Modulation of Angiogenic Phenotype Alters Tumorigenicity in Rat Ovarian Epithelial Cells

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Abstract

Vascular endothelial growth factor (VEGF) expression correlates with microvessel density, stage, malignant ascites, metastasis, and survival in ovarian cancer. By transducing VEGF165 into a nontumorigenic rat ovarian surface epithelial cell line (ROSE199), we investigated the direct effect of an angiogenic phenotype on tumor development. The neu oncogene, which is overexpressed in >30% of ovarian cancers, was used in comparison. Neu-transfected ROSE199 cells showed phenotypic characteristics of transformation in vitro with an abundance of focus-forming units in monolayer cultures and anchorage-independent growth in soft agar. In contrast, VEGF-secreting ROSE199 cells (VR) retained normal morphology and in vitro growth characteristics (e.g., proliferation rate) compared with parental ROSE199 cells. Interestingly, injection of VR cells into athymic mice formed malignant ascites in 100% of the animals when injected into the peritoneum and developed vascularized tumors in 85% of the mice when injected s.c. Furthermore, blocking VEGF-mediated signaling by the Flk-1/KDR receptor kinase inhibitor SU5416 significantly inhibited the growth of VR tumors. To validate that the proangiogenic switch is responsible for tumor development, the angiogenic phenotype was balanced by the inducible coexpression of endostatin under the control of Tet-activated promoter. Coexpression of endostatin along with VEGF reversed the tumorigenic phenotype of VR cells. These studies show that alterations in the angiogenic characteristics of ovarian surface epithelium may play an important role in the etiology of ovarian cancer, and that inhibition of angiogenesis can be effective in the treatment of epithelial ovarian cancer. [Cancer Res 2007;67(8):3683–90]

Introduction

The most common form of ovarian cancer develops from the malignant transformation of the ovarian surface epithelium (OSE), a single layer of cells that surround the ovaries. Ovarian epithelial cells secrete lysosomal proteases that aid in follicular rupture during ovulation. After ovulation, epithelial cells proliferate and migrate to heal the resultant wound. The etiology of ovarian surface epithelial cancer, however, remains poorly understood. Epidemiologic evidence supports two main theories: the "incessant ovulation" hypothesis and the "gonadotropin" hypothesis. The "incessant ovulation" hypothesis suggests that the risk of ovarian cancer increases with increased number of ovulations (1). This is supported by evidence that a decrease in occurrence of ovarian epithelial cancer (OEC) is found in women with reduced ovulatory cycles due to pregnancy, lactational amenorrhea, and oral contraceptive use. It is therefore suggested that repeated wounding and healing of the OSE increases the susceptibility for malignant transformation. Growth factors and cytokines influence post-ovulatory repair of the OSE and altered regulation of cytokine-mediated signaling may also be involved in the development of ovarian cancer (2). The "gonadotropin" hypothesis predicts that high levels of pituitary gonadotropins increase cancer risk by stimulation of the OSE (3). Studies have shown that gonadotropins stimulate OSE proliferation and induce expression of growth factors such as epidermal growth factor (4), keratinocyte growth factor (5), and vascular endothelial growth factor (VEGF; ref. 6), which may play a role in the onset of OEC. It is likely that the etiology of ovarian cancer is multifactorial, with genetic, hormonal, and environmental factors playing a role.

Although ovarian cysts have been suggested to be involved in ovarian cancer development, no potential precursor lesions have been identified. Normal OSE can be transformed by introducing SV40 large T antigen alone or with the catalytic subunit of telomerase (hTERT), resulting in immortalized cell lines (7), but have required additional factors such as c-erbB2 or mutant Ha-ras transduction to induce tumorigenicity (8). Conditional expression of SV40 large T antigen under the control of MISIIR promoter lead to ovarian cancer in transgenic mice (9). Other recent studies have characterized genetic models of familial ovarian cancer involving BRCA1 mutations. These studies have suggested that Myc activation is necessary and sufficient to transform BRCA1- and p53-deficient OSE (10, 11). We questioned whether phenotypic alterations are necessary for immortalized nontumorigenic ovarian surface epithelial cells to become tumorigenic. It is hypothesized that acquiring an angiogenic phenotype is a crucial and necessary step in the establishment of ovarian cancer.

Neoangiogenesis is an important step in tumor growth and metastasis (12, 13). To grow beyond a critical size, solid tumors must acquire an angiogenic phenotype to attract nourishing vasculature (14). Along with tumor cells, fibroblasts, macrophage, and endothelial cells secrete a number of factors that influence the angiogenic environment of a tumor. Acquisition of an angiogenic phenotype is ultimately regulated by the net balance between positive and negative regulators in the local environment (15, 16). Studies have shown that VEGF, a potent angiogenic growth factor, is up-regulated in ovarian tumors, and that high levels of VEGF are found in associated ascites fluid in ovarian cancer patients (17, 18). Furthermore, neutralization of VEGF with a monoclonal antibody inhibits ovarian tumor growth and ascites formation in athymic
mouse models (19). We therefore determined whether the acquisition of an angiogenic phenotype by OSE could provide survival advantage in vivo and confer tumorigenic phenotype. Using a nontumorigenic cell line derived from normal rat ovarian surface epithelial cells (ROSE199), we induced high-level expression of VEGF165 and characterized its effects on OSE both in vitro and in vivo. Constitutive expression of VEGF in ROSE199 cells led to tumor development and malignant ascites formation. Balancing the angiogenic phenotype with inducible expression of endostatin reversed the tumorigenic phenotype without affecting the in vitro growth characteristics.

**Materials and Methods**

Chemicals. **SU5416**, 3′-[2,4-dimethylpyrrol-5-yl]methylidencyl-indolin-2-one, was a gift from Sugen (San Francisco, CA; ref. 20). Zeocin-selective reagent was purchased from Invitrogen (Carlsbad, CA; R250-05); doxycycline hydrochloride (D-9891) and tissue culture grade DMSO were purchased from Sigma (St. Louis, MO; D-2650).

Cell culture. The parental ROSE199 cells were derived from a line of spontaneously immortalized rat OSE and cultured as previously described (21). Selection and maintenance of Neu/ROSE199 cells was accomplished by growing cells in the presence of 0.8 mg/ml G418 (Calbiochem, San Diego, CA). VR and control vector clones were selected and maintained in DMEM in the presence of 50 μg/ml Zeocin (Invitrogen). G418 (0.8 mg/ml; Calbiochem) was used for selection and maintenance of VR/2 cells, and 200 μg/ml Hygromycin B (Sigma) was added for selection and maintenance of VR/2-end cells.

Generation of transfected cell lines. Human VEGF cDNA was cloned into the cytomegalovirus promoter–driven mammalian expression vector pSecTag C (Invitrogen). The full-length VEGF165 cDNA (498 bp) was inserted directly between the cytomegalovirus and NotI sites downstream of the murine IgGc chain signal peptide (VR). ROSE199 cells were transfected with empty vector or VEGF expression construct by calcium phosphate method (22). Zeocin-resistant colonies were selected with optimized concentrations of Zeocin (50 μg/ml), and culture supernatants were assayed for VEGF165 by ELISA. The oncogenic Neu/ROSE199 cell line was generated using retrovirus transduction encoding the sequence for rat transforming neu inserted into retrovector pNL/A, kindly provided by Dr. A.W. Edwards (University of Cambridge, Cambridge, United Kingdom). The retroviral plasmid is a derivative of pgagneoSRV in which the rat β-actin promoter replaced the SV40 promoter (23).

Generation of tetracycline-regulated expression of endostatin in VR cells. A 104-bp secretory signal sequence of the VEGF165 gene was cloned upstream to the 533-bp human endostatin cDNA. An upstream primer was synthesized incorporating the entire secretory signal of VEGF165 gene and BamHI restriction enzyme site (5′-GGGTAATTGCTAGCTTTTCTCAGTT-CTTGGGTTGACATGCTCGGCTTGGTGTCACTCTACCTCCAACATGC-CAAGTGGTCCCAGGCTGCACCCCACAGCCACCGACTTC-3′). This primer was synthesized to introduce a HindIII site (5′-GGG-AAGCTTCTACTTGGAGCTCAT-3′). This cDNA contained nucleotide sequences 1504 to 2055 of human collagen XVIII for cloning into the BamHI/HindIII site of pREV-TRE retroviral response vector (Clontech, Palo Alto, CA). We used the RevTet System to produce infectious retroviral particles providing inducible expression of human endostatin.

Measurement of VEGF165 and endostatin protein expression. Secretion of VEGF165 protein from VR or vector only transfected ROSE199 cells in vitro was determined using a VEGF ELISA kit according to the manufacturer's protocol (R&D Systems, Inc., Minneapolis, MN). Tetracycline-regulated protein expression was verified using human endostatin specific ELISA (R&D Systems), similarly as described above, except for the presence or absence of doxycycline (1 μg/ml).

Proliferation rate of ROSE199 cell lines. Cell proliferation in vitro was determined by bromodeoxyuridine cell proliferation assay, according to manufacturer's instructions (Oncogene Research Products, Cambridge, MA; ref. 24).

**Characterization of transfected ROSE199 cells in vitro.** The propensity for focus formation was assessed in vitro. As a positive control, ROSE199 cells expressing the mutant rat neu oncogene were used. Briefly, 3.0 × 10^4 neu-transfected ROSE199, VR, or control cells were seeded into 10-cm tissue culture dishes and allowed to grow to 100% confluency. The cells were incubated at 37°C + 5% CO_2 for an additional 2 to 4 days before assessing focus (colony) formation. Cultures were stained with crystal violet, and the focal unit size was calculated. The potential for cells to form colonies in semi-solid medium was measured by suspending 4 × 10^4 VR, control, or Neu/ROSE199 cells in 1 ml of top agar consisting of 0.36% Bacto-agar in DMEM with 10% fetal bovine serum (FBS). The top agar was plated onto 1.5 mL of solidified bottom agar, consisting of 0.5% Bacto-agar in DMEM. After 21 days, the dishes were examined, and colonies containing more than 50 cells were counted. Cloning efficiencies were determined by the following ratio: number of colonies counted/number of cells seeded.

**In vivo Matrigel assays.** Matrigel plug angiogenesis assays and morphometric analysis of vessel density were carried out as previously described (25). When VR/2-end or VR/2 cells were used, mice were given water containing 2 mg/ml doxycycline plus 5% sucrose ad libitum.

**Tumorigenicity assays.** All animals used in this study were treated humanely, and the experiments were carried out by strictly following the protocols approved by the Institutional Animal Care and Use Committee. Female athymic mice, 6 to 8 weeks old, were obtained from National Cancer Institute. Control or VR cells (2 × 10^6) were suspended in 200 μL of sterile saline and injected either i.p. or s.c. into the right hind flank of the animal. For experiments, animals were treated once daily with a 50-μl ip. bolus injection of SU5416 (15 mg/kg/d), a selective Flk-1/KDR receptor tyrosine kinase inhibitor, in DMSO or vehicle alone for 14 days beginning 3 days after implantation. Treatment was initiated on the same day as cell transplantation in “preventive” studies or on day 7 for “therapeutic” studies.

To test the effect of endostatin on VR tumor development, 2 × 10^6 VR/2-end or VR/2 cells were resuspended in 200 μL of sterile PBS and injected s.c. into the right hind flank of the animals. After 7 days, half the mice injected with VR/2-end cells (n = 10) and animals injected with VR/2-end cells (n = 10) received drinking water supplemented with doxycycline (2 mg/ml) and sucrose (5%) ad libitum. The other half of the VR/2-end–injected mice (n = 10) received plain water to rule out possible effects of doxycycline supplementation. For all s.c. models, tumor growth was measured by vernier calipers. Tumor volume was calculated by the following formula: tumor volume (mm^3) = (π/6) × (a × b^2), where a represents the length (longest diameter in mm), and b represents the tumor width (shortest diameter in mm). For i.p. models, mice were monitored for ascites formation and signs of disease by palpation. Animals were kept until natural death, or until they became moribund and necessitated culling. Animals were autopsied for the presence of solid tumor and ascites. Statistical significance was determined by Student’s t test. To ascertain that the tumors were derived from the injected cells, parts of the tumor tissues were minced, treated with collagenase, and cultured in DMEM plus 10% FBS and 50 μg/ml Zeocin (VR cells) and 0.8 mg/ml G418 (VR/2-end) to verify maintenance of antibiotic resistance. Diagnostic toxicity was assessed by average body weight and hematocrit levels (% BWC).

**Telomerase levels.** Normal rat ovarian surface epithelial cells were isolated following published methods (21). Cytokeratin staining and cobble stone morphology confirmed epithelial cell characteristics (data not shown). Primary cells were grown to reach confluence and then harvested. Telomerase levels were determined by TelO-TAGG telomerase PCR ELISA assay method (Roche Applied Sciences, Indianapolis, IN) according to manufacturer's protocol. Telomerase levels were expressed as relative levels compared with positive control provided in the kit.

**Results**

Expression of VEGF165 in ROSE199 cells. We have previously found that human VEGF165 stimulates proliferation and migration of multiple endothelial cell lines, including human umbilical vascular endothelial cell, bovine capillary endothelium, and mouse microvascular heart endothelial cells (SMHEC4). Therefore,
ROSE199 cells were transfected with pSecTag C containing the entire coding sequence for VEGF165 (VR) or pSecTag C vector only (control). Zeocin-resistant clones transfected with VEGF165 secreted VEGF protein ranging from 300 to 3,200 pg/mL as measured by ELISA. No significant detection was observed in control transfected ROSE199. Clones expressing higher levels of VEGF (>2,000 pg/mL) were selected for further studies. Expression of neu in pneuINA-transfected ROSE199 cells was verified by immunohistochemical staining of G418-resistant cells using a rat-specific anti-neu antibody (Oncogene Research Products; data not shown).

Proliferation rate of ROSE199 cells in vitro. The ability of VEGF165 to influence the growth of ROSE199 cells in vitro was investigated. Viable cell counts were done on parental ROSE199, VR, and control cells at 24, 48, and 72 h time points. Viable cell counts by trypan blue exclusion revealed no significant difference in cell proliferation rate between the different ROSE199 cell lines in vitro. These data were further confirmed by determining viable cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. There was no significant difference in cell doubling time between the cell lines. Parental ROSE199 cells had a doubling time of 16 h (doubling time: control, 17.4 h; VR cells, 18.2 h). The marginal differences seen in doubling times were not statistically significant (Table 1).

Similarly, coexpression of endostatin under the inducible promoter Tet did not alter the growth rate of VR ovarian surface epithelial cells in vitro (data not shown). Furthermore, we compared the telomerase activity in the transduced cells with primary normal rat ovarian surface epithelial cells. These studies showed that the relative levels of telomerase activity of VR and

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**Table 1. Characteristics of ROSE-transfected cell lines**

<table>
<thead>
<tr>
<th>Life span</th>
<th>Doubling time (h)*</th>
<th>Focus formation †</th>
<th>Colony formation (%) ‡</th>
<th>Telomerase levels (ratio) §</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROSE</td>
<td>3rd passage</td>
<td>18.0</td>
<td>No</td>
<td>&lt;1</td>
</tr>
<tr>
<td>ROSE199</td>
<td>Immortal</td>
<td>16.0</td>
<td>No</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Empty</td>
<td>Immortal</td>
<td>17.4</td>
<td>No</td>
<td>&lt;1</td>
</tr>
<tr>
<td>VR</td>
<td>Immortal</td>
<td>18.2</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>Neu/ROSE199</td>
<td>Immortal</td>
<td>13.5</td>
<td>Yes</td>
<td>70</td>
</tr>
<tr>
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<td>Immortal</td>
<td>17.4</td>
<td>No</td>
<td>&lt;1</td>
</tr>
<tr>
<td>VRtet</td>
<td>Immortal</td>
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<td>Immortal</td>
<td>17.1</td>
<td>No</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.
*As determined by viable cells at 24-h intervals for 72 h of triplicate cultures.
†As determined by cultures displaying focal units ≥0.1 mm in diameter.
‡Expressed as ratio of colonies to total number of cells seeded. Colonies were defined as ≥50 cells.
§As determined by TelotAGGG telomerase PCR ELISA assay. Levels were expressed as normalized to manufacturer’s provided positive control. For this assay, freshly isolated ROSE were used.

ROSE199 and ROSE cells show similar characteristics regarding to laminin, cytokeratin-7, cytokeratin-8, cytokeratin-19, and 7-hydroxysteroid expression; both are nontumorigenic and substrate dependent and show contact inhibition.

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**Figure 1.** Induction of angiogenesis by transfected ROSE199 cells in Matrigel. A, representative phase-contrast photomicrographs and H&E and CD31 staining (arrows show RBC in blood vessels) of Matrigel plugs. Representative images of two of the five Matrigel plugs from each group. B, quantification of vessel density as measured by CD31-positive pixels. Mean vessel density was determined from 15 images obtained from each Matrigel section at ×200 magnification. Bar, 30 μm as indicated for H&E and CD31. †, P < 0.05, statistical significance as determined by Student’s t test.
VRTet-endo had relatively lesser telomerase activity when compared with the normal primary OSE cells (Table 1). Between ROSE199 and VR, there was 1.5-fold more telomerase activity. Between VR and VRTet-endo, there was no significant difference in telomerase activity.

**In vitro phenotypes of transfected ROSE199 cells.** Anchorage independence (growth in soft agar) and focus formation are generally considered phenotypic markers of transformation. Parental ROSE199 cells have previously been shown to retain characteristics of normal epithelium (21). We assayed transfected ROSE199 cells for focus formation in monolayer cultures and anchorage-independent growth in soft agar. Results summarized in Table 1 show that the oncogenic neu-transfected ROSE199 cells formed foci in vitro, but neither control, VR, nor pINA/ROSE199 cells were able to form foci after reaching confluence. Similar results were observed in anchorage-independent growth assays where the Neu/ROSE199 cells were able to form multiple large colonies in soft agar with a cloning efficiency of ~70%, whereas parental ROSE199 and control cells produced no colonies at any of the cell densities tested. VR cells, however, did form a small number of colonies in soft agar, with an average cloning efficiency of 5%.

**Angiogenesis induced by ROSE199 transfectants.** To assess angiogenic stimulation by the different ROSE199 cell lines in vitro, a Matrigel plug assay was done. Seven days after implantation, animals were euthanized and Matrigel specimens resected for histologic examination (Fig. 1A). H&E staining revealed microvessels dispersed throughout Matrigel containing VR and Neu/ROSE199 cells compared with nearly avascular Matrigel containing either the control or pINA/ROSE199 cells (Fig. 1A). In addition, Matrigel plugs were stained with an anti-CD31 phycoerythrin conjugate, and vessels were directly visualized by fluorescence microscopy (Fig. 1A). A traditional estimation of microvessel density was done by a manual count of tumor blood vessels per high power field (~200 magnification) as well as evaluation using computer-assisted image analysis, as first described by Wild et al. (25). VEGF- and Neu-transfected ROSE199 implanted Matrigel plugs had a 4- to 5-fold higher vessel count than Matrigel plugs containing control ROSE199 cells ($P < 0.01$; Fig. 1B).

**Tumorigenicity of VEGF-transfected ROSE199 cells.** In parallel experiments, mice were injected either s.c. or i.p. with control or VR cells. Tumor incidence was 20 out of 20 (100%) in mice injected i.p. with VR cells and 17 out of 20 (85%) in mice injected s.c. with VR within 2 months after cell transplantation. In contrast, no tumors (0 of 20) developed in any of the mice injected with control cells in either s.c. or i.p. models. However, one i.p. injected control mouse died early on without any apparent sign of disease. Animals injected s.c. with VR cells developed palpable, vascularized tumors that reached an average size of 2,500 mm$^3$ in volume (Fig. 2A and B). Tumors were locally invasive into muscle and adipose tissue, but no distant metastases were found. The animals injected i.p. with VR cells necessitated culling within 2 months after injection due to extensive ascites formation and animals becoming moribund (Fig. 2C and D). Examination of the peritoneum revealed widespread intra-abdominal tumor mass and up to 11 mL of ascites fluid per mouse.

**Inhibition of VR tumor growth by SU5416.** SU5416 is a selective inhibitor of VEGF receptor tyrosine kinase and has previously been shown to inhibit the growth of a variety of tumor types (20). We tested the ability of SU5416 to inhibit VR tumor growth in s.c. and i.p. models. In s.c. models, mice were treated i.p. with SU5416 (15 mg/kg/d) or vehicle alone starting on the day before cell implantation. A, gross appearance of s.c. tumors overexpressing VEGF165. Mice were injected with VR cells in right hind flank. Picture was taken 45 days after cell transplantation. B, average tumor volume in mice injected s.c. with either VR or control cells ($n = 10$). Points, mean tumor volume; bars, SE. C, malignant ascites development following i.p. transplantation of VR cells. Observation time point 42 d after cell transplantation. D, survival of athymic mice injected i.p. with either VR or control cells; 20 of 20 mice injected with VR cells i.p. developed ascites compared with 0 of 20 mice injected with control cells.
of cell transplantation (preventive), or 7 days after cell transplantation (therapeutic). SU5416 significantly inhibited VR tumor growth in both “preventive” and “therapeutic” treatment regimens by 9 weeks after cell transplantation (Fig. 3A and B). The average tumor volume for treated versus control in the “preventive” and “therapeutic” models were 56 versus 500 mm³ and 700 versus 2,300 mm³, respectively. The control, vehicle-treated group from the therapeutic model had different growth rate when compared with the prevention model (Fig. 3A). This can be in part attributable to the effect of vehicle on different sets of tumor microenvironment (stromal cells) between the prevention and therapeutic models. In mice receiving treatment immediately, tumor development was delayed by 4 weeks compared with controls. We next tested efficacy of SU5416 in an i.p. model to more closely resemble the physiologic environment of the disease. In the i.p. model, mice were treated i.p. with SU5416 (15 mg/kg/d) or vehicle starting on day 7 after cell transplantation. Treated animals had an increase in survival time of >3 weeks compared with control mice with 50% of mice in the control group dying by day 50 after cell transplantation compared with day 75 in the treated group (data not shown). Potential toxicities were assessed indirectly by body weight measurements and directly by determining the hematocrit levels in all animals at the end of treatment. There was no significant change in mean body weight (16–18 g) or hematocrit levels (52–55% RBC) between the treatment groups. These data show that treatment with SU5416 significantly inhibits ovarian surface epithelial tumor growth in both “preventive” and “therapeutic” models.

**Tet-operated inducible expression of endostatin in VR cells.** Endostatin expression construct was prepared as described before, and the reading frame and sequence was confirmed by DNA sequencing. After transduction, VRTet-endo clones were selected for G418 and Hygromycin B resistance, and culture supernatant was assayed for the presence of secreted endostatin protein in the presence or absence of doxycycline (1 mg/mL). Expression of endostatin protein was confirmed by ELISA. One clone in particular (clone 13) showed high-level endostatin expression in the presence of doxycycline with up to 5,000 pg per million cells. This clone was further analyzed for tetracycline regulated endostatin expression in the presence of increasing concentrations of doxycycline. ELISA results show a concentration-dependent increase in endostatin expression with >5,000 pg protein secreted per million cells at a concentration of doxycycline of 500 ng/mL (Fig. 4A). In addition, we verified the presence of endostatin mRNA expression by VRTet-endo clones using reverse transcription-PCR (data not shown).

**Biological activity of endostatin in vivo.** To determine if the recombinant endostatin retained angiogenic activity, we measured recruitment of microvessels into Matrigel plugs containing endostatin-secreting VR cells, as described above. Animals were treated with doxycycline for 1 week. H&E and CD31 staining revealed a high density of microvessels dispersed throughout Matrigel plugs containing VRTet control cells compared with significantly fewer microvessels recruited into Matrigel plugs containing VRTet-endo cells (Fig. 4B). Quantification of fluorescent pixels showed a statistically significant inhibition of microvessel recruitment in VRTet-endo containing Matrigel plugs compared with VRTet with an average of 34,125 ± 8,648 versus 13,672 ± 4,490 pixels as determined by morphometric computer-assisted analysis (Fig. 4C; *, P < 0.05).

**Effect of endostatin expression of VR tumor growth.** To determine the effect of endostatin expression on VR tumor development *in vivo*, 2 × 10⁶ VRTet-endo cells were injected s.c. into the right hind flanks of athymic mice with or without doxycycline supplementation in their drinking water. Animals receiving water without doxycycline, and therefore no endostatin induction, developed large tumors reaching an average tumor volume of 700 mm³ by 4 weeks after cell implantation (Fig. 5). In contrast, tumor growth was completely inhibited in animals when endostatin expression was induced by doxycycline supplementation. There was no evidence of toxicity in any of the animals tested. These data suggest that balancing the angiogenic phenotype of VR cells by the coexpression of endostatin can significantly inhibit tumor formation and reverse the tumorigenic phenotype *in vivo*.

**Discussion**

VEGF expression is implicated in the pathology of ovarian cancer, and a higher level of VEGF is associated with poor prognosis (26, 27). However, the role of VEGF in the etiology of ovarian cancer is not completely understood. A number of gain-of-function
mutations, such as the overexpression of neu/c-erbB2, and loss-of-function mutations in tumor suppressor genes such as BRCA1/2 (28) and p53 (29) are associated with ovarian cancer. Studies have also shown that the neu/c-erbB2 gene is frequently amplified and/or overexpressed in human epithelial ovarian cancers (30). However, the threshold of genetic changes needed to transform normal OSE to a tumorigenic cell is not defined.

Using murine fibroblasts as a model system, Weinberg et al. elegantly showed that at least two fundamental changes had to occur for malignant transformation. In their studies, perturbation of two signaling pathways involving p53 and Raf is sufficient to transform non-tumorigenic murine cells to acquire a tumorigenic phenotype (31). However, six different genetic changes, including telomerase activation and perturbed tissue-specific intracellular signaling pathways, were found necessary for normal human cells to become tumorigenic (32). Our studies show that ROSE199 cells had very similar levels of telomerase activity when compared with freshly isolated normal OSE. Telomerase activity could have helped in immortalization, but that was not sufficient to confer a tumorigenic phenotype to ROSE199 cells. Using this model system, we investigated whether perturbation in angiogenic phenotype could contribute to the establishment of ovarian cancer. ROSE199 cells transduced by retrovirus expressing mutated rat neu altered the growth characteristics resembling malignant phenotype and formed tumors in athymic mice (33). Additionally, activation of the neu oncogene has been shown to induce VEGF expression in tumor cells (34). To determine the role of angiogenesis in OSE, we compared in vitro and in vivo growth characteristics of parental ROSE199 (negative control) and Neu/ROSE199 (positive control) cells with VEGF- and vector only–transfected ROSE199 cells. Injection of VR cells s.c. or i.p. into female nude mice resulted in tumor formation. None of the mice injected with control cells developed tumors. These studies show that overexpression of the potent angiogenic stimulator VEGF confers a tumorigenic phenotype to the ROSE199 cell line. VEGF expression seems to provide in vivo survival advantage to ROSE199 cells because in vitro growth characteristics were not altered. Previous studies have also suggested a more functional role for VEGF in ovarian cancer development and progression in mice (35). Lee et al. studied the use of VEGF for therapeutic angiogenesis in ischemic myocardium and showed that unregulated and continuous expression of VEGF in murine myoblasts lead to formation of endothelial cell–derived vascular tumors in immunodeficient mice (36). Furthermore,
Arbriser et al. showed that overexpression of VEGF121 in MSI endothelial cells resulted in development of slowly growing endothelial tumors in nude mice (37). Moreover, this report underscores the importance of VEGF involvement in ascites development. VEGF is known to induce permeability of microvessels (38), and ascites formation is thought to be caused by an increased influx of fluid and blockage of lymphatics. In our study, i.p. injected VR, but not control cells, developed extensive abdominal ascites, providing distinct evidence for the role of VEGF in ascites formation in ovarian cancer.

Tumorigenic cells are known to have altered growth characteristics such as the loss of contact inhibition, focus formation, anchorage-independent growth in semisolid media, and invasion through extracellular matrix. Characterization of the VR cells revealed that in contrast to the Neu/ROSE199 cells, VEGF expression did not alter ROSE199 cell growth in vitro. In addition to cell proliferation, focus-forming assays were used to determine potential phenotypic changes in VR cells. These studies showed that Neu/ROSE199 cells formed multiple foci indicating oncogene-mediated transformed cell phenotype. VR cells however did not form foci to any significant levels. In addition, Neu/ROSE199 cells formed anchorage-independent growth in soft agar colony-forming assays. These studies again showed that VEGF expression in ROSE199 cells retained the phenotype of the parental ROSE199 cells (39) and did not lead to the acquisition of transformed phenotype often seen in oncogene-transduced cells. It is also true that bona fide cancer cells do not necessarily express some of the neoplastic properties in culture, or even grow in suspension, although they are malignant and invasive in vivo. For example, OVCAR3 cells do not invade Boyden chambers and are, to a high degree, anchorage dependent; yet, implanted in severe combined immunodeficient mice, they are highly malignant (40). Human cervical cancer cell line C-4111 displays contact inhibition and only forms monolayers in culture but develops invasive tumors in experimental animals (41). Most likely, the in vivo microenvironment contributes additional factors, such as hormones and stromal components, which benefits or even potentially promotes a neoplastic phenotype. Moreover, under certain conditions, ROSE199 cells themselves are able to deposit such stromal matrix and basal membrane components to confer a more invasive tumorigenic phenotype (39).

Another change often seen in tumorigenic cells is increased telomerase activity associated with immortalization. Unlike the human normal cells, murine cells are known to have higher levels of constitutive telomerase activity. A comparison of telomerase activity between freshly isolated rat OSE and VR cells showed similar levels of telomerase, indicating that VEGF transduction did not lead to overexpression of telomerase. Therefore, VEGF seems to confer an in vivo growth advantage for ROSE199 cells by sustained angiogenesis leading to tumorigenicity. Angiogenesis of course is necessary for even benign tumor cells and normal ovarian cells such as the granulosa cells of the corpus luteum (reviewed in ref. 42). Luteal angiogenesis (immediately following ovulation, early luteal phase) and vessel regression (late luteal phase, before the onset of next ovulatory cycle) are hormonally orchestrated by the modulation of complex sets of growth factors (VEGF and angiopoietin 2) and leukotriens. Therefore, the normal ovarian angiogenesis is highly regulated and balanced. In contrast, immortal ovarian epithelial cells acquiring sustained secretion of an angiogenic growth factor leads to tumorigenesis. This notion is further confirmed by the reversal of the tumorigenic phenotype by the coexpression of endostatin, an angiogenesis inhibitor.

It is possible that epithelial cells are capable of an autocrine-signaling pathway via VEGF and its receptors Flik-1/KDR or Flt-1. Abu-Jawdeh et al. showed expression of VEGF mRNA and protein in malignant and borderline tumors of the ovary but low to no expression in normal ovarian cortex or surface epithelium (17). This was further confirmed by Boocock et al. who showed expression of mRNAs encoding VEGF, Flt-1, and Flik-1/KDR in primary ascitic cells and in multiple ovarian carcinoma cell lines (43). To ascertain if autocrine stimulation was possible, we analyzed the different ROSE199 cell lines for VEGF receptor mRNA expression by real-time PCR and found low-level expression of both Flk-1 and Flt-1 receptor transcripts (data not shown). However, when we analyzed the proliferation rate of the VEGF-transfected cells compared with parental ROSE199 and control vector-transfected cells, with and without exogenous VEGF administration, we observed no change in growth rate compared with parental ROSE199 cells. Therefore, VEGF does not seem to act as an autocrine growth factor for ROSE199 cells in vitro. VEGF has been shown to be an important survival factor for endothelial cells under chemical or physical stress as well as reduced cardiomycyte apoptosis in hypertrophied myocardium (44). Moreover, recent studies show that stress-induced changes in VEGF secretion in ovarian cancer cells are mediated by β-adrenergic receptors (45). Use of β-blockers has reduced VEGF production and tumor growth in athymic mice. In other studies, inhibition of VEGF-mediated angiogenic response (13, 46) and interference with VEGF-mediated signaling could inhibit tumor growth. Ke-lin et al. showed significant reduction in solid tumor growth and ascites fluid development in mice treated with antibodies to VEGF (47). A later study by Lin et al. showed a 75% inhibition of tumor growth and 50% reduction in vascular density in carcinoma transplant models using a recombinant soluble VEGF receptor (48). In our studies, SU5416, a potent and selective inhibitor of Flk-1/KDR receptor tyrosine kinase, was found to inhibit VR cell–induced tumors in mice. These studies suggest that VEGF secreted by the ROSE199 cells is necessary for the tumor growth. Similarly, SU6668, a kinase inhibitor with broader specificity, was effective in limiting ovarian tumor growth (20, 49). Because SU5416 targets host endothelial cells, this treatment may be effective in escaping development of drug resistance common to many chemotherapeutic agents.

These studies show that triggering the angiogenic “switch” by overexpressing a potent angiogenic growth factor is an important step in development of ovarian surface epithelial cancer. The angiogenic phenotype of a cell or tissue is controlled by a balance between of positive and negative regulators (15, 16). Several studies have shown that altering the balance of these factors can significantly influence tumor development and growth (50, 51). Here, we also investigated whether expressing an endogenous antiangiogenic molecule (endostatin) was sufficient to reverse the angiogenic phenotype and consequently tumorigenicity of VR cells in athymic mice. Using a tetracycline-inducible retroviral expression system, we were able to dose-dependently induce of endostatin protein expression in VR cells upon treatment with doxycycline. We used clones expressing high levels of endostatin, up to 7,000 pg per million cells, and found that the recombinant endostatin was able to suppress angiogenesis in vivo as seen in Matrigel assays where microvessel recruitment into Matrigel plugs was significantly reduced.
In the study by Fathalla MF, the term "inconsecutive ovulation" was used to describe a factor in ovarian tumors with cumulative tumor volumes similar to those observed in doxycycline supplementation study. The study also showed that coexpression of endostatin and human ferritin, and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. The results of multiple studies support the notion that angioptosis mediates the suppression of angiogenesis, and the suppression of tumor growth. The study further suggests that by countering the proangiogenic tilt by angioptosis can be a therapeutic target for ovarian cancer.

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