Akt-activated endothelium and bevacizumab in ovarian cancer

**Akt-activated endothelium constitute the niche for residual disease and resistance to bevacizumab in ovarian cancer**

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ABSTRACT

Ovarian cancer is the second leading cause of cancer-related death in women worldwide. Despite optimal cytoreduction and adequate adjuvant therapies, initial tumor response is often followed by relapse suggesting the existence of a tumor niche. Targeted therapies have been evaluated in ovarian cancer to overcome resistant disease. Among them anti-angiogenic therapies inhibit new blood vessel growth, induce endothelial cell apoptosis, and block the incorporation of haematopoietic and endothelial progenitor cells into new blood vessels. Despite in vitro and in vivo successes, anti-vascular therapy with bevacizumab targeting VEGF-A has limited efficacy in ovarian cancer. The precise molecular mechanisms underlying clinical resistance to anti-VEGF therapies are not yet well understood. Among them, tumor and stromal heterogeneity might determine the treatment outcomes. The present study investigates whether abnormalities in the tumor endothelium may contribute to treatment resistance to bevacizumab and promote a residual microscopic disease. Here we showed that ovarian cancer cells (OCC) activate Akt phosphorylation in endothelial cells inducing resistance to bevacizumab leading to an autocrine loop based on FGF-2 secretion. Altogether our results point out the role of an activated endothelium in the resistance to bevacizumab and in the constitution of a niche for a residual disease.
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Introduction

Neo-angiogenesis is a primordial step for tumor growth and metastasis and its targeting is under investigation in many solid tumors (1-4). Human cancer cells express elevated levels of proangiogenic factors (5). Among them the role of vascular endothelial growth factor (VEGF-A) in tumor progression has been clearly established (6). Elevated expression of circulating VEGF-A is associated with poor prognosis in metastatic colorectal, lung, and renal cancer (7). Subsequently the first anti-angiogenic molecule developed to impair neo-angiogenesis targeted VEGF. Bevacizumab (Avastin, Genentech Inc., San Francisco, CA) is a humanized monoclonal antibody directed against all isoforms of VEGF-A (8). Bevacizumab prevents the binding of VEGF-A to its receptor VEGF-R1 (Flt-1) and R2 (Flt-2, or KDR). Although the function of VEGF-R2 in tumor angiogenesis has been characterized thoroughly, the function of VEGF-R1 is not yet well defined (9). Bevacizumab has been found clinically active in many solid tumors, such as colon, non-small cell lung, metastatic renal cell carcinoma or glioblastoma (10-13). Currently the Food and Drug Administration approves its use in combination with chemotherapy/immunotherapy in colon, lung and renal cancers and in glioblastoma as a single agent. The mechanisms of action of bevacizumab are not perfectly understood. It could act by sequestering VEGF-A in the blood and/or tumor interstitium or induce normalization of VEGF-A concentration in these compartments (14). Despite initial efficacy, the withdrawal of bevacizumab can be associated to rapid tumor re-growth or a “rebound” phenomenon with accelerated clinical decline in patients (15, 16). Moreover, in case of tumor progression under bevacizumab, salvage
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Chemotherapy has been quite inefficient, suggesting the emergence of a treatment-resistant phenotype (17). Most tumors will develop resistance to bevacizumab on a long term (18). While vessels were perceived as passively conducting nutrients to tumor cells, recent reports underline the perfusion-independent role of the endothelium. In such context endothelial cells (ECs) participate to an active cross-talk with tumor cells, resulting in the modification of their phenotype (19). ECs isolated from various tumors acquired genotype alterations as aneuploidie, or abnormal multiple chromosomes (20) or displayed an activated profile through Pi3/Akt or MAPK pathway (21).

Ovarian cancer usually presents as locally advanced disease with involvement of the peritoneal cavity and the use of bevacizumab is under investigation in several clinical trials. Here we hypothesized that the cross-talk between cancer and endothelial cells would induce an activated ECs profile responsible for bevacizumab resistance and the constitution of a vascular niche. We demonstrated that the cross-talk between ovarian cancer cells and established human umbilical vein endothelial cell line (HUVECs), resulted in an activation of Akt pathway among others. To model tumor endothelium, we used the Akt-activated endothelial cells (E4+ECs) to study the involvement of the endothelial component in the resistance to anti-angiogenic therapy (22). We demonstrated that in the setting of tumor-endothelial cross-talk, ECs activation of Pi3k/Akt pathway induces an autocrine loop through the pro-angiogenic factor fibroblast growth factor 2 (FGF-2), bypassing the VEGF-R pathway. We demonstrated that blocking FGF-2 would efficiently reverse the resistance to bevacizumab. These results propose the use of stroma directed synthetic therapy to overcome resistance in ovarian cancer.
Material and methods

Reagents and chemicals. Bevacizumab (trade name Avastin, Genentech) or 100 mg / 16.6 ml paclitaxel (Ebetaxel®, EBEWE Pharma, Unterach, Austria) were obtained from National Center for Cancer Care and Research (NCCCR, Doha, Qatar) pharmacy. The bevacizumab doses used for patients' treatment (5-15 mg/kg), correlates to a mean plasma concentrations value between 0.1 and 0.5 mg/mL; we diluted bevacizumab in serum free medium to obtain final concentrations of 0.5/ 1.0/ 1.5/ 2/ and 2.5 mg/mL. Ly294002 (L9908), FGF receptor (FGFR) inhibitors PD17034 (P2499) and SU-5402 (SML0443) were purchased from Sigma-Aldrich and resuspended in DMSO.

Immunohistochemistry. Immunohistochemistry (IHC) was performed at the Segal Cancer Centre Research Pathology Facility (Jewish General Hospital, Montreal, Quebec, Canada). Tissue samples were cut at 4-µm, placed on Superfrost Plus stain slides (Fisher), and dried overnight at 37°C. The slides were then loaded onto the Discovery XT Autostainer (Ventana Medical System). All solutions used for automated immunohistochemistry were from Ventana Medical System unless otherwise specified. Immunostaining for phospho-Akt was performed using a heat protocol. Briefly, rabbit monoclonal anti-phospho-Akt (Ser473) (D9E) (Cell Signaling Technology) diluted 1:50 in Antibody diluent solution, was manually applied for 30 min, and then followed by the appropriate detection kit (Omnimap anti-Rabbit HRP). Slides were counterstained with hematoxylin for four minutes, blued with Bluing Reagent for four minutes, removed from the autostainer, washed in warm soapy water,
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dehydrated through graded alcohols, cleared in xylene, and mounted with Permount. Sections were analyzed by conventional light microscopy.

**Cell culture.** Established Human umbilical vein endothelial cells (HUVECs) were purchased and grown as recommended by the American Type Culture Collection (ATCC, Manassas, VA, USA). E4+ECs were obtained as previously described (22). Briefly, isolated HUVEC were transduced with the early 4 region (E4) of the adenoviral vectors (AdE4+) to generate durable PEC feeders. HUVECs and E4+EC cells were cultured in M199 medium supplemented with 20% fetal bovine serum (FBS), 1% penicillin / streptomycin, 0.1% heparin and 0.1% endothelial cell growth supplement (ECGS) and maintained at 37 °C in a humidified 5% CO2 atmosphere. HUVECs cells were seeded on plates coated with 0.1% gelatin and allowed to grow. Treatments were performed in serum- growth factor- free medium over 24 hours. Human Ovarian Microvascular Endothelial Cells (HOMEC) were purchased from ScienCell research Laboratories (#7300) and grown according to the manufacturer instructions. Ovarian cancer cell lines SKOV-3 (HTB-77), OVCAR-3 (HTB-161) were purchased from ATCC (Manassas, VA, USA). Cells were grown in DMEM high glucose (Hyclone, Thermo Scientific) supplemented with 10% FBS (Hyclone, Thermo Scientific), 1% Penicillin-Streptomycin-Amphotericin B solution (Sigma), 2 mM L-glutamine (Sigma), 1X Non-Essential Amino-Acid (Hyclone, Thermo Scientific). All cell lines were obtained between 2011 and 2013 from American Type Culture Collection (ATCC) or ScienCell research laboratory. Upon receipt, cells were expanded and aliquots of cells at passage number <10 were stored frozen in liquid...
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nitrogen. Cells from one aliquot were kept in culture for less than 2 months. The authors did not authenticate identity of each cell line purchased. GOC-2 and GOC-A2 are primary cell lines that were derived by Dr Gotlieb’s laboratory. GOC-2 cells were isolated from a papillary serous ovarian cancer obtained after 4 courses of neo-adjuvant chemotherapy with carboplatin / taxol; and GOC-A2 from a stage IIIc serous ovarian cancer.

**Cell proliferation assay.** Cellular viability and proliferation was analyzed by both trypan blue exclusion and tetrazolium dye assay. HUVECs and E4*ECs cells were allowed to grow to 80% confluence in 96 well plates. Cells were then incubated with the different concentrations of bevacizumab in serum, cytokine free medium for 24 hours and subjected to XTT assay as previously described by the manufacturer (TACS XTT cell proliferation assay kit, Trevigen, Inc.). Optical density was measured at 540 nm. Alternatively, treated cells were then collected and diluted in trypan blue dye (Sigma) for manual counting with hemocytometer.

**Wound Healing and Tube Formation Assays.** Cells were grown to confluency in starvation medium (serum- growth factor- free). Using a pipette tip, cells were scraped from the culture dish leaving a void space. Cells were washed with PBS and then incubated for 36 hours in the presence of the treatment. After incubation, wound closure was imaged by phase contrast microscope and analyzed using Image J software. Five random fields per well were examined. For the tube formation assay, 24-well culture plates were coated with Matrigel according to the manufacturer's instructions. HUVECs
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and E4⁺ECs were seeded on coated plates at $5 \times 10^4$ cell/well in serum- growth factor- free medium, treated and incubated at 37 °C for 8 hours. Cells were then analyzed for tube formation with an inverted light microscope. The criteria taken into account were the branching point number being formed. Five random fields per well were examined. Cells seeded on coated plate in complete medium served as positive control.

**Generation of a treatment-resistant HUVEC line.** A treatment-refractory HUVEC line was developed by continuous exposure to bevacizumab. Briefly, HUVECs were exposed to stepwise increases in bevacizumab concentrations. The initial bevacizumab exposure was at a concentration of 0.1 mg/mL. After cells had regained their exponential growth rate, bevacizumab concentration was doubled; this procedure was repeated until selection of a clone resistant to 1 mg/mL of bevacizumab. The resulting bevacizumab-refractory cell line was sub-cultured every 5 days and treated every 15 days with 1 mg/mL of bevacizumab to maintain a high level of anti-angiogenesis resistance.

**Sphere formation assay.** E4⁺EC-eGFP and SKOV-3 were dissociated into single cell suspension by trypsinization and further sieving through 40-µm cell strainers. E4⁺ECeGFP and SKOV-3 cells (ratio 10/1) were then resuspended in 3D media consisting of DMEM F-12 supplemented with 2% B27, 20 ng/mL EGF, 20 ng/mL bFGF, and 5 μg/mL insulin in ultralow attachment plates. Primary cancer spheroids and angiospheres (SKOV-3 / E4⁺ECeGFP, ratio 1:10) started to form at day 3 and maintained up to day 7. Primary spheres...
were dissociated into single cell suspension and re-plated as mentioned above to form secondary spheres. 7 days old secondary spheres were used for inducing tertiary spheres. Sphere formation was evaluated by ImageJ64 software based on the area of the sphere covered by SKOV-3. To evaluate E4\textsuperscript{+}EC-eGFP survival under bevacizumab treatment, the area of the sphere covered by E4\textsuperscript{+}EC-eGFP was majored.

**Analysis of viability and apoptosis detection by flow cytometry.** Viability was assessed by flow cytometry evaluation of calcein AM staining as described by the manufacturer (Live Dead Viability/cytotoxicity Kit, Molecular Probes, Invitrogen). Cells treated with bevacizumab (1.5mg/mL), were harvested after 24h. Analyses were performed on SORP FACS Aria2 (BD Biosciences). Calcein AM fluorescence was acquired with 488 nm blue laser and 525/50 nm emission, ethidium homodimer was acquired with 640 nm red laser and 670/14 nm emission. Cytotoxicity was assessed by flow cytometry evaluation of annexin V, propidium iodide (PI) staining cells as described by the manufacturer (FITC Annexin V Apoptosis Detection Kit I, BD Pharmingen). Cells were treated overnight with varying concentrations of Bevacizumab (0.1, 1, 1.5, 2, 2.5 mg/ml) and/or Ly294002 (10 µM). PI and Annexin V were added and cells incubated at room temperature for 15 min. Stained cells were analyzed on SORP FACS Aria2 using excitation at 535 nm and emission at 582/15 nm for PI and 488 nm excitation, 525/50 nm emission for annexin-FITC. Data were processed with FACSDiva 6.3 (BD Biosciences). Doublets were excluded by FSC-W x FSC-H and SSC-W x SSC-H analysis, single stained channels were used for compensation, and fluorophore minus
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one (FMO) controls were used for gating (23). Single stained channels were used for compensation and fluorophore minus one (FMO) controls were used for gating.

**Phospho-flow analysis and intracellular detection.** After treatment or co-culture, cells were fixed using BD Cytofix™ Fixation Buffer (BD Biosciences), followed by permeabilization at 4°C with BD™ Phosflow Perm Buffer III (BD Biosciences). Conjugated antibodies were added to the cells for 45 min 4°C. Phospho-Akt S473 PE-conjugated antibody (BD Biosciences) and VEGF-A PE-conjugated antibody (R&D Systems) was acquired with 535 nm green laser and 582/15 nm emission. Phospho-STAT-3 (Y705) alexa Fluor 647 (BD Biosciences) was acquired with 640 nm red laser and 670/14 nm emission. 50000 events were acquired per sample. Single stained channels were used for compensation and FMO controls were used for gating.

**Western blot analyses, antibodies and immunoprecipitation.** Immunoblot analysis were performed using the following antibodies from Cell Signaling Technology (Beverly, MA): phospho-Akt (S473) (#9271), Akt isoform sampler kit (#9940), FGF-2 (#3196), PARP (Poly [ADP-Ribose] Polymerase, #9532), cleaved PARP (#9541), phospho-FGFR1 (#3472), phospho STAT-3 (Y705) (#9145), phospho-STAT-3 (S727) (#9134), STAT-3 (#4904), phospho-ERK1/2 (#9101), phospho-Src (Y416) (#2101), actin (#3700) and VEGF-A from Santa Cruz Biotechnology (#SC-152). Immunoreactive bands were visualized by using a developing solution (Western Lightening, Perkin Elmer LAS, NEL104, Boston). For immunoprecipitation: equal amount of cell lysate were
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preabsorbed with 50 μL of protein A/G Plus agarose (Santa Cruz, Sc-2003) and then incubated overnight at 4°C with anti-Akt1, anti-Akt2, and anti-Akt3 antibody. Immune complexes were precipitated with 50 μL of Protein A/G Plus agarose and then washed 3 times with lysis buffer. Immunoprecipitated proteins were eluted in Laemmli sample buffer and subjected to western blot analysis.

**RT-PCR analysis.** Total RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Briefly, addition of chloroform, after the centrifugation, separates the solution into aqueous and organic phases. RNA remains only in the aqueous phase. After transferring the aqueous phase, RNA is recovered by precipitation with isopropyl alcohol. A quantitative analysis of RNA was performed using a two-step reverse transcription-quantitative PCR (GoTaq® 2-Step qRT-PCR System qRT-PCR, Promega, USA) protocol according to the manufacturer’s instructions. cDNA was synthesized from 2μg total RNA, using the GoScript™ Reverse Transcription System and amplified using the GoTaq® qPCR Master Mix. Cycling conditions were 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, 72°C for 10 seconds, 72°C for 5 minutes and cooled to 4°C. The final extension step was continued for 5 min. Variation in cDNA loading was normalized to GAPDH expression and relative expression values were determined using ∆∆Ct method of relative quantification. Primer sequences are listed as supplementary table and were purchased from IDT.
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Neutralizing anti FGF-2 assay. A monoclonal neutralizing mouse anti-FGF-2 antibody (Millipore, clone bFM-1) was used for FGF-2 neutralization at 1 µg/ml and 5 µg/ml with or without bevacizumab. Cell viability was determined by XTT assay after 2 days neutralization. E4+ECs stimulated with 20 ng/ml of FGF-2 were used as positive controls. Isotypic IgG control was used as internal control, and serum-free medium as negative control.

FGF-2 and VEGF-A secretion. We used a Human VEGF-A Quantikine ELISA Kit (DVE00, R&D Systems) and Human FGF-2 Quantikine ELISA Kits (DFB50, R&D Systems). Plates were read at 450 nm. Concentrations were interpolated from a standard curve.

Statistical analysis. All quantitative data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed, using SigmaPlot 11 (Systat Software Inc., Chicago, IL, USA). A Shapiro-Wilk normality test, with a p=0.05 rejection value, was used to test normal distribution of data prior further analysis. All pairwise multiple comparisons were performed by one way ANOVA followed by Sidak-Holm Post Hoc tests for data with normal distribution or by Kruskal-Wallis analysis of variance on ranks followed by Tukey Post Hoc tests, in case of failed normality test. Paired comparisons were performed by Student’s t-tests or by Mann-Whitney rank sum tests in case of unequal variance or failed normality test. Statistical significance was accepted for p < 0.05 (*), p < 0.01 (**) or p < 0.001 (***)
Results

Cancer cell cross-talk with endothelial cells induces pro-survival signaling. Tumor endothelial heterogeneity and in particular endothelial Akt activation has been previously reported (24). Ovarian tumor vessels displayed heterogeneous phospho-Akt staining (Fig. 1A). To investigate the role of soluble factors in tumor-endothelial cells communication, we established a trans-well co-culture system of HUVEC and ovarian cancer cells (OCCs). We used (i) established OCCs lines (OVCAR-3, SKOV-3), (ii) and two primary OCCs isolated from papillary serous ovarian cancers (GOC-2 and GOC-A2). As shown in Figure 1B, we observed heterogeneous Akt phosphorylation in HUVECs. OVCAR-3 cells induced less Akt phosphorylation than the other cell lines tested. SKOV-3, which are highly resistant to chemotherapy and have metastatic features, induced the higher level of Akt activation. We confirmed the 'activated' phenotype in HUVEC by western blot (Supplementary Fig. S1).

E4*ECs display resistance to bevacizumab. We hypothesize that endothelium activation might play a role in bevacizumab resistance. The use of serum and cytokines to maintain HUVECs in culture might hinder cell autonomous effect. Thus to model a tumor associated endothelium, we used our recently generated endothelial cell line E4*ECs that can survive in serum-cytokine-free media (22) to study further mechanisms of resistance to bevacizumab. We first verified the presence of VEGF-A and VEGFR (mRNA and protein levels) in HUVECs and E4*ECs (supplementary Fig. S2-A and B). We conducted several functional assays with E4*ECs, their control HUVEC and HUVEC co-cultured with ovarian cancer cells (Figure 2A). We evaluated
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the cell viability by XTT Cell proliferation assay and FACS, after bevacizumab treatment. Doses of bevacizumab between 1 and 2.5 mg/mL did not significantly affect the number of viable E4+ECs contrary to HUVECs at 24h and 48h (Fig. 2B, 2C and data not shown). Addition of VEGF-A (10, 25, 50 ng/mL) could not completely protect HUVEC from the decrease of viability (supplementary Fig. S3-A, upper panel). Interestingly, HUVEC pretreated with VEGF-A before treatment with Bevacizumab displayed higher survival (supplementary Fig. S3-A, bottom panel). We could illustrate the absence of an apoptotic phenomenon in E4+ECs and HUVEC co-cultured with SKOV-3 conditioned medium by the absence of cleaved PARP upon bevacizumab treatment (Fig. 2D). Besides the pro-survival signaling, VEGF-A also induces endothelial cell migration and vessel formation (25). Under serum- growth factor- free condition, we were not able to evaluate the motility of HUVEC in the presence or absence of VEGF-A (10, 25, 50ng/mL), or bevacizumab treatment. But we observed that in E4+ECs, or HUVEC co-cultured with SKOV-3 condition medium, bevacizumab was not able to prevent wound closure (Fig. 2E and supplementary Fig. S3-B, S3-C).

In a tube formation assay, while HUVECs treated by bevacizumab alone or supplemented with VEGF-A resulted in unramified tube-like structures with dead ends, E4+ECs or HUVEC co-cultured with SKOV-3 condition medium maintained the capacity to form assembled capillary-like structures (Figure 2F and Supplementary Fig. S3-D and S3-E). Concordantly, E4+ECs displayed the same profile of resistance than HUVECs co-cultured with SKOV-3 supernatants, confirming the use of E4+ECs as a model. Interestingly, we also showed that bevacizumab had a profound effect on HOMEC compared to
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E4+ECs (Supplementary Fig. S3-F).

**Inhibition of Akt-mediated survival pathways increases bevacizumab effect.** We first showed that Akt phosphorylation was abrogated as early as 10 minutes after treatment with the pan-PI3K inhibitor LY294002 (10 µM) (Fig. 3A and Supplementary Fig.S4-A). LY294002 treatment alone did not decrease E4+ECs viability, but co-treatment with bevacizumab reversed E4+ECs resistance to anti-angiogenic therapies (Fig. 3B and supplementary S4-B). This underlines the central role of Akt pathway in the resistance to bevacizumab treatment. To illustrate the essential role of Akt, we developed a bevacizumab-refractory HUVEC line. Treatment of this cell line with Ly294002 decreased Akt phosphorylation and restored the sensitivity to bevacizumab (Supplementary Fig. S4-C). Western blot analysis and immunoprecipitation showed that Akt1 is the predominant isoform expressed in E4+ECs as well as the main form phosphorylated (supplementary Fig. S4-D). Additional angiogenic factors such as FGF-2 (26) can compensate the blockade of VEGF (27). Interestingly, transcriptomic and RT-PCR analysis showed that FGFR1 mRNA was increased by 2.5 fold in E4+ECs compared to HUVECs (Supplementary Fig. S4-E). In all OCCs used in this study, while bevacizumab efficiently neutralizes VEGF-A, the expression of FGF-2 could still be detected (Supplementary Fig. S4-F). We observe that E4+ECs treatment by bevacizumab is accompanied by a three-fold increase in FGF-2 mRNA and protein level. We also observed an increase in FGFR1 mRNA and in FGFR1 phosphorylation (Fig. 3C). We then verified if FGF-2 was able to induce Akt activation and found that Akt phosphorylation could be detected in HUVEC
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as soon as 10 minutes after treatment with FGF-2 (10ng/mL) (Fig. 3D, left panel). An overnight treatment with FGF-2 shows that Akt phosphorylation in HUVEC cells is similar to the Akt phosphorylation observed in E4⁺EC as shown by phospho-flow analysis (Fig. 3D, right panel). Concordantly, the treatment of HUVEC by FGF-2 induced resistance to bevacizumab and reversed the inhibition of angiogenesic attributes (Supplementary Fig.S5-A, B, C). We investigated the molecular events underlying the potential mechanisms of resistance through FGF-2 expression in endothelial cells. As previously reported (28), endothelial FGF-2 synthesis is regulated by a signaling cascade involving ERK1/2 and STAT-3 activation. Under bevacizumab treatment, FGF-2/FGFR1 axis mediates Src activation and downstream activation of the pro-angiogenic ERK1/2-STAT-3 signaling pathway as shown by the increase phosphorylation of Src (Y416), STAT3 and ERK1/2 (Fig. 3E). The secretion of FGF-2 has been mainly attributed to cancer cells (27, 29), we showed the existence of an autocrine loop maintaining Akt activation in E4⁺ECs in the context of VEGF-A inhibition. Akt inhibition by Ly294002 resulted in FGF-2 inhibition at mRNA and protein levels suggesting that Akt activation is responsible for FGF-2 secretion (Fig. 3F).

Inhibition of FGF2 signaling reversed bevacizumab resistance in E4⁺ECs. To investigate if the inhibition of FGF-2 signaling would reverse bevacizumab resistance, we used a FGF-2 blocking antibody. We showed that neutralization of FGF-2 activity decreases Akt phosphorylation in E4⁺EC. We then showed a synergistic effect when anti-FGF-2 was added to
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bevacizumab treatment. This result suggests that Akt activation of E4*EC depend on both VEGF-A and FGF-2 secretion (Figure 4A). We verified that blocking FGF-2 alone did not impact E4*ECs survival. However, in the context of FGF-2 deprivation, bevacizumab resistance was reverted (Fig. 4B). Finally, we also investigated the role of FGF-2 in the angiogenic attributes. Blocking FGF-2 alone has a minor effect on motility, but a drastic effect on tube formation, confirming the role of FGF-2 as a potent angiogenic factor (Fig.4C and 4D). We then assessed the effect of the inhibition of FGFR activity using two FGFR-selective inhibitors, PD17034 (30) and SU5402 (31) in combination with bevacizumab. While treatment with PD17034 (10nM), or SU5402 (1µM) alone resulted in a modest decrease of E4*EC growth, a bevacizumab-dose-dependent reduction of E4*EC proliferation was observed after co-targeting VEGF-A and FGFR, confirming the synergistic role of the two cytokines (Fig. 4E). These data suggests that the FGF/FGFR axis influences the sensitivity of the E4*ECs to bevacizumab therapy.

Residual Disease. The rebound effect described in the literature and observed in the clinical setting can from anendothelial niche. We set-up a model of co-culture with endothelial and cancer cells and tried to reproduce a residual disease. Both HUVECs and E4*ECs integrated tumor nodules of SKOV-3 produced on matrigel. We then sequentially treated these nodules with the microtubule-damaging agent taxol (10µM) and bevacizumab (1mg/mL). After taxol treatment, few cancer and endothelial cells survived. E4*ECs presented significant resistance to the treatment. The treatment with bevacizumab completely eliminated all tumor and endothelial cells in the
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HUVEC control group. However E4+ECs and SKOV-3 were able to grow and produce a “recurrent nodule” (Fig. 5A). We went further and created structures called angiospheres. We have recently illustrated the ability of the endothelium to form 3D structures that can sustain tissue specific cell growth (32). We hypothesize that the same phenomenon could be relevant to cancer biology where within 3D structures the endothelial cells can support the expansion of the cancer propagating cells. We were able to demonstrate an increase ability of cancer cells co-cultured with E4+ECs to form spheres comparing to cancer cells alone. The results were similar when secondary and tertiary spheres were induced (Figure 5B). To confirm that E4+ECs are responsible for the expansion of the spheroids under bevacizumab treatment, we treated daily the spheroids and showed that after 7 days, E4+ECs can still support sphere formation (Figure 5C).

Discussion

This study demonstrates that an endothelial FGF-2 autocrine loop induce resistance to bevacizumab and lead the creation of an Akt-dependant endothelial niche.

Angiogenesis supports tumor progression through delivery of oxygen and nutrients and provides a point of entry into circulation that enables blood born metastases (33). Bevacizumab is the first anti-angiogenic agent available in the clinical setting. Despite a number of clinical results and reports showing improvements in progression-free survival in patients with advanced ovarian cancer (34), some patients do not respond to bevacizumab or gradually develop resistance. Converging data support that tumor microenvironment
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modulates cancer cells sensitivity to treatment. Our study describes a comprehensive observational and functional investigation on the pivotal role played by the endothelium in the resistance to bevacizumab. Anti-angiogenic agents are able to induce or aggravate hypoxia, which up-regulates the production of other pro-angiogenic factors, leading to a ‘VEGF-independent’ revascularization. This phenomenon is called evasive resistance (35). Le Page et al. reported that FGF-2 levels are elevated in the serum of patients with ovarian cancer compared to cancer-free individuals and in tumoral tissue compared to non-tumoral tissue (36). Concordantly Lin et al. and Zhang et al. reported that FGF-2 stimulates proliferation, migration, angiogenesis and invasion in ovarian cancer cell lines OVCAR-3 and SKOV-3 (37-39). They also reported that treatment with an FGF-2 antibody could inhibit FGF-2 dependent proliferation and angiogenesis. Furthermore, Gan et al. reported that high FGF-2 tumor levels reduced drug sensitivity, in part due to the direct effects of FGF-2 on proliferation and apoptosis (40). FGF-2 activates the phosphatidylinositol-3 kinase (PI3-K) in endothelial cells (41), which regulate Akt or protein kinase B (PKB) activity (42). Our data indicate that FGF-2 is able to revert the effect of bevacizumab on HUVEC and highlight FGF-2 participation to E4°EC survival under bevacizumab treatment. We demonstrated that FGF-2 blocking would efficiently reverse the resistance to bevacizumab in E4°EC. We showed that primary ovarian cancer cells and ovarian cancer cell lines express FGF-2, this data supports the FGF-2/FGFR mediated cross-talk between cancer and endothelium.

The cross-talk between the tumor and endothelium supports the tumor vasculature after bevacizumab treatment in a paracrine manner. Butler and
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colleagues recently proposed that endothelial cells produce “angiocrine” factors that could enable tumor growth, motility, and ultimately metastasis (26). Autocrine circuit for FGF-2 as well other angiocrine factors may be responsible for the acquisition of autonomous proliferation of endothelial cells in contact with the tumor. It has been suggested that activated endothelium provide a vascular ‘niche’ inducing the angiogenic switch in micrometastases and allowing the persistence of a residual disease. These data are compatible with our findings, as the activated endothelium resists to anti-angiogenic therapy. The concept that a physical niche protects tumor cells during drug treatment has also been suggested for the perivascular space within the tumors. Indeed, recent work has highlighted a tumor re-initiating cells along tumor vessels suggesting that these locations have a predictive role in tumor sensitivity to treatment (43, 44). Bissel group demonstrated that ECs and factors deposited within their surrounding basement membrane may be a prime player within the dormant niche, and revealed that stable microvasculature constitutes a dormant niche whereas sprouting neovasculature sparks micrometastatic outgrowth (45). Moreover, bevacizumab-treated spheroid cells could form more spheres than the control, indicating that they have an increased self-renew capacity. Patient’s therapeutic responses remain a significant challenge, and therapeutic resistance to bevacizumab reflects active tumor evolution and also microenvironmental mediated resistance.

In summary, our study used the E4+ECs as a surrogate for tumor associated endothelium. The tumor-protective effect of the endothelium can be attributed to the ability to support tumor vasculature after bevacizumab treatment,
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leading to the relapse of the tumor. An FGF-2/PI3K-Akt autocrine loop is required in ECs to perturb bevacizumab treatment and involve the pro-angiogenic ERK1/2-STAT-3 signaling pathway (Figure 6). The FGF/FGFR/Akt signaling pathway is involved in an angiocrine switch and may be a target for therapeutic strategies against bevacizumab resistant ovarian cancer.

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Figure legends

Figure 1. Akt pathway is activated in vivo and in vitro in ovarian cancer endothelium. A. Representative immunohistochemical staining of phospho-Akt (Ser473) and CD31 in papillary serous adenocarcinomas specimens. Sections exhibit intense CD31 immunostaining co-localizing with pAkt staining (arrows) 20X. B. Co-culture with established OCCs lines and isolated primary OCCs induced Akt activation in HUVECs. HUVECs co-cultured 24h with ovarian cancer cell lines were stained for phospho-flow cytometry analysis targeting Akt (S473) phosphorylation. A representative experiment depicting phosphorylation of Akt at S473 after co-culture with OCCs is shown. The percentage of cells scoring positive for pAkt-Ser473 (red area) was determined by comparing co-cultured HUVECs with their control (non-co-cultured HUVECs).

Figure 2. Akt-activated endothelial cells (E4*ECs) display resistance to bevacizumab. A. Schematic representation of the experimental design used B. Effect of bevacizumab on the viability of E4*ECs; their control HUVECs and HUVECs incubated with SKOV-3 conditioned media (CM) after 24h treatment with bevacizumab. Growth inhibition was assessed by XTT assay
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and normalized to the control (non treated cells). C. E4+ECs and HUVECs monolayers were incubated 24h in serum and growth factor free medium and treated with bevacizumab (2 mg/mL). The number of cells was visualized by phase-contrast microscopy (4X, Scale bar= 500μm). Viability was evaluated by flow cytometry using Live/Dead kit. Live cells are represented in green. D. Effect of bevacizumab on cleavage of PARP in HUVECs, E4+ECs, and HUVECs incubated with SKOV-3 CM. Cells were treated 24h with bevacizumab (1.5 mg/mL), harvested, and lysates were subjected to western blotting using anti-PARP, anti-cleaved PARP or anti-β-actin antibody as a loading control. E. Effect of bevacizumab on E4+EC cells migration. E4+EC cells were grown as monolayer in triplicate. Confluent cultures were starved overnight, scratched with a pipette tip and incubated in serum free media alone (control) or with bevacizumab (1.5mg/mL). Pictures displayed the wound at time T0 and T36h (4X, scale bar= 1000μm). F. Organization of endothelial cells into vascular tubes. HUVECs and E4+ECs were incubated on growth factor-reduced matrigel, in serum-cytokines-free medium +/- bevacizumab (1.5 mg/mL). Phase contrast evaluation of the networks was performed after 8h. Five random fields were analyzed based on the branching point number. Data were means from three independent experiments (4X, scale bar =100 μm).

Figure 3. Akt inhibition restores sensibility to bevacizumab and activation of FGF-2 signaling contributes to bevacizumab resistance via an activation of the Akt pathway. A. Flow cytometry and western blot analysis of Akt phosphorylation in E4+ECs and its inhibition by Ly294002 (10μM, 1h). Histograms represent mean fluorescence intensity (MFI) of pAkt
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(S473). HUVECs (heavy line) used as a negative control, E4+ECs treated with Ly294002 (red) display an inhibition in pAkt levels as shown by the leftward shift of the histogram when compared to unstimulated E4+ECs (grey filled histogram). The SEM was calculated from 3 different experiments (HUVEC: 1%± 0.5%, E4+ECs control: 26.3%± 3% and E4+ECs treated with Ly294002: 5.2%± 3.7%). Cell lysates from HUVECs, E4+ECs control or treated with Ly294002 (10μM) were subjected to western blot analysis using anti-pAkt (S473) or anti-β-actin antibody as a loading control. B. Involvement of Akt in E4+ECs resistance to bevacizumab. E4+ECs were treated 24h with bevacizumab +/- Ly294002 (10μM). Phase-contrast microscopy representative pictures displayed cells after treatment (4X, scale bar= 500μM). Survival determined by XTT assay. C. E4+ECs treatment with bevacizumab induces an increase in FGF-2 and FGFR1 expression. Cells were treated overnight with bevacizumab (1mg/mL). RNA was extracted and relative expression of FGF-2 or FGFR1 was determined by RT-PCR and normalized to GAPDH. Lysates were subjected to western blotting using anti-FGF2, anti-phospho FGFR1 or anti-β-actin antibody as a loading control. D. FGF-2 induces Akt activation in HUVEC. HUVECs incubated in 96-well plates were incubated for 10 and 60 minutes with FGF-2 (10 ng/mL). Phosphorylation of Akt on S473 was determined using phospho-Akt (S473) cell-based ELISA. Values represent the mean ± the range of triplicate determinations (right panel). Flow cytometry analysis of Akt phosphorylation in HUVEC incubated overnight with FGF-2 (10 ng/mL). Histogram represent mean fluorescence intensity of pAkt (S473) in HUVECs (grey) used as a negative control, HUVECs in the presence of FGF-2 (red) display a similar
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activation of pAkt levels than unstimulated E4⁺ECs (heavy line) (left panel). **E.** Analysis of the molecular events underlying the resistance of E4⁺ECs. Cells were treated with bevacizumab (1mg/mL), collected for western blot analysis using antibodies against phosphorylated form of STAT3 (Y705), Src (Y416), ERK1/2, and STAT3 (S727). **F.** Ly294002 (10μM) decreased FGF-2 expression in E4⁺ECs. Relative expression of FGF-2 was determined by RT-PCR and normalized to GAPDH (right panel). E4⁺ECs lysate were subjected to western blot using anti-FGF2 or anti-β-actin antibody as a loading control (left panel).

**Figure 4. Inhibition of FGF-2 signaling reversed bevacizumab resistance in AKT-activated endothelial cells.** **A.** Akt phosphorylation was assessed in E4⁺ECs under FGF-2 inhibition. Cells were treated with bevacizumab, anti-FGF-2, or both and akt (S473) phosphorylation was evaluated by phosphoflow cytometry analysis. The results shown in the bar graphs are representative of three independent experiments. **B.** Neutralizing FGF-2 sensitizes E4⁺ECs to bevacizumab treatment. Escalating doses of neutralizing agent were tested concomitant to bevacizumab treatment (1.5 mg/mL). The growth inhibition was assessed by XTT assay and compared to the control. **C.** Neutralizing FGF-2 reverse the effect on E4⁺ECs migration. Phase-contrast images of cells migrating into the wounded area were analyzed. Phase contrast pictures displayed the wound at time T0 and T36h (4X, scale bar=1000μm). **D.** Neutralizing FGF-2 influences the organization of E4⁺ECs into vascular tubes. E4⁺ECs were incubated on growth factor-reduced matrigel, in serum- cytokines- free medium +/- bevacizumab or anti-FGF-2. Images of the formed network were taken after 8h (4X, scale bar =100 μm).
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showing the branching point numbers. Data were means from three independent experiments. E. Involvement of FGFR1 in E4+ECs resistance to bevacizumab. E4+ECs were treated 24h with bevacizumab and PD17034 (10nM) or SU5402 (1µM). Survival was assessed by XTT assay and normalized to the control.

Figure 5. Residual disease and re-growth of the nodule due to Akt-activated endothelium. A. Residual disease model. Endothelial cells (E4+EC-eGFP and HUVECs labeled with PKH26 tracker dye) were co-cultured with SKOV-3 on top of growth factor-reduced matrigel. The nodules were successively treated with taxol (24h), followed by bevacizumab (5 days). Images of formed-structures were taken by phase-contrast microscopy (4X) (Taxol: 10µM, bevacizumab: 1.5mg/mL) B. E4+EC-eGFP increase the ability of SKOV-3 to form 3D structures comparing to SKOV-3 alone. Confocal imaging representing cancer cells spheroids and the interaction of SKOV-3 with E4+EC-eGFP (angiospheres). Scale bar= 20 μm. Primary spheres (cancer spheroids or angiospheres alone) were maintained up to 7 days. Pictures of the entire well were taken and the surface area of SKOV-3 integrated in the angiospheres or SKOV-3 spheroids alone was determined by ImageJ64 software. Secondary and tertiary spheres were produced as indicated in Material and Methods and the surface area measured as described. Graph bars display the values representing the mean ± the range of triplicate determinations. C. E4+EC-eGFP supports sphere formation under bevacizumab treatment. E4+EC-eGFP and SKOV-3 dissociated into single cell suspension by trypsinization were suspended in 3D media in the presence or absence of bevacizumab (1.5mg/mL). Spheroids were formed
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and bevacizumab was added daily to the culture during 7 days. E4+EC-eGFP survival was analyzed by ImageJ64 software based on the area covered by E4+EC-eGFP spheroids. Graph bars display the values representing the mean ± the range of triplicate determinations (4X, scale bar= 500 μm).

**Figure 6. Schematic representation of the FGF-2/PI3K-Akt autocrine loop in E4+ECs.** Cross-talk between Akt and FGF-2 mediates an autocrine pathway leading to E4+ECs survival and angiogenesis during bevacizumab treatment.
Figure 1.

A. 

B. Ovarian Cancer cells

Co-culture under starvation for 24 h

HUVEC cells collection

Phospho-Flow: pAkt (S473)

HUVEC control

HUVEC cells

HUVEC pAkt (S473)

SKOV-3

GOC2

GOC-A2

OV CAR-3

pAkt (S473) quantification (normalized to control)

0  5  10  15  20  25  30

Cln  SKOV-3  GOC2  GOC-A2  OVCAR-3

*** *** ***
Figure 2.

A. Functional Assays
- Cell proliferation (XTT)
- Cell viability (Live / Dead)
- Apoptosis (PARP/Cleaved PARP)
- Migration (Wound closure assay)
- Angiogenesis (Tube formation)

B. Mean Absorbance (Normalized to control)
- HUVEC
- E4+EC
- HUVEC CM SKOV-3

C. Ethidium homodimer-1

D. PARP Cleavage
- HUVEC
- E4+EC
- HUVEC-CM

E. Control
- Bevacizumab

F. Branching point Number
- HUVEC
- E4+EC
Figure 4.

A. Phospho-Akt (S473) quantification (normalized to control)

B. Absorbance (Fold Decrease)

C. % of dense area

D. Branching point number

E. Absorbance (Fold Decrease)
Figure 5.

A. HUVEC PKH Red

B. SKOV-3 E4*EC-eGFP

C. SKOV-3 E4*EC-eGFP

Table: Initial tumor nodule, Taxol (24h), Residual Disease, Beva (5 Days), Re-growth.

Graph: Spheroids Size (Pixel) for Primary spheres, Secondary spheres, Tertiary spheres. SKOV-J, SKOV-3, SKOV-3 / E4*EC.
Figure 6. Schematic presentation of the FGF-2/PI3K-Akt autocrine loop in endothelial cells

Angiogenic Factors (VEGF-A, PDGFB, FGF1, FGF2…)

Blood vessels

Activated-vascular Endothelial cell

Tumor

FGF-2

VEGF-A

Bevacizumab

FGFR

VEGFR

PI3k

AKT

ERK1/2

FGF-2

Gene Expression

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