Supplemental Experimental Procedures

Reagents

Vandetanib was kindly provided by AstraZeneca (Macclesfield, United Kingdom). Erlotinib and BV were both obtained from the institutional pharmacy. For in vivo studies, phosphate-buffered saline (PBS) was used as vehicle, erlotinib and vandetanib were dissolved in 1% Tween-80, and BV was dissolved in PBS. The following primary antibodies were used for immunohistochemistry and immunofluorescence: rabbit anti-EGF, rabbit anti-bFGF and rabbit anti-Bek (FGFR2) (C-17, all from Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phosphorylated VEGFR2 (Flk-1; PC460, Oncogene, Boston, MA), rabbit anti-phosphorylated EGFR (pEGFR; Tyr1173; Biosource, Camarillo, CA); rabbit anti-EGFR (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-desmin (Molecular Probes, Eugene) for double staining with rabbit anti-pEGFR, and rabbit anti-desmin (Abcam Inc., Cambridge, MA) for double staining with rat anti-CD31; and rat anti-mouse CD31 (BD PharMingen, San Diego, CA); rabbit anti–mouse F4/80 (MCAP497; Serotec, Raleigh, NC), and mouse anti-alpha-SMA (Clone 1A4, Dako, Carpinteria, CA). The goat anti-mouse IgG F(ab) fragment (Jackson ImmunoResearch Laboratories, West Grove, PA) was used in protein block (1:10 dilution) at 4 °C overnight before using any primary antibody made in mouse. The following secondary antibodies were used for colorimetric immunofluorescent analyses: peroxidase conjugated goat anti-rabbit IgG and Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories,

West Grove, PA), and Cy5-conjugated goat anti-rat IgG goat anti-rabbit Alexa 488 (A-11034) and goat anti-rat Alexa 594 (A-11007) (Molecular Probes).

Tumor Cell Lines and Culture Conditions

NIH-H1975 lung adenocarcinoma cells (L858R-T790M EGFR), A549 and H441 lung adenocarcinoma cells (EGFRWT, KRAS mutant) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). H1975 and H441 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine and 50 units/mL each of penicillin and streptomycin (all from Life Technologies, Grand Island, NY). A549 tumor cells were maintained in Dulbecco's minimum essential medium (DMEM) (Life Technologies) containing 10% FBS and antibiotic solution. Both A549 cells and H1975 cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air and determined to be free of mycoplasma and pathogenic murine viruses (assayed by Science Applications International Co., Frederick, MD).

Quantitative real-time PCR

Total RNA was prepared from frozen tumor tissue using RNeasy Mini kit (250) (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Human GAPDH was used as an endogenous control for human EGFR and human VEGF, and mouse Tubulin was used as an endogenous control for murine EGFR and murine VEGF. Lewis lung carcinoma (LLC) cells grown under

normoxic or hypoxic conditions were used as negative and positive controls for mouse VEGF, respectively. A549 cells cultured in normoxic or hypoxic conditions were used as a negative and positive control for human VEGF, respectively. Quantitative Real-time PCR (qRT-PCR) was performed using TaqMan one-step RT-PCR master mix kit and gene specific taqman gene expression assay kits (Applied Biosystems, Inc. Foster City, CA; Hs99999905_m1GAPDH, Mm00495804_1Tubb5, Hs00900054_m1VEGFa, Mm00437306_m1Vegfa, Hs0107609_m1EGFR, Mm01187858_m1Egfr, Hs00256527_m1FGFR2, and Mm00438541_m1) in a 7300 real-time PCR system (Applied Biosystems). Samples were prepared in duplicate, and 200ng mRNA was added to each PCR tube containing the reaction mixture. Real-time cycler conditions were programmed according to the recommended protocols as follows: 48 degrees for 30 min., 95 degrees for 10 min. followed by 45 cycles of 95 degrees for 15 sec. and 60 degrees for 1 min.

Immunohistochemistry

Paraffin-embedded tumors from mice were used for immunohistochemical examination of EGF and basic-FGF expression. The sections were deparaffinized in xylene, treated with a graded series of alcohol, and rehydrated in PBS at pH 7.5. Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS for 12 minutes. Slides were placed in a humidified chamber and incubated with protein blocking solution (5% normal horse serum and 1% normal goat serum in PBS) for 20 min at room temperature. Samples were incubated

overnight at 4°C with antibodies against EGF (1:100) or basic-FGF (1:100). Slides were washed three times with PBS and incubated with the appropriate peroxidase-conjugated secondary antibody (1:500) for 1 hour at room temperature. Slides were exposed to stable 3,3'-diaminobenzidine (Phoenix Biotechnologies, Huntsville, AL) and counterstained with Gill's hematoxylin (Sigma Chemical Co., St. Louis, MO). Sections were examined using a Nikon Microphot-FXA photomicroscope (Nikon Instruments, Inc., Melville, NY) equipped with a Leica DFC320 camera (Leica Microsystems, Inc., Bannockburn, IL). Five to ten representative images, depending on the tumor size, were captured using Photoshop software (Adobe Systems, Inc., Mountain View, CA).

Laser Scanning Cytometry

Laser scanning cytometry (LSC) was used to quantify immunofluorescence intensity of EGFR staining and TUNEL positive cells in control and BV-resistant tumors, as previously described (21). In brief, frozen sections were stained in a two-step procedure with EGFR as primary antibody and Cy5 as secondary antibody, or CD31 as primary antibody and TUNEL, using the same staining procedure as described above. Nuclei were labeled with a sytox orange dye (Molecular Probes). The slides were placed onto the computer-controlled motorized stage of an Olympus BX50 fluorescent microscope that was coupled to an argon and HeNe laser. Tumor regions that were devoid of normal and necrotic regions were identified and selected for analysis. Analytic gates were established based on the properties of control samples that were labeled with an

isotype-matched control primary antibody. For quantitative analysis of EGFR, the threshold contour was set to optimize single cell contours. Cell nuclei were contoured for cell counting purposes and the slides were scanned using x20 objective. The instrument was adjusted to count a total of 2000 cells in each sampels and the results for EGFR were represented by the ratio of EGFR+ cells to total number of sytox orange cells. The relocation feature was used to ensure that positively labeled cells were included in the appropriate gate.

Supplemental Table 1

UNIQUE ID	GENE SYMBOL	FOLD CHANGE	p-VALUE
ILMN_1225071	*Fgfr2*	6.04	9.46E-05
ILMN_2759371	*Fgfbp1*	5.98	8.80E-06
ILMN_2753623	*Epgn*	5.59	1.19E-05
ILMN_1238547	*Areg*	4.62	2.55E-05
ILMN_2972748	*Epha1*	4.19	9.09E-05
ILMN_2726837	*Nppb*	2.18	0.0005309
ILMN_2693922	*Egfr*	1.88	0.0013425
ILMN_2745480	*Fgf13*	1.77	0.0019072
ILMN_2946520	*Npy*	1.69	0.0003833
ILMN_1240323	*Dnajb1*	1.66	0.0033161
ILMN_2424721	*Pdgfa*	0.60	0.0008159
ILMN_2748966	*Tgfb3*	0.51	0.0032832
ILMN_2788593	*Nos3*	0.49	0.0009875
ILMN_2624938	*Pea15*	0.48	0.0006909
ILMN_1243212	*Sparc*	0.45	0.0012674
ILMN_2907655	*Bax*	0.45	2.47E-05
ILMN_2707967	*Man2b1*	0.39	0.000729
ILMN_2849449	*Itga5*	0.37	0.0004719
ILMN_2618714	*Pdgfb*	0.36	0.0002237
ILMN_2635229	*Thbs2*	0.35	1.07E-05
ILMN_2486573	*Vegfc*	0.32	0.0005823
ILMN_2903972	*Pdgfrb*	0.32	0.0040339
ILMN_2771550	*Cdh5*	0.29	0.0002098
ILMN_1215919	*Egfl7*	0.29	0.0006389
ILMN_1227926	*Flt1*	0.27	0.0002043
ILMN_1220697	*Notch4*	0.15	3.96E-05

Table S1. Stromal mouse angiogenic genes significantly modulated in H1975 BV-resistant xenografts compared with controls.
In H1975 BV-resistant xenografts 10 stromal genes related to angiogenesis were significantly upregulated, whereas 16 genes were downregulated compared with controls. Significant genes were determined using selection criteria of a $P < 0.005$ and a fold-change ≥1.5.

Supplemental Table 2

UNIQUE ID	GENE SYMBOL	FOLD CHANGE	p-VALUE
ILMN_1725139	*CA9*	4.54	5.60E-06
ILMN_2219767	*MYCN*	3.55	0.0020786
ILMN_1796094	*CD36*	2.39	0.0008565
ILMN_1776602	*RNASE4*	2.14	0.0031742
ILMN_2173451	*GPI*	1.73	0.0036727
ILMN_1802205	*RHOB*	0.61	0.0003186

Table S2. Tumor human angiogenic genes significantly modulated in H1975 BV-resistant xenografts compared with controls.
In H1975 BV-resistant xenografts 5 human genes related to angiogenesis were significantly upregulated, whereas 1 gene was found downregulated compared with controls. Significant genes were determined using selection criteria of a $P < 0.005$ and a fold-change ≥1.5.

Supplemental Figure 1

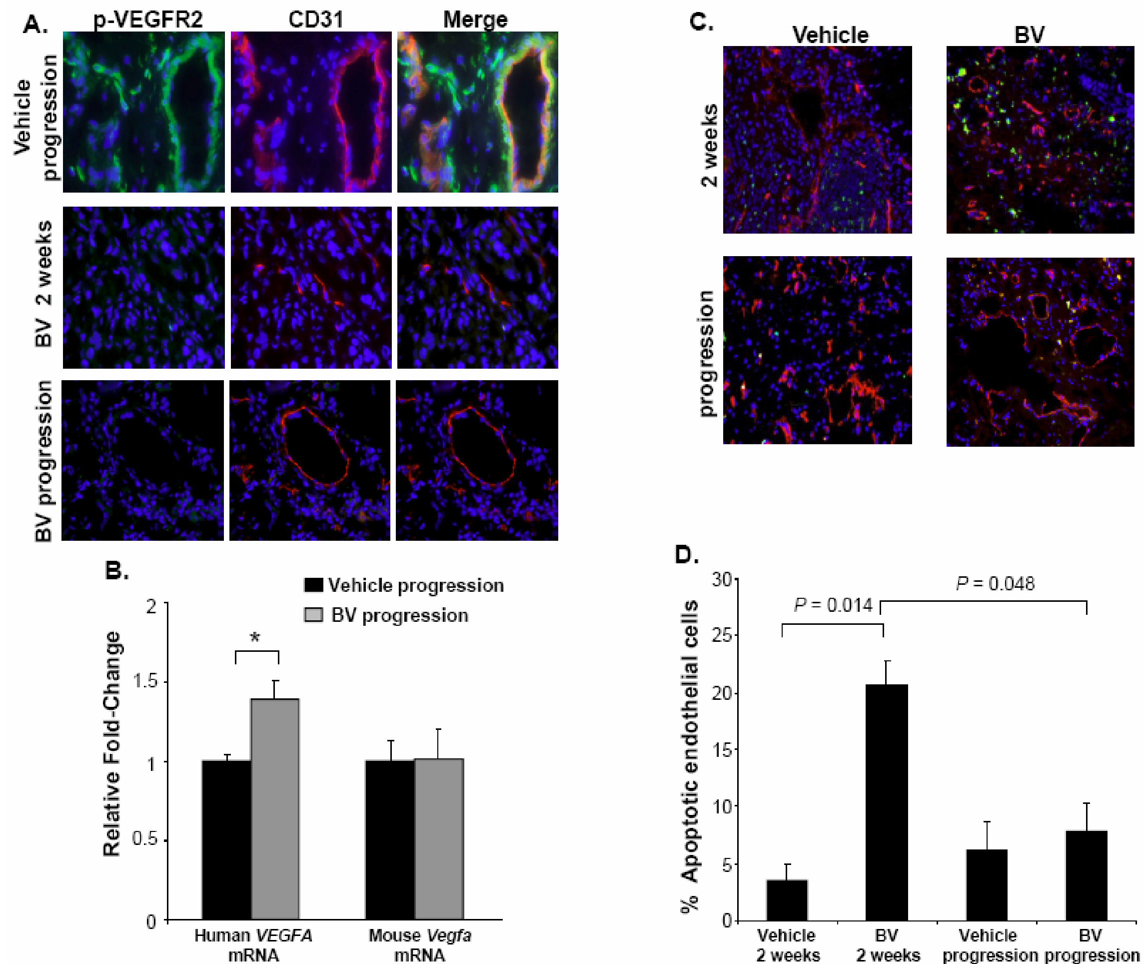

Figure S1. BV resistance is associated with sustained p-VEGFR2 inhibition and decreased endothelial cell apoptosis.

A. Representative IF microphotographs of H1975 xenografts treated with Vehicle and BV for 2 weeks and at progression showing CD31 (red), p-VEGFR-2 (green) and nuclei (blue) staining at original magnification 400×. Five random fields were collected from each of 4 specimens per group of treatment at each time point. ***B.*** Human *VEGFA* and mouse *Vegfa* mRNA expression in H1975 xenografts that progressed while receiving Vehicle and BV using qRT-PCR (N = 4/group). Human *GAPDH* and mouse *tubulin* were used as housekeeping controls for human and mouse mRNA, respectively. Data were normalized relative to mRNA levels in Vehicle-treated samples at progression and are graphed as relative fold change ± SEM, $^*P < 0.05$ (t-test). ***C.*** Representative IF images of CD31 (red) and TUNEL (green) staining in H1975 xenografts treated with Vehicle and BV after 2 weeks and at progression (200×). Five microphotographs were collected from each of 4 specimens per group. ***D.*** Five random microscopic fields (200×) were quantified as percentage of apoptotic (TUNEL+) endothelial cells (CD31+) in each of 3 specimens, each group. Data are graphed as percentage ± SEM; *P* values are calculated using t-test.

Supplemental Figure 2

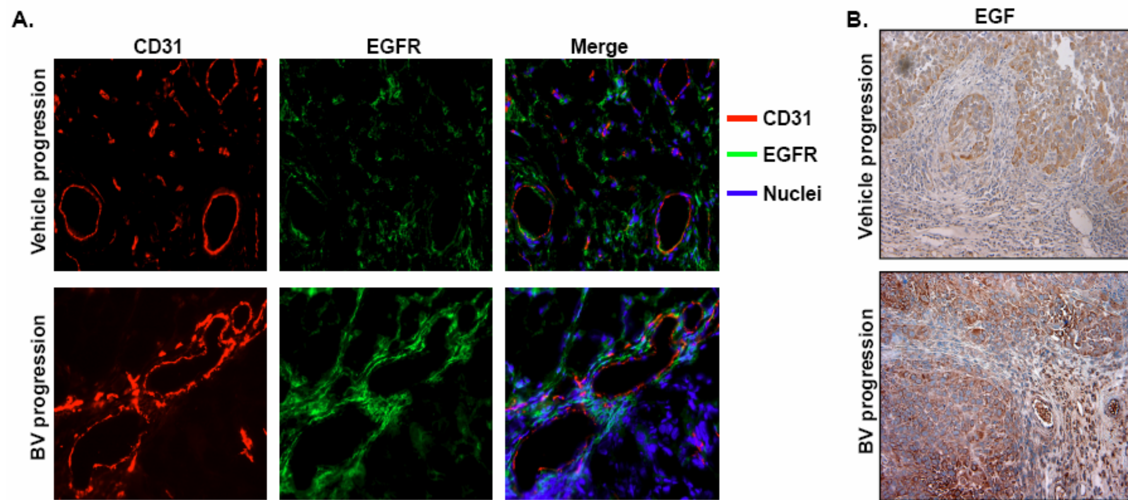

Figure S2. EGFR and EGF protein levels are increased in H1975 BV-resistant xenografts compared with controls.
A. Representative immunofluorescent staining images (200×) of CD31 (red), total EGFR (green) and nuclei (blue) in Vehicle and BV- resistant H1975 xenografts. ***B.*** Representative immunohistochemical images (100×) of EGF staining in Vehicle- and BV-resistant H1975 xenografts. Five microphotographs were collected from 4 stained tumor sections per treatment group.

Supplemental Figure 3

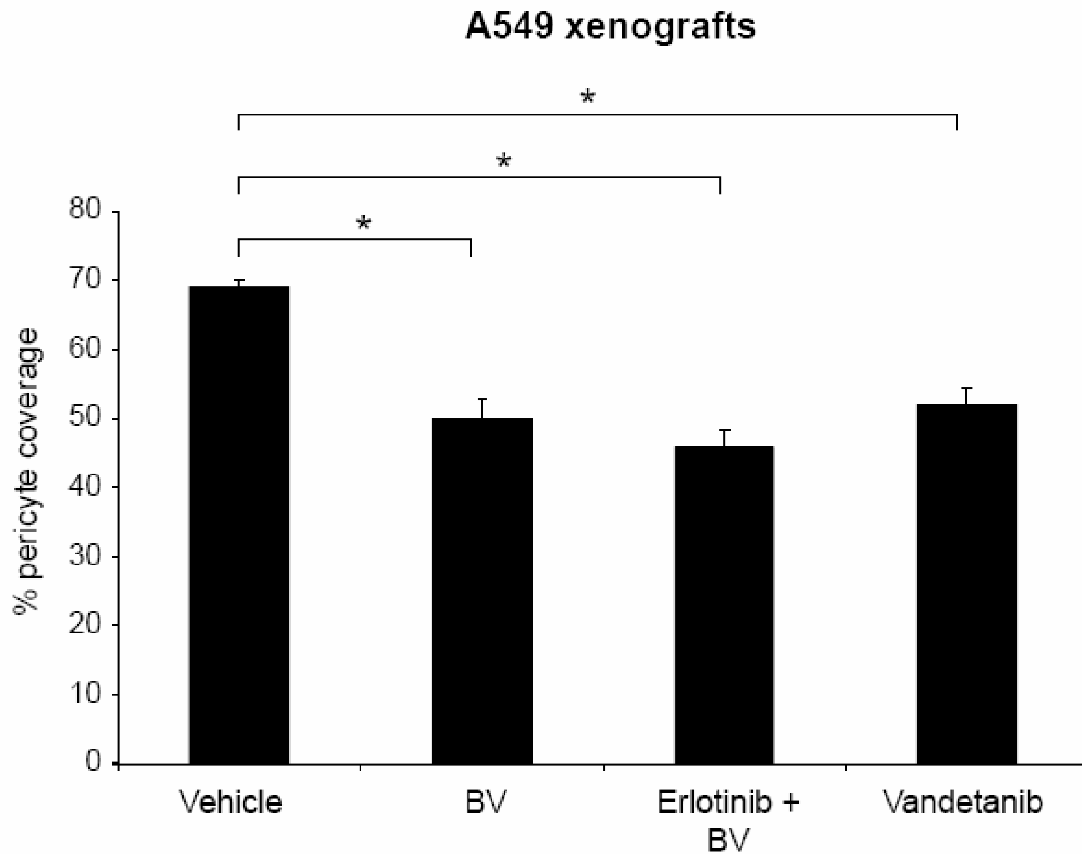

Figure S3. Pericyte coverage is reduced in A549 xenografts treated with VEGFR/EGFR inhibitors.

Pericyte coverage in A549 xenografts was quantified as described in Materials and Methods. A minimum of 5 microscopic fields (200×) in tumors that received long-term treatment with Vehicle (N = 4), BV (N = 4), Erlotinib + BV (N = 4) and Vandetanib (N = 3) were quantified. Data are graphed as percentage ± SEM, $*P < 0.01$ (t-test).

Supplemental Figure 4

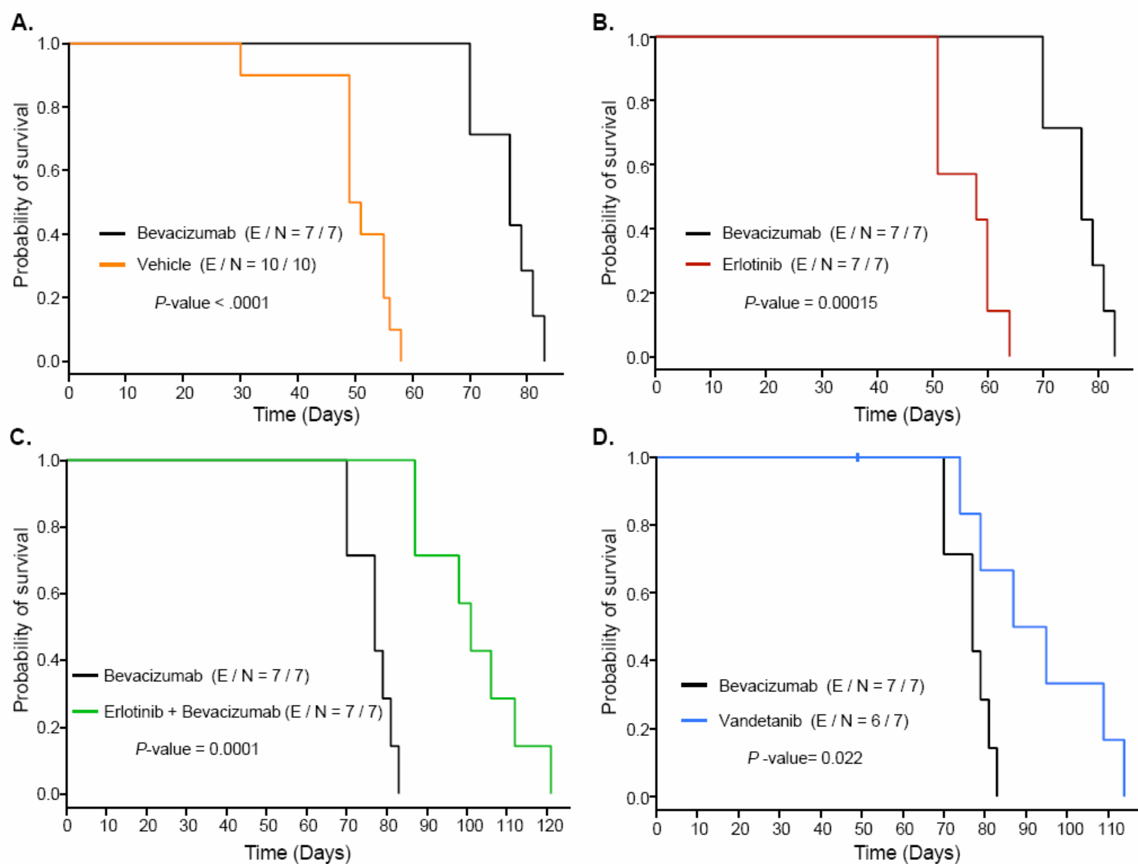

Figure S4. Dual EGFR/VEGFR inhibitors prolong survival and delay onset of resistance in H441 orthotopic tumors.

A, B. Kaplan-Meier plots show the increased survival in H441 tumors receiving BV compared with Vehicle (A) and Erlotinib (B). ***C, D.*** Kaplan-Meier plots show the increased survival of H441 tumors receiving the combined treatment with Erlotinib + BV compared with BV alone (C) and Vandetanib compared with BV alone (D). E = Events, N = Number of animals in a determined group of treatment. *P* values are calculated using the log-rank (Mantel-Cox) test.

Supplemental Figure 5

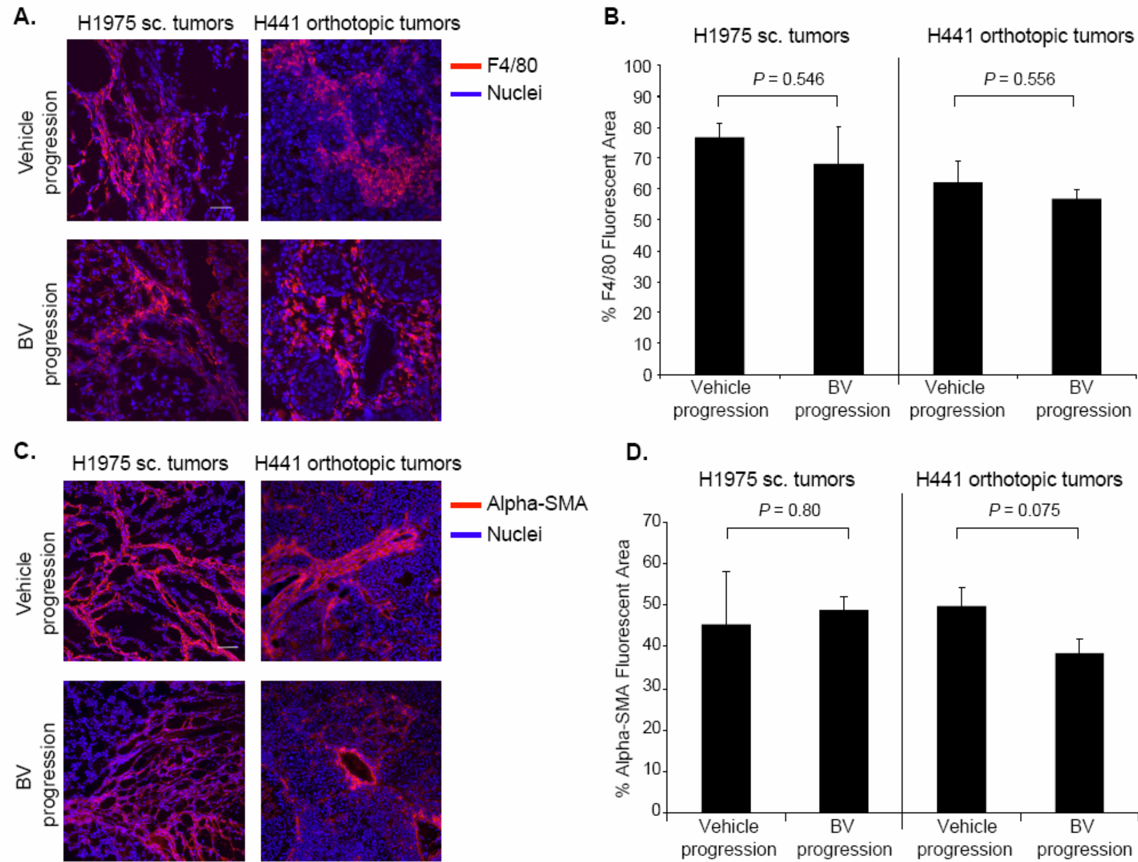

Figure S5. Characterization of macrophage infiltration and myofibroblast expression in H1975 and H441 NSCLC murine models of acquired resistance to VEGF blockade.

A. Representative IF images (200×) of F4/80 (red) and nuclei (blue) in Vehicle- and BV-resistant H1975 subcutaneous and H441 orthotopic tumors, using confocal microscopy. Scale bar: 50 µm. ***B.*** Quantification of F4/80 (red) fluorescent area in H1975 and H441 tumors that progressed while receiving either Vehicle or BV, using Alpha Innotech Software. Five random microphotographs (200×) of F4/80 (red) and nuclei (blue) fluorescence were collected in a minimum of 4 specimens per group. Data are shown as mean percentage ± SEM; *P* values are calculated using t-test ***C.*** Representative IF images (100×) of alpha-SMA (red) and nuclei (blue) in Vehicle- and BV-resistant H1975 and H441 tumors (confocal microscopy). Scale bar: 100 µm. ***D.*** Quantification of alpha-SMA (red) fluorescent area in H1975 subcutaneous and H441 orthotopic tumors that progressed while receiving either Vehicle or BV. Five random microphotographs (100×) were collected from 4 to 6 specimens per each group. Data are shown as mean percentage ± SEM; *P* values are calculated using t-test.