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GROWTH FACTORS, CYTOKINES, AND CELL CYCLE MOLECULES

Angiopoietins Promote Ovarian Cancer Progression by Establishing a Procancer Microenvironment

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Despite decades of research, the survival rate of ovarian cancer patients is largely unchanged. Current chemotherapeutic drugs are effective only transiently because patients with advanced disease eventually develop resistance. Thus, there is a pressing need for identifying novel therapeutic targets in ovarian cancer. Mounting evidence suggests that angiopoietins (Angpts) may play an essential role in cancer progression; however, the expression profiles and biological effects of Angpts on ovarian cancer remain largely unknown. Here, we show that, compared with their normal counterparts, expressions of Angpt1, Angpt2, and Angpt4 are increased in ovarian cancer cells and tissues and that human ovarian cancer cells also express the Angpt receptor Tie-2—receptor tyrosine kinase. We show that increased expression of Angpt1, Angpt2, or Angpt4 promotes intraperitoneal growth of ovarian cancers and shortens survival of the experimental mice. We further show, for the first time, that Angpts promote accumulation of cancer-associated fibroblasts and tumor angiogenesis in the ovarian cancer microenvironment, as well as enhance ovarian cancer cell proliferation and invasion *in vivo*. In addition, we establish a novel function of Angpts in promoting proliferation and invasion and inducing Tie-2 and extracellular signal-regulated kinase 1/2 activation in ovarian cancer—associated fibroblasts. Taken together, these data suggest that the Angpt—Tie-2 functional axis is an important player in ovarian cancer progression and an attractive target for ovarian cancer therapy. *(Am J Pathol 2014, 184: 2285—2296; http://dx.doi.org/10.1016/j.ajpath.2014.05.006)*

Ovarian cancer is the most lethal gynecologic malignancy.[1] Current chemotherapies for ovarian cancer patients are effective only transiently because patients with advanced disease eventually develop resistance despite significant initial responses.[2,3] Extensive studies have shown a critical role for angiogenesis in the progression of ovarian cancer, and targeted anti—vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) therapies have shown a clinical benefit for ovarian cancer patients.[4—7] However, these effects often were transient and followed by therapeutic resistance.[5,6] One of the potential mechanisms underlying the observed resistance is activation of alternative angiogenic signaling pathways that bypass these inhibitors.[8—10] Thus, simultaneously targeting multiple angiogenic signaling pathways likely will achieve better clinical outcomes for cancer patients.

In addition to the VEGF—VEGFR signaling pathway, the Tie-2—receptor tyrosine kinase (RTK), and their ligands, angiopoietin (Angpt)1, Angpt2, and Angpt4, are

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attractive targets for cancer therapy.[11] Altered expression profiles of angiopoietins have been detected in several cancer types and studies have shown the essential roles of angiopoietins in angiogenesis and progression of certain cancer types.[11–13] However, the expression profiles and biological effects of angiopoietins on ovarian cancer remain largely unknown. Therapeutic agents targeting this functional axis are currently in different stages of preclinical and clinical development. Most of the agents in development selectively target Angpt2, and the remaining few target Angpt1 and Tie-2 with more broad specificity. Little has been done to investigate the comparative effects of Angpt1, Angpt2, and Angpt4 on cancer progression, and the mechanisms underlying these effects are not fully understood. There are still several urgent questions that need be answered to guide effective targeting of the Angpt–Tie-2 functional axis. For example, it remains to be determined whether merely targeting Angpt2 is enough to block the procancer progression effect of this functional axis, and it remains to be determined if simultaneously targeting Angpt2 and Angpt1/Angpt4 will be a more effective therapeutic strategy that leads to better clinical outcomes for cancer patients. To provide guidance to the ongoing drug development efforts that target this functional axis, it is necessary to understand the relative contributions of Angpt1, Angpt2, and Angpt4 to ovarian cancer progression and the mechanisms underlying their effects. The studies reported herein answer these questions.

We show that expressions of Angpt1, Angpt2, and Angpt4 are increased in human serous ovarian cancers, including primary and secondary cancer lesions, compared with normal ovary, and that a panel of human ovarian cancer cells express Angpt1, Angpt2, and Angpt4, as well as their receptor, Tie-2. We establish for the first time that increased expression of Angpt1, Angpt2, and Angpt4 significantly promotes the intraperitoneal growth of ovarian cancers with comparable efficiency and results in reduced survival of experimental mice. On the contrary, knockdown of Angpt1 or Angpt4 inhibits the progression of ovarian cancer *in vivo* and extends survival of the experimental mice. Immunohistochemical analyses of orthotopic tumors show that in addition to a proangiogenic effect, angiopoietins, especially Angpt2 and Angpt4, promote accumulation of ovarian cancer–associated fibroblasts (OCAFs) within the tumors. We establish for the first time that OCAFs express Tie-2 RTK, and Angpt1, Angpt2, and Angpt4 promote OCAF proliferation and invasion and induce activation of Tie-2 and extracellular signal-regulated kinase (ERK)1/2 in these CAF cells. These results establish a novel role for angiopoietins in promoting accumulation and/or expansion of CAFs and suggest a broad role for angiopoietins in establishing a pro–ovarian cancer microenvironment beyond simply promoting tumor angiogenesis. Furthermore, we show that ovarian cancer cells also express Tie-2 RTK and that Angpt2 and Angpt4 promote ovarian cancer cell proliferation *in vivo* and enhance invasion of ovarian cancer cells into host organs. These results suggest that the Angpt–Tie-2 functional axis works in a paracrine fashion on endothelial cells and CAFs to establish a protumor microenvironment as well as in an autocrine fashion on ovarian cancer cells. Together, these results suggest that the Angpt–Tie-2 functional axis is an important player in ovarian cancer progression and Angpt1, Angpt2, and Angpt4 are important targets for ovarian cancer therapy. A therapeutic strategy that targets Angpt1/Angpt2/Angpt4 or Angpt2/Angpt4 simultaneously should be developed to safely and more effectively block this functional axis and to achieve better clinical outcomes for ovarian cancer patients.

Materials and Methods

Patient Ovarian Cancer Samples, Cells, and Reagents

OVCAR-3ip and SKOV-3ip cells were derived as described.[3] Ovarian cancer samples were obtained from the Cooperative Human Tissue Network at the University of Pennsylvania and Ohio State University, and details of the human tissues are provided in the legend to Figure 1. OCAFs were derived from fresh human ovarian cancer samples from the Cooperative Human Tissue Network and cultured in fibroblast medium (FM; ScienCell Research Laboratories, Carlsbad, CA). Human umbilical vein endothelial cells (HUVECs) were from All-Cells, LLC (Alameda, CA). Human ovarian surface epithelial cells were from the ScienCell Research Laboratories. OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, IGROV1, and SKOV-3 cells were from the National Cancer Institute (Developmental Therapeutics Program; Division of Cancer Treatment and Diagnosis Tumor Repository, Bethesda, MD).

Anti-v5 epitope (Life Technologies, Grand Island, NY), anti–Tie-2, and anti-Angpt1, anti-Angpt2, and anti-Angpt4 (Santa Cruz, Dallas, TX, and R&D Systems, Minneapolis, MN), anti-CD31 (Millipore, Billerica, MA, and BD Biosciences, San Jose, CA), anti–α-smooth muscle actin (α-SMA) (Sigma, St. Louis, MO, and R&D Systems), anti-ERK1/2, anti–phospho-ERK1/2 (Santa Cruz Biotechnology and Cell Signaling Technology, Danvers, MA) antibodies, and the Premix WST-1 Cell Proliferation Assay System (Takara Bio, Mountain View, CA) were used in the experiments. Purified Angpt1, Angpt2, and Angpt4 were obtained from R&D Systems.

RT-PCR and Expression Constructs

RT-PCR was performed and full-length human Angpt1, Angpt2, and Angpt4 cDNAs were generated and cloned along with their COOH-terminal v5-epitope tags to the retroviral expression vector pQCXIP (BD Biosciences) as described.[12,14,15] All expression constructs were verified by DNA sequencing. Retroviruses were generated using these expression constructs and pVSVG in GP2-293 cells following the manufacturer's instructions (BD Biosciences).

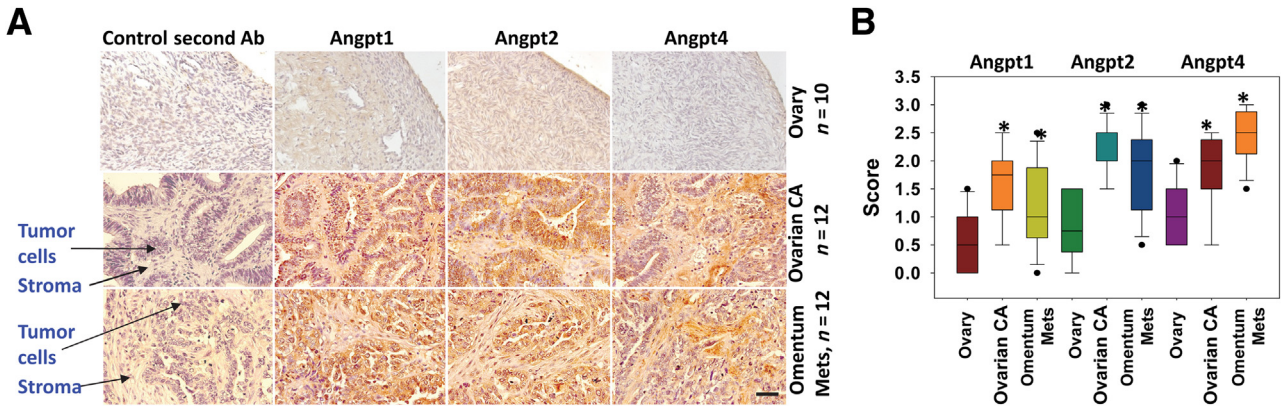

Figure 1 Expression of angiopoietins by ovarian cancers. **A:** Angiopoietin expression in normal ovary, primary serous ovarian cancer, and serous omental metastases was assessed by immunohistochemistry using anti-human Angpt1, Angpt2, or Angpt4 antibodies (Santa Cruz Biotechnology). **B:** Scoring of the immunohistochemistry results. The intensity of immunoreactivity to anti-Angpt1, Angpt2, and Angpt4 antibody in 12 cases of primary serous ovarian cancer, 12 cases of omental metastases of serous ovarian cancer, and 10 cases of normal ovaries were scored. $*P < 0.05$. Scale bar = 100 μm. Ab, antibody; Met, metastases.

Retrovirus Transduction and Real-Time Quantitative PCR

OVCAR-3ip and SKOV-3ip cells were transduced with the retroviruses carrying the empty retroviral expression vector, the Angpt1-, Angpt2-, or Angpt4-infected cells were selected for their resistance to puromycin and pooled populations of puromycin-resistant cells transduced with empty expression vector or expressing Angpt1, Angpt2, or Angpt4 were used in the experiments. Anti-v5 monoclonal antibody (Life Technologies) was used to detect expression of exogenous v5-tagged Angpt1, Angpt2, and Angpt4.

Quantitative real-time PCR (qPCR) was performed as described[12,16] by using SYBR Green PCR Master Mix (Roche Diagnostics, Indianapolis, IN) and the Mx3005P Real-Time PCR Machine (Agilent Technologies, Santa Clara, CA). The cycling parameters used were 95°C for 10 minutes followed by 45 cycles of 95°C (15 seconds), 60°C (30 seconds), and 72°C (15 seconds), and a melting curve analysis. Relative quantification of the targets was normalized with an endogenous housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase) and data analyses were performed using a comparative (ΔΔCt) method according to the manufacturer's instructions (Agilent Technologies).

Western Blot Analysis and ERK Phosphorylation

Serum-free cell culture supernatants were collected from cultured ovarian surface epithelial cells and ovarian cancer cells after 48 hours. Serum-free supernatant (2 mL) was concentrated by NanoSep Centricon-3K (Pall Corporation, Port Washington, NY) to 100 μL. Cells were extracted with 4 × SDS Laemmli sample buffer without the dye. Protein concentrations from all of the samples were determined using D_c Protein Assay Reagents (Bio-Rad, Hercules, CA). Equal amounts of proteins were analyzed by Western blot as described.[17] Ponceau S staining was used as a control for loading and transfer efficiency for secreted proteins in cell culture supernatants. Ten percent β-mercaptoethanol was added to samples as indicated to reduce disulfide bonds.

OCAF cells and HUVECs were cultured until subconfluence and switched to serum-free medium for 24 hours. Purified Angpt1, Angpt2, and Angpt4 (R&D Systems), or basic fibroblast growth factor/VEGF (as positive controls), were applied to the serum-starved CAF cells and HUVECs for different lengths of time. The cells then were lysed and equal amounts of extracted proteins were analyzed by Western blot using anti–phospho-ERK1/2 and anti-ERK1/2 to detect phosphorylated and total amounts of ERK1/2 proteins, respectively.

Cell Proliferation and Invasion Assays

Cell proliferation assays were performed by seeding CAFs or ovarian cancer cells at 2×10^3 cells/well in 96-well plates in triplicate in FM medium or 10% fetal bovine serum RPMI-1640. After 24 hours, the cells were switched to fresh 1:1 mixture of FM:Dulbecco's modified Eagle's medium or 2% fetal bovine serum RPMI 1640 medium in the absence or presence of 250 ng/mL of angiopoietins. Cell proliferation assays were performed using the Premix WST-1 Cell Proliferation Assay System, following the manufacturer's instructions.

Invasion assays (Transwell; Corning, Corning Inc., NY) were performed as described.[17] Briefly, 0.5×10^6 of ovarian cancer cells or 1×10^6 of OCAFs in 2% fetal bovine serum RPMI 1640 medium or a 1:1 mixture of FM:Dulbecco's modified Eagle's media, respectively, were placed in the upper chambers of the Matrigel-coated (BD Biosciences) Transwell inserts (Corning) in triplicate. Two percent fetal bovine serum RPMI 1640 medium or a 1:1 mixture of FM:Dulbecco's modified Eagle's media in the presence or absence of 250 ng/mL of Angpt1, Angpt2, or Angpt4 was added to the bottom chambers. After incubation at 37°C for 30 hours, the ovarian cancer cells and OCAFs that migrated through the Transwell and reached the underside of the inserts

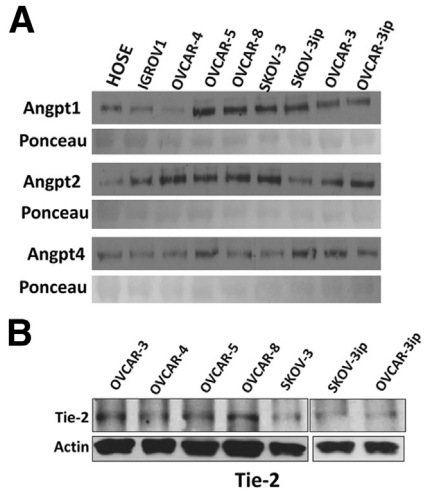

Figure 2 Expression of angiopoietins and Tie-2 protein by ovarian cancer cells. **A:** Western blot shows the levels of endogenous Angpt1, Angpt2, and Angpt4 secreted by HOSEs and a panel of human ovarian cancer cells. The intensities of approximately 50-kDa Ponceau staining bands on the transferred membranes were used as the control for protein loading and transferring efficiency. **B:** Western blot shows the levels of endogenous Tie-2 RTK. Western blot analysis was performed using whole-cell lysates derived from a panel of human ovarian cancer cells. Total protein (50 μg) was loaded in each lane. Actin was used as a loading control.

were fixed and stained using the Diff-Quick Stain Set (Polysciences Inc., Warrington, PA). The stained cells in 20 randomly selected 200× microscopic fields then were counted using QCapture Suite Software (Qimaging, Surrey, BC).

Intraperitoneal Ovarian Cancer Growth Experiments

Pooled populations of OVCAR-3ip and SKOV-3ip cells transduced with empty expression vector or overexpressing Angpt1, Angpt2, or Angpt4 were used for intraperitoneal tumor growth experiments. Briefly, 2×10^6 cells were injected intraperitoneally into each immunocompromised Rag-2/Il2rg mouse (Rag2; Taconic, Hudson, NY). For each type of infected ovarian cancer cell, 6 to 10 mice were used. Following an approved Institutional Animal Care and Use Committee at the Icahn School of Medicine at Mount Sinai protocol, after injection of the cancer cells, mice were monitored closely and a mouse survival analysis was performed. Mice were sacrificed when they appeared moribund or displayed signs of distress, at which time they were recorded as dead. At the conclusion of the experiments, tumors and adjunct mouse tissues were removed, fixed, and sectioned for further histologic and immunologic analyses.

Histology, Immunohistochemistry, and Immunofluorescence

Histology was performed as described.[12] Paraffin sections derived from ovarian cancer patients and the ovarian cancer and adjacent normal mouse tissues derived from the *in vivo* experiments were stained immunohistochemically. Immunohistochemical analysis of Ki-67, a proliferation marker, was performed on the tumor sections derived from OVCAR-3ip control cells and OVCAR-3ip cells expressing v5-tagged angiopoietins. Ki-67$^+$ cells were counted in five randomly selected 400× microscopic fields derived from three different tumors for each condition. The percentage of Ki-67$^+$ tumor cells was determined by dividing the number of positive tumor cells by the total number of tumor cells per microscopic field. Paraffin sections were derived from 12 cases of primary serous ovarian cancer, 12 cases of omental metastases of serous ovarian cancer, and 10 cases of normal ovaries, and reacted with anti-Angpt1, anti-Angpt2, or anti-Angpt4 antibody (Santa Cruz Biotechnology). The immunoreactive intensity of each case was scored by two people independently as follows: 0 indicates negative; 1, weak; 2, intermediate; and 3, strong staining.[12] The scores were averaged, and standard deviations and *P* values were calculated.

The frozen sections of the ovarian cancer tissues derived from the *in vivo* experiments were stained with the fluorescein isothiocyanate—conjugated anti-v5 monoclonal antibody (Life Technologies) and/or the Cy3-conjugated anti—α-SMA antibody (Sigma). The single- and double-stained immunofluorescent images were taken and compared.

Statistical Analysis

Other than survival experiments, the Student's *t*-test was used to analyze statistical differences between the control and experimental groups. For mouse survival experiments, log-rank statistical analysis (SigmaPlot version 11.0; Systat

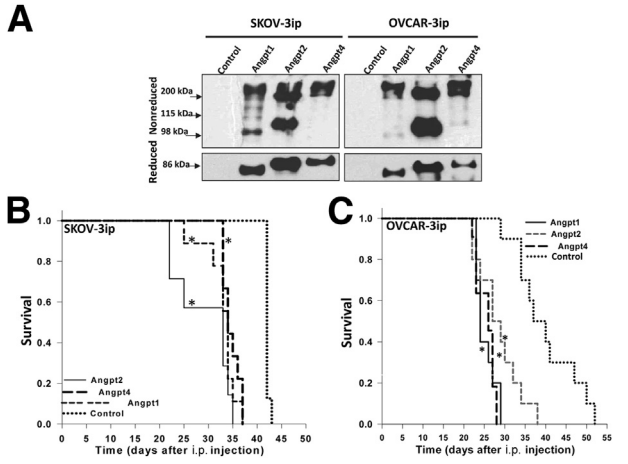

Figure 3 Angiopoietins promote *in vivo* growth of ovarian cancer cells and significantly shorten the survival length of experimental mice. **A:** Establishment of SKOV-3ip and OVCAR-3ip cells expressing v5 epitope—tagged Angpt1, Angpt2, and Angpt4 or transduced with the empty expression vector alone (control). Secreted v5-tagged angiopoietins were detected by anti-v5 monoclonal antibody under nonreducing and reducing conditions. The multiple banding patterns are consistent with multimerization of angiopoietins; angiopoietin monomers are formed under the reducing condition. The effects of angiopoietins on intraperitoneal progression of SKOV-3ip (**B**) and OVCAR-3ip (**C**) cells and survival curves of the experimental mice are shown. At least six mice were used for each type of transduced ovarian cancer cell. $^*P < 0.05$.

Software Inc., San Jose, CA) was performed. Differences were considered statistically significant at $P < 0.05$.

Results

Human Ovarian Cancer Tissues and Cells Express Endogenous Angiopoietins

Expression of angiopoietins has been reported in a variety of non−vasculature-associated cell types, including monocytes[18] and myocytes,[19] and a variety of tumor cells[12,20−22]; however, the expression profiles of angiopoietins in ovarian cancer are largely unknown. We therefore performed immunohistochemical analyses of the sections derived from 12 cases of primary serous carcinoma, 12 cases of omental metastases of serous carcinoma, and 10 cases of normal human ovaries. Compared with normal ovary, expression of Angpt1, Angpt2, and Angpt4 was elevated in primary serous carcinoma as well as in omental metastatic lesions of serous carcinoma, one of the most common sites of ovarian cancer metastasis (Figure 1).[23] Interestingly, there are distinct localization patterns of angiopoietins within the tumor: Angpt1 and Angpt2 are more concentrated in the tumor cells whereas Angpt4 has a higher expression level in the tumor stroma (Figure 1A). This pattern is consistent in both primary and metastatic lesions.

To compare the levels of angiopoietins and Tie-2 transcripts in human ovarian cancer cells and human ovarian surface epithelial cells (HOSEs) (ScienCell Res Lab), we performed real-time RT-qPCR analyses and observed higher expression levels of Angpt1, Angpt2, Angpt4, and Tie2 in OVCAR-3ip and SKOV-3ip human ovarian cancer cells compared with normal HOSEs (Supplemental Figure S1). RT-qPCR results show only the relative transcript levels in ovarian cancer cells and HOSEs. To confirm that ovarian cancer cells produce higher levels of endogenous angiopoietin proteins, we collected and concentrated serum-free cell culture supernatants derived from a variety of ovarian cancer cell lines and HOSEs and observed higher levels of secreted Angpt1, Angpt2, and Angpt4 in ovarian cancer cells (Figure 2A). Although earlier studies indicated that Tie-2 is expressed largely by endothelial cells and bone marrow progenitor cells,[24,25] consistent with other findings,[12] our RT-qPCR results showed that cancer cells express Tie-2

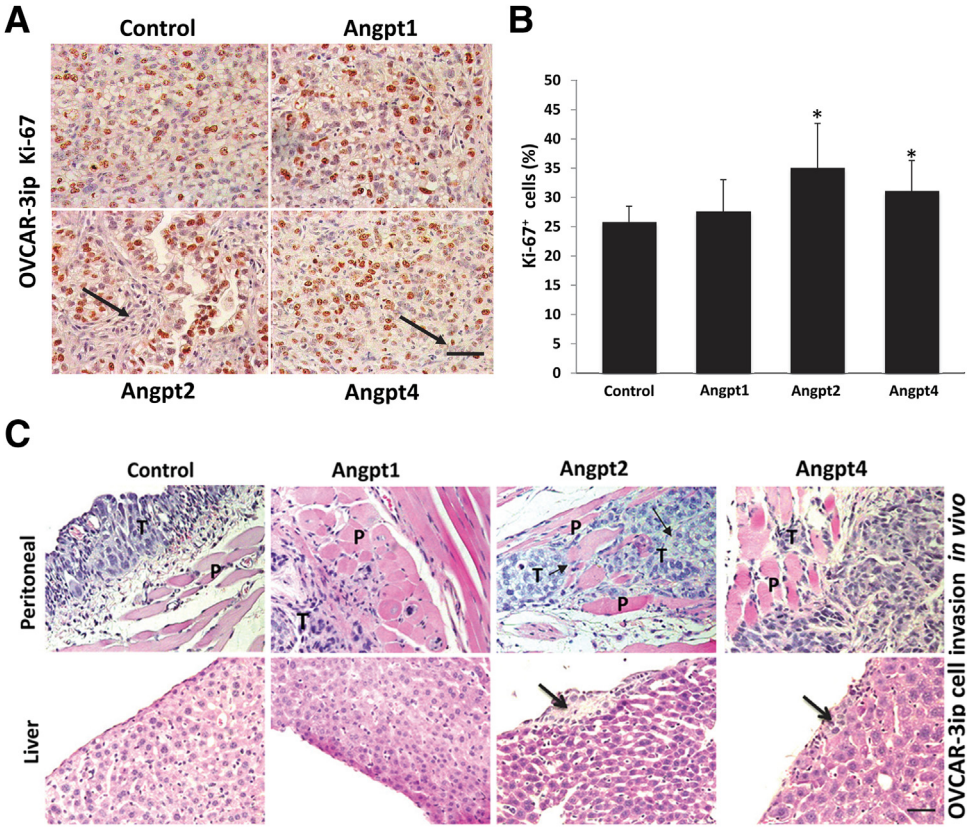

Figure 4 Angpt2 and Angpt4 promote ovarian cancer cell proliferation and invasion *in vivo*. The tumor sections of intraperitoneal tumors derived from the transduced OVAR-3ip cells reacted with anti−Ki-67 antibody. **A:** Representative images of Ki-67−stained tumor sections derived from OVCAR-3ipControl, OVCAR-3ipAngpt1, OVCAR-3ipAngpt2, and OVCAR-3ipAngpt4 cells. **B:** Quantification of Ki-67$^+$ cells per microscopic field. Ki-67$^+$ cells were counted in five randomly selected 400× microscopic fields derived from three different tumors for each condition. The percentage of Ki-67$^+$ tumor cells was determined by dividing the number of Ki-67$^+$ tumor cells by the total number of tumor cells per microscopic field. **C:** Angpt2 and Angpt4 promote invasion of ovarian cancer cells. Representative images of H&E-stained intraperitoneal tumor sections derived from OVCAR-3ip cells transduced with empty expression vector or overexpressing angiopoietins. **A** and **C: Arrows** respectively indicate stroma and invaded ovarian cancer cells in the liver tissues. *$P < 0.05$. Scale bars: 50 μm (**A**); 40 μm (**B**). T, tumor cells; P, peritoneal tissues.

transcripts, as well (Supplemental Figure S1). To establish that Tie-2 protein is expressed by human ovarian cancer cells, we performed Western blot analysis using whole-cell lysates derived from a panel of ovarian cancer cells. We found that these ovarian cancer cells indeed express endogenous Tie-2 protein (Figure 2B).

Angiopoietins Promote Intraperitoneal Ovarian Cancer Growth

To establish roles of the angiopoietins in ovarian cancer progression and compare their relative contributions, we established orthotopic mouse models that mimic the development of intraperitoneal tumors and metastasis of ovarian cancer cells to various organs within the abdominal cavity, the condition associated with the late-stage disease.[3] To study the effects of angiopoietins on ovarian cancer growth and progression, we expressed exogenous Angpt1, Angpt2, and Angpt4 in SKOV-3ip and OVCAR-3ip cells. SKOV-3ip and OVCAR-3ip cells were derived from *in vivo* selections of their parental cells, SKOV-3 and OVCAR-3 cells, based on their intraperitoneal growth ability.[3] SKOV-3ip and OVCAR-3ip cells transduced with the empty expression vector were used as negative controls. The expression of v5-epitope–tagged angiopoietins was validated by Western blot analyses of cell culture supernatants of the transduced ovarian cancer cells (Figure 3A). The multiple banding patterns of angiopoietins are typical and reflect the multimerization status of the angiopoietins.[12,14,15] These multimers can be dissociated into monomers under reducing conditions with 10% 2-mercaptoethanol (Figure 3A).

To determine the effects of angiopoietins on ovarian cancer progression *in vivo*, the pooled populations of SKOV-3ip and OVCAR-3ip cells expressing angiopoietins or transduced with the empty expression vector were injected intraperitoneally into each immunocompromised Rag-2/II2rg mouse. Increased expression of Angpt1, Angpt2, and Angpt4 in OVCAR-3ip and SKOV-3ip cells significantly promoted the cancer progression and reduced the survival length of experimental mice compared with the controls and there were no statistically significant differences among the pro-ovarian cancer activities of these angiopoietins in both ovarian cancer cells (Figure 3, B and C). In SKOV-3ip cells, Angpt2 showed the most potent promotive effect on intraperitoneal growth of the ovarian cancer cells because its overexpression reduced the median mouse survival to less than 5 weeks, whereas in OVCAR-3ip cells all three angiopoietins showed similar pro-ovarian cancer growth activities (Figure 3, B and C). Taken together, these results suggest that all three angiopoietins promote the progression of ovarian cancer *in vivo*.

To confirm the pro-ovarian cancer activity of angiopoietins, we knocked down Angpt1 and Angpt4 expression in OVCAR-3ip and SKOV-3ip cells (Supplemental Figure S2A). We showed that knockdown of Angpt1 or Angpt4 expression inhibited the intraperitoneal growth of OVCAR-3ip and SKOV-3ip cells and significantly extended survival of the experimental mice (Supplemental Figure S2, B and C). Together, these results established the pro-ovarian cancer progression effects of angiopoietins and established that angiopoietins are potential targets for ovarian cancer therapy.

Angiopoietins Promote Ovarian Cancer Cell Proliferation *in Vivo*

Given the fact that increased expression of angiopoietins significantly promotes ovarian cancer progression and

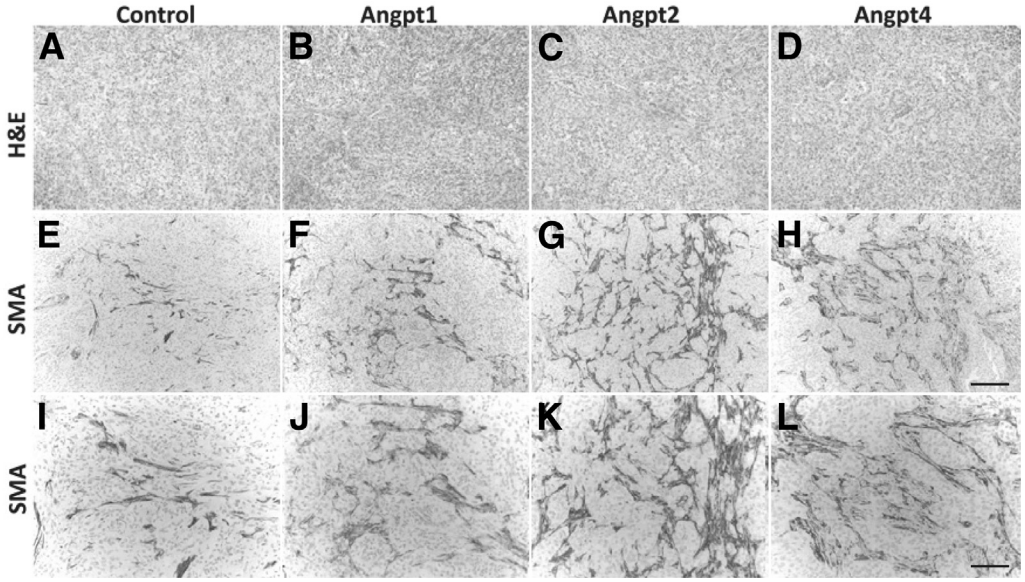

Figure 5 Angiopoietins promote accumulation of the CAFs in ovarian tumors. Tumor sections derived from abdominal OVCAR-3ip ovarian cancers transduced with empty expression vector or overexpressing angiopoietins were stained with H&E (**A**–**D**) and anti–α-SMA (**E**–**L**), highlighting CAFs in these tumors. Scale bars: 100 μm (**A**–**H**); 50 μm (**I**–**L**).

reduces the survival of experimental mice, we assessed the biological effects of angiopoietins on ovarian cancer cells by investigating whether angiopoietins are capable of promoting *in vivo* proliferation of the ovarian cancer cells. Immunohistochemical analysis of Ki-67, a proliferation marker, was performed on the tumor sections derived from OVCAR-3ip control cells and OVCAR-3ip cells with increased expression of angiopoietins. These data showed that increased expression of Angpt2 and Angpt4 significantly increased the number of Ki-67$^+$ and proliferating ovarian cancer cells (Figure 4, A and B). The percentage of Ki-67$^+$ tumor cells was determined by dividing the number of Ki-67$^+$ tumor cells by the total number of tumor cells per microscopic field.

Angiopoietins Promote Ovarian Cancer Invasion into Host Tissues

To investigate the effects of angiopoietins on ovarian cancer invasion, we performed histologic analyses of peritoneal and liver tissues derived from the tumor-bearing experimental mice. We found that increased expression of Angpt2, and, to a lesser extent, Angpt4, promotes invasion of the ovarian cancer cells into host peritoneal (Figure 4C) and liver tissue parenchyma (Figure 4C). The borders of host peritoneal tissues in mice with tumors derived from OVCAR-3ip with increased Angpt2 expression were nearly indistinguishable from tumor tissues that invaded and literally mixed with the host tissue (Figure 4C).

Angiopoietins Promote Tumor Angiogenesis and Accumulation of CAFs in Ovarian Tumors

Protumor angiogenesis effects of angiopoietins have been well established in other cancer types.[11,12,26,27] Immunohistochemical analyses confirmed that Angpt1, Angpt2, and Angpt4 promote tumor angiogenesis during ovarian cancer progression (Supplemental Figure S3). We also observed that the tumor sections derived from the ovarian cancer cells with increased expression of angiopoietins appeared to have more infiltrating host stroma, recognizable by its distinct swirling pattern between clusters of epithelial-like ovarian cancer cells (data not shown). It is well established that tumor stroma plays important roles in promoting cancer invasion, metastasis, progression, and chemoresistance,[28–30] and that the CAFs are associated with an increased risk of invasion and metastasis of a variety of tumor types.[31–33] To assess the presence of CAFs in these ovarian cancers, we performed immunohistochemical analyses on these tumor sections to detect α-SMA, an intermediate-filament–associated protein that routinely is used as a marker for CAFs.[34,35] Results from theses analyses showed a striking increase in the amount of α-SMA–positive CAFs in the ovarian cancers derived from OVCAR-3ip and SKOV3-ip cells expressing increasing amounts of angiopoietins compared with their control counterparts (Figure 5 and Supplemental Figure S4), suggesting a

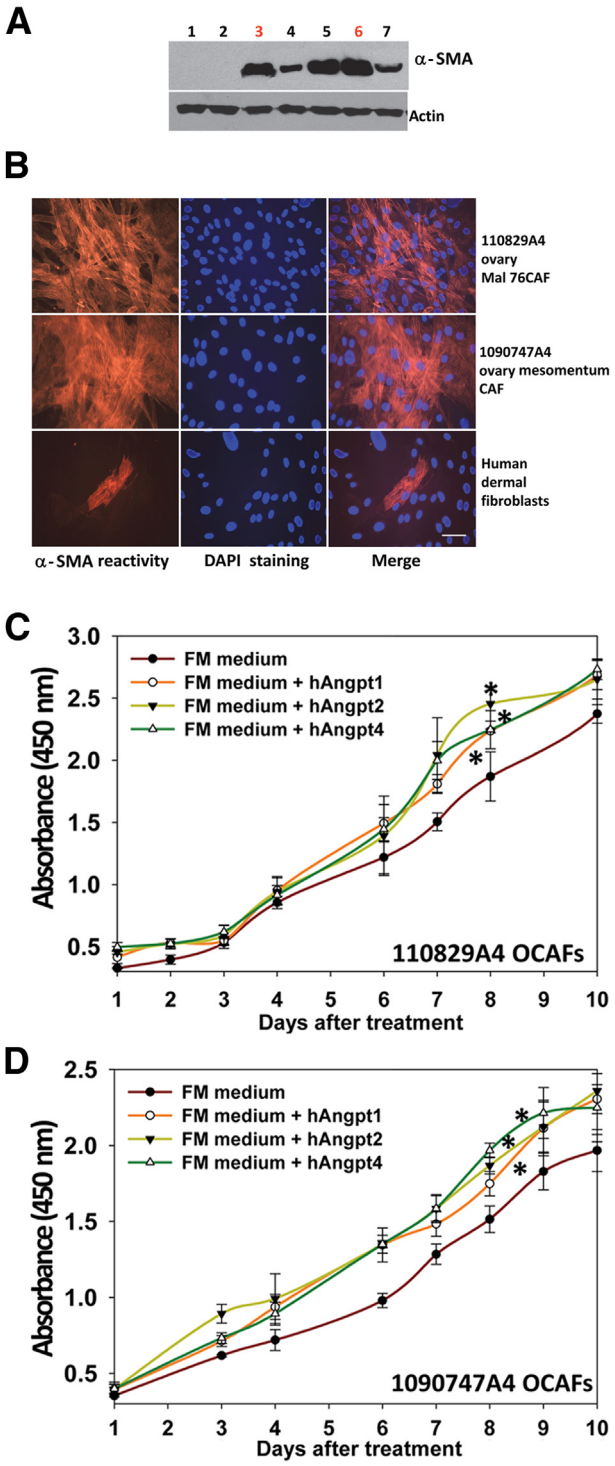

Figure 6 Angiopoietins promotes OCAF proliferation. **A** and **B:** Establishment and characterization of primary OCAFs. **A:** Western blot analysis of whole-cell lysates of five preparations of primary OCAFs (lanes 3 to 7) and human dermal skin fibroblasts (lanes 1 to 2) using anti–α-SMA antibody. Actin was used as a loading control. **B:** Immunocytochemical analyses of α-SMA expression by OCAFs and human dermal skin fibroblasts. **C** and **D:** OCAF proliferation assays in the presence or absence of 250 ng/mL of Angpt1, Angpt2, or Angpt4. Two different preparations of OCAFs were used. The results show representative means ± SDs of one of the two independent experiments performed in triplicate. $^*P < 0.05$. Scale bar = 20 μm. hAngpt, human Angpt.

novel role of angiopoietins in regulating CAF infiltration and/or expansion.

To further assess the relative localization of angiopoietin-expressing ovarian cancer cells and α-SMA–positive CAFs, and taking advantage of our knowledge that Angpt4 is localized on the surface of ovarian cancer cells (Supplemental Figure S5), we performed co-immunofluorescence localization experiments using the fluorescein isothiocyanate–conjugated anti-v5 antibody to detect the ovarian cancer cells expressing v5-tagged Angpt4 and the Cy3-conjugated anti–α-SMA antibody to detect CAFs. Results showed that Angpt4-positive ovarian cancer cells are infiltrated by the α-SMA$^+$ CAFs (Supplemental Figure S6), suggesting a potential role of angiopoietins in attracting CAFs to the angiopoietin-expressing ovarian cancer cells.

Angiopoietins Promote OCAF Proliferation and Invasion

The observed increase in the amount of CAFs in the ovarian cancer tissues that express higher levels of angiopoietins suggests that angiopoietins directly or indirectly (through other soluble factors) affect cellular behaviors of OCAFs. To investigate this possibility, we first established several preparations of primary OCAFs from fresh ovarian cancer samples (The Cooperative Human Tissue Network, University of Pennsylvania and Ohio State University). We validated these cells by assessing their expressions of the CAF marker α-SMA. Two preparations of CAFs from two different ovarian cancer samples that express higher levels of SMA (Figure 6A) were validated further by immunocytochemistry using the anti-SMA antibody to establish that most, if not all, of the cells in the preparations are highly SMA positive (Figure 6B).

These CAF cells were used in proliferation assays and the results showed that Angpt1, Angpt2, and Angpt4 promote OCAF proliferation (Figure 6, C and D). We then assessed the effects of angiopoietins on invasion of these OCAFs through Matrigel in the Transwell invasion assays. Our results showed that Angpt4, and, to a lesser extent, Angpt2 and Angpt1, promote OCAF invasion through Matrigel (Figure 7, A–C). These results suggest that angiopoietins promote infiltration and expansion of OCAFs in ovarian cancers.

To assess whether the pro-ovarian cancer activities of angiopoietins are exerted through their direct effects on ovarian cancer and/or their indirect effect on OCAFs, we performed similar cell proliferation and Matrigel invasion assays using OVCAR-3ip and SKOV-3ip cells in the presence and absence of angiopoietins. Although angiopoietins did not lead to a statistically significant increase of proliferation of OVCAR-3ip and SKOV-3ip cells *in vitro*, angiopoietins significantly promoted invasion of these ovarian cancer cells through Matrigel (Supplemental Figure S7), suggesting that angiopoietins affect ovarian cancer cells directly and indirectly through OCAFs.

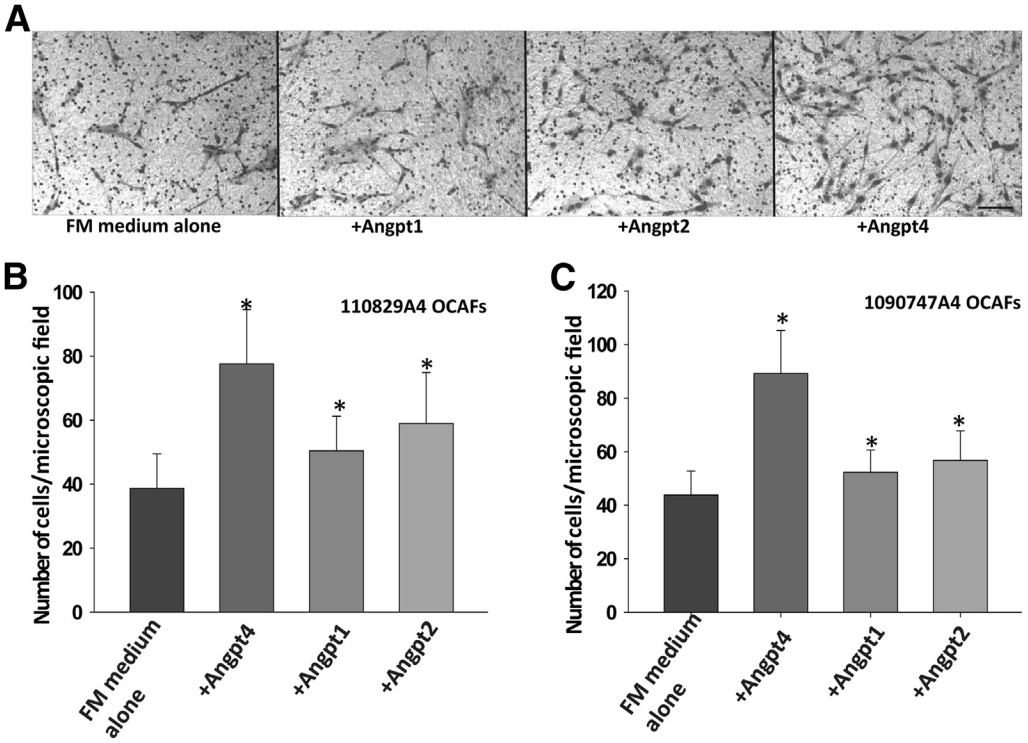

Figure 7 Angiopoietins promote OCAF invasion through Matrigel. Invasion of OCAFs was assessed in the presence or absence of 250 ng/mL of Angpt1, Angpt2, or Angpt4. **A:** Representative images of the OCAF cells show migration through the Matrigel-coated Transwell inserts. **B** and **C:** The OCAFs that migrated through the Transwell inserts in 20 randomly selected 200× microscopic fields were counted and their mean values with SD were quantitated. Two different preparations of OCAFs were used in the studies. The results show representative means ± SDs of one of the two independent experiments performed in triplicate. $^*P < 0.05$. Scale bar = 150 μm.

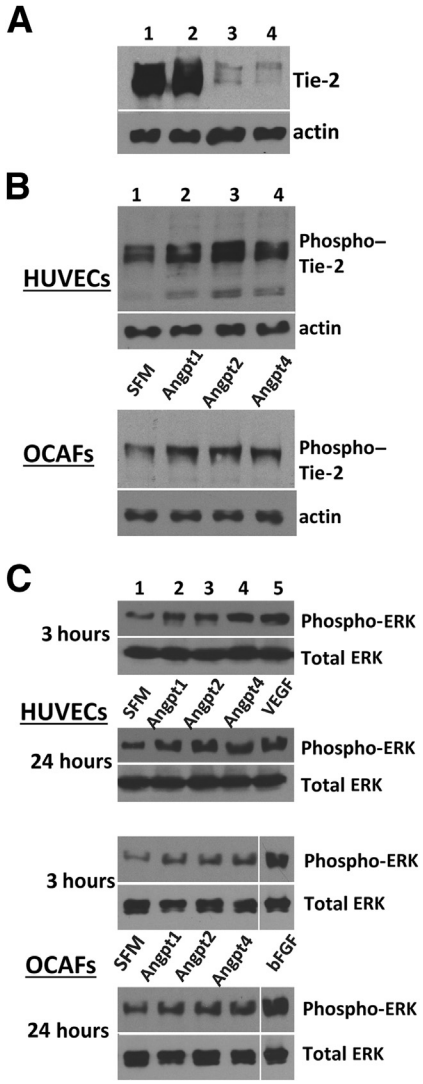

Figure 8 Angiopoietins promote activation of Tie-2 RTK and ERK1/2 kinases in OCAFs. **A:** OCAFs express Tie-2 RTK. Expression of Tie-2 by OCAFs (lanes 3 to 4) and HUVECs (lanes 1 to 2) was determined by Western blot using anti—Tie-2 antibody (Santa Cruz Biotechnology). Fifty microgram of total protein was loaded in each lane. Actin was used as a loading control. Serum-starved OCAF cells and HUVECs were supplied with serum-free medium (SFM) or SFM containing 250 ng/mL of Angpt1, Angpt2, or Angpt4, or 20 ng/mL of basic fibroblast growth factor (bFGF) (for OCAFs)/VEGF (for HUVECs) for 1 hour (**B**), 3 hours (**C**), and 24 hours (**C**). The cells were lysed and proteins were analyzed by Western blot with anti—phospho-Tie2 (**B**) and phospho-ERK1/2 (**C**), with anti-actin (**B**) or anti-ERK1/2 (**C**) antibodies.

Angiopoietins Induce Activation of Tie-2 and ERK1/2 in OCAFs

To assess how angiopoietins affect OCAF behavior, we first investigated whether OCAFs express the Tie-2 receptor and found that OCAFs indeed express Tie-2 RTK, although the level of Tie-2 in OCAFs is much lower than that in endothelial cells (Figure 8A). To determine and compare the effects of angiopoietins on activation of Tie-2 RTK and its downstream signaling pathway in these OCAFs and HUVECs, we treated serum-starved CAFs and HUVECs with Angpt1, Angpt2, Angpt4, or basic fibroblast growth factor/VEGF for various lengths of time. Angpt1, Angpt2, and Angpt4 induced Tie2 activation and activation of downstream ERK1/2 kinase in OCAFs and HUVECs (Figure 8, B and C). Together, these results establish a novel function for angiopoietins and suggest that in addition to endothelial cells, Angpts can regulate CAF function directly and promote their proliferation and invasion. These data also suggest that angiopoietins affect ovarian cancer progression through modulating the tumor microenvironment as well as affecting tumor cells through Tie-2 RTK.

Discussion

Angiopoietins Play Key Roles in Ovarian Cancer Progression and Are Attractive Therapeutic Targets for Ovarian Cancer

Tumor angiogenesis is regulated by proangiogenic and antiangiogenic factors produced by tumor cells and the tumor-associated host cells.[36,37] Sufficient angiogenesis is essential for ovarian cancer growth and progression. Anti—angiogenesis-based therapies such as anti-VEGF/VEGFR therapies have shown clinical benefit for ovarian cancer patients; however, resistance to anti-VEGF and -VEGFR therapies often occurs.[4–7] One of the potential mechanisms of the resistance is activation of alternative angiogenic signaling pathways, which lead to demands of better understanding of other key angiogenic factors such as angiopoietins.

The expression profiles of angiopoietins in various cancer types have been established; however, their expression, effects, and the mechanisms underlying their effects on ovarian cancer previously were unknown. Here, we show for the first time the angiopoietin expression pattern/profiles in human ovarian cancer tissues/cells and establish that Angpt1, Angpt2, and Angpt4 promote ovarian cancer growth and progression. Our results show that Angpt2 and Angpt4 promote proliferation and invasion of ovarian cancer cells and that in addition to promoting tumor angiogenesis, angiopoietins stimulate accumulation of CAFs in ovarian cancers. Furthermore, we show that angiopoietins promote proliferation and invasion of OCAFs and that angiopoietins induce activation of Tie-2 and ERK1/2 in OCAFs, which likely mediates the effects of angiopoietins on OCAFs. Together, our results establish that all three angiopoietins play important roles in promoting ovarian cancer progression and the angiopoietin—Tie-2 functional axis provides a promotive signal to cancer cells in addition to their positive effects on the tumor microenvironment (CAFs and endothelial cells). Our results further suggest that the angiopoietin/Tie2 functional axis is an ideal target for ovarian cancer therapy because it affects cancer cells and their microenvironment simultaneously.

A Novel Promotive Role of Angiopoietins in Regulating CAFs

It has been well established that CAFs have wide-ranging effects on various aspects of the tumor phenotype, including promotion

of proliferation, invasion, and angiogenesis.[29,30,33,38–41] Our data indicated that angiopoietins, especially Angpt2 and Angpt4, promote accumulation of CAFs in ovarian cancer. The results are consistent with the previous reports that another angiogenic factor, VEGF, can induce reactive stroma.[42] A study has shown that conditioned medium from SKOV-3 ovarian cancer cells is capable of inducing the transdifferentiation of normal ovarian fibroblasts to a reactive population positive for α-SMA,[41] although the factor(s) that mediate(s) this effect was not identified. The data presented here suggest that angiopoietins play important roles in promoting infiltration and/or proliferation of CAFs. Angiopoietins modestly promote *in vitro* proliferation of OCAFs and enhance OCAF invasion through Matrigel, suggesting that angiopoietins promote CAF infiltration as well as expansion. Further studies are required to distinguish the effects of angiopoietins definitively on recruitment and expansion of CAFs *in vivo*. We showed that OCAFs express Tie-2 RTK and that angiopoietins induce activation of Tie-2 and a Tie-2 downstream signaling component, ERK1/2, suggesting that angiopoietins affect OCAFs through Tie-2 RTK.

It has been established that CAFs can secrete proteases to degrade the extracellular matrix, literally paving the way for cancer cell migration and invasion.[43] Once localized within the tumor, these OCAFs can promote invasive and angiogenic phenotypes. In addition to promoting tumor angiogenesis, angiopoietins may enhance ovarian tumor progression by simulating CAF accumulation, which in turn promotes invasion, metastasis, and progression of the cancer cells. Thus, blockage of the angiopoietin-induced CAF accumulation may be an effective therapeutic strategy to combat ovarian cancer.

Expression of Tie-2 by a Variety of Different Cell Types Suggests Wider Roles of the Angpt–Tie-2 Functional Axis

Although Tie-2 expression originally was discovered in endothelial cells and hematopoietic stem cells, recent studies have shown Tie-2 expression in a variety of cell lineages, including monocytes,[18] megakaryocytic cells,[44] and cancer cells.[12,21,45] Similarly, our studies showed that ovarian cancer cells express Tie-2. These data are consistent with previous reports indicating that tumor cells exploit vascular proteins in a cell-autonomous manner because Lichtenberger et al[46] reported that autocrine VEGF signaling is required for epithelial tumor cell proliferation. We previously showed that glioblastoma multiforme cells express Tie2 and angiopoietins, which in turn can induce signaling in a cell-autonomous manner,[12] and here we show that angiopoietins promote ovarian cancer invasion *in vitro* (Supplemental Figure S7), supporting a direct role of angiopoietins in ovarian cancer cells.

In addition to signaling through their receptor Tie-2, angiopoietins also are known to function through integrins. Angpt1 was found to induce PC12 neurite outgrowth in a Tie2-independent and β1-integrin–dependent manner,[47] and to reduce cardiac hypertrophy through integrin, but not Tie-2.[48] Furthermore, Angpt2 stimulates breast cancer metastasis through the α5β1 integrin-mediated, but not Tie-2–mediated, signaling pathway.[49] Additional detailed studies are required to determine whether integrins are involved in mediating the effects of angiopoietins on OCAFs and ovarian cancer cells.

Targeting Angpt2 and Angpt4 Simultaneously Is a New Cancer Therapy Strategy

Because of the potential important roles of Angpt1 in the maintenance of normal vasculature and the limited knowledge of Angpt4, the efforts of targeting angiopoietins have been focused on Angpt2.[11,50] Unlike Angpt1, Angpt4 has limited expression in normal tissues.[51] We recently established that Angpt4 is up-regulated in human glioblastoma multiforme and plays a key role in glioma growth and progression.[12] Our current study showed that in addition to Angpt1 and Angpt2, Angpt4 is highly expressed by human ovarian cancer and promotes ovarian cancer growth and progression. Our results suggest that simply blocking Angpt2 function/bioactivity may not be sufficient to interfere with the Angpt–Tie-2 functional axis in ovarian cancer/glioblastoma multiforme and other cancer types in which Angpt4 plays an essential protumor role. Safely and more effectively blocking protumor activity of the Angpt–Tie-2 functional axis likely requires simultaneously targeting Angpt2 and Angpt4 in human cancers.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2014.05.006*.

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