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Tyrosine kinase A receptor (trkA): A potential marker in epithelial ovarian cancer

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ABSTRACT

Objectives. To evaluate the role of trkA receptor as a potential tumor marker in serous epithelial ovarian cancer and its relationship with the angiogenic factors expression as vascular endothelial growth factor (VEGF) and nerve growth factor (NGF). Additionally, to examine whether NGF and VEGF secreted by epithelial ovarian cancer (EOC) explants and from epithelial ovarian cancer cell line (A2780) are involved in the process of angiogenesis, such as cellular proliferation, migration and differentiation of the human endothelial cell line (EA.hy926).

Methods. The mRNA levels of VEGF, NGF and trkA receptors were measured using PCR in 60 ovarian samples. Cellular localization and semi-quantitative estimation of VEGF, NGF, total trkA and p-trkA was performed using IHC in epithelial cells. NGF, total trkA and p-trkA protein were also evaluated in endothelial cells from the same tissues. Human endothelial cell line EA.hy926 was cultured with conditioned media obtained from both EOC explants and from the A2780 cell line, with or without NGF stimulus.

Results. Significantly higher levels of NGF, total trkA and p-trkA protein expressions were observed in epithelial and endothelial cells in poorly differentiated EOC versus normal ovary. Interestingly, the p-trkA receptor expression level showed the most significant difference and its presence was only found in borderline tumor and EOC samples indicating the importance of trkA receptor in EOC as a potential tumor marker.
A significant increase in proliferation, migration and differentiation of EA.hy926 cells was observed with NGF, and this effect was significantly reverted when NGF was immuno-blocked and when a trkA inhibitor was used, showing that NGF is an important angiogenic factor in EOC by activating its trkA receptor.

Conclusion. These results indicate that p-trkA may be considered as a new potential tumor marker in EOC, and that NGF may also act as a direct angiogenic factor in EOC.

Introduction

About 80–90% of ovarian cancers are of epithelial origin (EOC). These cancers tend to be highly invasive, respond poorly to therapies and are usually detected at advanced stages, resulting in low survival [1,2]. EOC etiology has not been adequately determined, although the ovarian surface epithelial-mesenquimal conversion hypothesis has gained acceptance recently [3].

Cancer cells display uncontrolled growth partly due to signals generated by growth factor receptors including tyrosin-kinase receptors [4,5]. The tyrosin-kinase receptor, trkA, is over-expressed in various carcinomas, including ovarian cancer [6,7]. Its ligand, the nerve-growth factor (NGF), is involved in angiogenesis [8], a key feature of tumor development and progression. NGF indirectly acts in ovarian angiogenesis by increasing vascular endothelial growth factor (VEGF) expression [9,10]. Over-expression of VEGF isoforms 121, 165 and 189 has been found in EOC [9], whereas further expression of all isoforms occurs with NGF stimulation [9–11]. Nevertheless, NGF and trkA involvement in EOC initiation and progression have not been adequately established.

Increased p-trkA expression has been described in advanced ovarian carcinomas [12], and the co-expression of NGF with angiogenesis-related molecules and p-trkA expression in endothelial cells suggest that both the *in vitro* and *in vivo* pro-angiogenic roles of NGF could be relevant in ovarian cancer. Therefore, the objective of the present study was to examine if trkA receptor may be used as a potential tumor marker in EOC. Furthermore, our interest was also to evaluate the role of NGF as a direct angiogenic

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factor in the ovarian angiogenesis. For this purpose, we examine the trkA, p-trkA and NGF expression in ovarian epithelium and endothelium, and also the effect of NGF and its receptor on endothelial proliferation, migration and differentiation, using an *in vitro* cell culture system.

Materials and methods

Tissue samples

Sixty human ovarian tissues were obtained from Hospital Clinico, Universidad de Chile and Clinica Las Condes. Patients signed an informed consent, which was approved by the Ethics Committee of both institutions. Ten normal ovarian samples (I-OV) were derived from women subjected to hysterectomy with oophorectomy due to non-ovarian pathologies. Remaining samples were from women diagnosed with ovarian tumors: ten benign tumors (Ben-T) and ten borderline tumors (Bord T). Ten of the epithelial ovarian cancer tissues were classified as well differentiated serous epithelial ovarian cancer (EOC I), ten as moderately differentiated serous epithelial ovarian cancer (EOC II) and ten as poorly differentiated serous epithelial ovarian cancer (EOC III). The classification of each sample was performed by two experienced pathologists.

All participants in this study were peri-menopausal or post-menopausal women, age range 46–68 ± 3,5 years old.

Immunohistochemistry

Tissues were previously formalin fixed, paraffin included and sliced into several 4–6 μm sections. One section was selected for morphological analysis and the remaining for immunohistochemistry, performed as described previously [9]. The primary antibodies and working dilutions used were the following: VEGF (Abcam plc, Cambridge, UK; 1:1000), NGF (as described previously [9], 1:750), trkA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:300), p-trkA (Abcam plc, Cambridge, UK; 1:100). Sections were examined under an Olympus BX51 optical microscope (Olympus Corporation, Tokyo, Japan). Images were acquired with a MicroPublisher 3.3 RTV camera (Q Imaging, Surrey, BC, Canada). The immunohistochemical evaluation for each protein was evaluated by three independent observers blinded to samples category, and the positive staining was assessed in at least 1000 cells per sample The semiquantitative analysis of the immunohistochemistry was performed by the percentage of positive cells.

RT-PCR

Total RNA extraction, primers for NGF, VEGF and β-actin mRNA amplification and PCR conditions have been reported previously [9,11]. Primers for trkA mRNA amplification and PCR conditions are described in Table 1.

PCR products were resolved using agarose gel electrophoresis. To visualize PCR bands, we used a transilluminator UVP with Doc-it Image Acquisition and 1 D Analysis software (UVP, Inc. Laboratory Products, Upland, CA, USA). Human granulosa cells were employed as a positive control, as previously reported [11,13].

EOC explant cultures and cell line cultures

EOC specimens were cut into pieces (approximately 100 mg) and incubated for 2 h at 37 °C with NGF (100 ng/ml) in a serum-free DMEM/Ham-F12 [9].

EA.hy926: This cell line was obtained by hybridizing human umbilical vein endothelial cells with the A549/8 human lung carcinoma cell line [14].

A2780: A2780 is a drug-sensitive human ovarian cancer cell line with epithelial morphology that was established using tumor tissue from an untreated patient [15].

EA.hy926 cells were cultured in Iscove's modified Dulbecco's medium (IMDM, GIBCO® Invitrogen Corporation, Camarillo, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone™ Thermo Fisher Scientific, Rochester, NY, USA), in the presence of 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate and 25 g/ml Amphoterecin B (Hyclone™ Thermo Fisher Scientific, Rochester, NY, USA) at 37 °C with 5% CO_2.

A2780 cell line was cultured in DMEM/Ham-F12 medium (Sigma-Aldrich Co. Saint Louis, MO, USA) supplemented with 10% FBS in the presence of the same antibiotics and antimycotic described above.

Conditioned medium preparation

A2780 cells were cultured in DMEM/Ham-F12 medium (Sigma-Aldrich Co. Saint Louis, MO, USA) with 10% fetal bovine serum (Hyclone™ Thermo Fisher Scientific, Rochester, NY, USA), until the culture reached 80% confluence. Cells were then washed twice with Dulbecco's Phosphate Buffered Saline (DPBS, GIBCO® Invitrogen Corporation, Camarillo, CA, USA) and cultured in serum-free medium for additional 24 h. Under these conditions, cells were exposed to NGF 50 ng/mL; (Sigma-Aldrich Co. Saint Louis, MO, USA), 100 nM tyrosine kinase inhibitor K252a ($IC_{50} = 3$ nM; Sigma-Aldrich Co. Saint Louis, MO, USA) and NGF antibody (NGF Ab, dilution 1:1000). After 18 h incubation, the culture media (conditioned medium) was collected for cell proliferation, migration, and differentiation assays. One aliquot was stored at −80 °C until ELISA for VEGF assay (Quantikine® human VEGF Immunoassay, R&D Systems, Inc. Minneapolis, Mn, USA) as previously described [9].

The conditioned media from EOC explants were obtained by treatment with NGF and a combination of NGF plus anti-NGF or NGF plus K252a. After incubation, the cultured medium was collected and one aliquot was stored at −80 °C to measure VEGF using ELISA.

Immunocytochemistry

In order to evaluate the protein expression and localization of NGF, trkA receptor, VEGF and VEGF-R1/VEGF-R2 receptors, both EA.hy926 and A2780 cells were fixed in iced cold methanol for 30 min at −20 °C. Immunocytochemistry was carried out subsequently as previously described [11]. The primary antibodies and working solutions used were the following: NGF [9] (1:700), trkA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 0.4 μg/ml), VEGF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 20 μg/ml), VEGF receptor 1 (Abcam plc, Cambridge, UK; 1:250), VEGF receptor 2 (Abcam plc, Cambridge, UK; 50 μg/ml). DAB was used as a chromogen. The cells were counter-

Table 1
Conditions for PCR.

Gene	Forward and reverse primers 5′-3′	PCR program (35 cycles)		PCR product size (bp)	Accession no.
		T°	Time		
trkA	F: AACCTCACCATCGTGAAGAGTGGT	94 °C	1 min	900	NM_001012331.1
	R: GGTTGAACTCGAAAGGGTTGTCCA	55 °C	1 min		
		72 °C	2 min		
		72 °C	10 min		

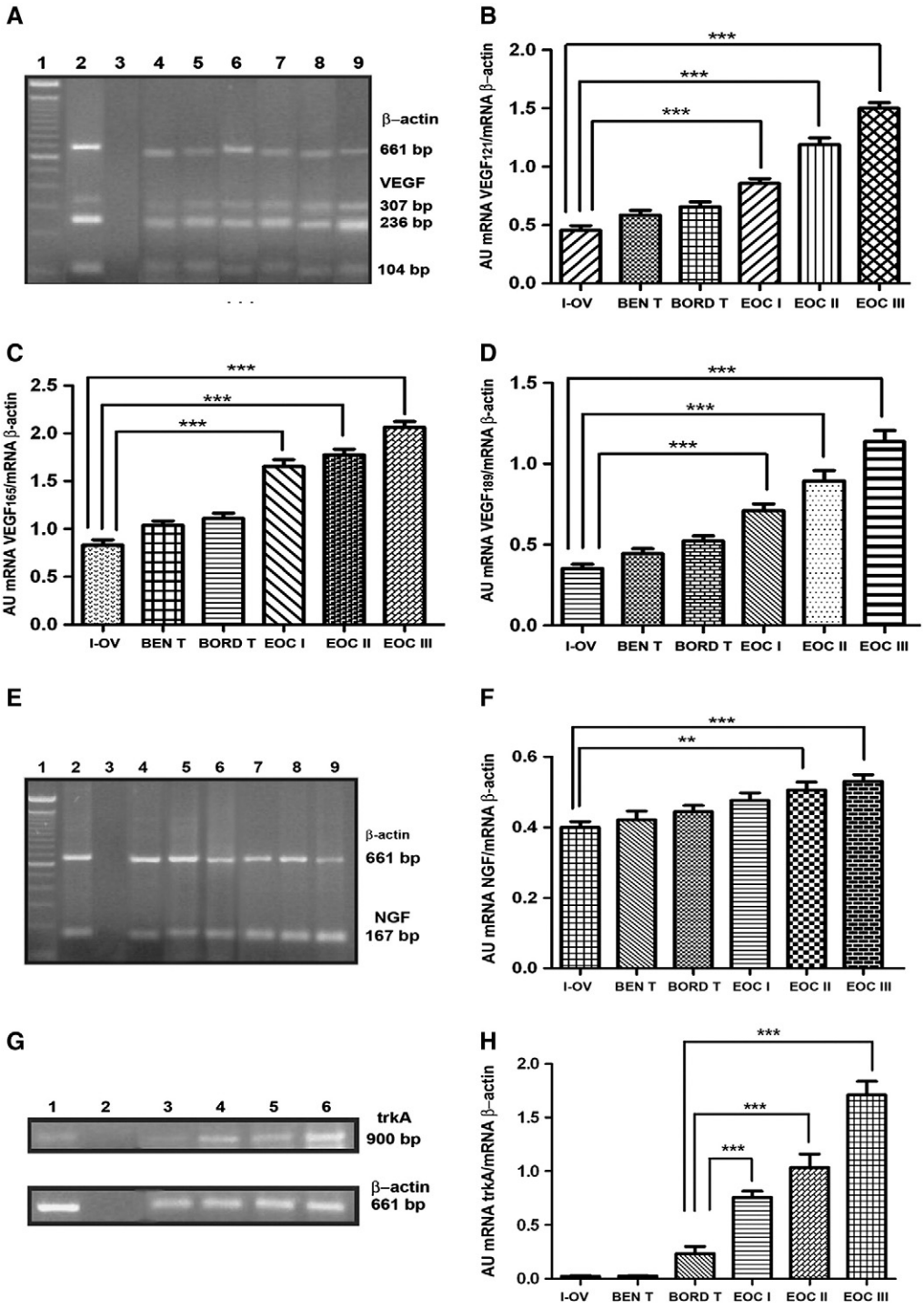


Fig. 1. NGF, trkA and VEGF mRNA levels in serous ovarian tumors. A: Representative agarose gel of ten independent experiments for the gene expression of VEGF. Line 1: Ladder 100 bp; 2: Positive control corresponding to human granulosa cells; 3: Negative control for the PCR; 4: Normal ovaries (OV-I); 5: Benign tumors (Ben T); 6: Borderline tumor (Bord T); 7: Well differentiated epithelial ovarian cancer (EOC-I); 8: Moderately differentiated epithelial ovarian cancer (EOC-II); 9: Poorly differentiated epithelial ovarian cancer (EOC-III). The PCR product for β-actin corresponds to the 661 bp band, the 104 bp band corresponds to the VEGF121 transcript, the 236 bp band corresponds to the VEGF165 transcript and the 307 bp band corresponds to VEGF189 transcript. B: Semi-quantitative analysis of mRNA levels of VEGF 121. ***$p<0.001$ I-OV. vs. EOC I, II and III. C: Semi-quantitative analysis of the VEGF 165 mRNA levels. ***$p<0.001$ I-OV. vs. EOC I, II and EOC III. D: Semi-quantitative analysis of the VEGF 189 mRNA levels. ***$p<0.001$ I-OV. vs. EOC I, EOC II and EOC III. E: Representative agarose gel of the NGF mRNA levels from 10 independent experiments. Line 1: Ladder 100 bp; 2: Positive control corresponding to human granulosa cells; 3: Negative control for the PCR; 4: I-OV; 5: Ben T; 6: Bord T; 7: EOC-I; 8: EOC-II; 9: EOC-III. The 661 bp band corresponds to -actin and the 167 bp band corresponds to the NGF transcript. F: Semi-quantitative analysis of the NGF mRNA levels from ten ovarian tissues corresponding to the six study groups. **$p<0.01$ I-OV vs. EOC II, ***$p<0.001$ I-OV vs. EOC III. G: Representative agarose gel of the trkA mRNA levels from 10 independent experiments. Line 1: Ladder 100 bp; 2: Positive control corresponding to human granulosa cells; 3: Negative control for the PCR; 4: I-OV; 5: Ben T; 6: Bord T; 7: EOC-I; 8: EOC-II; 9: EOC-III. The PCR product for β-actin corresponds to the 661 bp band, the 900 bp band corresponds to the trkA transcript. H: Semi-quantitative analysis of the trkA mRNA levels from the ovarian tissues corresponding to the six study groups. **$p<0.01$ I-OV vs. EOC II, ***$p<0.001$ I-OV vs. EOC III. **$p<0.01$ Bord T vs. EOC I, ***$p<0.001$ Bord T vs. EOC II y III, ***$p<0.001$ EOC I vs. EOC III, **$p<0.01$ EOC II vs. OEC III. Bars represent the mean ± SEM of ten samples from each group of study, expressed in arbitrary units (AU) of transcript of interest mRNA/β-actin mRNA.

stained with hematoxylin, dehydrated in ethanol and cleared in xylene, coverslipped and examined under an Olympus optical microscope with a repeatability of ± 1.0% (Olympus BX51, Olympus Corporation, Tokyo, Japan). Images were acquired with a MicroPublisher 3.3 RTV camera (Q Imaging, Surrey, BC, Canada).

Cell proliferation assay

CellTiter 96®

To measure the amount of cell proliferation, we used the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA). Briefly, EA.hy926 cells were kept in serum-free Iscove's medium for 24 h. After this time, EA.hy 926 cells were plated on a 96-well plate (5×10^3 cells/well) and incubated in a conditioned medium for 24 h. The assay was then performed following manufacturer instructions. The absorbance at 570 nm was recorded in a microtiter plate reader with a repeatability of ± 0.5% (EL800, BioTek Instruments, Inc. Winooski, Vermont, USA).

BrdU incorporation

The cell proliferation assay was performed using BrdU Cell Proliferation Assay (Chemicon International, Inc. Temecula, CA, USA) in a 96-well plate. EA.hy926 cells were cultured in serum-free medium and 5×10^3 cells per well were seeded and incubated in a conditioned medium for 24 h. BrdU was added for incorporation into proliferating cells and incubated for additional 24 h. BrdU was detected immunochemically following manufacturer instructions. Finally, the absorbance at 450 nm was measured using a microtiter plate reader (EL800, BioTek Instruments, Inc. Winooski, Vermont, USA).

Migration assay

Transwell polycarbonate membrane inserts (10 mm diameter, 8 μm pore size; Nunc™ Thermo Fisher Scientific, Rochester, NY, USA) were coated with 50 μl Matrigel (Growth Factor Reduced BD Matrigel™ Matrix, BD Biosciences, Bedford, MA, USA) and allowed to gel for 3 h at 37 °C in the upper chamber. After solidification, EA.hy926 cells (1×10^5) were suspended in 400 μl conditioned medium and plated on the upper chambers, which were then mounted to the lower chambers (24-well plate with 1 ml Iscove's medium supplemented with 10% fetal bovine serum/well). Cell movement towards lower chamber was evaluated. The cells were incubated at 37 °C for 24 h. After incubation, non-migrated cells on the upper surface of the membrane were removed with a cotton swab. The cells that had migrated through would be found on the undersurface of the membrane after fixation with methanol. Once the membranes were detached from the transwell system, they were washed three times with DPBS (Dulbecco's Phosphate Buffered Saline, GIBCO® Invitrogen Corporation, Camarillo, CA, USA), and immunocytochemistry was performed as described above. Incubation with cytokeratin antibody (pan-cytokeratin antibody, sc-53405, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was carried out overnight at 4 °C in a 1:100 dilution in PBS containing 2% BSA. The immunoreaction was developed with 3,3′-diaminobenzidine as the chromogen. Membranes were counterstained with hematoxylin, mounted with gelatin, coverslipped and evaluated with Image-Pro Plus software (Media Cybernetics, Inc. Silver Spring, MD, USA) under an Olympus optical microscope (Olympus BX51, Olympus Corporation, Tokyo, Japan).

Differentiation assay (In vitro capillary formation)

A differentiation assay known as *in vitro* capillary formation was performed to evaluate tube formation of EA.hy926 in Matrigel. Cells were cultured for 24 h in serum-free Iscove's medium prior to the assay. Matrigel (180 μl/well) (Growth Factor Reduced BD Matrigel™ Matrix, BD Biosciences, Bedford, MA, USA) was dispensed into 24-well tissue culture plates according to the manufacturer's instructions. EA.hy926 cells were dispersed by trypsinization and counted, and 4×10^4 cells were re-suspended in 1 ml conditioned medium and plated on the surface of the Matrigel. The cells were then placed in a CO_2 incubator at 37 °C and periodically observed using an Olympus inverted routine microscope with a 10× objective (Olympus CKX41, Olympus Corporation, Tokyo, Japan) and photographed with a digital camera (Canon PowerShot A640, Canon Inc., Tokyo, Japan). The usual period of observation was 24 h. The extent of angiogenesis was scored as described in a previous report [16].

Statistical analysis

The data showed a parametric distribution, as assessed by the Kolmogorov–Smirnov test. An ANOVA and Bonferroni post-test were used to identify significant differences between groups. Significance was set at $p<0.05$. The statistical analysis was done with the program Ghaph Pad Prism 5

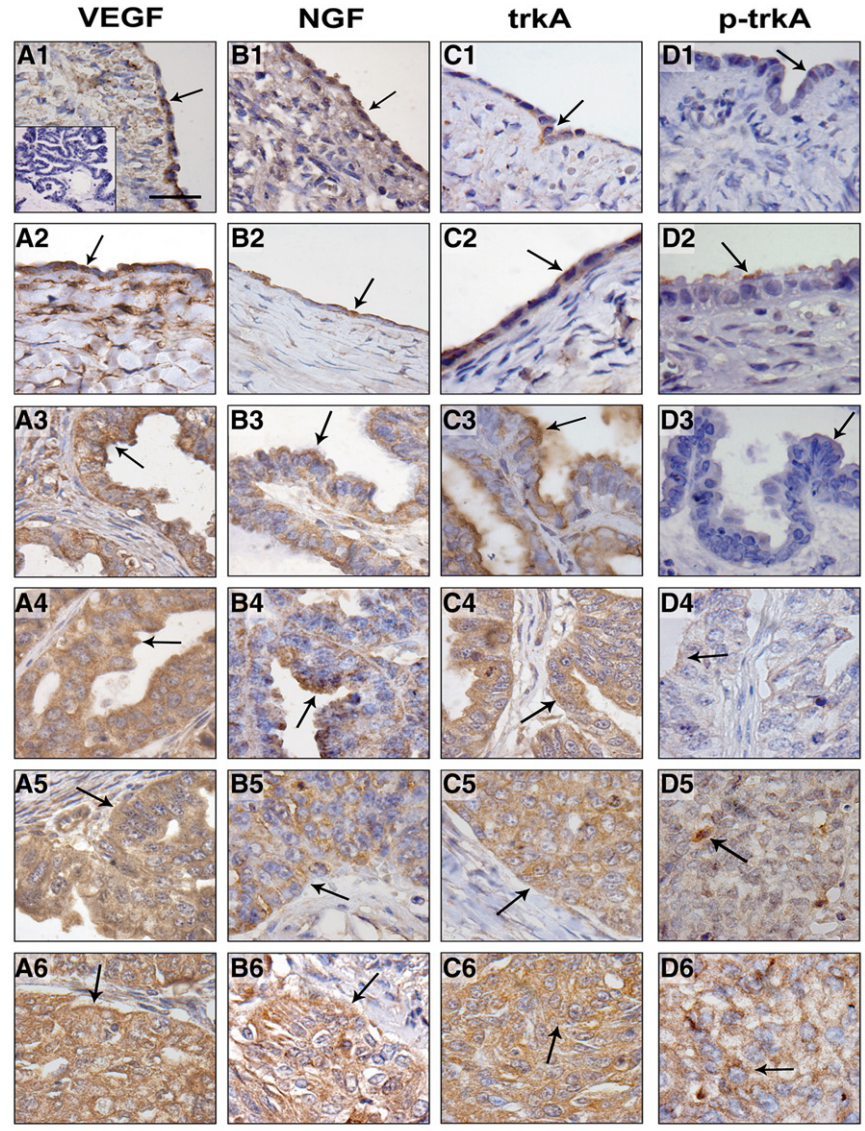
Results

VEGF, NGF and trkA mRNA levels in the different study groups

We found increased levels in the three alternative splicings for VEGF expressed in human ovarian tissues (transcript for VEGF121, VEGF165 and VEGF189). A representative agarose gel from ten independent experiments carried out in the ovarian tissues from the six study groups is shown in Fig. 1-A, and the semi-quantitative analysis for VEGF121 transcript is shown in Fig. 1-B. The three study groups with epithelial ovarian cancer (EOC-I, EOC-II and EOC-III) had significantly higher levels than inactive normal ovaries (I-OV) ($p<0.001$ with confidence interval of 0.89 ± 0.09 EOC-I; 1.22 ± 0.14 EOC-II; 1.54 ± 0.12 EOC-III and 0.45 ± 0.09 I-OV). In addition, significant differences were found among the three types of EOC ($p<0.001$ with confidence interval of 1.54 ± 0.12 EOC III vs 0.89 ± 0.09 EOC I and $p<0.01$ with confidence interval of 1.54 ± 0.12 EOC III vs 1.22 ± 0.14 EOC II).

Fig. 1-C shows that mRNA levels for VEGF165 were significantly higher in EOC-I, EOC-II and EOC-III as compared to inactive normal ovaries ($p<0.001$ with confidence interval of 1.75 ± 0.16; 1.89 ± 0.13; 2.19 ± 0.14 vs 0.88 ± 0.09), and that there were significant differences among different EOC grades, i.e. grade 3 versus grade 1 and grade 2 ($p<0.001$ and $p<0.01$, respectively with confidence interval 2.19 ± 0.14 vs1.75 ± 0.16 and 1.89 ± 0.13 respectively). Furthermore, significantly higher levels of VEGF189 product (Fig. 1-D) were found for well differentiated EOC versus inactive normal ovaries ($p<0.001$ with confidence interval of 0.73 ± 0.09EOC-I vs 0.36 ± 0.06), and for EOC-II

Fig. 2. Representative microphotographs and semi-quantitative analysis of VEGF, NGF, trkA and p-trkA immunodetection in the ovarian epithelium from inactive normal ovary to epithelial ovarian cancer grade III. Panel A: Immunodetection of VEGF. Panel B: Immunodetection of NGF. Panel C: Immunodetection of trkA. Panel D: Immunodetection of phosphorylated trkA (p-trkA). A1, B1, C1, D1: Normal ovary; A2, B2, C2, D2: Benign tumor; A3, B3, C3, D3: Borderline tumor; A4, B4, C4, D4: EOC-I; A5, B5, C5, D5: EOC-II; A6, B6, C6, D6: EOC-III. In the left inferior frame of the first microphotograph, a representative negative control for the IHC is shown. The arrows indicate positive epithelial stain in each of the microphotographs. The bar represents 50 m with a magnification of 1000×. E: Semi-quantitative analysis of the VEGF IHQ in epithelial cells of ovarian samples from the six study groups. *p<0,01 I-OV vs. EOC I, *p<0,01 I-OV vs. EOC II,**p<0,001 I-OV vs. EOC III. F: Semi-quantitative analysis of the NGF immunodetection in epithelial cells of ovarian samples from the six study groups. *p<0,01 I-OV vs. EOC II,**p<0,001 I-OV vs. EOC III. G: Semi-quantitative analysis of the total trkA immunodetection in epithelial cells of ovarian samples from the six study groups. ***p<0,001 I-OV vs. Bord T, EOC I, EOCII and EOC III. ***p<0,001 Ben T vs. EOC I, EOC II and EOC III. ***p<0,001 Bord T vs. EOC I, EOC II and EOC III. ***p<0,001 EOC I vs. EOC III. H: Semi-quantitative analysis of the p-trkA immunodetection in epithelial cells of ovarian samples from the six study groups, **p<0,01 EOC I vs. EOC III. The bars represent the mean ± SEM of 10 individual samples from each study group, expressed as the percentage of cells with positive stain.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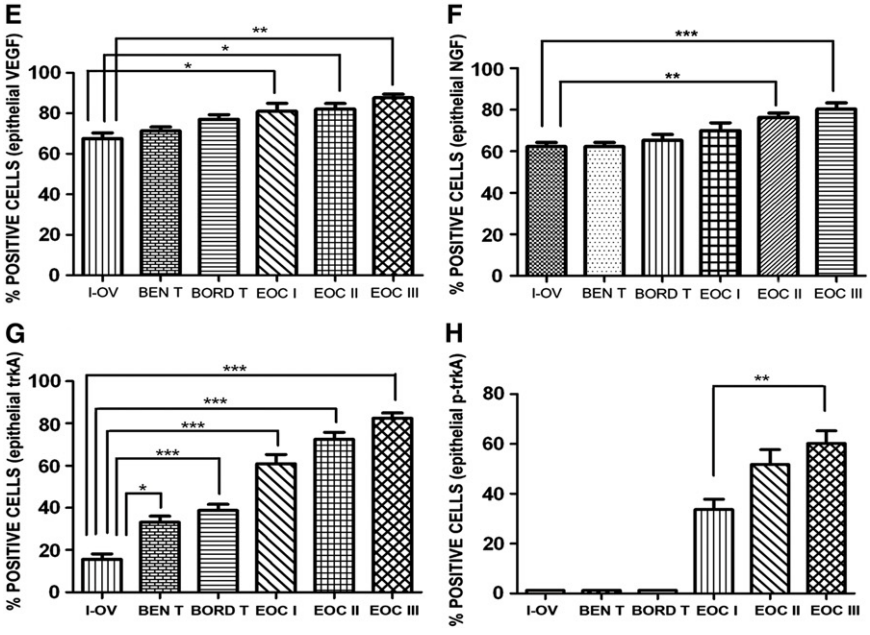
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% POSITIVE CELLS (epithelial VEGF)
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I-OV
BEN T
BORD T
EOC I
EOC II
EOC III

and EOC-III versus normal ovaries ($p<0.001$ and $p<0,001$, respectively with confidence interval 0.93 ± 0.15 and 1.17 ± 0.15 vs 0.36 ± 0.06 respectively). In addition, we found significant differences among epithelial ovarian cancers with different grades of differentiation: EOC-III versus EOC-I and EOC-II ($p<0.001$ and $p<0.05$, respectively with confidence interval 1.17 ± 0.15 and 0.93 ± 0.15 vs 0.36 ± 0.06 respectively).

The mRNA for VEGF165 and 121 were the predominant transcripts observed in all samples. However, the greatest difference (three-fold increase) was detected for VEGF189 transcript levels in poorly differentiated EOC versus normal ovary.

To determine the NGF mRNA levels in the different ovarian samples, PCR was carried out in ten independent experiments, which are shown in a representative agarose gel (Fig. 1-E). The semi-

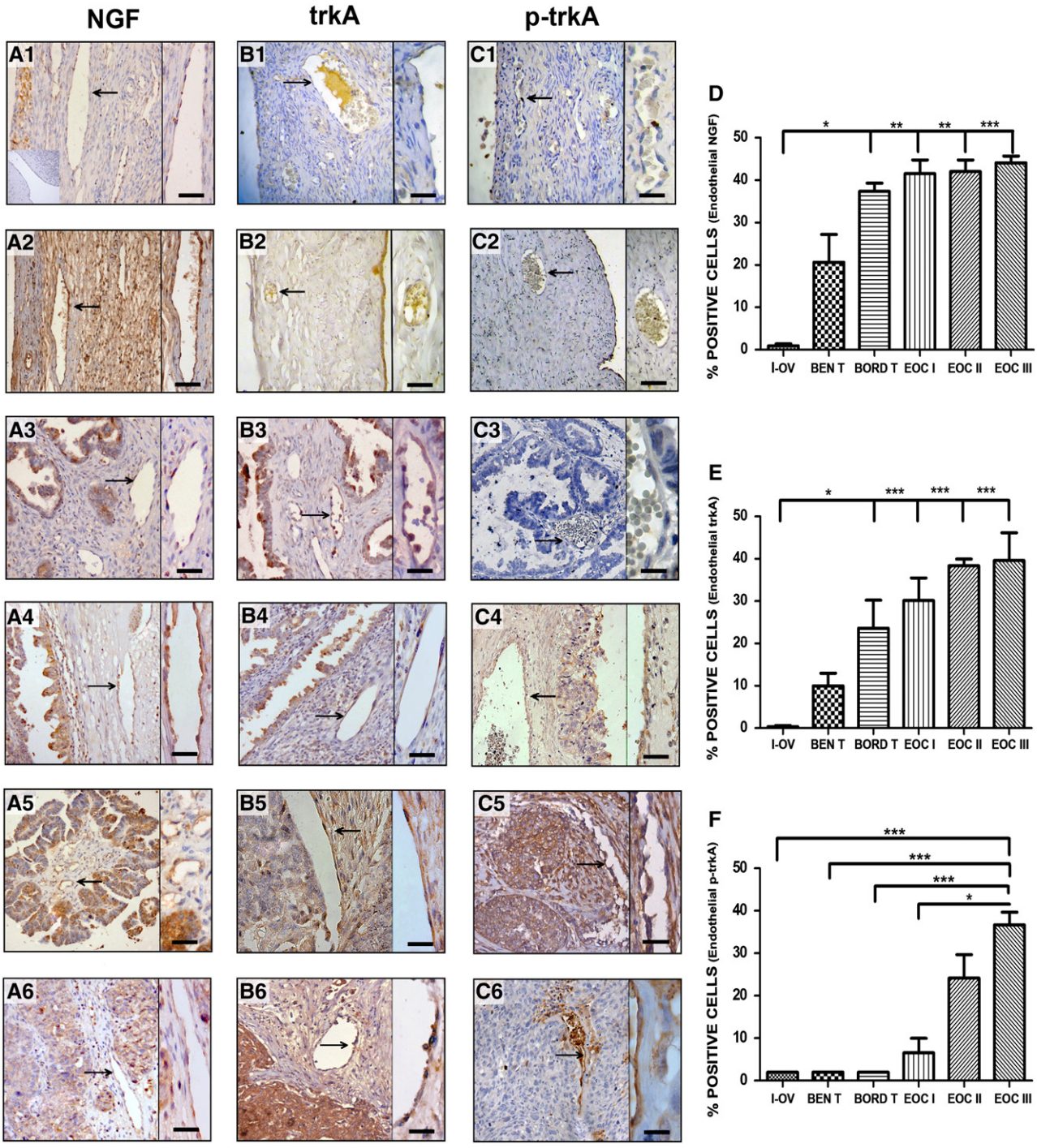

Fig. 3. Representative microphotographs and semi-quantitative analysis of NGF, trkA and p-trkA inmunodetection in endothelial cells of ovarian tissues from inactive normal ovary to poorly differentiated serous epithelial ovarian cancer. Panel A: Immunodetection of NGF. Panel B: Immunodetection of total trkA. Panel C: Immunodetection of phosphorylated trkA (p-trkA). A1, B1, C1, D1: Normal ovary; A2, B2, C2, D2: Benign tumor; A3, B3, C3, D3: Borderline tumor; A4, B4, C4, D4: EOC-I; A5, B5, C5, D5: EOC-II; A6, B6, C6, D6: EOC-III. The arrows indicate positive endothelial stain in each of the microphotographs. The bar represents 50 μm with a magnification of 1000×. D: The semi-quantitative analysis of the NGF IHQ in endothelial cells of ovarian samples from the six study groups. *$p<0.01$ I-OV vs. EOC I, *$p<0.01$ I-OV vs. EOC II,*$p<0,001$ I-OV, Ben T vs. EOC III. E: The semi-quantitative analysis of total trkA IHQ in endothelial cells of ovarian samples from the six study groups. **$p<0.01$ I-OV vs. EOC II, ***$p<0.001$ OV-I vs. COE III, *$p<0.05$ Ben T vs. COE III. F: The semi-quantitative analysis of p-trkA IHQ in endothelial cells of ovarian samples from the six study groups. *$p<0.05$ EOC I vs. EOC III. The bars represent the mean ± SEM of 10 individual samples from each study group, expressed as the percentage of cells with positive stain.

quantitative analysis for NGF mRNA is shown in Fig. 1-F, where we found that levels increased in accordance with loss of cell differentiation in ovarian cancer. Significantly higher levels were detected for EOC-II and EOC-III versus normal ovary ($p<0.01$; $p<0.001$ respectively with confidence interval of 0.527 ± 0.05 and 0.550 ± 0.04 vs 0.414 ± 0.04 respectively).

A representative agarose gel of trkA mRNA levels in ovarian tissues is shown in Fig. 1-G. In the semi-quantitative analysis for trkA mRNA levels, (Fig. 1-H), we found significantly higher levels in EOC than in borderline tumors ($p<0.001$ with confidence interval 0.793 ± 0.13 EOC-I; 1.104 ± 0.24 EOC-II and 1.779 ± 0.23 vs 0.236 ± 0.13 I-OV). Moreover, levels were significantly higher in EOC III than EOC-I and EOC-II ($p<0.01$ and $p<0.01$, respectively with confidence interval of 1.779 ± 0.23 vs 0.793 ± 0.13 and 1.104 ± 0.24 respectively) Also, trkA mRNA levels were significantly higher in EOC-I, EOC-II and EOC-III than in normal ovaries and benign tumors.

Immunodetection and semi-quantitative analysis of VEGF protein in epithelial cells of the ovarian tissues

Immunohistochemical detection of VEGF (Figs. 2: A1–A6) shows a positive granular staining uniformly distributed in the cytoplasm of ovarian epithelial cells of the samples from the six study groups. The semi-quantitative analysis of VEGF protein levels was assessed in each group by the Image Pro Plus 6.2 computational program and the results exhibit a significant increase between carcinoma samples EOC-I, EOC-II and EOC-III versus normal ovary ($p<0.05$, $p<0.05$ and $p<0.01$ respectively with confidence interval of 66 ± 25.8 and 87.2 ± 4.8; 86.4 ± 14.9 and 91 ± 6.8 respectively) (Fig. 2-E).

Immunodetection and semi-quantitative analysis of NGF, total trkA and p-trkA in epithelial and endothelial cells in ovarian tissues

Immunohistochemical evaluation for NGF was performed in ovarian samples from ten patients of each study groups which demonstrated a positive granular staining uniformly distributed in the cytoplasm of epithelial cells (Figs. 2B1 to B6). By the semi-quantitative analysis of NGF protein levels in epithelial cells, a significant increase was found between poorly and moderately differentiated EOC samples versus normal ovary ($p<0.01$ and $p<0.05$, respectively with confidence interval of 62.4 ± 16.3 and 79.6 ± 6.11 and 72.7 ± 13.4, respectively).(Fig. 2-F). Importantly, the Immunodetection of NGF was also found in endothelial cells of the same samples as shown in Figs. 3 (A1 to A6). The stained endothelial cells were measured as percentage of positive cells, where a significant increase was detected between borderline tumors, EOC I, EOC II and EOC III samples versus normal ovary ($p<0.05$, $p<0.01$, $p<0.01$ and $p<0.001$, respectively with confidence interval of 37.36 ± 9.44; 41.52 ± 9.72; 42.0 ± 5.99 and 44.09 ± 6.58 vs 0.92 ± 0.89 respectively). (Fig. 3-D).

The localization of trkA protein was detected in the cytoplasm of epithelial cells, because the antibody used recognizes the intracellular domain and it was found a positive granular staining for trkA. Representative micro-photography is shown in Figs. 2 (C1 to C6), and the localization of this protein in endothelial cells is shown in Figs. 3: B1 to B6 in all groups under study. The semi-quantitative analysis of the total trkA protein in epithelial and endothelial cells from all studied samples was performed in order to understand if the expression of this receptor may be useful as a possible tumor marker in EOC. The results in epithelial cells are shown in Fig. 2-G, where a steady, significant increase was observed between borderline tumors

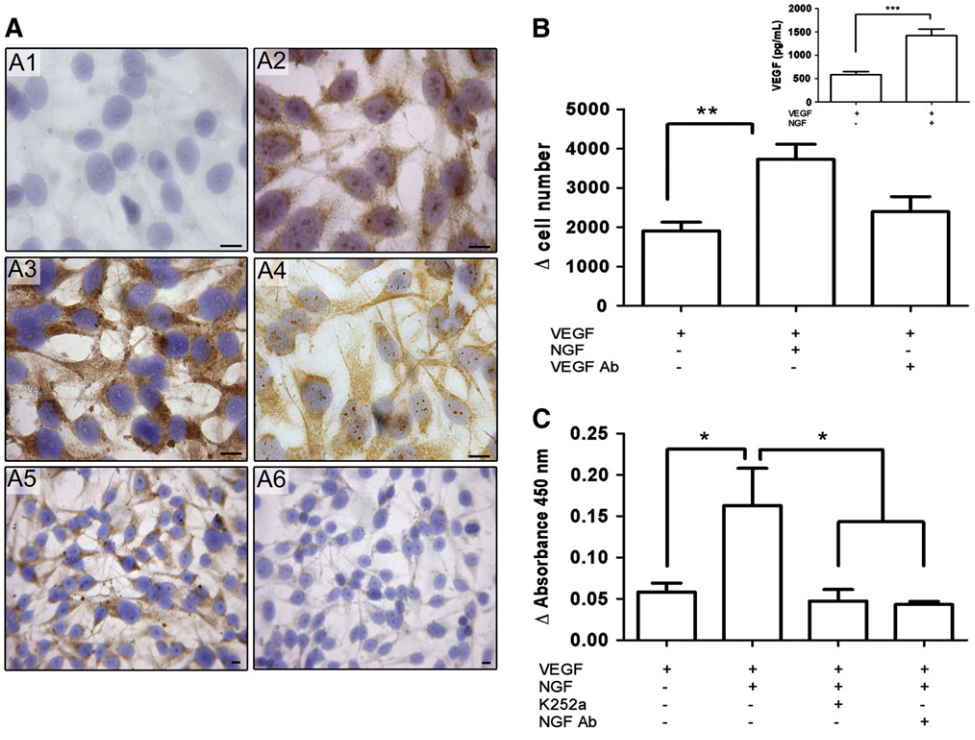

Fig. 4. Immunodetection on EA.hy926 cell line and evaluation of EOC cell proliferation. A: Representative microphotographs of trkA (A2), NGF (A3), VEGF (A4), VEGFR1 (A5) and VEGFR2 (A6) inmunodetection in EA.hy926 cell line. A negative control (A1) shows lack of staining in control cells incubated in the absence of the primary antibody. The bar represents 10 μm with a magnification of 1000× and 400× (VEGFR1 and VEGFR2). B: Effect of 24 h incubation with conditioned medium from EOC explants on EA.hy926 cell proliferation. A number of 5×10^3 EA.hy926 cells were plated, cultured and incubated with conditioned medium for 24 h and CellTiter 96® assay was used to evaluate cell proliferation. The results represent the mean ± SEM from a minimum of 3 independent experiments. **$p<0.01$. Insert: VEGF level detected by ELISA in conditioned medium from EOC explants. VEGF levels of conditioned medium with NGF 50 ng/mL showed a significant increase with respect to the control condition, ***$p<0.001$. The results represent the mean ± SEM from seven independent experiments. C: BrdU proliferation assay was used to evaluate EA.hy926 cell proliferation. The results represent the mean ± SEM from a minimum of 3 independent experiments. *$p<0.05$.

and all groups of EOC versus normal ovary ($p<0.001$ with a confidence interval of 37.7 ± 19.7 for Bord T; 59.5 ± 10.9 for EOC I; 80.1 ± 19.0 EOC II; 85.2 ± 27.2 EOC III and 11.96 ± 8.8 for I-OV) and a significant increase ($p<0.05$ with a confidence interval 27.7 ± 19.7 and 11.96 ± 8.8, respectively) between benign tumor and normal ovary. The semi-quantitative analysis for total trkA in endothelial cells was shown in (Fig. 3-E), where a significant increase was found between poorly, moderately and well differentiated EOC versus normal ovary ($p<0.001$ with confidence interval of 39.59 ± 6.11 EOC-III; 38.35 ± 6.94 EOC-II; 30.13 ± 17.77 EOC-I vs 0.37 ± 0.26 I-OV), also a significant increase was found between borderline versus normal ovary ($p<0.05$ with confidence interval of 26.6 ± 8.21 vs 0.37 ± 0.26).

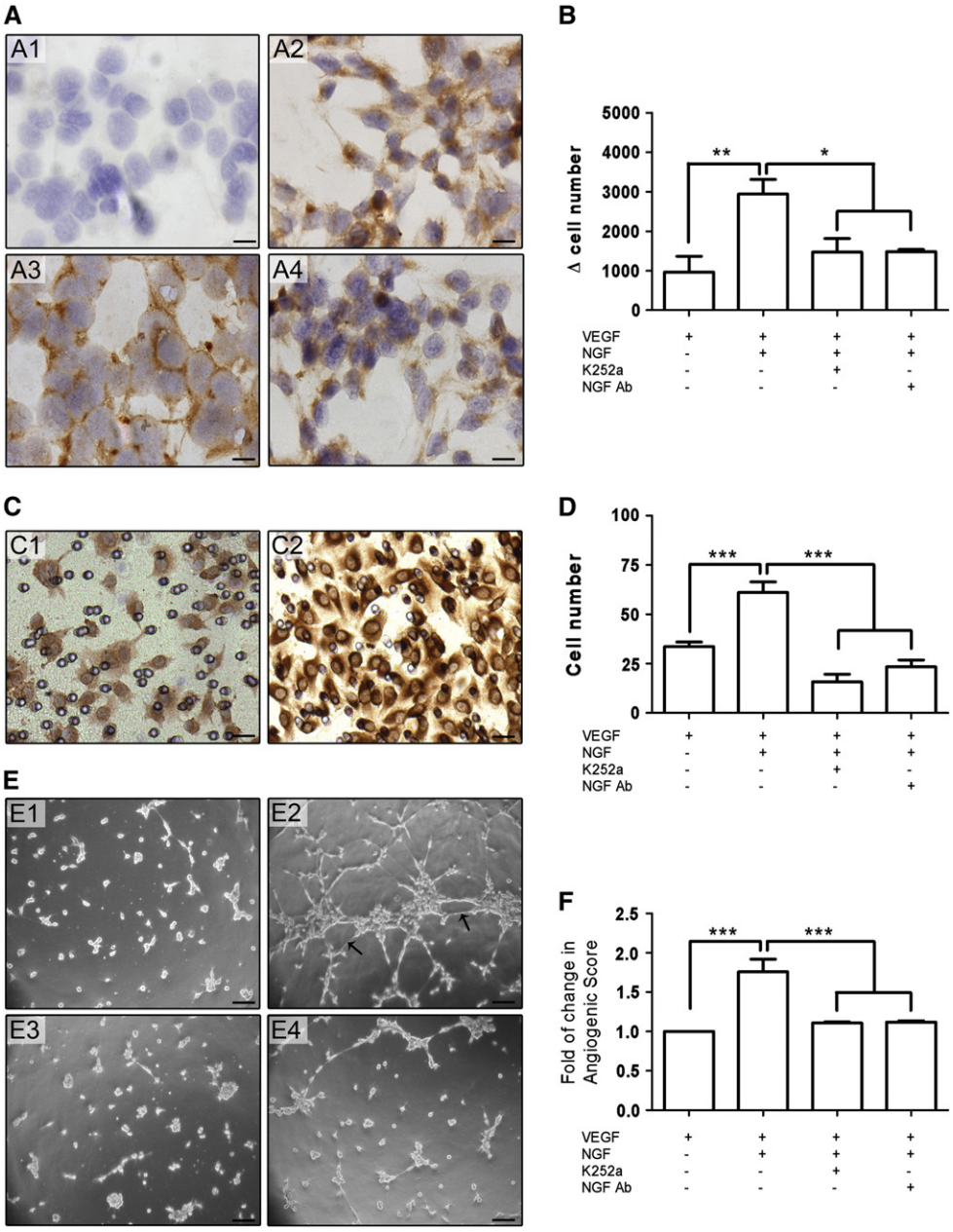

Fig. 5. Immunodetection on A2780 cell line and evaluation of EA.hy926 cell proliferation, migration and differentiation. A: Representative microphotographs of trkA (A2), NGF (A3), and VEGF (A4), immunodetection in A2780 cell line. A negative control (A1) shows lack of staining in control cells incubated in the absence of the primary antibody. The bar represents 10 m with a magnification of 1000×. B: Effect of 24 h incubation with conditioned medium from A2780 cell line on EA.hy926 cell proliferation. A number of 5×10^3 EA.hy926 cells were plated, cultured and incubated with conditioned medium for 24 h and CellTiter 96® assay was used to evaluate cell proliferation. The results represent the mean ± SEM from a minimum of 3 independent experiments. *$p<0.05$, **$p<0.01$. C: Representative micro-photography of migration assay. EA.hy926 cells were plated on Transwell inserts with 8 μm size pores, coated with Matrigel substratum and incubated with conditioned medium from A2780 cell line for 24 h. EA.hy926 cell treated with control (C1) or NGF stimulated (C2) conditioned medium. Bars: 20 m. D: Migration assay. A statistically significant increase in EA.hy926 cell migration was observed when comparing the control condition vs. NGF 50 ng/mL stimulated conditioned medium, ***$p<0.001$. The increase in cell migration induced by NGF was completely blocked when either the tyrosine kinase inhibitor K252a or NGF antibody was present in the conditioned medium, ***$p<0.001$. The results represent the mean ± SE from a minimum of 5 independent experiments. E: Differentiation assay. EA.hy926 cells were incubated on Matrigel substratum with conditioned medium from A2780 cell line and the angiogenic score was calculated 24 h later. There were significant differences in EA.hy926 cell differentiation under the microscope. After applying conditioned medium from the A2780 cell line under NGF stimulation (E2), EA.hy926 cells are highly differentiated, elongated, and organized into vascular networks with hollow lumen (arrows), while in the control (E1) and in other conditions (E3: NGF + K252a; E4: NGF + NGF antibody), cells are distributed and alive but not organized into capillary-like structures. Bar 50 μm. F: Differentiation assay: A statistically significant increase in EA.hy926 cell differentiation was observed when comparing the control condition vs. NGF 50 ng/mL stimulated conditioned medium, ***$p<0.001$. The increase in cell differentiation induced by NGF was completely blocked when either the tyrosine kinase inhibitor K252a or NGF antibody were present in the conditioned medium, ***$p<0.001$. Values are expressed as percent change with respect to the control condition from a minimum of three independent experiments.

A positive immunohistochemical signal for p-trkA in the cytoplasm of both transformed epithelial cells (Figs. 2 D1 through D6) and endothelial cells (Figs. 3, C1 through C6) was detected in ovarian samples from the six groups studied. The semi-quantitative analysis of p-trkA protein levels in the epithelium from the same samples shows a significantly higher level in well differentiated versus poorly differentiated EOC ($p<0.01$ with confidence interval of 25.9 ± 9.5 and 59.7 ± 14.9). Epithelium cells from inactive normal ovaries, benign, and borderline tumors showed a very weak staining, which indicates low p-trkA levels in epithelial cells in these types of samples. In contrast, all EOC samples showed high p-trkA levels (Fig. 2-H). Furthermore, the semi-quantitative analysis of p-trkA in endothelial cells in all samples from the six groups studied (Fig. 3-F) showed a similar expression as the epithelial cells, in which p-trkA was significantly higher for ovarian carcinomas (EOC I; EOC II and EOC III) versus normal ovary and benign and borderline tumors ($p<0.001$). Levels were also significantly higher in EOC III versus EOC I ($p<0.05$with confidence interval of 38.2 ± 7.3 vs 6.6 ± 7.6).

Immunodetection of NGF, VEGF and trkA receptor in EA.hy926 and A2780 cells lines

In order to evaluate the effect of NGF and VEGF on endothelial proliferation, migration and vasculogenesis of the endothelial cell line EA.hy926 under stimulus of conditioned media from the human ovarian cancer cell line A2780, we first needed to establish their presence and localization in both cell lines.

EA.hy926 cells showed a granular staining uniformly distributed in the cytoplasm for both NGF and trkA (Fig. 4-A). The expression of NGF and its receptor trkA had not been previously characterized. VEGF also showed a cytoplasm-positive immunoreactions, indicating that this cell line expresses non-secreted VEGF isoforms (VEGF189 and VEGF165). In this cell line, we also evaluated the protein expression of VEGF receptors R1 and R2, where only VEGF R1 receptor showed a positive immunodetection in the EA.hy926 cell line (Fig. 4-A).

It has been demonstrated that VEGF, NGF and its receptor trkA are expressed in epithelial ovarian cancer; therefore, it was necessary to evaluate whether the human ovarian cancer cell lines have the same characteristics. Immunocytochemical localization for trkA, NGF and VEGF was performed in the A2780 cell line, showing that these three proteins were uniformly distributed, with positive granular staining detected in the cytoplasm of the ovarian cancer cells (Fig. 5-A).

NGF in conditioned medium from ovarian cancer cells induces EA.hy926 cell proliferation

To evaluate the effect of conditioned medium on endothelial cell proliferation, EA.hy926 cells were incubated with either conditioned medium obtained from EOC explants or conditioned medium obtained from A2780 cells, both cultured in different conditions, as described above. The proliferation was performed using the CellTiter 96® and BrdU proliferation assays. We found a significant increase of up to 95% ($p<0.01$ with confidence interval of 3728 ± 932 for NGF and 1908 ± 534 for control condition) in EA.hy926 cell proliferation when conditioned medium from EOC explants under NGF stimulation was used (Fig. 4-B). After EA.hy926 cell proliferation was evaluated using BrdU proliferation assay, conditioned medium under NGF stimulation showed an increase of 1.8-fold with respect to the control condition ($p<0.05$ with confidence interval of 0.163 ± 0.101 for NGF and 0.058 ± 0.024 for control condition) (Fig. 4-C). In both cases, the NGF stimulatory effect on EA.hy926 cell proliferation was inhibited when explants were cultured in the presence of VEGF antibody, NGF antibody ($p<0.05$ with confidence intervals of 0.163 ± 0.101 and 0.048 ± 0.031) or tyrosine kinase receptors inhibitor K252a ($p<0.05$) with confidence interval of 0.163 ± 0.101 and 0.043 ± 0.007 (Figs. 4-B and C).

When conditioned medium from A2780 under NGF 50 ng/mL stimulation was used, we found a two-fold increase ($p<0.01$ with confidence interval of 2942 ± 907 for NGF and 964 ± 977 for control) in EA.hy926 cell proliferation with respect to the control condition (Fig. 5-B). As above, the NGF stimulatory effect on EA.hy926 cell proliferation was inhibited when the A2780 cell line was cultured in the presence of tyrosine kinase receptors inhibitor K252a ($p<0.05$ with confidence interval of 2942 ± 907 and 1468 ± 854), showing that the NGF may participate in cell proliferation via its high-affinity receptor trkA, A similar result was observed when the A2780 cell line was cultured in the presence of NGF antibody ($p<0.05$ with confidence interval of 2942 ± 907 and 1483 ± 124) indicating that either endogenous NGF or exogenous NGF has a direct role in endothelial cell proliferation.

NGF present in conditioned medium increases secreted VEGF

When EOC explants were stimulated by NGF (100 ng/mL), it was found a 1.4-fold increase of the VEGF secretion and showed statistical differences with respect to a conditioned medium in the control condition ($p<0.001$ with confidence interval of 584.1 ± 162.9 and 1421 ± 337 (insert in Fig. 4-B)). The same effect was observed in a conditioned medium from A2780 cell line. Interestingly, the stimulatory effect that NGF showed on VEGF protein expression level was inhibited when both EOC and the A2780 cell line were cultured in the presence of either NGF antibody or K252a, dramatically decreasing the VEGF secretion by 91.9% ($p<0.001$) (data not shown).

NGF induces EA.hy926 cell migration

Conditioned media from A2780 cells under NGF stimulation produced a significant increase of 0.8-fold ($p<0.001$ with confidence interval of 61.14 ± 12.96 and 33.6 ± 5.8) in the chemotactic migration of endothelial cells with respect to the control condition (Figs. 5-C and D). Interestingly, the NGF stimulatory effect on EA.hy926 cell migration was inihibited when the A2780 cell line was cultured in the presence of either K252a ($p<0.001$ with confidence interval of 61.14 ± 12.96 and 15.67 ± 9.78) or NGF antibody ($p<0.001$ with confidence interval of 61.14 ± 12.96 and 23.33 ± 8.61).

Effect of NGF on in vitro capillary formation

Plating EA.hy926 cells on a Matrigel in the presence of conditioned media with NGF resulted in a rapid and profound morphological rearrangement, leading to the formation of tube or cordlike structures that resemble capillaries to some degree (Fig. 5-F), and increasing the angiogenic score up to 0.8-fold ($p<0.001$ with confidence interval of 1.27 ± 0.35 and 2.31 ± 0.38) (Fig. 5-E). The beginning of the process were visually detectable as early as 5 h after plating the cells on Matrigel, and the process was essentially completed within 24 h. Cells moved from their initial uniform pattern of dispersal and associated to form a series of cell clusters (nodes) joined by long, multicellular processes (cords or tubes). The NGF stimulatory effect on EA.hy926 cell differentiation was inhibited when the A2780 cell line was cultured in the presence of either K252a ($p<0.001$ with confidence interval of 2.31 ± 0.38 and 1.46 ± 0.11) or NGF antibody ($p<0.001$ with confidence interval of 2.31 ± 0.38 and $1.462.\pm0.15$). Under these stimulus conditions, EA.hy926 showed similar negative trends in the tubular forming ability of the endothelial cells (Figs. 5-E and F).

Discussion

To better understand the changes in EOC epithelial transformation, we studied the participation of trkA receptor and its ligand NGF in this pathology, since we and others have demonstrated an overexpression of NGF and trkA in EOC [6,9]. In the present study we

found that NGF and trkA expression also increased significantly with level of differentiation in the epithelium and also in the endothelium of the ovarian samples. Interestingly, we also found very low levels of trkA protein within the epithelium and endothelium of normal ovaries, but a gradual yet sustained and significant increase along the spectrum from benign tumor to poorly differentiated EOC. In fact, the high levels of trkA protein and mRNA in epithelial ovarian cancer suggest a role of trkA during epithelium transformation in EOC, based on the fact that trkA mRNA was undetectable in normal tissue, but detectable in borderline lesions and cancerous tissues, gradually but significantly increasing with grade of malignancy. Also, the most interesting result was to find that the active form of this receptor (p-trkA) was identified in the epithelium and endothelium; however, in inactive ovaries, benign tumors and borderline lesions staining for this protein was too weak, suggesting low levels of the active form of the trkA receptor in these groups. In contrast, in EOC we observed high levels of p-trkA, suggesting that immunodetection of p-trkA might serve as a potential tumor marker. Eventhough, total trkA receptor is also present in the benign tumor samples, the possibility exists that this receptor could be inactive in these samples. Nevertheless, the most important finding is that p-trkA receptor is only present in the neoplasic tissues.

The data of the present work is in agreement with previous reports showing that high levels of trkA receptor have been found in a vast number of cancers such as breast and pancreatic cancer [4,5]. Indeed, breast tumors present with high levels of trkA and p-trkA compared to normal breast tissue. Importantly, trkA overexpression has been shown to enhance tumor growth, angiogenesis and metastasis of xenografted breast cancer cells in immunodeficient mice [17].

On the other hand, to determine whether NGF participates indirectly and directly in ovarian angiogenesis, we cultured EA.hy926 using conditioned media from EOC explants and A2780 cells and we observed that VEGF and NGF participate in EA.hy926 cell proliferation, and that NGF-induced proliferation was blocked by the NGF/trkA antagonist K252a, indicating that NGF has a direct effect via trkA receptor activation on endothelial cell proliferation.

In the present work, we have also shown for the first time that A2780 and EA.hy926 cell lines also express NGF and its high affinity receptor trkA, which suggest that these two cell lines provide a good *in vitro* model to study the angiogenesis process in ovarian cancer.

Our data show that endogenous NGF from A2780 cells induces VEGF secretion and that when A2780 cells are treated with NGF, the VEGF level increases in conditioned media. These results indicate that in this cell type, NGF acts as an indirect angiogenic factor promoting VEGF secretion. This effect on VEGF secretion was partially reverted in the presence of K252a, suggesting that NGF participates in the control of VEGF expression through the activation of its high affinity trkA receptor. These results support the proposition of NGF participation in the control of VEGF expression in EOC explants, as we have previously reported [9].

Therefore, NGF and trkA expression in endothelial cells from EOC, as previously described by Davidson et al. [18], suggest a direct action of NGF on ovarian endothelial cells. Thus, the results of the present study, in addition to the data from the literature, clearly indicate that both VEGF and NGF might play important roles as angiogenic factors during the development of ovarian cancer, contributing to its metastatic potential.

As known, EOC present a high angiogenesis that may contribute to its growth, aggressiveness, and low survival rates [19–22]. The angiogenesis process is reflected in the high VEGF expression levels found in the epithelium of EOC; in fact, VEGF transcripts that codify different isoforms of this protein were highly expressed in EOC compared with the normal ovary [9]. Furthermore, in this investigation we have now shown that these VEGF isoforms also varied with epithelium transformation.

Based on the fact that NGF may act as a direct angiogenic factor in EOC, these findings suggest that the new combined therapy for ovarian cancer (chemotherapy plus anti-angiogenic therapy, with an antibody against VEGF) may be insufficient for satisfactory therapeutic results.

Despite the important findings of the present work, it is important to note that the limitations of this investigation are: the relatively small sample size and a carefully selected cohort of ovarian specimens and cell lines; therefore, the results observed in this work have to be confined within the boundaries of the serous epithelial ovarian cancer without generalisation to ovarian cancer. Therefore, it will be of interest to examine: a) trkA receptor expression in other types of ovarian cancer, b) the correlation of p-trkA receptor with prognosis and reliable prediction value, c) the correlation between the trkA expressions in ovarian cancer samples with the response of the patients to the actually therapies used.

Conflict of interest statement
The authors declare no conflict of interest.

Acknowledgments

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