Gonadotropin-Regulated Lymphangiogenesis in Ovarian Cancer Is Mediated by LEDGF-Induced Expression of VEGF-C

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Abstract

The risk and severity of ovarian carcinoma, the leading cause of gynecologic malignancy death, are significantly elevated in postmenopausal women. Ovarian failure at menopause, associated with a reduction in estrogen secretion, results in an increase of the gonadotropic luteinizing hormone (LH) and follicle-stimulating hormone (FSH), suggesting a role for these hormones in facilitating the progression of ovarian carcinoma. The current study examined the influence of hormonal stimulation on lymphangiogenesis in ovarian cancer cells. *In vitro* stimulation of ES2 ovarian carcinoma cells with LH and FSH induced expression of vascular endothelial growth factor (VEGF)-C. *In vivo*, ovariectomy of mice resulted in activation of the *VEGF-C* promoter in ovarian carcinoma xenografts, increased *VEGF-C* mRNA level, and enhanced tumor lymphangiogenesis and angiogenesis. Seeking the molecular mechanism, we examined the role of lens epithelium-derived growth factor (LEDGF/p75) and the possible contribution of its putative target, a conserved stress-response element identified *in silico* in the *VEGF-C* promoter. Using chromatin immunoprecipitation, we showed that LEDGF/p75 indeed binds the *VEGF-C* promoter, and binding is augmented by FSH. A corresponding hormonally regulated increase in the *LEDGF/p75* mRNA and protein levels was observed. Suppression of *LEDGF/p75* expression using small interfering RNA, suppression of LH and FSH production using the gonadotropin-releasing hormone antagonist cetrorelix, or mutation of the conserved stress-response element suppressed the hormonally induced expression of *VEGF-C*. Overall, our data suggest a possible role for elevated gonadotropins in augmenting ovarian tumor lymphangiogenesis in postmenopausal women. [Cancer Res 2009;69(24):9306–14]

Introduction

Ovarian carcinoma is the leading cause of gynecologic malignancy death (1). The risk for the disease as well as its severity are higher in postmenopausal women. Epidemiologic studies suggested that whereas the initial transformation occurs during ovulation, tumors remain dormant throughout the fertile age and become clinically evident only at menopause. Hormonal alternations, which are associated with menopause such as a decrease in estrogen and progesterone secretion, and a consequent increase in the systemic level of the gonadotropic hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH), could have a role in the manifestation of the disease.

Ovarian cancer cells were reported to express LH and FSH receptors (2, 3). Stimulation by these hormones affected gene expression and steroid production in a way that enhanced invasiveness, cell growth, and angiogenesis (4–7). Clinical significance of gonadotropins in ovarian cancer patients was shown by the correlation of increased LH and FSH concentrations in both serum and ascitic fluid with increased disease severity (8).

Ovarian cancer metastasis occurs by direct multifocal seeding in the peritoneum as well as by migration through the lymphatic system. Extra-abdominal metastases of ovarian cancer were reported in the para-aortic, jugular, and supraclavicular lymph nodes (9–11), and lymphatic vessels were detected in human ovarian tumors (12–14). The expression level of vascular endothelial growth factor (VEGF)-C, a key regulator of lymphangiogenesis, was found to correlate significantly with lymph node and peritoneal metastasis as well as with poor survival (15, 16). In preclinical studies, downregulation of VEGF-C activity, either by a specific small interfering RNA (siRNA), by blockage of its receptor VEGF receptor-3, or by usage of soluble VEGF receptor-3 as a trap, was found to inhibit tumor lymphangiogenesis and metastasis and to enhance survival (17–20). These studies implicate lymphatic vessels in ovarian tumor progression. The aim of the current study was to investigate the possible role of LH and FSH stimulation on lymphangiogenesis in ovarian cancer. We show that gonadotropin stimulation induced *VEGF-C* promoter activation and increased *VEGF-C* mRNA and protein levels *in vitro*. Accordingly, elevation of gonadotropin levels *in vivo* by ovariectomy resulted in VEGF-C activation, enhanced lymphangiogenesis, and angiogenesis.

We recently reported an *in silico* analysis of the *VEGF-C* promoter region as well as *in vitro* and *in vivo* studies of hyperthermia and oxidative stress-induced expression of VEGF-C, suggesting a role for lens epithelium-derived growth factor LEDGF/p75 as a putative upstream regulator of VEGF-C expression (21). LEDGF/p75, a member of the hepatoma-derived growth factor family, was shown to protect cells from stress-induced cell death by activation of several stress-related genes, such as *Hsp27, αB-crystallin, AOP2, ADH, ALDH,* and *PKCγ* (22–24). Transcriptional coactivation by LEDGF/p75 was reported in several studies to be mediated by binding of the protein to heat shock elements and stress-response elements (STRE) found in the promoter region of its downstream targets (23–28). Alternatively, LEDGF/p75 was reported to induce MYC-regulated transcription by interaction with JPO2 (29), through a *STRE*-independent DNA binding mediated by the LEDGF/p75 nuclear localization signal and a dual copy of the AT-hook DNA-binding motif (30). Recent findings indicate that LEDGF/p75 also plays a role in survival of cancer cells, resistance to chemotherapy, and tumor progression (31, 32).

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Table 1. *In vitro* luciferase assay as well as RT-PCR analysis of VEGF-C show LEDGF/p75-dependent hormonal regulation of *VEGF-C* promoter activity

	No stimulation	LH	FSH	LH + FSH
Luciferase				
Wild-type promoter	1	1.96 ± 0.07[a] (P = 0.03)	1.98 ± 0.17[a] (P = 0.04)	3.4 ± 0.27[a] (P = 0.007)
Control siRNA	0.96 ± 0.03	1.7 ± 0.02[b] (P = 0.03)	1.81 ± 0.02[b] (P = 0.01)	2.69 ± 0.2[b] (P = 0.03)
LEDGF/p75 siRNA	0.75 ± 0.06	1.14 ± 0.02[c] (P = 0.2)	1.17 ± 0.03[c] (P = 0.19)	1.53 ± 0.06[c] (P = 0.08)
Mutated promoter	1	1 ± 0.05[d] (P = 0.49)	1.06 ± 0.04[d] (P = 0.11)	1.06 ± 0.007[d] (P = 0.07)
RT-PCR				
Control siRNA: *VEGF-C*	1	2.3[b]	1.9[b]	3.3[b]
Control siRNA: *LEDGF*	1	2.04[b]	1.7[b]	2.9[b]
LEDGF-p75 siRNA: *VEGF-C*	1	0.99[c]	1.12[c]	0.96[c]
LEDGF-p75 siRNA: *LEDGF*	1	1.25[c]	1.17[c]	1.22[c]

NOTE: All values are presented as fold induction compared with control ± SE. Statistical significance was determined by Student's t tests as follows: a, compared with nonstimulated cells; b, compared with nonstimulated, control siRNA-transfected cells; c, compared with nonstimulated, *LEDGF/p75* siRNA-transfected cells; d, compared with nonstimulated cells transfected with a mutated construct.

The results reported here, showing that lymphangiogenesis is hormonally regulated in ovarian cancer and the coactivation of VEGF-C and LEDGF/p75 by gonadotropins, provide better understanding of the disease pathophysiology in postmenopausal women and suggest LEDGF/p75 as a possible target for intervention.

Materials and Methods

Cell culture and *in vitro* hormonal stimulation. For *in vitro* hormonal stimulation studies, human epithelial ovarian carcinoma ES2 cells (kindly provided by Prof. Hauptmann, Charite) were serum-starved 24 h before hormonal stimulation and then administered with 1 ng/mL human LH or human FSH (kindly provided by Dr. Fortune Kohen, Weizmann Institute).

Reverse transcription and real-time PCR. Total RNA was extracted using PerfectPure RNA Cultured Cell or Tissue kit (5 PRIME). Total RNA (1.5 μg) was used for reverse transcription using SuperScript II RNase H-reverse (Invitrogen). Real-time PCR (RT-PCR) was done using StepOnePlus RT-PCR System (Applied Biosystems) with the following primers: human *VEGF-C* (accession no. NM005429.2) 5′-tgccagcaacactaccacag and 5′-gtgattattccacatgtaattggtg, human *LEDGF/p75* (accession no. NM_033222.3) 5′-gggccaaacaaaaagctaga and 5′-ttcattgctctccccgttat, and human B2M (accession no. NM_004048.2) 5′-ttctggcctggaggctatc and 5′-tcaggaaatttgactttccattc.

Immunoblot assays. Whole-cell lysates were prepared in ice-cold radioimmunoprecipitation assay buffer [20 mmol/L Tris (pH 7.4), 137 mmol/L NaCl, 10% glycerol, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1% Triton X-100, 2 mmol/L EDTA] containing 1 mmol/L phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma) and fractionated by SDS-PAGE. Primary antibodies were used for the detection of VEGF-C (C-20; Santa Cruz Biotechnology), LEDGF/p75 (C16; Santa Cruz Biotechnology), and β-tubulin (Santa Cruz Biotechnology). Horseradish peroxidase–conjugated anti-goat or anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories) were used. Densitometric evaluation was carried out using ImageJ software.

***In vitro* luciferase assay.** To create a reporter plasmid for the *VEGF-C* promoter region, human genomic DNA was used for PCR amplification of a 468-bp sequence upstream to the *VEGF-C* cds using the following primers: 5′-ccgccgcagcgcccgcg and 3′-gggccaggaaggtggtac. The PCR product was inserted into the pLuc plasmid, which encodes for the firefly luciferase gene. Another construct was used, in which two *STRE*s and an AGG box in the promoter region of *VEGF-C* were disrupted by nine mutations using specific PCR primers (5-gccagagccctcgtttttctcctttcttttcttccccgaagtgagag) as reported previously (21). For *in vitro* luciferase assay, ES2 cells were co-transfected with the luciferase reporter plasmid and with pSV-*Renilla* using Lipofectamine 2000 (Invitrogen). Following transfection, cells were hormonally stimulated (1 ng/mL LH or FSH in serum-free medium; 18 h). The luciferase assay was done using Dual-Luciferase Reporter Assay System (Promega). Measurement of *Renilla* luciferase activity was used for calibration. Experiments were done three times in triplicates. Downregulation of LEDGF/p75 was achieved by transfection of the cells with a specific siRNA (5′-agacagcaugaggaagcgdtdt; Dharmacon). A nonspecific sequence was used as a control.

***In vivo* tumor xenografts.** All experiments were approved by the Weizmann Institutional Animal Care and Use Committee.

Tumors were generated by s.c. injection of 1.75×10^6 ES2 cells stably transfected with the *pVEGF-C-Luc* construct (see *In vitro* Luciferase Assay) to the hind limb of 7.5-week-old CD1 nude female either control or ovariectomized mice. Blockade of gonadotropin-releasing hormone–induced secretion of LH and FSH was achieved by s.c. daily injection of 0.5 mg/kg cetrorelix (as Cetrotide; Merck Serono), starting 15 days before induction of the tumors and throughout the whole period of the experiment. For time-lapse luciferase bioluminescence analysis, mice were i.p. injected with 1.5 mg D-luciferin (50 μg/g body weight; Caliper Life Sciences) and imaged after 15 min using the IVIS100 system (Caliper Life Sciences). The total flux of photons from the tumor area was measured. Measurements of the tumor three dimensions were conducted manually using a caliper. Values below the detection threshold were defined as zero. Sixteen days following tumor induction, mice were euthanized, and tumors were removed for RNA analysis or fixed in 4% paraformaldehyde for histologic analysis.

Histology. Following fixation in 4% paraformaldehyde, tissues were embedded in paraffin and sectioned serially at 4 μm thickness. Staining was carried out using anti-CD34 (Cedarlane Laboratories), anti–LYVE-1 AP (Fitzgerald Industries), or anti-Prox1 AP (Novus Biologicals) antibodies. Detection of the anti-CD34 antibody was carried out using a biotin-SP–conjugated anti-rat antibody and Cy3-conjugated streptavidin (both from Jackson ImmunoResearch Laboratories). For the detection of the anti–LYVE-1 AP and anti-Prox1 AP antibodies, we used an alkaline phosphatase–conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories) and Fast Red substrate (Sigma). Staining was detected using a fluorescence microscope (Zeiss Axioscope II, Simple PCI software). The percentage of fluorescent staining coverage in each field of view was calculated using ImageJ software.

***In situ* hybridization.** For preparation of *VEGF-C* and *LEDGF/p75* mRNA probes, RT-PCR was carried out using the following pairs of primers: 5′-ctgtgtccagcgtagatgagc and 5′-gtagacggacacacatggagg and 5′-cacacagagatgattactacactg and 5′-ccatcttgagcatcagatcctc, respectively. Following cloning of the fragments using the pGEM-T Easy Vector System (Promega),

digoxigenin-labeled riboprobes were prepared by *in vitro* transcription using the DIG RNA Labeling kit (Roche).

Tissues fixed in 4% paraformaldehyde were embedded in paraffin and sectioned serially at 7 μm thickness. Sections were deparaffinized, hydrated, digested with proteinase K (Sigma), rinsed with TBS, incubated in 4% paraformaldehyde, dehydrated, and incubated with a hybridization mixture for 30 min at 55°C. Hybridization with the specific probes was carried out overnight at 65°C using 1 μg/mL riboprobes. Following the hybridization procedure, sections were washed with SSC and TBS and then blocked with 1% bovine serum albumin. For detection of the digoxigenin-labeled riboprobes, sections were incubated with antidigoxigenin alkaline phosphatase (Roche) and stained with nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (Roche). Staining was detected using E800 microscope and digital camera DXM 1200 (Nikon).

Chromatin immunoprecipitation assays. ES2 cells were serum-starved for 24 h and then stimulated with 1 ng/mL LH or FSH for either 1 or 4 h. Chromatin immunoprecipitation was done with the EZ-ChIP kit (Upstate) using anti-LEDGF/p75 antibodies (C16; Santa Cruz Biotechnology). PCR was done using primers designed for the *VEGF-C* promoter region (see *In vitro* Luciferase Assay).

Statistical analysis. Data are presented as mean ± SE. Statistical significance ($P < 0.05$) was assessed by *t* test unless indicated differently.

Results

Hormonal stimulation induces VEGF-C elevation *in vitro*. To study the effects of gonadotropin stimulation on lymphangiogenesis in ovarian cancer, we first examined the regulation of the *VEGF-C* promoter activity by LH and FSH stimulation *in vitro*. ES2 human ovarian carcinoma cells were transfected with a luciferase reporter plasmid in which the *VEGF-C* promoter region regulates the firefly luciferase transcription. Cotransfection with a *Renilla* luciferase construct served for calibration. Following transfection, cells were stimulated for 18 h with either 1 ng/mL LH, 1 ng/mL FSH, or a combination of the two hormones together in a serum-free medium. Measurements of luciferase activity showed ~2-fold increase in the activation level of the *VEGF-C* promoter following stimulation with either LH or FSH (Table 1; $P = 0.034$ and 0.036, respectively; $n = 6$). Stimulation of the cells with both LH and FSH resulted in a 3.4-fold increase, suggesting that the effect of the hormonal stimulation is additive ($P = 0.007$).

Hormonal stimulation induces expression of LEDGF/p75. LEDGF/p75 was reported previously to induce expression of several downstream target genes (23, 26, 25), including VEGF-C (21), possibly by binding to *STRE* in the promoter of the target genes. We hypothesized that LEDGF/p75 may mediate VEGF-C activation as a response to hormonal stimulation. To study this hypothesis, we downregulated *LEDGF/p75* using a specific siRNA. ES2 cells transfected with the siRNA were further stimulated with LH, FSH, or a combination of both hormones. Luciferase assay revealed an attenuated enhancement of the *VEGF-C* promoter following hormonal stimulation in cells transfected with the siRNA compared with untransfected cells or cells transfected with a nonspecific siRNA sequence (Table 1). These results were further verified by RT-PCR. Whereas hormonal stimulation of cells transfected

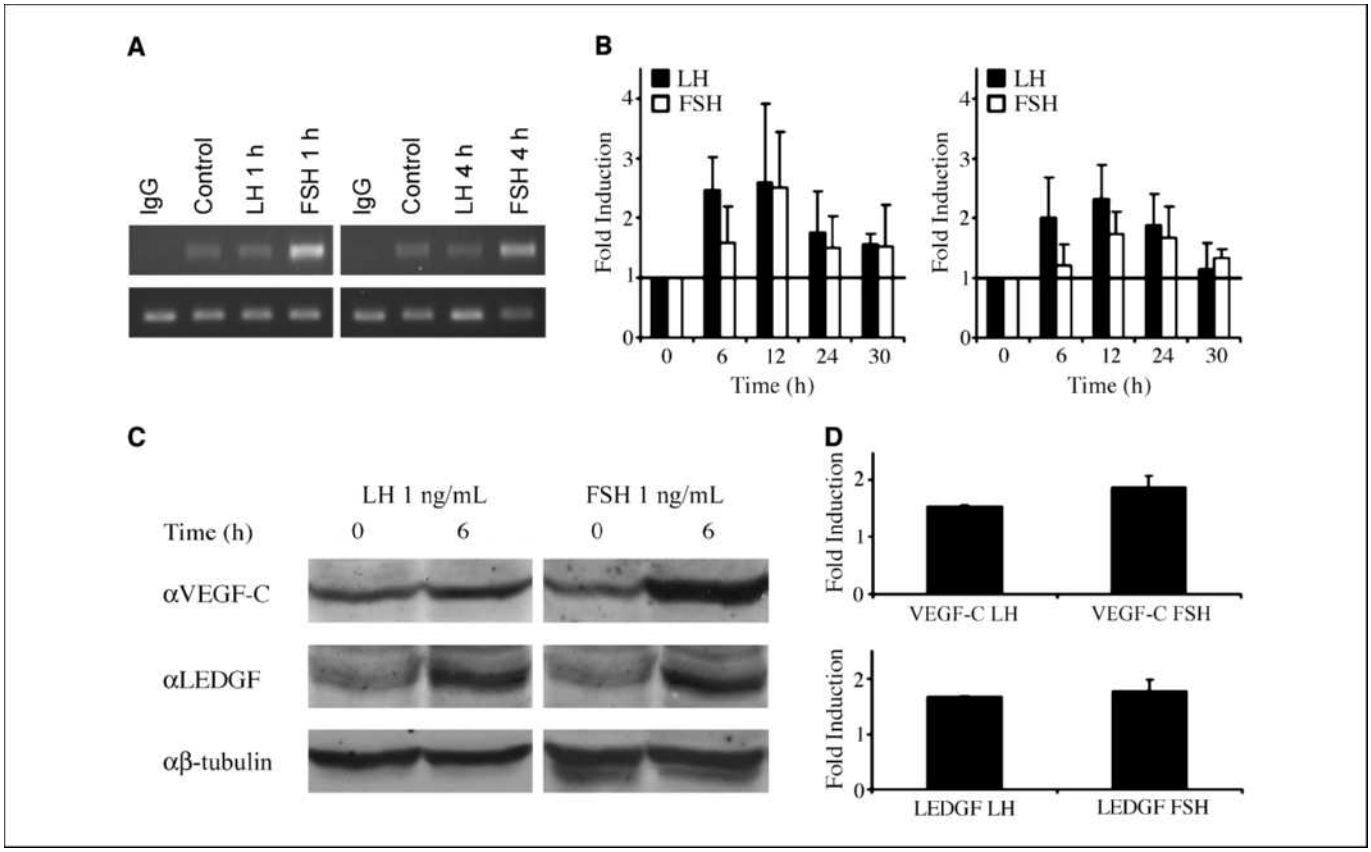

Figure 1. Hormonal stimulation induces VEGF-C elevation *in vitro*. *A*, chromatin immunoprecipitation carried out on hormonally stimulated or control ES2 cells using anti-LEDGF/p75 antibody and primers specific for the *VEGF-C* promoter area (*top*) or glyceraldehyde-3-phosphate dehydrogenase for input samples (*bottom*). IgG antibody was used as a negative control. *B*, RT-PCR using *VEGF-C*–specific primers (*left*) or *LEDGF/p75*-specific primers (*right*) carried out on RNA derived from ES2 hormonally stimulated cells ($n = 3$). *C*, Western blot analysis using anti-VEGF-C, anti-LEDGF/p75, or anti–β-tubulin antibodies carried out on total cell lysate derived from ES2 hormonally stimulated cells ($n = 3$). *D*, densitometric analysis of *C*.

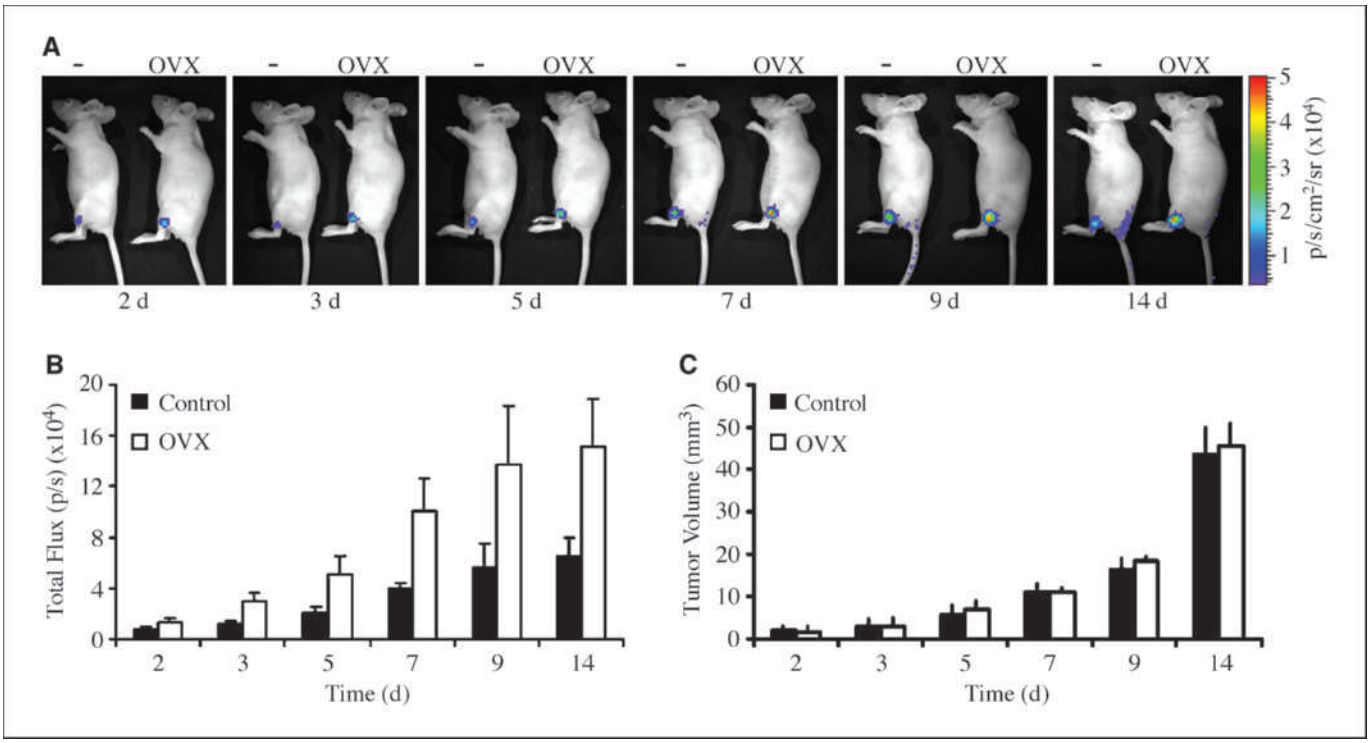

Figure 2. Ovariectomy induces *VEGF-C* promoter activation in ovarian tumors. *A,* time-lapse bioluminescence imaging of ovariectomized (*OVX*) and control (–) mice s.c. injected with ES2 cells that stably express the firefly luciferase gene under *VEGF-C* promoter regulation. *B,* total flux of photons derived from the tumor area of ovariectomized and control mice s.c. injected with ES2 cells that stably express the firefly *luciferase* gene under *VEGF-C* promoter regulation. *C,* volume of tumors induced in ovariectomized and control mice (ovariectomized $n = 5$ and control $n = 5$).

with control nonspecific siRNA resulted in a significant increase in both *VEGF-C* and *LEDGF/p75* mRNA levels, cells transfected with the specific *LEDGF/p75* siRNA showed only attenuated response to the hormonal stimulation (Table 1). Treatment with *LEDGF/p75* siRNA was found to be very effective and resulted in a 22-fold reduction in the *LEDGF/p75* mRNA level. The slight increase observed in the *VEGF-C* promoter activity following hormonal stimulation of *LEDGF/p75* siRNA-treated cells might suggest the minute involvement of other molecules in this pathway. The role of the conserved STRE in the *VEGF-C* promoter in hormonal-mediated VEGF-C activation was analyzed using ES2 cells transfected with a construct in which the luciferase gene was regulated by a mutated *VEGF-C* promoter sequence, with nine mutations disrupting the conserved STRE (21). Using this construct, the hormonal activation of the *VEGF-C* promoter was abolished, suggesting the *STRE* sequence to be crucial for VEGF-C activation by LH and FSH (Table 1).

Following administration of either 1 ng/mL LH or 1 ng/mL FSH to ES2 cells for 1 or 4 h, chromatin immunoprecipitation was carried out using anti-LEDGF/p75 antibodies as well as *VEGF-C* promoter-specific primers. An increase in the amount of *VEGF-C* promoter attached to LEDGF/p75 was detected following stimulation with FSH but not with LH (Fig. 1*A*; $n = 2$).

We next examined whether the enhanced activation of the *VEGF-C* promoter following hormonal stimulation results in a correlated elevation of the *VEGF-C* and *LEDGF/p75* mRNA and protein levels. Following 24 h starvation in a serum-free medium, ES2 cells were hormonally stimulated for varying periods (0, 6, 12, 24, and 30 h). For examination of the mRNA levels, total RNA was extracted, reverse-transcribed, and analyzed by RT-PCR using *VEGF-C*–specific and *LEDGF/p75*-specific primers. An elevation of the *LEDGF/p75* mRNA level was detected in ES2 cells following stimulation with either LH or FSH, reaching its maximal values (2.3- and 1.7-fold increase, respectively) 12 h following hormones application (Fig. 1*B, right*). Signed-rank test showed statistical significance for both LH and FSH stimulation ($P = 0.003$ and 0.008, respectively). Detection of the LEDGF/p75 protein level by Western blot analysis showed an increase in the amount of LEDGF/p75 protein compared with control (Fig. 1*C* and *D*; $P = 0.004$ and 0.035 for LH and FSH stimulation, respectively). Similarly, an elevation of the *VEGF-C* mRNA level was observed following stimulation with either LH or FSH, starting as early as 6 h following treatment and reaching its maximal values (2.6- and 2.5-fold increase, respectively) 12 h following hormones application (Fig. 1*B, left*). Signed-rank test showed statistical significance for both LH and FSH stimulation ($P = 0.00025$ and 0.0261, respectively). For detection of VEGF-C protein levels, a whole-cell lysate was prepared and analyzed by Western blot analysis using a VEGF-C–specific antibody. An increase in the protein level was observed for both LH and FSH treatments (Fig. 1*C* and *D*; $P = 0.027$ and 0.028 for LH and FSH stimulation, respectively).

Ovariectomy induces *VEGF-C* promoter activation in ovarian tumors. The effect of menopause-induced ovarian failure along with the corresponding changes in the hormonal milieu was tested *in vivo* using ovariectomized mice. Two weeks following ovariectomy, s.c. hind limb tumors were initiated by inoculation of ES2 cells stably expressing a luciferase reporter regulated by the *VEGF-C* promoter. Bioluminescence imaging using the IVIS100 system enabled a follow-up of the *VEGF-C* promoter activation level (Fig. 2*A* and *B*; ovariectomized $n = 5$ and control

n = 5). Bioluminescence was detected as early as 2 days post-injection and was restricted to the tumor area. At all time points examined, the total flux of photons from the tumor was higher in the ovariectomized group compared with untreated controls, indicating a higher activation level of the *VEGF-C* promoter. To statistically analyze the data, we calculated for each animal the rate of signal enhancement by deriving the total photon flux as a function of time. Comparing the results by t test resulted in a significant (P = 0.013) difference between the ovariectomized and the control groups.

The tumor length, width, and height were measured manually, and analysis of the tumor volume showed an increase of the tumor mass in both the ovariectomized and the control groups (Fig. 2*C*; ovariectomized n = 5 and control n = 5). Interestingly, no significant differences in tumor volume were found among the groups (P = 0.88, 0.96, 0.68, 0.96, 0.55, and 0.82 for 2, 3, 5, 7, 9, and 14 days following tumor induction respectively), indicating that the higher bioluminescence signal observed in the ovariectomized group is indeed a consequence of an increased *VEGF-C* promoter activity and not a mere result of tumor size differences.

To evaluate metastatic spread of the tumors, we carried out autopsies. Histological sections from the adjacent lymph nodes were taken. No definite lymph node involvement in either control or ovariectomized mice could be detected at these early time points (data not shown).

To verify the role of gonadotropins elevation in VEGF-C promoter activation, either ovariectomized or control mice were s.c. injected daily with 0.5 mg/kg cetrorelix, a gonadotropin-releasing hormone antagonist (Fig. 3*A* and *B*; ovariectomized n = 5, ovariectomized + cetrorelix n = 5, control n = 4, and control + cetrorelix n = 4). *VEGF-C* promoter activity, as reflected by the total flux of photons from the tumor area, was found to be higher in the ovariectomized group compared with all other groups in each of the time points examined. Gonadotropin-releasing hormone blockade showed no effect in the control group, whereas in the ovariectomized group this treatment prevented the induction of *VEGF-C* expression, suggesting that the *VEGF-C* promoter is indeed activated in a gonadotropin-dependent manner. The statistical significance of the data was verified using a t test by comparing the rate of signal enhancement in each of the groups (ovariectomized versus control P = 0.04, ovariectomized versus ovariectomized + cetrorelix P = 0.04, and ovariectomized versus control + cetrorelix P = 0.03).

Ovariectomy induces an elevation in *VEGF-C* and *LEDGF/p75* mRNA levels in tumors. Following mRNA extraction from tumors derived from each of the experimental groups, *VEGF-C* and *LEDGF/p75* mRNA levels were determined (Fig. 4*A* and *B*; ovariectomized n = 4, control n = 4, ovariectomized + cetrorelix n = 5, and control + cetrorelix n = 3). In the ovariectomized group, *VEGF-C* mRNA was 1.9-fold higher compared with the nonovariectomized control (P = 0.0009). Treatment with cetrorelix significantly blocked the increase in the *VEGF-C* mRNA (ovariectomized versus ovariectomized + cetrorelix P = 0.0006 and ovariectomized versus control + cetrorelix P = 0.006). A 1.8-fold increase was observed in the *LEDGF/p75* mRNA levels in the ovariectomized mice compared with control (P = 0.005). This increased transcription was blocked by treatment with cetrorelix (ovariectomized versus ovariectomized + cetrorelix P = 0.0003 and ovariectomized versus control + cetrorelix P = 0.005).

To further investigate the influence of ovariectomy on *VEGF-C* and *LEDGF/p75* transcription in ovarian tumor-bearing mice, an

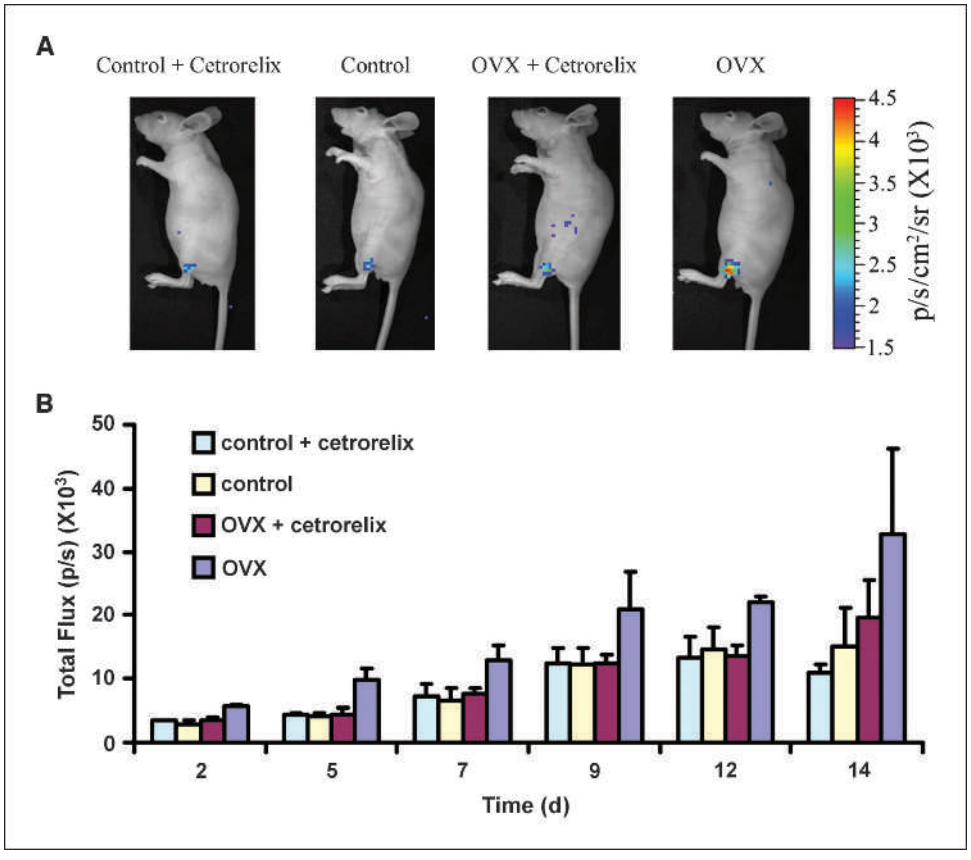

Figure 3. Gonadotropin-releasing hormone blockade prevents the *VEGF-C* promoter–enhanced activation in ovariectomized mice. *A,* bioluminescence imaging of ovariectomized and control tumor-bearing mice either treated or untreated with cetrorelix. Mice were imaged 7 d following tumor induction. *B,* total flux of photons derived from the tumor area of ovariectomized and control tumor-bearing mice either treated or untreated with cetrorelix (ovariectomized n = 5, ovariectomized + cetrorelix n = 5, control n = 4, and control + cetrorelix n = 4).

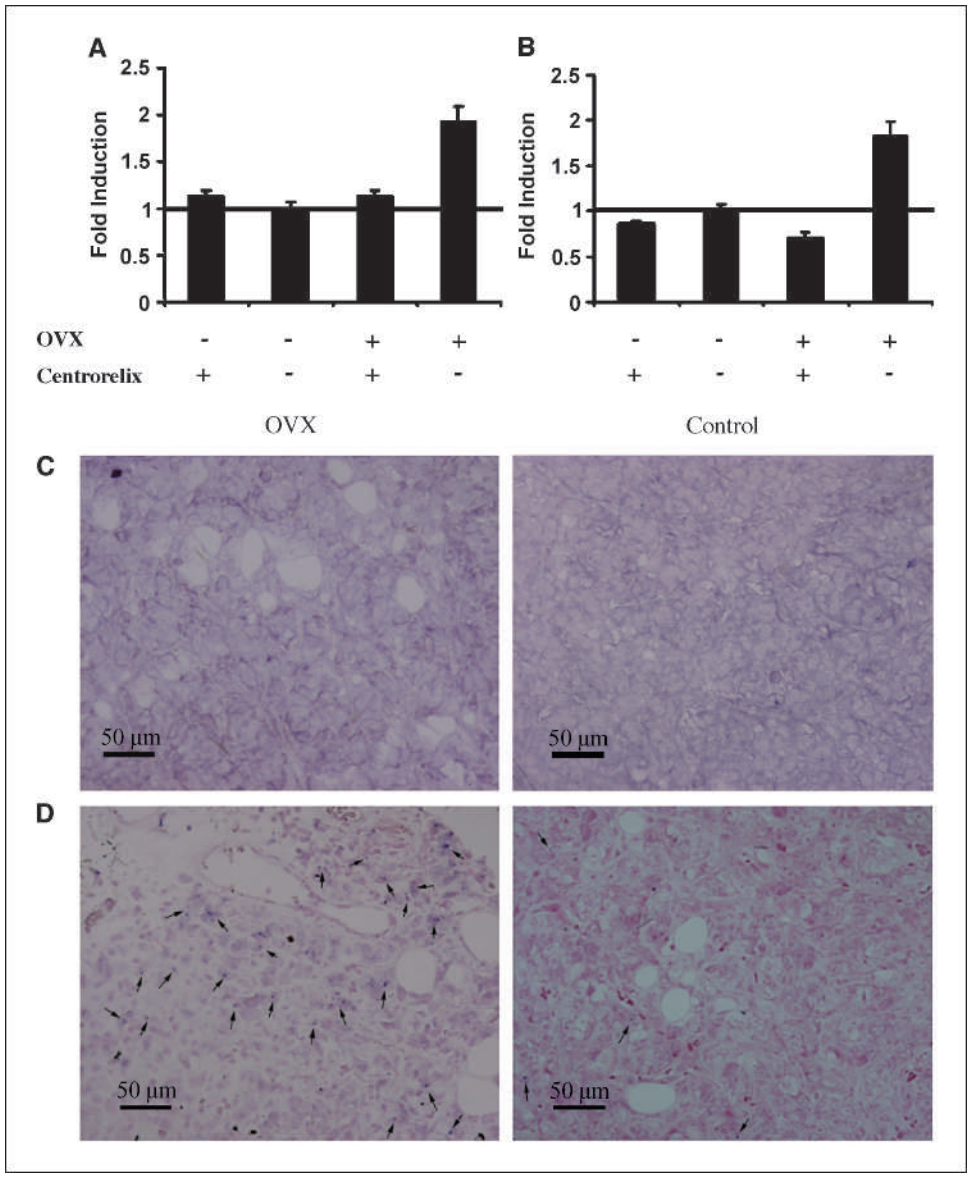

Figure 4. Ovariectomy induces an elevation in *VEGF-C* and *LEDGF/p75* mRNA levels in tumors. *A,* RT-PCR using *VEGF-C*–specific primers carried out on RNA extracted from tumors. Data are fold induction compared with control group (ovariectomized $n = 4$, control $n = 4$, ovariectomized + cetrorelix $n = 5$, and control + cetrorelix $n = 3$). *B,* RT-PCR using *LEDGF/p75*-specific primers carried out on RNA extracted from tumors. Data are fold induction compared with control group (ovariectomized $n = 4$, control $n = 4$, ovariectomized + cetrorelix $n = 5$, and control + cetrorelix $n = 3$). *C, in situ* hybridization using a *VEGF-C*–specific probe of histologic sections derived from tumors induced in ovariectomized and control mice (ovariectomized $n = 5$ and control $n = 5$). *D, in situ* hybridization using a *LEDGF/p75*-specific probe of histologic sections derived from tumors induced in ovariectomized and control mice (ovariectomized $n = 5$ and control $n = 5$).

in situ hybridization was carried out on histologic sections derived from tumors induced in ovariectomized versus control mice. The staining indicated an increase both in the *VEGF-C* and *LEDGF/p75* mRNA levels in the ovariectomized group compared with untreated control (Fig. 4*C* and *D*; ovariectomized $n = 5$ and control $n = 5$).

Ovariectomy induces lymphangiogenesis and angiogenesis in ovarian tumors. Histologic sections of the tumors were stained for blood and lymphatic vessels (Fig. 5). Analyzing both the tumor area and the skin area in the vicinity of the tumor by an immunohistochemical staining using an anti–LYVE-1 antibody, we found an enhanced staining in the ovariectomized group, indicating enhanced lymphangiogenesis (Fig. 5*A*; ovariectomized $n = 5$ and control $n = 5$). Analysis of the data was carried out by calculating the percentage of fluorescent staining coverage in each field of view, showing a significant difference between the ovariectomized and the control groups for both the tumor and skin areas ($P = 0.04$ and 0.03, respectively, t test). To verify the specificity of the LYVE-1 staining as a marker for lymphatic vessels, an anti-PROX1 antibody was used for immunohistologic staining of adjacent sections (Fig. 5*C*). A similar pattern of vascular staining was observed for the two antibodies.

Having some proangiogenic activity, we hypothesized that the VEGF-C activation in ovariectomized tumor-bearing mice may result in enhanced tumor angiogenesis. Immunohistochemical staining of both the tumor area and the skin area in the vicinity of the tumor using an anti-CD34 antibody showed an enhanced staining in the ovariectomized group (Fig. 5*B*; $P = 0.02$ and 0.04 for tumor and skin areas, respectively).

Discussion

Lymphangiogenesis, an important step in tumor progression, serves as a route for metastasizing cells. Lymphangiogenesis was reported to play a role in ovarian cancer progression both clinically and in experimental models (12, 20). In addition, the risk and aggressiveness of ovarian cancer were found to increase with menopause. In the current study, we combined both findings, showing the role of the gonadotropic hormones LH and FSH, whose level

increase at menopause, in the regulation of lymphangiogenesis in ovarian cancer.

In vitro stimulation of ovarian cancer cells by gonadotropins was reported previously to enhance cell invasiveness and to induce cell growth, activation of oncogenes, and inhibition of apoptosis (1, 5, 6, 33). We have shown previously that gonadotropin stimulation of ovarian cancer induced expression of VEGF, integrins, and CD44 (7, 34). In the current study, we show that *in vitro* stimulation by gonadotropins activated the *VEGF-C* promoter and augmented *VEGF-C* mRNA and protein levels in human ovarian carcinoma ES2 cells.

The role of VEGF-C in peritumor lymphatic remodeling and lymph node metastatic spread was shown previously for multiple types of cancers (35–37). Overexpression of VEGF-C increased tumor growth, lymphatic involvement, and metastasis (38–40). Clinically, high levels of VEGF-C were correlated with metastasis, low survival, and poor prognosis (15, 16).

The involvement of gonadotropins elevation in VEGF-C activation was examined in ovariectomized mice and was further verified using cetrorelix, a gonadotropin-releasing hormone antagonist. In previous studies, this drug was effectively used to reduce gonadotropins levels *in vivo* in mice (41, 42). Treatment with the cetrorelix significantly suppressed the ovariectomy-induced activation of the *VEGF-C* promoter in the ovarian carcinoma tumors.

Consistent with the *in vivo* bioluminescence imaging, *VEGF-C* mRNA level was found to be higher in tumors derived from

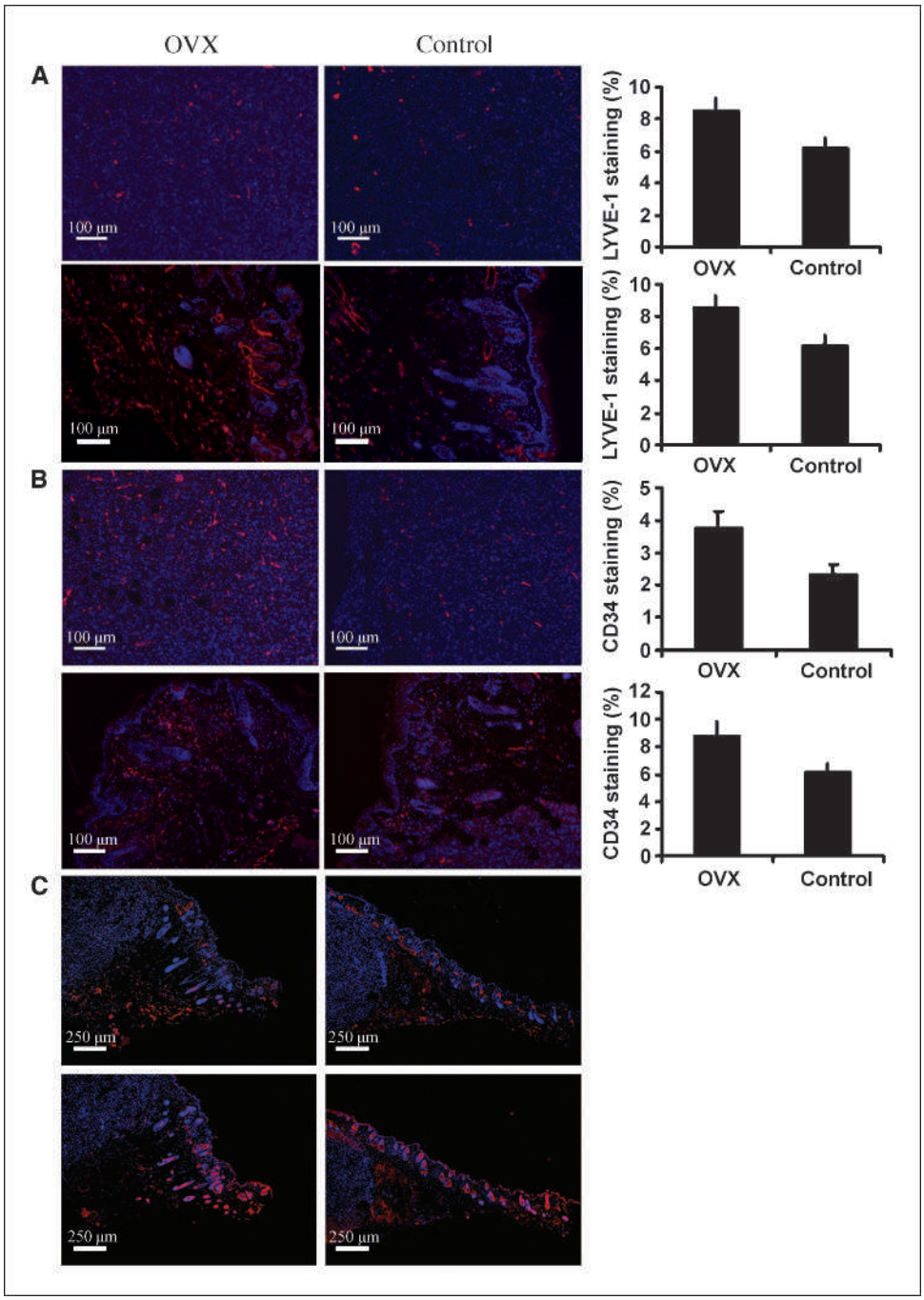

Figure 5. Ovariectomy induces lymphangiogenesis and angiogenesis in ovarian tumors. *A,* immunohistochemical staining using anti–LYVE-1 antibody of histologic sections derived from tumors (*top*) or the skin area in the vicinity of the tumors (*bottom*) induced in ovariectomized and control mice (ovariectomized $n = 5$ and control $n = 5$). Analysis of the data was carried out by calculating the percentage of fluorescent staining coverage in each field of view. *B,* immunohistochemical staining using anti-CD34 antibody of histologic sections derived from tumors (*top*) or the skin area in the vicinity of the tumors (*bottom*) induced in ovariectomized and control mice (ovariectomized $n = 5$ and control $n = 5$). Analysis of the data was carried out by calculating the percentage of fluorescent staining coverage in each field of view. *C,* immunohistochemical staining using anti–LYVE-1 antibody (*top*) or anti-PROX1 antibody (*bottom*) of histologic sections derived from ovariectomized and control mice.

ovariectomized mice compared with control mice. Accordingly, the density of lymph vessels detected by immunohistochemistry was elevated. In addition to its lymphangiogenic activity, VEGF-C is known to bind VEGF receptor-2 and to induce endothelial cell survival, mitogenesis, and migration. We therefore hypothesized ovariectomy to induce angiogenesis of the tumors. Indeed, immunohistochemical staining with an anti-CD34 antibody revealed enhanced density of blood vessels in the ovariectomized group. This may result as well from VEGF-A enhanced activation shown previously to take place in ovariectomized mice (7).

The role of the LEDGF/p75 in initiation and progression of malignant tumors was exemplified in different cancer types. It was found to inhibit the caspase-independent lysosomal cell death pathway in various human cancers (31), to confer resistance to chemotherapy in acute myelogenous leukemia patients (32), and to play a role in the initiation of leukemic transformation by MLL oncoproteins (43). High levels of LEDGF/p75 autoantibodies were found in prostate cancer patients (44), and the level of the protein was found to be elevated in various human cancers (31). We reported recently the role of LEDGF/p75 in stress-induced expression of *VEGF-C* in response to hyperthermia and oxidative stress and the contribution of a conserved *STRE* in the *VEGF-C* promoter in this stress-induced expression (21).

As reported here, chromatin immunoprecipitation showed that LEDGF/p75 binds the *VEGF-C* promoter. Strong enhancement of this binding was observed following stimulation with FSH. Interestingly, no such enhancement was observed following LH stimulation, suggesting a different pattern of regulation regarding these two hormones. Downregulation of *LEDGF/p75* by siRNA as well as mutations in the *STRE* binding site located in the *VEGF-C* promoter sequence resulted in an attenuated enhancement of the *VEGF-C* promoter following hormonal stimulation, emphasizing the contribution of LEDGF/p75 to the hormonally mediated VEGF-C activation. *In vitro* studies conducted in ES2 cells showed LH and FSH stimulation to increase both LEDGF/p75 mRNA and protein levels. *LEDGF/p75* mRNA levels were measured at different time points following hormonal administration, starting from 6 h stimulation in which an elevation in the *LEDGF/p75* mRNA level was already detected. The chromatin immunoprecipitation data, however, show FSH-induced binding to LEDGF/p75 as early as 1 h following gonadotropin administration, suggesting that the initial stages of VEGF-C activation do not require *de novo LEDGF/p75* mRNA synthesis. In accordance with the *in vitro* data, *LEDGF/p75* mRNA levels were found to be elevated in ovariectomized tumor-bearing mice, and the enhanced transcription could be blocked by treatment with cetrorelix.

In summary, we showed here that enhanced LH and FSH stimulation, both *in vitro* or by ovariectomy, enhanced LEDGF/p75-dependent activation of VEGF-C expression and resulted in enhanced lymphangiogenesis and angiogenesis of ovarian carcinoma tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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