawa cell cultures. The results obtained from this study are supported by some similar studies in the literature [4, 14]. Further studies could be designed *in vivo* using the OPN inhibitors. Also, whether preoperative and postoperative blood OPN levels can be used to detect recurrences in patients with endometrial cancer remains to be resolved.

## 5. Conclusions

The finding that rhOPN stimulates proliferation and migration in cells and the formation of tube-like structures *in vitro* in HUVEC and Ishikawa cultures suggests that this molecule can be a candidate for a prognostic marker in endometrial cancer and may be an important molecule to bring metastasis under control. *In vitro* cellular migration and angiogenesis that are triggered by PI3K and ERK1/2 molecules increase depending on rhOPN concentrations.

### AVAILABILITY OF DATA AND MATERIALS

The data are contained within this article.

### AUTHOR CONTRIBUTIONS

EK—designed the research study, performed the research, analyzed the data, wrote the manuscript. TA—designed the research study, performed the research, analyzed the data. He advised Evrim Kardelen at every stage of the study. HY—wrote the manuscript. He provided consultancy to Evrim Kardelen at every stage of the study. All authors read and approved the final manuscript.

### ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval is not required for cell culture studies. Therefore, ethical approval was not obtained for this study.

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### REFERENCES

[1] Braun MM, Overbeek-Wager EA, Grumbo RJ. Diagnosis and management of endometrial cancer. American Family Physician. 2016; 93: 468–474.

[2] Du X, Jiang T, Sheng X, Gao R, Li Q. Inhibition of osteopontin suppresses *in vitro* and *in vivo* angiogenesis in endometrial cancer. Gynecologic Oncology. 2009; 115: 371–376.

[3] Kerenidi T, Kazakou AP, Lada M, Tsilioni I, Daniil Z, Gourgoulianis KI. Clinical significance of circulating osteopontin levels in patients

with lung cancer and correlation with VEGF and MMP-9. Cancer Investigation. 2016; 34: 385–392.
[4] Li Y, Xie Y, Cui D, Ma Y, Sui L, Zhu C, *et al*. Osteopontin promotes invasion, migration and epithelial-mesenchymal transition of human endometrial carcinoma cell HEC-1A through AKT and ERK1/2 signaling. Cellular Physiology and Biochemistry. 2015; 37: 1503–1512.
[5] Ramachandran S, Kwon K, Shin S, Kwon S, Cha S, Lee H, *et al*. Regulatory role of osteopontin in malignant transformation of endometrial cancer. Molecular Biology Reports. 2013; 40: 3623–3629.
[6] Auerbach R, Lewis R, Shinners B, Kubai L, Akhtar N. Angiogenesis assays: a critical overview. Clinical Chemistry. 2003; 49: 32–40.
[7] Grant DS, Yenisey C, Rose RW, Tootell M, Santra M, Iozzo RV. Decorin suppresses tumor cell-mediated angiogenesis. Oncogene. 2002; 21: 4765–4777.
[8] Norrby K. Angiogenesis: new aspects relating to its initiation and control. APMIS. 1997; 105: 417–437.
[9] Brooks PC. Cell adhesion molecules in angiogenesis. Cancer and Metastasis Reviews. 1996; 15: 187–194.
[10] Güllüoğlu MG, Dizdaroğlu F. Tumor angiogenesis. Turkish Journal of Surgery. 2000; 16: 279–290.
[11] Cho H, Kang ES, Kim YT, Kim J. Diagnostic and prognostic impact of osteopontin expression in endometrial cancer. Cancer Investigation. 2009; 27: 313–323.
[12] Wai PY, Kuo PC. The role of osteopontin in tumor metastasis. Journal of Surgical Research. 2004; 121: 228–241.
[13] Yang M, Jiang C, Chen H, Nian Y, Bai Z, Ha C. The involvement of osteopontin and matrix metalloproteinase-9 in the migration of endometrial epithelial cells in patients with endometriosis. Reproductive Biology and Endocrinology. 2015; 13: 95.
[14] Kang Y, Forbes K, Carver J, Aplin JD. The role of the osteopontin-integrin $\alpha v\beta 3$ interaction at implantation: functional analysis using three different *in vitro* models. Human Reproduction. 2014; 29: 739–749.
[15] Hahne JC, Meyer SR, Kranke P, Dietl J, Guckenberger M, Polat B, *et al*. Studies on the role of osteopontin-1 in endometrial cancer cell lines. Strahlentherapie Und Onkologie. 2013; 189: 1040–1048.
[16] Wang J, Wei Q, Wang X, Tang S, Liu H, Zhang F, *et al*. Transition to resistance: an unexpected role of the EMT in cancer chemoresistance. Genes & Diseases. 2016; 3: 3–6.
[17] Sun T, Qin Y, Zhong W. Epithelial-mesenchymal transition and its regulation in tumor metastasis. In Xu K (ed.) Tumor metastasis (pp. 217–239). 1st edn. IntechOpen: Rijeka. 2016.
[18] Fedarko NS, Jain A, Karadag A, Van Eman MR, Fisher LW. Elevated serum bone sialoprotein and osteopontin in colon, breast, prostate, and lung cancer. Clinical Cancer Research. 2001; 7: 4060–4066.
[19] Carvalho MS, Cabral JM, da Silva CL, Vashishth D. Synergistic effect of extracellularly supplemented osteopontin and osteocalcin on stem cell proliferation, osteogenic differentiation, and angiogenic properties. Journal of Cellular Biochemistry. 2019; 120: 6555–6569.
[20] Bast RC, Raamanathan A, Gentry-Maharaj A, Skates S, Baggerly KA, Fourkala R, *et al*. Validation of a multi-marker panel for early detection of ovarian cancer. Journal of Clinical Oncology. 2016; 34: 5570–5570.
[21] Guo J, Yang WL, Pak D, Celestino J, Lu KH, Ning J, *et al*. Osteopontin, macrophage migration inhibitory factor and anti-interleukin-8 autoantibodies complement CA125 for detection of early stage ovarian cancer. Cancers. 2019; 11: 596.
[22] Rani S, Sehgal A, Kaur J, Pandher D, Punia R. Osteopontin as a tumor marker in ovarian cancer. Journal of Mid-Life Health. 2022; 13: 200.
[23] Al-Maghrabi H, Gomaa W, Al-Maghrabi J. Increased osteopontin expression in endometrial carcinoma is associated with better survival outcome: Ginekologia Polska. 2020; 91: 73–78.
[24] Shi L, Hou J, Wang L, Fu H, Zhang Y, Song Y, *et al*. Regulatory roles of osteopontin in human lung cancer cell epithelial-to-mesenchymal transitions and responses. Clinical and Translational Medicine. 2021; 11: e486.
[25] Qin S, Yi L, Liang Y, Chen Y, Wang W, Liao Y, *et al*. Biological and clinicopathological characteristics of OPN in cervical cancers. Frontiers in Genetics. 2022; 13: 836509.
[26] Poleboyina PK, Alagumuthu M, Pasha A, Ravinder D, Pasumarthi D, Pawar SC. Entrectinib a plausible inhibitor for osteopontin (SPP1) in cervical cancer—integrated bioinformatic approach. Applied Biochemistry and Biotechnology. 2023; 195: 7766–7795.
[27] Kariya Y, Kariya Y. Osteopontin in cancer: mechanisms and therapeutic targets. International Journal of Translational Medicine. 2022; 2: 419–447.

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