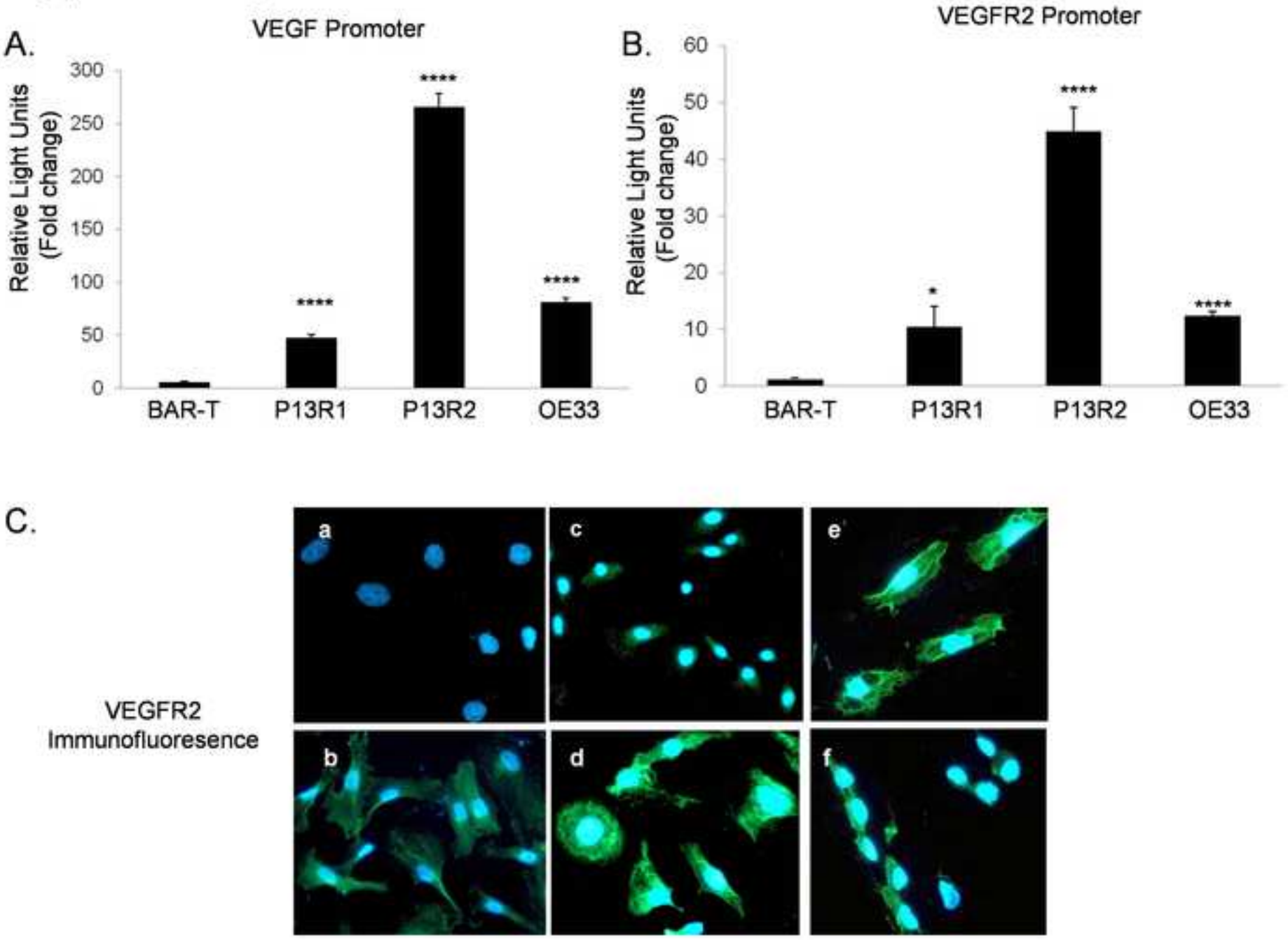
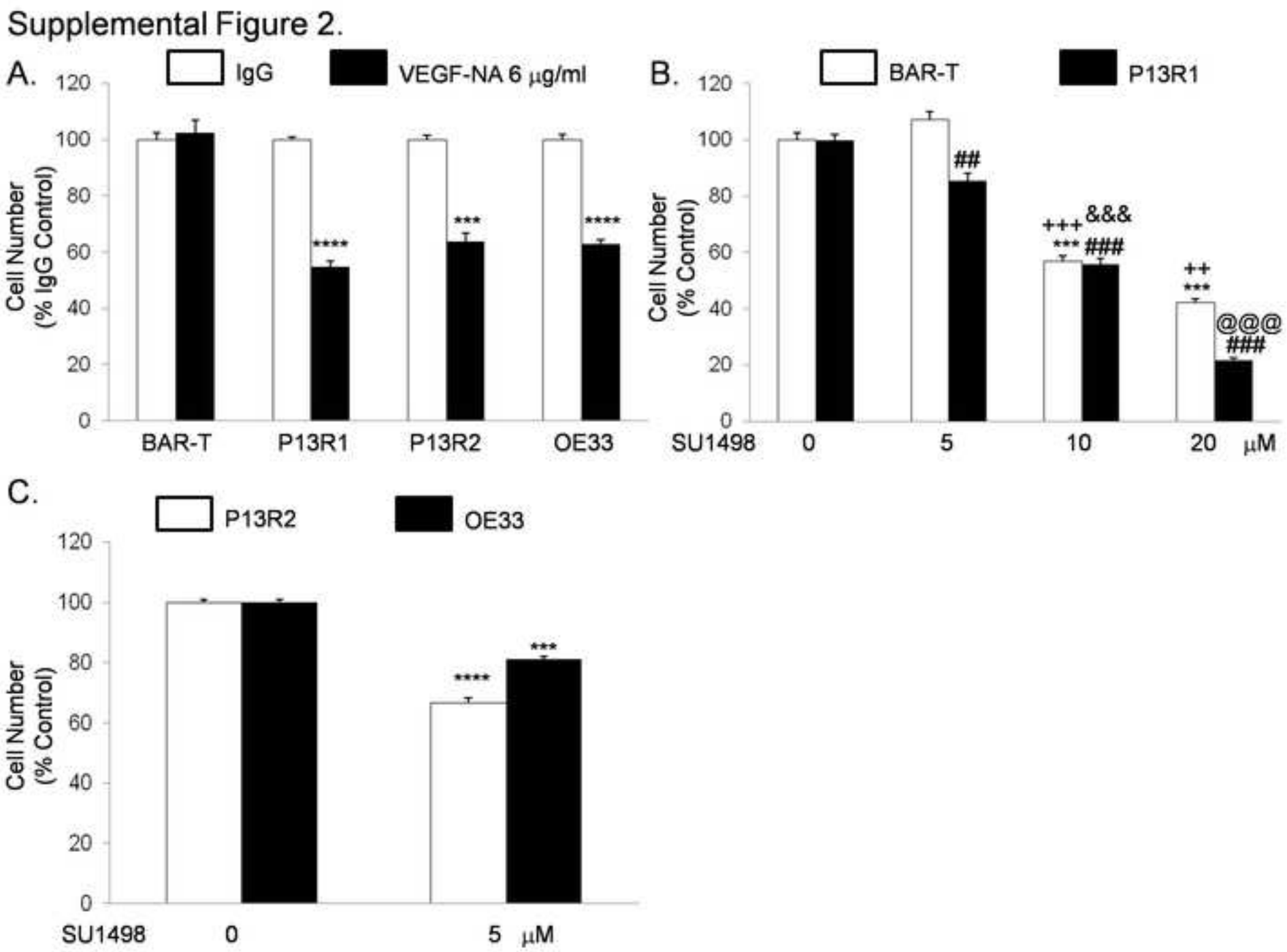
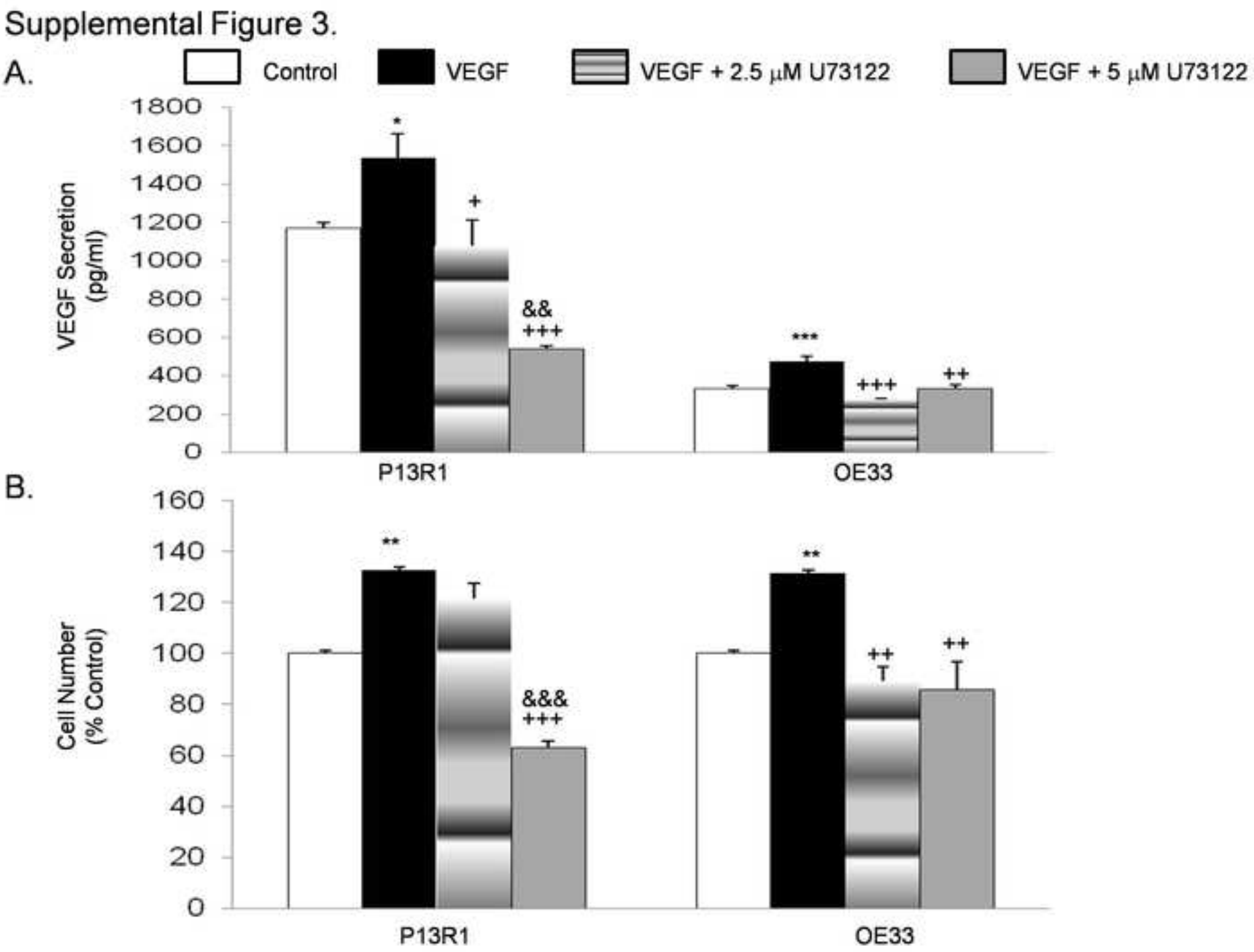
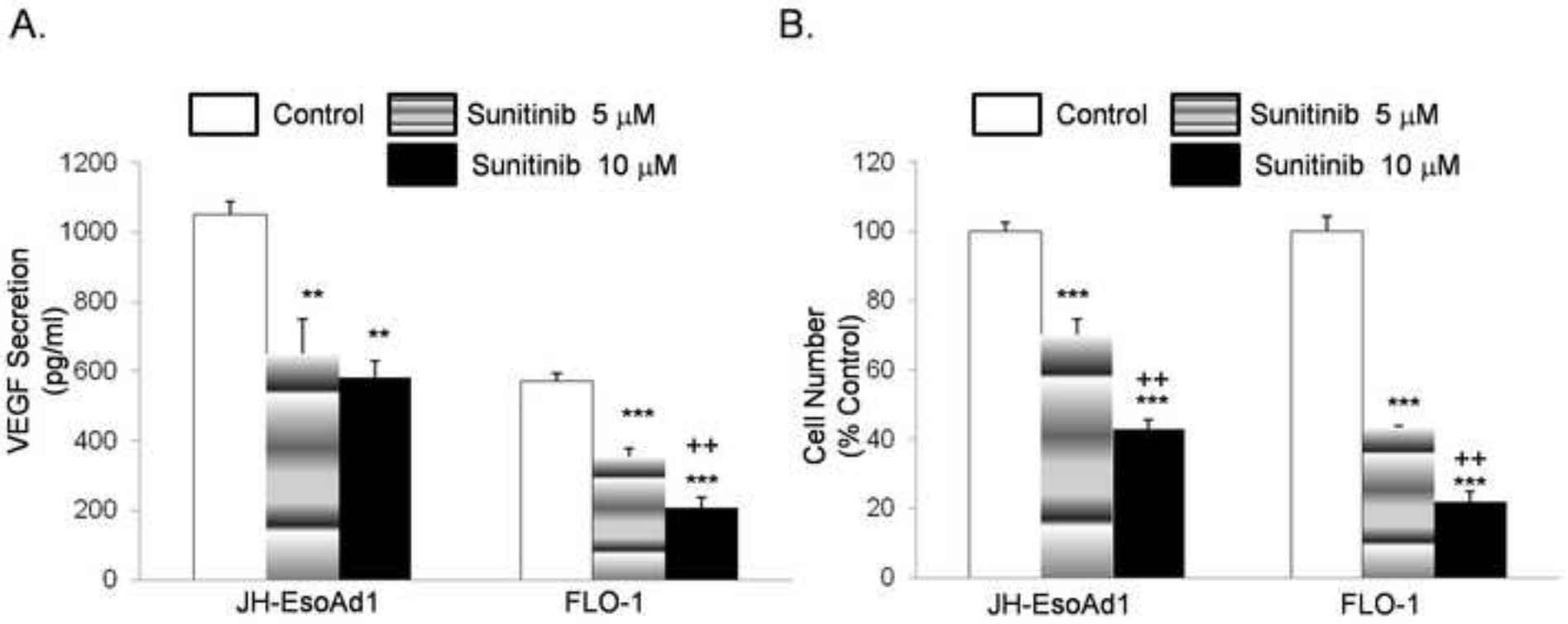
# Supplemental Figure 1.




Supplemental Figure 2.
A.
IgG
VEGF-NA 6 μg/ml
Cell Number (% IgG Control)
120
100
80
60
40
20
0
****
***
****
BAR-T
P13R1
P13R2
OE33
B.
BAR-T
P13R1
Cell Number (% Control)
##
+++
***
&&&
###
++
***
@@@
###
SU1498
0
5
10
20
μM
C.
P13R2
OE33
Cell Number (% Control)
****
***
SU1498
0
5
μM

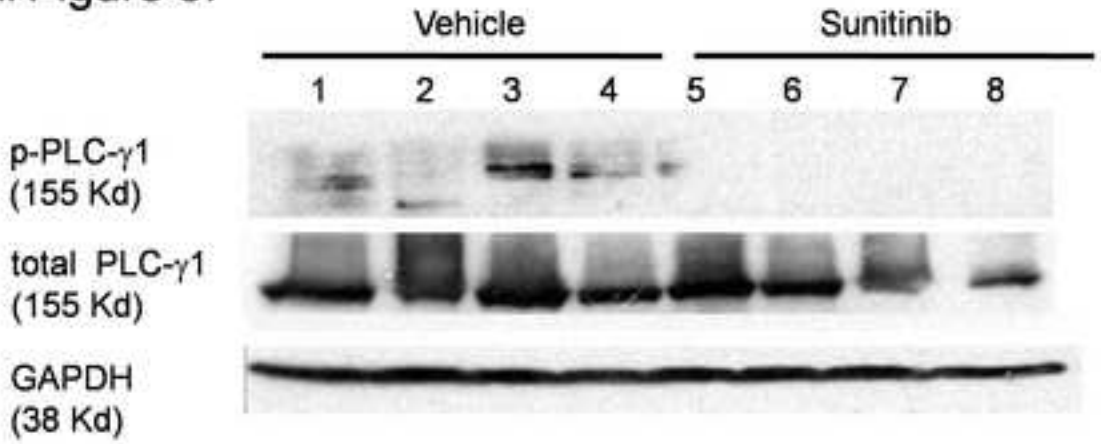

Supplemental Figure 3.
A.
Control
VEGF
VEGF + 2.5 μM U73122
VEGF + 5 μM U73122
VEGF Secretion (pg/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
*
+
&&
+++
***
+++
++
P13R1
OE33
B.
Cell Number (% Control)
160
140
120
100
80
60
40
20
0
**
T
&&&
+++
**
++
T
++
P13R1
OE33

Supplemental Figure 4.

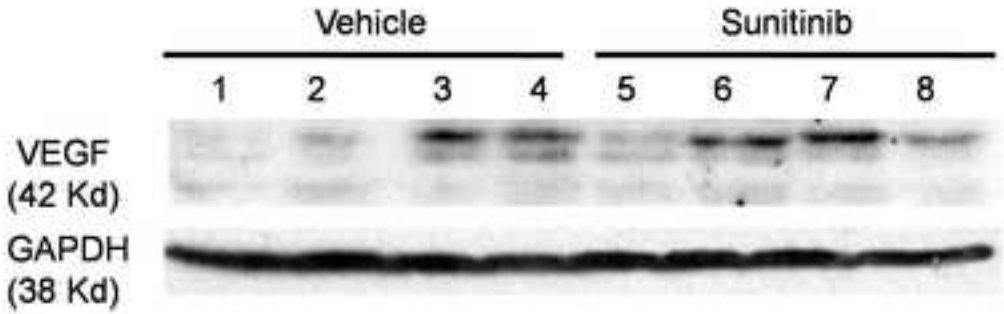


# Supplemental Figure 5.

A.




B.



**Supplemental Figure Legends**

**Supplemental Figure 1.** Upregulation of VEGF (A) and VEGFR2 (B) promoter activity in transformed Barrett's cells (P13R1 and P13R2) and OE33 adenocarcinoma cells. Data are the means ± SEM of at least 2 separate experiments. *, p≤ 0.05; ****, p≤ 0.0001 compared to BAR-T cells. (C) VEGFR2 immunofluorescence in (c) benign Barrett's epithelial cells, transformed Barrett's (d) P13R2 and (e) P13R1 cells, and (f) OE33 adenocarcinoma cells. VEGFR2 was stained with anti-human VEGFR2 antibody (green) and nuclei were counterstained with DAPI (blue). Human endothelial cells stained with (a) normal IgG and (b) VEGFR2 served as negative and positive controls, respectively.

**Supplemental Figure 2.** Cell numbers in BAR-T cells, in transformed Barrett's cells (P13R1 and P13R2), and in OE33 adenocarcinoma cells treated with (A) VEGF neutralization antibody (VEGF-NA); normal IgG served as a control. Data are the means ± SEM of 3 separate experiments. ***, p≤ 0.001; ****, p≤ 0.0001 compared to corresponding IgG controls. (B) SU1498 in concentrations ranging from 5-20 μM in BAR-T and P13R1cells. Data are the means ± SEM of 3 separate experiments. ***, p≤ 0.001 compared to untreated BAR-T cells; +++, p≤ 0.001 compared to BAR-T treated with 5 μM; ++, p≤ 0.01 compared to BAR-T treated with 10 μM; ##, p≤ 0.01 compared to untreated P13R1; ###, p≤ 0.001 compared to untreated P13R1; &&&, p≤ 0.001 compared to P13R1 treated with 5 μM; @@@, p≤ 0.001 compared to P13R1 treated with 10 μM. (C) 5 μM SU1498 treatment in transformed P13R2 and OE33 cells. Data are the means ± SEM of 3 separate experiments. ***, p≤ 0.001 compared to untreated control; ****, p≤ 0.0001 compared to untreated control.

**Supplemental Figure 3.** Treatment with U73122, a PLC-γ inhibitor, decreases (A) VEGF secretion and (B) cell number in transformed Barrett's cells and OE33 adenocarcinoma cells. Data are the means ± SEM of 3 separate experiments. *, p≤ 0.05; **, p≤ 0.01; and ***, p≤ 0.001 compared to corresponding controls. +, p≤ 0.05; ++, p≤ 0.01; +++, p≤ 0.001 compared to corresponding VEGF treated cells. &&, p≤ 0.01; &&&, p≤ 0.001 compared to corresponding cells treated with 2.5 μM U73122.

**Supplemental Figure 4.** Sunitinib decreases (A) VEGF secretion, and (B) cell number in esophageal adenocarcinoma cell lines JH-EsoAd1 and FLO-1. Data are the means ± SEM of at least 3 separate experiments. **, p≤ 0.01; ***, p≤ 0.001 compared to corresponding controls; ++, p≤ 0.01 compared to corresponding 5 μM treated cells.

**Supplemental Figure 5.** Sunitinib decreases (A) phospho-PLC-γ1 protein expression, but not (B) VEGF protein expression in mouse xenografts of transformed Barrett's cells compared to vehicle-treated tumors. Individual mouse tumors are numbered 1-8.

## Supplemental materials and methods

### *Cell Lines*

Briefly, we started with a non-transformed, telomerase-immortalized, p16-deficient cell line (BAR-T) that we established from biopsy specimens of non-dysplastic Barrett's metaplasia. BAR-T cells maintain morphological and immunohistochemical characteristics of a mucin-producing columnar cell type exhibiting intestinal-type differentiation with the expression of cytokeratins 8 and 18 and mucin that stains positive with Alcian-blue.[13] To knockdown p53, we infected BAR-T cells with the retroviral vector pSUPER-RNAi-p53 (OligoEngine, Seattle, WA). To express human oncogenic H-Ras, we infected BAR-T cells with the retroviral vector pBabe-H-Ras$^{G12V}$ (obtained from Dr. Robert Weinberg, Whitehead Institute, Cambridge, MA).[14] In immunodeficient mice, BAR-T cells containing both pSUPER-p53RNAi and pBabe- H-Ras$^{G12V}$ (clones P13R1 and P13R2) form glandular tumors typical of esophageal adenocarcinoma, and thus were transformed.[14] The BAR-T cells were co-cultured with a fibroblast feeder layer as previously described[13]; the transformed P13R1 and P13R2 did not require a fibroblast feeder layer.[14] The OE33 Barrett's-associated esophageal adenocarcinoma cell line was purchased from Sigma (St. Louis, MO); the JH-EsoAd1 esophageal adenocarcionoma cell line was a generous gift of Drs. James Eshleman and Anirban Maitra (Johns Hopkins University); the FLO-1 esophageal adenocarcinoma cell line was a generous gift of Dr. David G. Beer (University of Michigan).[15;16] All cell lines were maintained in monolayer culture at 37˚C in humidified air with 5% $CO_2$ in growth media as previously described.[14-16] For individual experiments, the BAR-T cells were seeded into collagen IV-coated wells (BD Biosciences, San Jose, CA), while P13R1, P13R2, OE33, JH-EsoAd1, and FLO-1 cells were seeded in uncoated wells in standard culture plates.

**Patients.** This study was approved by the institutional review board of the Dallas VA Medical Center. Patients scheduled for elective endoscopy who had a history of esophageal carcinoma

or Barrett's esophagus were invited to participate. During endoscopy, biopsy specimens of esophageal carcinoma or Barrett's esophagus were taken and snap frozen in liquid nitrogen; biopsies of normal areas of esophagus, and/or duodenum, and/or stomach were taken as control tissues.

### *RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (PCR)*

Total RNAs were isolated using RNeasy Mini kit (Qiagen, Valencia, CA) per manufacturer's instructions, and quantitated using the Nanophotometer (IMPLEN, Westlake Village, CA). The integrity of total RNA was examined by using an ethidium bromide-stained, formamide RNA gel. Reverse transcription was performed using QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA) per manufacturer's instructions. The primer sequences (Supplemental Table 1) were designed using Primer Express (Applied BioSystems, Foster City, CA) and manufactured by Integrated DNA Technologies (Coralville, IA). Real-time PCRs for VEGF and VEGFR2 mRNA were carried out with the StepOnePlus Real-Time PCR System and SYBR Green mix (Applied Biosystems, Foster City, CA) using an established protocol[27], esterase D was used as a reference gene. The relative quantity of mRNA with respect to the reference gene was calculated as described previously[27] and the result expressed as a ratio to the mean value in the control group.

### *Total Protein Extraction and Immunoblotting*

Total protein was extracted using 1X cell lysis buffer according to manufacturer's instruction (Cell Signaling Technology, Danvers, MA). The lysis buffer was supplemented with phosphatase inhibitor cocktail 2 (Cat# P8340) and cocktail 3 (cat# P0044) (Sigma**,** St. Louis, MO) as needed. Nuclear and cytoplasmic extracts were isolated from cell lines using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, Rockford, IL) per the manufacturer's instructions. Protein concentrations were determined using the BCA-200

Protein Assay kit (Pierce, Rockford, IL). Esophageal adenocarcinoma, Barrett's esophagus, and control patient tissues (30–60 mg wet weight) were minced on ice and homogenized by polytron in buffer containing 300 mM sucrose, 20 mM Tris, pH 8.0, 10 mM HEPES, 5 mM EGTA, and 2 mM 2-mercaptoethanol with supplementation of protease inhibitors as above. The homogenates were cleared by centrifugation at 15,000 *g*, the supernatant was ultracentrifuged at 150,000 *g* to separate membrane-bound and soluble cytosolic protein, and the pellet was dissolved in radioimmunoprecipitation assay (RIPA) buffer for use as crude nuclear extracts. Membrane pellets were re-suspended in buffer containing 5 mM Tris, pH 6.8, 10% glycerol, 1% 2-mercapto- ethanol, and 1% SDS. The membrane-bound and cytosolic protein fractions were quantified by Brad-ford assay (Bio-Rad Laboratories, Hercules, CA). Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and incubated with primary antibodies (Supplemental Table 2) overnight at 4$^{o}$C. Secondary antibody was either horse anti-rabbit or horse anti-mouse IgG conjugated with horseradish peroxidase (Cell Signaling Technology), and chemiluminescence was determined using the ECL detection system (Pierce, Rockford, IL). The membranes were stripped and re-probed with mouse anti-β-tubulin (Sigma, St. Louis, MO) as a loading control; lamin A/C (Cell Signaling Technology, Danvers, MA) was used as a loading control for nuclear fractions**.** Proteins were quantified by densitometry (ImageQuant Version 5, Amersham Biosciences), the relative quantity of protein with respect to the loading control was calculated as described previously[27], and the results were expressed as a ratio to the mean value in the control group.

***Promoter Reporter Gene Assays***

The mouse VEGF (−807/+118, genebank accession# NW_001030618) and VEGFR2 (−620/+304, genebank accession# NW_001030787) promoter regions were isolated by PCR amplification of mouse genomic DNA and inserted into the firefly luciferase reporter pGL3-Basic. Cells were transfected with 200 ng/well of one of the promoter constructs or with pGL3 empty

vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) per manufacturer's instructions. After 24 hours, cell extracts were assayed for luciferase activities. Within each transfection set, luciferase activity of the VEGF or VEGFR2 promoter was normalized to luciferase activity in the empty vector control as previously described.[28]

### *Cell Growth, Proliferation, Apoptosis, and Enzyme-Linked Immunosorbent Assays (ELISA)*

Equally seeded wells of cells were incubated overnight in starvation medium (composed of basal medium containing 10 μM $CaCl_2$ but without serum or growth factors) followed by a 48-hour treatment with the pharmacologic inhibitors. Cell number and proliferation were then determined as described. For apoptosis, cells were stained with Annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining kits (BD Biosciences, San Jose, CA) as previously described.[29] Cells stained only with Annexin-V only were considered to be apoptotic. For VEGF ELISA assays, the absorbance of each well was read at 450 nm using a POLARstar Omega Multimode plate reader (BMG Labtech, Cary, NC).

### *Inhibition of VEGF/VEFGR2 Signaling*

Cells were treated with (a) SU1498 (cat# 572888, Calbiochem, Billerica, MA), (b) a VEGF neutralization antibody (VEGF-NA; R&D Systems, Minneapolis, MN) or (c) sunitinib (S-8803, LC Laboratories, Woburn, MA). In preliminary experiments, cells were treated with the sunitinib in doses ranging from 5-15 μM, and we found that doses ≥15 μM decreased cell viability. Therefore, we selected to use sunitinib at doses ≤10 μM for all further experiments. Cells were also transfected with non-targeting control siRNA (cat# 37007, Santa Cruz Biotechnology, Santa Cruz, CA) or specific VEGFR2 siRNA (cat# J-003148-12, Thermo Fisher Scientific, Pittsburgh, PA) using an established method.[14] To establish stable cell lines with

VEGFR2 knockdown, we purchased custom shRNA against human VEGFR2 from Origene Technologies (Rockville, MD). Retroviral particles were generated using a previously established method.[14] P13R1 cells were infected at approximately 50% confluence in the presence of 4 mg/ml of Polybrene (Sigma, St. Louis, MO) for 10–12 hours. After recovery for 72 hours, cells were selected in 40 μg/ml blasticidin for 14 days. Single colonies or a population of surviving cells were selected, and VEGFR2 knockdown was confirmed by Western blotting.

***Inhibition of the PLC Signaling Pathway***

In preliminary experiments, cells were treated with the PLC-γ inhibitor U73122 (Tocris Bioscience, Ellisville, MO) at doses ranging from 5- 15 μM, and we found that doses ≥10 μM decreased cell viability. Therefore, we selected to use U73122 at doses ≤5 μM for all further experiments. Cells were pre-treated for 2 hours with DMSO or with 2.5 or 5 μM U73122 prior to treatment with recombinant human VEGF as described below. The inhibitor was maintained in the medium throughout the entire course of the experiment. Cells were also transfected with non-targeting control siRNA (Santa Cruz) or specific PLC-γ1 siRNA (cat# J-003559-08, Thermo Fisher Scientific) as previously described.[14]

***Activation of VEGF Pathway Signaling***

Equally seeded wells of cells were placed overnight into starvation medium (basal medium supplemented with 1% FBS). The following day, the starvation medium was supplemented with or without 30 ng/ml of recombinant human VEGF (rhVEGF) for 15 minutes (P13R1) or 1 hour (OE33). For ELISA assays and cell counts, cells were rinsed twice with PBS, and incubated with starvation medium for an additional 24 hours, after which the medium was aspirated for ELISA assays and the cells were harvested for cell counts. For analysis of VEGFR2 signaling pathways, cell were treated with rhVEGF for 0, 15 , 30, 60, 180, or 360

minutes; for siRNA experiments, cells were treated with rhVEGF for 0, 1, or 3 hours. Proteins were harvested immediately for analysis following rhVEGF treatment.

***Immunofluorescense***

Cells were seeded at a density of $1 \times 10^5$ cells per well onto glass cover slips placed in six-well plates. Cells were fixed in 4% paraformaldehyde for 20 min at $4^{o}$ C and incubated with primary antibodies against total VEGFR2 (1:100 dilution, Cell Signaling), phospho-VEGFR2 (1:100 dilution, Abcam, Cambridge, MA) or normal IgG (Santa Cruz) at $4^{o}$ C overnight (Supplemental Table 2). After three washes in phosphate buffered saline (PBS), cells were incubated with Alexa Fluor® 488-conjugated donkey-anti-rabbit IgG (1:500, Invitrogen) for 1 hr at room temperature. Images were recorded with a fluorescence microscope (DM6000B, Leica Microsystem Ltd., Heerbrugg, Switzerland).

***Immunohistochemistry***

Human esophageal adenocarcinoma tissue arrays were purchased from Biomax USA (Rockville, MD) containing 20 esophageal adenocarcinoma tissues with 20 adjacent histologically normal esophageal epithelial tissues as controls, arranged in duplicate on microscopic slides. Deparaffinized sections were pretreated with 10 mM sodium citrate buffer for antigen unmasking (pH 6.0, boiling temperature, 30 min), blocked in goat serum (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA), incubated with rabbit polyclonal anti-human phospho-VEGFR2 at $4^{o}$ C overnight, rinsed, and incubated with anti-rabbit secondary antibody (Vectastain ABC kit). Signals were amplified using Vectastain ABC kit per manufacturer's instruction. Targeted protein was visualized using diaminobenzidine as substrate. The tissues were simultaneously evaluated by two gastrointestinal pathologists (SDM, RMG), and the areas of viable tissues with strongest labeling for phospho-VEGFR2 (hot spots) were scored. For CD31 staining of total tumor burden from xenograft experiments, we used a pre-diluted rabbit

polyclonal anti-mouse CD31 and stained 5μ formalin-fixed paraffin sections using a standardized automated techniques on a Ventana Medical Systems Automated immunohistochemistry staining machine (Tucson, AZ). Briefly, following heat-mediated antigen retrieval, the tissue was formaldehyde-fixed and blocked with serum. The tissue was incubated with the primary antibody for 20 minutes. A biotinylated goat anti-rabbit antibody was used as the secondary antibody. External control was mouse kidney; internal control was the endothelium of vessels in the tissues adjacent to the tumors. The vessel density in CD31-stained tumors was simultaneously evaluated by two gastrointestinal pathologists using an established method.[30] After selecting three hot spots (i.e. vessel-rich areas) within the viable portions of each tumor, positive-staining vessels were counted in 3 high-power fields (a 237-square micron area) within each hot spot, and averaged. Then, the averages of the counts of each hot spot were in turn averaged. Thus, the counts for each tumor reflect the mean number of vessels in a total of 9 equal areas in each tumor.

For Ki67 staining of total tumor burden from xenograft experiments, we used a pre-diluted rabbit monoclonal antibody and stained by standardized automated techniques using the Ventana BenchMark XT. Briefly for image analysis, 10 fields were selected from the tumor and an average score of the percent positive staining nuclei in the 10 fields were taken as the final score. Only viable areas of the tumor were selected for analysis.

***Tumor Xenografts***

All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Xenograft experiments were done as described previously.[14] In brief, 2-3 × $10^6$ P13R1 cells or P13R1 cells infected with VEGFR2 shRNA (population or selected clone B3) were suspended in 50% Matrigel and 50% culture medium, and implanted subcutaneously into the dorsal skin of female NOD/SCID mice. Each mouse received 3 implants in order to minimize the number of animals needed for the study. When the mice first developed a

palpable mass (deemed the “index” tumor), tumor measurements were begun. Mice were sacrificed at 4 months or earlier if their total tumor burden (index tumor plus any other tumors) exceeded 2 cm or if they demonstrated signs of distress (including weight loss). For sunitinib experiments, mice were randomized to either the sunitinib treatment or control group when they first developed a palpable mass. Mice in the treatment group were administered a daily oral gavage with 4 mg/kg sunitinib (Sutent®, Pfizer) resuspended in an aqueous solution of 0.5% carboxyethylcellulose and 0.25% Tween 80, whereas mice in the control group were administered a daily oral gavage with vehicle-only solution as previously described.[31] At sacrifice, all tumors were removed, weighed, and processed using standard histological procedures.

***Data Analyses***

Quantitative data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using an unpaired Student’s t-test with the Instat for Windows statistical software package (GraphPad Software, San Diego, CA). For multiple comparisons, an ANOVA and the Student-Newman-Keuls multiple-comparisons test was performed using the Instat for Windows statistical software package (GraphPad).

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31. Bauerschlag DO, Schem C, Tiwari S, Egberts JH, Weigel MT, Kalthoff H, Jonat W, Maass N, Meinhold-Heerlein I. Sunitinib (SU11248) inhibits growth of human ovarian cancer in xenografted mice. Anticancer Res 2010;30:3355-3360.

**Supplemental Table 1. Oligonucleotide primers**

| Primer | Sequence (5' to 3') | Location | Use |
|---|---|---|---|
| VEGF-5' | ATCTTCAAGCCATCCTGTGTGC | sense | Real-time PCR |
| VEGF-3' | CAAGGCCCACAGGGATTTTC | antisense | Real-time PCR |
| VEGFR2-5' | AGGCAGCTCACAGTCCTAGAGC | sense | Real-time PCR |
| VEGFR2-3' | GTCTTTTCCTGGGCACCTTCTA | antisense | Real-time PCR |
| Esterase D-5' | TGATCAAGGGAAAGATGACCA | sense | Real-time PCR |
| Esterase D-3' | AACCCTCTTGCAATCGAAAA | antisense | Real-time PCR |

**Supplemental Table 2. Antibodies Used**

| antibody | Source information(cat#, vendor) | dosage | Usage |
|---|---|---|---|
| VEGF | ab1316, Abcam | 1:800 dilution | WB |
| VEGF-NA | AB293-NA, R&D Systems | 6 μg/ml | neutralization |
| VEGFR2 | sc-315, Santa Cruz Biotechnologies | 1 μg/ml | WB |
| VEGFR2 | #2479, Cell Signaling | 1:100 | IF |
| pVEGFR2 | Ab5473, Abcam | 1 μg/ml, IHC; 0.5 μg/ml WB; 1:100 dilution IF | IHC, WB, IF |
| PLC-γ1 | #2822, Cell Signaling | 0.1 μg/ml | WB |
| pPLC-γ1 | #2821, Cell signaling | 0.1 μg/ml | WB |
| PKC-α | #2056, Cell signaling | 0.1 μg/ml | WB |
| pPKCα/β | #9375, Cell signaling | 0.1 μg/ml | WB |
| Erk1/2 | #9102, Cell signaling | 0.1 μg/ml | WB |
| pErk1/2 | #9101, Cell signaling | 0.1 μg/ml | WB |
| Lamin A/C | #2032, Cell signaling | 1:3000 | WB |
| β-tubulin | T-5293, Sigma | 1:3000 | WB |

| | | | |
|---|---|---|---|
| pAkt | #9271, Cell signaling | 0.1 μg/ml | WB |
| Akt | #9272, Cell signaling | 0.1 μg/ml | WB |
| GAPDH | MAB374 | 1:3000 | WB |
| normal IgG | Sc-2027 L, Santa cruz | 6 μg/ml<br>1 μg/ml | Neutralization<br>IHC |
| CD31 | Ab28364, Abcam | Pre-diluted at 100 μg per .2 mg/ml | IHC |
| Ki67 | Clone: 30-9, Ventana | Pre-diluted into .1 ml delivery per sample | IHC |