Supporting Information

Antagonistic VEGF variants engineered to simultaneously bind to and inhibit VEGFR2 and $\alpha_v\beta_3$ integrin

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SI Materials and Methods

Preparation of scVEGF constructs and libraries. PCR assembly with overlapping primers was used to prepare two inserts: one encoding for VEGF chain 1 (amino acids E13 to D109), and one encoding for a 14-amino acid linker (GSTSGSGKSSEGKG) and VEGF chain 2 (amino acids E13 to D109). Residue numbers used here correspond to the numbering scheme of wild-type $VEGF_{121}$. Amplification was performed using primers containing *Nhe*I and *Bam*HI restriction sites for chain 1, and *Bam*HI and *Mlu*I restriction sites for the 14-amino acid linker and chain 2. The two inserts were introduced into the yeast display vector pCT using a two-step cloning procedure. scVEGFmut was created in a similar manner and contained four amino acid mutations corresponding to key ligand binding residues at one pole of the molecule: chain 1 F17A, E64A; chain 2 I46A, I83A (Fig. 1*B* and Table S1). The construct for scVEGF m27I was obtained using custom gene synthesis (Biomatik Corporation). Libraries were prepared starting with the scVEGFmut construct. Full-length genes were created with varying numbers of NNS degenerate codons for substitution of loop 1 in chain 1, or loops 2 and 3 in chain 2, where N = A, C, T, or G and S = C or G. The pCT vector was digested with *Nhe*I/*Bam*HI (loop 1) or *Bam*HI/*Mlu*I (loops 2 or 3) restriction enzymes and electroporated along with each insert into EBY100 yeast cells as previously described (1). Libraries containing the RGD sequence and randomized flanking residues in scVEGFmut loop 3 were prepared analogously. Typical library sizes were 0.5-2 x 10^7 transformants, as estimated by dilution plating on selective media.

Screening of scVEGF loop libraries. Yeast-displayed scVEGF libraries were grown in selective media and induced for expression with galactose according to established protocols (1). Approximately 5-20 x 10^6 yeast (depending on sort round) were labeled with detergent-solubilized $\alpha_v\beta_3$ integrin (R&D Systems, octyl-glucopyranoside formulation) and a 1:200 dilution of chicken anti-cMyc antibody (Invitrogen) in integrin binding buffer (20 mM Tris pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $CaCl_2$, and 1 mg/mL bovine serum albumin (BSA)) for 2 h at room temperature. Cells were washed and resuspended in ice-cold PBSA (phosphate buffered saline + 1 mg/mL BSA) containing a 1:25 dilution of FITC-labeled mouse anti-α_v integrin (BioLegend) and a 1:100 dilution of phycoerythrin (PE)-conjugated anti-chicken-IgY (Santa Cruz Biotechnology). After 20 min on ice, yeast were washed in PBSA and sorted using a Becton Dickinson FACSVantage SE instrument (Stanford FACS Facility). In each sort, 1-2% of the yeast population was collected; after sort 1 the number of yeast analyzed was at least 10-fold excess of the remaining library diversity. To ensure retention of VEGFR2 binding, one intermediate sort was performed against VEGFR2-Fc (R&D Systems), using a 1:25 dilution of FITC-conjugated mouse anti-human Fc (Sigma) for detection of receptor binding. Concentrations of receptor used in each sort round were as follows: sort 1 – 200 nM $\alpha_v\beta_3$, sort 2 – 100 nM $\alpha_v\beta_3$, sort 3 – 100 nM $\alpha_v\beta_3$, sort 4 – 100 nM VEGFR2-Fc, sort 5 – 50 nM $\alpha_v\beta_3$, sort 6 – 50 nM $\alpha_v\beta_3$, sort 7 – 25 nM $\alpha_v\beta_3$. Plasmid DNA was extracted from yeast clones using a Zymoprep kit (Zymo Research) and transformed into XL-1 blue supercompetent *E. coli* cells (Stratagene) for plasmid miniprep. DNA sequencing of resulting clones was performed by MCLab (South San Francisco, CA).

Soluble production and purification of scVEGF proteins. Soluble protein production was performed using the Multi-Copy *Pichia* Expression Kit (Invitrogen K1750-01) as previously described (2). The open reading frames of scVEGF proteins were cloned into the pPIC9K vector for expression in the *P. pastoris* strain GS115. Proteins were prepared with an N-terminal FLAG

epitope tag and a C-terminal hexahistidine tag as handles for cell binding studies and protein purification, respectively. scVEGF proteins were purified from yeast culture supernatant by metal chelating chromatography using Ni-NTA agarose beads (Invitrogen). Eluted protein fractions were concentrated and buffer exchanged to phosphate buffered saline (PBS), pH 7.4, and ~4 mg was treated with Endo H_f (1,000 Units, New England Biolabs Inc.) overnight at 37 °C to remove N-linked glycosylation. Gel filtration chromatography was performed using a Superdex 75 column (GE Healthcare) equilibrated with PBS, pH 7.4 at a flow rate of 0.4 ml/min on a Varian Prostar instrument. Proteins were analyzed by SDS-PAGE (NuPAGE 4-12% Bis-Tris, Invitrogen) under non-reducing conditions. Protein concentrations were determined by Bradford protein assay and UV-Vis absorbance at 280 nm (ε_{278}= 13616 $M^{-1}cm^{-1}$ for scVEGFwt, scVEGFmut, and scVEGF m27I and 7H, and ε_{278}= 12216 $M^{-1}cm^{-1}$ for scVEGF 7I and 7P). Purification yields for all proteins were 1-10 mg per one-liter flask culture. Proteins were filter-sterilized before use in cell assays and mouse models and were stored at 4 °C.

Far-UV circular dichroism spectroscopy. Circular dichroism (CD) spectra were recorded on a Jasco J-815 spectropolarimeter over a range of 190–250 nm in PBS, pH 7.4 using a quartz cuvette with a pathlength of 10 mm. Protein spectra were collected with a scanning speed of 50 nm/min and a data interval of 1 nm. Three scans of 10 μM protein solutions were averaged to obtain smooth data. All spectra were background corrected with respect to PBS and converted to units of mean residue ellipticity.

Direct cell binding assays. K562 cells transfected with individual α and β integrin subunits were grown as described (3). PAE/KDR cells were grown in F-12 (Ham's) Nutrient Media (Gibco) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HUVECs (ATCC) were grown in full EGM-2 media (Lonza) containing 2% FBS and growth factor supplements. SVR cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco) with 10% FBS and 1% penicillin/streptomycin. For binding assays, 10^5 cells were suspended in integrin binding buffer. Varying concentrations of scVEGF proteins were added and cells were incubated at 4 °C for 4-6 h with gentle agitation. Cells were centrifuged at 1000 rpm (0.1 rcf) at 4 °C for 3 min and resuspended in 20 μL PBSA containing a 1:50 dilution of FITC-conjugated anti-His antibody (Bethyl) for K562 cells, or a 1:50 dilution of PE-conjugated anti-FLAG antibody (Prozyme) for other cell lines. After 20 minutes on ice, cells were washed in PBSA and analyzed by flow cytometry with a BD FACSCalibur instrument (Becton Dickinson). Mean fluorescence values were generated using FlowJo software (Treestar, Inc) and plotted versus log concentration. Data were fit to sigmoidal curves and apparent equilibrium dissociation constants were determined using KaleidaGraph (Synergy Software). Data points for scVEGFwt, where a decrease in signal was observed at high concentrations, were not included in apparent K_D calculations. Error is reported as the standard deviation of triplicate measurements.

Competition cell binding assays. For competition binding assays, 10^5 SVR cells were suspended in integrin binding buffer in volumes that were sufficient to avoid ligand depletion. A VEGFR2 inhibitor (scVEGF-mA) or integrin inhibitor c(RGDfV) (Peptides International) were added at concentrations of 10 μM, followed by the addition of 100 nM Alexa488 labeled scVEGF proteins. scVEGF-mA is an affinity-matured version of the scVEGFmut antagonist, while c(RGDfV) is a backbone-cyclized pentapeptide that contains an RGD integrin-binding motif. Cells were incubated at 4 °C for 3 h with gentle agitation to prevent settling, washed with

1 ml PBSA, and analyzed by flow cytometry. To further probe dual receptor binding, a mixture of soluble human VEGFR2 (500 nM, Calbiochem EMD Chemicals) and scVEGF proteins (20 or 200 nM) were added to 10^5 K562-$\alpha_v\beta_3$ cells and incubated at 4 °C for 2 h. Cells were washed in PBSA and labeled with a 1:25 dilution of fluorescein-conjugated anti-human VEGFR2/KDR (R&D Systems) for 20 min at 4 °C. Cells were analyzed by flow cytometry as above. Error bars represent the standard deviation of experiments performed in triplicate.

Surface plasmon resonance experiments.

(i) Kinetic binding experiments with immobilized VEGFR2. The binding interactions of VEGFR2 to $VEGF_{121}$ (R&D Systems), scVEGFwt, scVEGFmut, and scVEGF mutants 7H, 7I, and 7P were analyzed in real-time by surface plasmon resonance using a Biacore 3000 instrument (Biacore Life Sciences, Stanford PAN facility). Recombinant human VEGFR2 extracellular domain (rhVEGFR2) (Calbiochem EMD Chemicals) was immobilized on a Biacore CM5 sensor chip by amine coupling according to the following protocol. The CM5 dextran matrix was activated using a 1:1 solution of EDC (0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in water) and NHS (0.1 M N-hydroxysuccinimide in water). Next, rhVEGFR2 (30 μg/mL in 10 mM sodium acetate pH 5.5) was flowed over the activated surfaces of flow cells two and four at a flow rate of 5 μL/min until the target immobilization levels (500 and 2,000 RU for flow cells two and four, respectively) were reached. These two different levels of VEGFR2 immobilization were selected to provide versatility of chip use while maintaining the maximum binding capacity within the range appropriate for kinetic analysis. Uncoupled receptor was removed and unreacted moieties on the dextran matrix were blocked with 1 M ethanolamine-HCl pH 8.5. Flow cells one and three were activated and blocked without exposure to rhVEGFR2 and served as background control surfaces for the binding interactions.

All kinetic binding experiments were performed at 25 ^oC in degassed PBS running buffer (phosphate buffered saline, pH 7.4, containing 0.1 mg/mL BSA and 0.005% Surfactant P20). A range of concentrations (two-fold dilutions from 1.6 μM to 1.6 nM for scVEGF mutants 7H, 7I, and 7P, and from 200 nM to 0.2 nM for $VEGF_{121}$, scVEGFwt and scVEGFmut) of the protein analytes were flowed over the surface-immobilized rhVEGFR2 at 50 μL/min for 3 min and the binding interaction was monitored. Following association, the dissociation of the various ligand-receptor complexes was monitored for 6 min. Finally, the rhVEGFR2 surfaces were regenerated with two sequential pulses (30 sec and 15 sec) of 10 mM NaOH at an increased flow rate of 100 μL/min and the sensorchip surfaces were allowed to equilibrate in PBS running buffer for 5 min at a flow rate of 50 μL/min. Kinetic binding experiments were performed in triplicate over all flow cells of the rhVEGFR2 immobilized sensorchip. Final sensorgrams were generated by first subtracting the background response of flow cells one and three from the binding response generated against immobilized rhVEGFR2 in flow cells two and four, respectively, and next subtracting the bulk refractive index shift of cycles of PBS running buffer alone from cycles containing protein analyte.

Mass transfer control experiments were performed for each protein analyte at a single concentration (200 nM for scVEGF mutants 7H, 7I, and 7P, and 50 nM for scVEGFwt, scVEGFmut, and $VEGF_{121}$) following the experimental sequence outlined above, but with association phase flow rates of 5, 15, and 75 μL/min. Linked reaction control experiments were performed for each protein analyte at the highest concentration of the kinetic series tested (1.6 μM for scVEGF mutants 7H, 7I, and 7P and 200 nM for scVEGFwt, scVEGFmut, and $VEGF_{121}$) according to the protocol above, but at an association flow rate of 20 μL/min and with

association phases of 1, 3, and 9 min. Mass transfer control experiments showed no differences in binding kinetics with changes in flow rate, confirming the absence of mass transport limitations under the experimental conditions used. Additionally, linked reaction control experiments showed that ligand dissociation rates were independent of the length of the association phase, indicating a lack of kinetic heterogeneity.

Representative data sets for each ligand are shown. Sensorgram binding data was analyzed with BIAevaluation Software version 4.1 using global fitting procedures and a 1:1 Langmuir binding model to simultaneously calculate the equilibrium binding affinities and the on- and off-rates of the interactions of rhVEGFR2 with $VEGF_{121}$, scVEGFwt, scVEGFmut, or scVEGF mutants 7H, 7I, or 7P (Table 1). In addition to fitting the binding interactions with a 1:1 Langmuir model, we also explored the use of other models including a bivalent analyte model. We found that the 1:1 Langmuir model gave the best fit for all ligand-analyte interactions tested. The 1:1 binding interaction was expected for the scVEGFmut and scVEGF mutants 7H, 7I, and 7P because their second binding interfaces have been modified to prevent binding to more than one receptor. For $VEGF_{121}$ and scVEGFwt, the observed 1:1 binding interaction is likely an artifact of the experimental design. Because the VEGFR2 was immobilized on the sensorchip surface at low densities to allow for determination of kinetic binding parameters, the distance between immobilized receptors likely precluded bivalent binding of the protein analyte.

(ii) Dual receptor binding experiments with immobilized VEGFR2. A second CM5 sensorchip surface with higher density of immobilized rhVEGFR2 was created following the amine coupling protocol above, but using 50 μg/mL rhVEGFR2 in 10 mM sodium acetate pH 5.5 and with a target immobilization level of 10,000 RU. As before, a paired flow cell without rhVEGFR2 was created as a background control surface. Dual receptor binding experiments were performed at 25 ^{o}C in degassed PBS running buffer or integrin binding (IB) running buffer (20 mM Tris pH 7.5 with 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $CaCl_2$, 100 mM NaCl containing 0.1 mg/mL BSA and 0.005% Surfactant). A single concentration of scVEGFmut or the scVEGF mutants 7H, 7I, and 7P (400 nM in PBS running buffer) was flowed over the immobilized VEGFR2 at a flow rate of 20 μL/min for 2 min. Immediately following this first association phase, soluble $\alpha_v\beta_3$ integrin extracellular domain (200 nM in IB running buffer) was flowed over the surface for 1 min. The dissociation of the complex in PBS running buffer was monitored for 5 min. Complete regeneration of the immobilized rhVEGFR2 surface was achieved at a flow rate of 100 μL/min using two 30 sec injections of 50 mM EDTA with 1 M NaCl to chelate residual ions from the IB running buffer and wash them out of the dextran matrix followed by two 30 sec injections of 10 mM NaOH. Injections of PBS running buffer followed by $\alpha_v\beta_3$ integrin in IB running buffer, or injections of scVEGF proteins in PBS running buffer followed by IB running buffer served as negative controls. Final sensorgrams were obtained by first subtracting the background response from the flow cell without immobilized receptor from the binding response generated against immobilized rhVEGFR2, and then subtracting the bulk refractive index shifts of injections of PBS running buffer and IB running buffer from injections in like buffer containing protein analyte.

(iii) Dual receptor binding experiments with immobilized $\alpha_v\beta_3$ integrin. Three identical Biacore CM5 sensorchip flow cells were prepared with immobilized $\alpha_v\beta_3$ integrin extracellular domain (R&D Systems); 30 μg/mL $\alpha_v\beta_3$ integrin in 10 mM sodium acetate, pH 5.0 was immobilized on the flow cell surface by amine coupling with target immobilization levels of

4,000 RU. Again, a paired flow cell without immobilized receptor was generated for each of the three immobilized surfaces and served as background control surfaces. As with the dual binding assays using rhVEGFR2 immobilized surfaces, experiments were performed at 25 ^{o}C in degassed PBS running buffer or IB running buffer. A single concentration of scVEGFmut or scVEGF mutants 7H, 7I, and 7P (400 nM in IB running buffer) was flowed over the integrin immobilized surface at a flow rate of 20 µL/min for 2 min. Following a short dissociation period of approximately 20 sec, soluble rhVEGFR2 (400 nM in PBS running buffer) was flowed over the surface for 1 min. The dissociation of the complex in IB running buffer was monitored for 5 min. No suitable regeneration conditions were found for the surface with immobilized $\alpha_v\beta_3$ integrin; therefore, a separate flow cell was used to test the binding of each scVEGF protein. Injections of IB running buffer followed by rhVEGFR2 in PBS running buffer served as a negative control. Final sensorgrams were generated as described above.

VEGFR2 phosphorylation assays. Assays were performed as previously described with minor modifications (4). Subconfluent HUVECs were cultured in growth factor and serum-depleted EBM-2 medium for 20 h at 37 °C/5% CO_2 prior to experimentation. Alternatively, wells were coated with vitronectin as described below, and rinsed twice with Dulbecco's PBS (DPBS, Gibco) before cell plating. After pretreatment with 1 mM sodium orthovanadate (Na_3VO_4, Sigma) for 20 min, cells were co-incubated with 1 nM of $VEGF_{121}$ and different concentrations of scVEGF proteins for 10 min at 37 °C. Cells were then washed in PBS with 1 mM Na_3VO_4 and lysed for 2 h in ice-cold 1% Triton X-100 lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1% TritonX-100, 1× APC, 1× AEBSF (R&D Systems), 1 mM Na_3VO_4, and 1x complete protease inhibitor tablet (Roche)). The lysates were clarified by centrifugation (13,000 rpm for 10 min at 4 °C). Protein concentration was measured by BCA assay (ThermoFisher Scientific) and equivalent amounts of each lysate sample was analyzed by 4-12% SDS-PAGE and transferred to nitrocellulose (Invitrogen). Blots were blocked (5% milk, 20 mm Tris-HCl pH 7.4, 150 mm NaCl, 0.3% Tween 20) for 2 h at RT and probed with a phospho-specific rabbit polyclonal antibody (1:1000 dilution; Y951-VEGFR2, Abcam) overnight at 4 °C. Immunoreactive bands were visualized using a HRP-conjugated anti-rabbit secondary antibody (1:2000 dilution; Santa Cruz Biotechnology) and chemiluminescence (ECL plus, Amersham), and were quantified on a BioRad Chemidoc instrument. Blots were stripped and re-probed with a rabbit polyclonal VEGFR2 antibody (Abcam) to determine total amounts of VEGFR2 present. Unstimulated cells were used as a negative control. Cells stimulated with 1 nM $VEGF_{121}$ or 1 nM scVEGFwt were used as positive controls. The intensities of the phospho-VEGFR2 bands were adjusted for total VEGFR2 expression to account for differences in gel loading, and were normalized against the 1 nM $VEGF_{121}$-stimulated positive control. Data are presented as average values and error bars represent the standard deviation of measurements performed in triplicate.

Cell proliferation assays. Cell proliferation was assayed using an established protocol (5). Briefly, $4x10^3$ HUVECs were added to each well of a microtiter plate in EBM-2 media containing serum and growth factors, incubated overnight, then exchanged to growth factor and serum-free EBM-2 medium for 20 h at 37 °C/5% CO_2. Alternatively, wells were coated with vitronectin as described below, and rinsed twice with DPBS before cell plating. Cells were incubated with varying concentrations of scVEGF proteins and 1 nM $VEGF_{121}$ for 48 h at 37 °C/5% CO_2. For the last 24 h of incubation, 1 µCi (20 Ci/mmol) [^{3}H]thymidine (MP Biomedicals) was added to each well in 50 µl of EBM-2 media. Plates were then frozen at -80

°C, and after thawing at room temperature, [^{3}H]thymidine incorporation was measured by harvesting the cells onto glass fiber filtermats using a Mach IIIM harvester 96 (Tomtec) and performing scintillation counting with a Wallac MicroBeta instrument (Perkin Elmer). Unstimulated and $VEGF_{121}$-stimulated cells were used as negative and positive controls, respectively. Data was normalized against the 1 nM $VEGF_{121}$-stimulated positive control. Error bars represent the standard deviation of measurements performed in triplicate.

Cell adhesion assays. Inhibition of HUVEC adhesion to vitronectin was performed as described with slight modifications (2). Microtiter plates were coated with 0.2 μg/cm^2 human vitronectin (Promega) for 2 hrs at 37 °C in DPBS. Wells were blocked with 2 mg/ml BSA for 1 hr at room temperature. Varying concentrations of scVEGF proteins and 5 x 10^4 HUVECs were added to vitronectin-coated wells, incubated at 37 °C/5% CO_2 for 2 h, and washed two times with PBS. A solution of 0.2% crystal violet in 10% ethanol was added to the wells for 10 min, and then washed three times with PBS. Solubilization buffer (a 1:1 mixture of 0.1 M NaH_2PO_4 and ethanol) was added and the plate was gently rocked for 15 min. Absorbance was measured at 600 nm using a microtiter plate reader (BioTek Instruments), and data were background subtracted with a negative control containing no cells. IC_{50} values were generated by fitting a sigmoidal curve to plots of log concentration protein versus percent adhesion. Data was normalized using samples containing no competing scVEGF proteins. Data are presented as average values and error bars represent standard deviations of measurements performed in triplicate.

Matrigel endothelial cell tube formation assay. Assays were carried out as previously described with minor modifications (6). Briefly, Matrigel (12.5 mg/ml; BD Biosciences) was thawed overnight at 4 °C, and 50 μl was added to each well of a microtiter plate and allowed to solidify for 10 min at 37 °C. Wells were incubated with 2 × 10^4 HUVECs with 10 nM $VEGF_{121}$ and varying concentrations of scVEGF proteins for 20 h at 37 °C/5% CO_2. After incubation, cells were labeled with 2 μm calcein-AM for 30 min at 37 °C in the dark. Cells were washed in PBS and capillary tube formation was observed using an ImageXpress 5000A epiflourescent microscope (Molecular Devices). Images were taken with a 4X S Fluor objective using S470/30X excitation, S510/30 emission with 150 msec exposure. Four individual optical sections were collected (with a Z-section that was 100 μm apart) and the average was calculated. Thread length was quantified by analysis of digitized images using ImageJ software to determine the extent of capillary-like structures. Data was normalized against a 10 nM $VEGF_{121}$-stimulated positive control. Error bars represent the standard deviation of measurements performed in triplicate.

***In vivo* Matrigel angiogenesis assay.** Animal procedures were performed according to a protocol approved by the Stanford University Administrative Panel on Laboratory Animal Care (APLAC). Anesthetized C3H/HeN mice (n=3) were injected subcutaneously with a liquid Matrigel-PBS mixture (650 μl/injection) as previously described (7). Matrigel contained 20 nM human $VEGF_{165}$ (R&D Systems) and 42 Units of heparin (Hospira) to stimulate angiogenesis, and scVEGFmut, scVEGF m27I, or scVEGF 7I at a final concentration of 20 nM or 200 nM. Matrigel plus PBS only or human $VEGF_{165}$/heparin served as negative and positive controls, respectively. After 10 days, Matrigel plugs were removed and photographed. For histological evaluation, excised Matrigel plugs from controls or samples treated with 200 nM scVEGF

proteins were fixed in 4% buffered formaldehyde, embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin (H&E). Hemoglobin content in Matrigel plugs was quantified as using an established method (8). Briefly, dissected Matrigel plugs in PBS were treated with a Polytron homogenizer. Matrigel homogenate (15 μl) was added to 135 μl of 90% glacial acetic acid and incubated for 20 min. Samples were clarified (5 min at ~5000 × g) and 10 μl of supernatant was added to 140 μl of 5 mg/ml 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) in 90% glacial acetic acid in a microtiter plate. Hydrogen peroxide (150 μl of 0.3%, Sigma) was added to each well and the absorbance at 600 nm was measured. Relative hemoglobin content was calculated based on a standard curve (Sigma), and was normalized based on the weight of the Matrigel plug. To calculate % hemoglobin, data was normalized against samples containing 20 nM human $VEGF_{165}$/heparin. Error bars represent the standard deviation of measurements performed on at least three Matrigel plugs.

Histology and immunofluorescent staining. Immunofluorescent staining was performed as previously described (9, 10). Briefly, paraffin-embedded 5-μm Matrigel sections were blocked with 5% normal goat serum (Abcam) overnight at 4 °C and stained with phycoerythrin (PE) labeled rat-anti–msCD34 monoclonal antibody (Abcam; 1:100 dilution) against blood vessels, rabbit anti-mouse VEGFR2 monoclonal antibody (Cell Signaling; 1:50 dilution), or rabbit anti-mouse β_3 integrin polyclonal antibody (Abcam; 1:50 dilution). Sections were visualized with Alexa488 labeled anti-rabbit antibody (Invitrogen; 1:5000 dilution) and DAPI (Fisher Scientific), or rabbit HRP-polymer followed by incubation with 3,3'-diaminobenzidine peroxidase substrate (Vector) and hematoxylene (Fisher Scientific).

Table S1. Amino acid sequences of: (A) scVEGFmut scaffold, (B) Libraries used to evaluate scVEGFmut loop tolerance, and (C) Engineered dual-specific scVEGF mutants.

(A) Sequence of scVEGFmut:

EVVK**A**MDVYQRSYCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGGCCND**A**GLECVPTEESNIT
MQIMRIKPHQGQHIGEMSFLQHNKCECRPKKDGSTSGSGKSSEGKGEVVKFMDVYQRSYCHPIE
TLVDIFQEYPDEIEY**A**FKPSCVPLMRCGGCCNDEGLECVPTEESNITMQIMR**A**KPHQGQHIGEMS
FLQHNKCECRPKKD

VEGF chain 1: dark blue
VEGF chain 2: cyan
Loop 1: purple
Loop 2: orange
Loop 3: green
Black: flexible linker

Underlined, bold residues are point mutations introduced into scVEGFmut (chain 1: F17A, E64A; chain 2: I46A, I83A).

Note: The scVEGF proteins used in this study have truncations at the N- and C-terminus relative to $VEGF_{121}$, and start at amino acid 13 and end at amino acid 109. However, for consistency with the literature we have used the $VEGF_{121}$ numbering scheme.

(B) Libraries used to evaluate scVEGFmut loop tolerance:

Loop 1A: ΔNDAGLE (Replace with loop sizes 6, 7, 8, 9)
Loop 1B: ΔNDAGL (Replace with loop sizes 6, 7, 8, 9)

Loop 2A: ΔYPDEIEYA (Replace with loop sizes 7, 8, 9)
Loop 2B: ΔYPDEIEY (Replace with loop sizes 7, 8, 9)
Loop 2C: ΔPDEIEYA (Replace with loop sizes 7, 8, 9)
Loop 2D: ΔPDEIEY (Replace with loop sizes 7, 8, 9)
Loop 2E: ΔDEIEYA (Replace with loop sizes 7, 8, 9)
Loop 2F: ΔDEIEY (Replace with loop sizes 7, 8, 9)

Loop 3A: ΔIKPHQGQ (Replace with loop sizes 7, 8, 9)
Loop 3B: ΔIKPHQG (Replace with loop sizes 7, 8, 9)

(C) Loop 3 sequences of scVEGF mutants isolated from sort round 7:

7I: PSVRRGDSPAS
7K: PTTRGDCPD
7H: PGGRGDSAY
7B: PHDRGDAGV
7F: STDRGDASA
7G: ASGRGDGGV
7P: PASRGDSPP

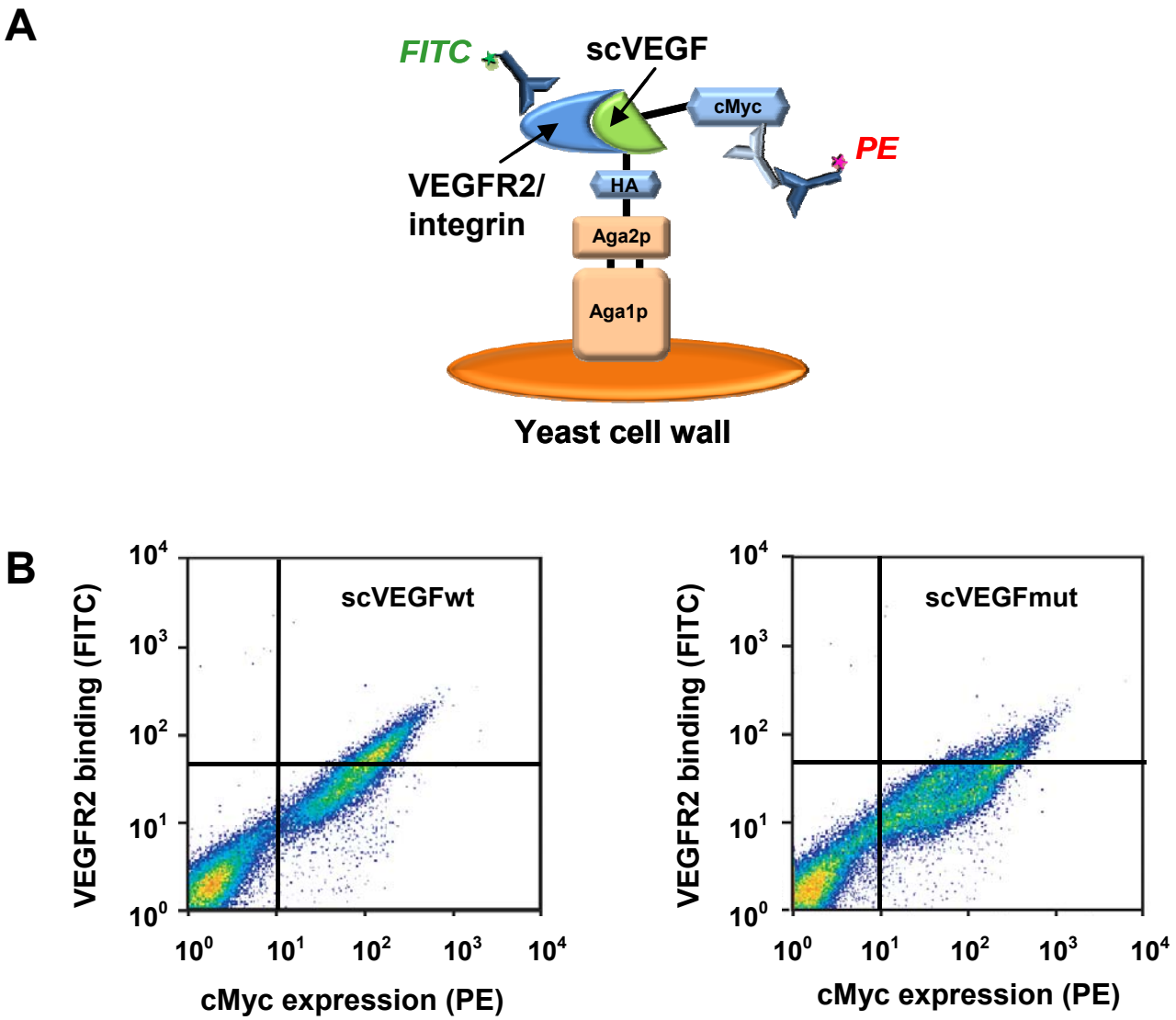

Fig. S1. (A) Yeast surface display construct. scVEGF is tethered to the cell surface as a fusion to yeast agglutinin proteins. Display levels were quantified using a primary antibody against the C-terminal cMyc tag and a phycoerythrin (PE)-labeled secondary antibody. VEGFR2 or $\alpha_v\beta_3$ integrin binding were measured using a fluorescent antibody. Two-color flow cytometry allowed for normalization of display levels with binding to isolate scVEGF mutants with the highest receptor binding affinity. (B) Flow cytometry density dot plots of yeast cell surface expression levels and VEGFR2 binding of scVEGFwt and scVEGFmut. Yeast-displayed proteins were labeled with 25 nM VEGFR2 overnight at room temperature, and a 1:250 dilution of chicken anti-cMyc antibody for 45 min on ice, followed by a 1:25 dilution of fluorescein (FITC)-labeled mouse anti-VEGFR2 and a 1:100 dilution of PE-conjugated anti-chicken-IgY. After 20 min on ice, yeast were washed and analyzed by flow cytometry. While both constructs were well expressed on the yeast cell surface, scVEGFwt exhibited higher levels of binding to 25 nM VEGFR2 compared to scVEGFmut. Quadrant markers are included for comparison purposes.

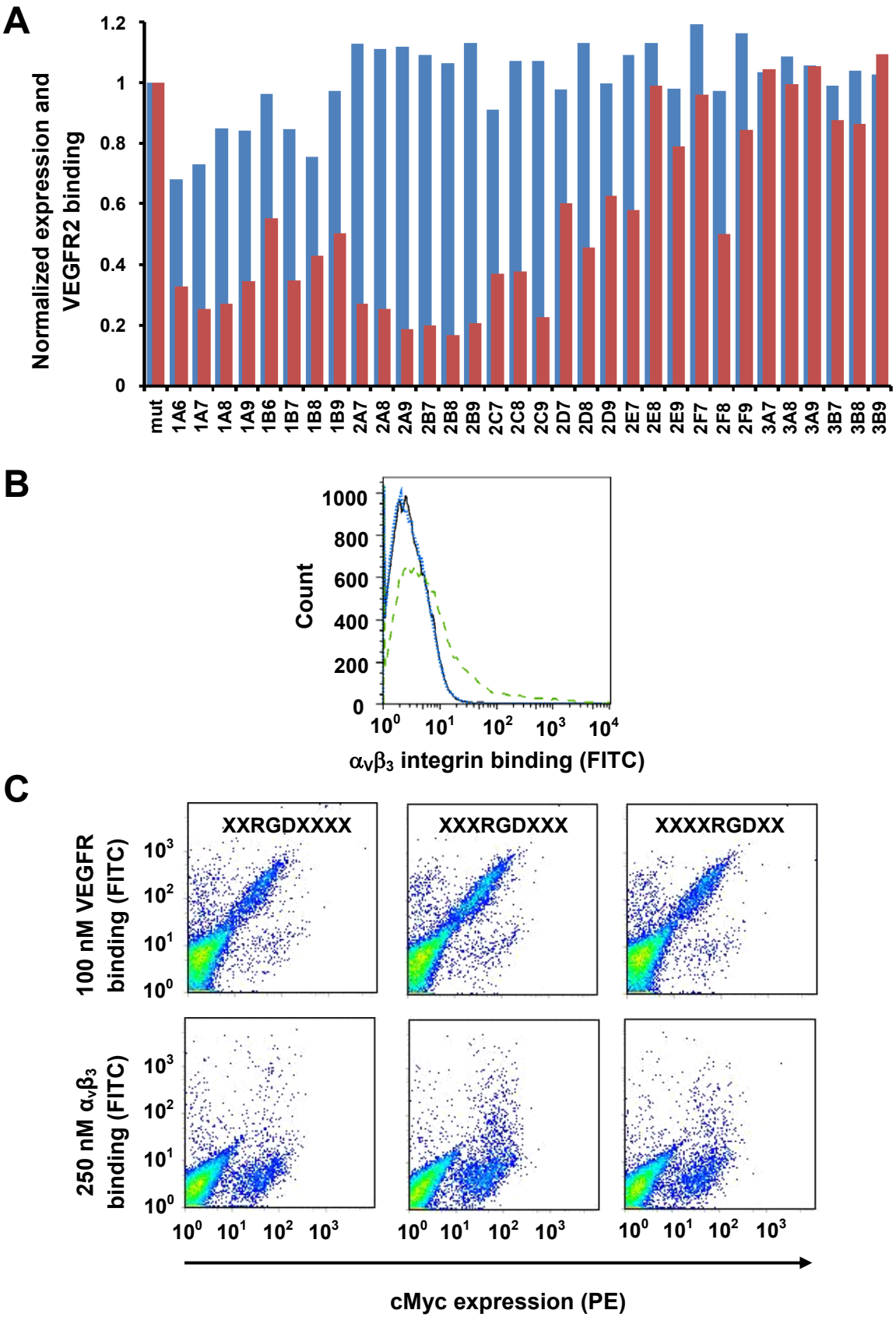

Fig. S2. (A) Thirty-two different loop libraries (Table S1) were tested for yeast cell surface expression levels and binding to VEGFR2, and normalized to scVEGFmut (mut). Blue bars: cMyc expression; Red bars: VEGFR2 binding. (B) Flow cytometry histograms of $\alpha_v\beta_3$ integrin binding to a variant of scVEGFmut, in which a fibronectin-derived sequence (TGRGDSPAS) was substituted in place of loop 3. Integrin binding was detected using a 1:25 dilution of fluorescein (FITC)-labeled mouse anti-α_v integrin antibody. Binding to 500 nM (green dashed line) or 100 nM (blue dotted line) $\alpha_v\beta_3$ integrin, or cells labeled with FITC-α_v antibody alone (black solid line). (C) Individual RGD loop libraries were tested for yeast cell surface expression (cMyc; x-axis) and binding to VEGFR2 or $\alpha_v\beta_3$ integrin (y-axis).

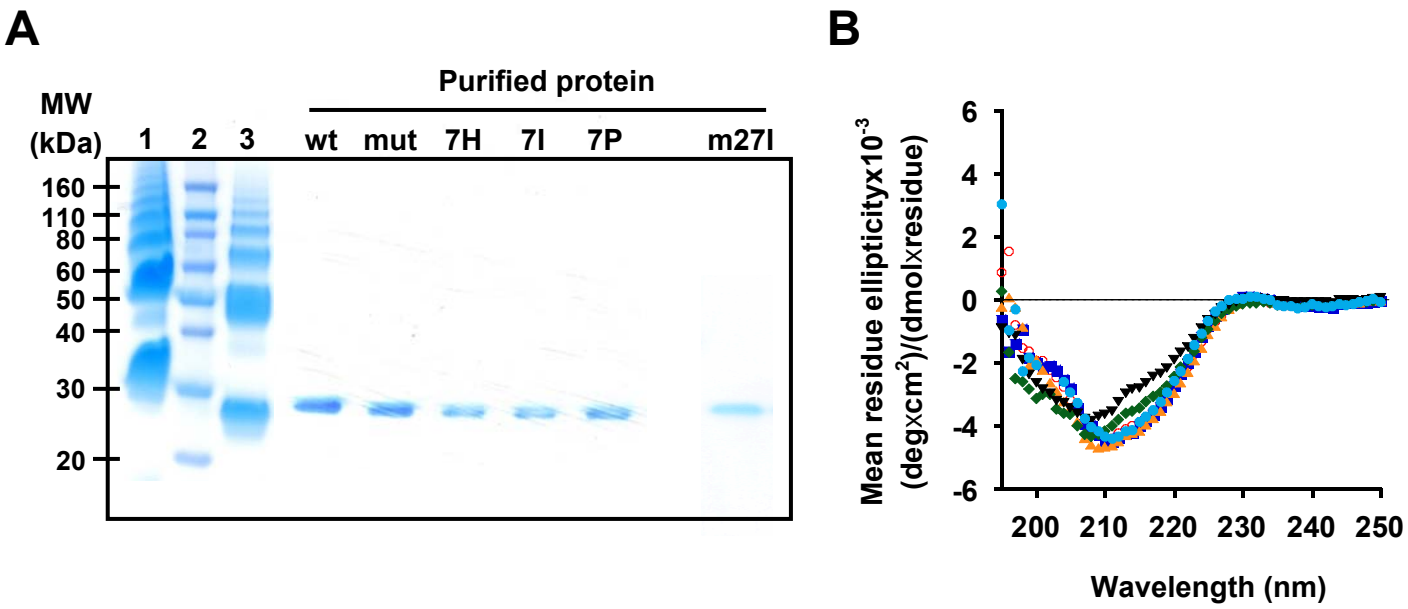

Fig. S3. Purification and characterization of scVEGF proteins. (A) SDS-PAGE analysis under non-reducing conditions. Bands were visualized with SimplyBlue SafeStain (Invitrogen). Lane 1: scVEGF 7I post Ni-NTA column; lane 2: molecular weight markers; Lane 3: scVEGF 7I post Endo H_f treatment. Purified scVEGF proteins are shown post gel filtration chromatography. N-linked glycosylation was effectively removed upon Endo H_f treatment, and monomeric-species were able to be separated from disulfide-linked multimers by gel filtration chromatography. The purified proteins appeared as a single peak on analytical-scale gel filtration chromatography and did not revert to multimers. (B) Far UV CD spectra at 20 °C of the purified scVEGF proteins (50 μM) in PBS pH 7.4. Symbols: scVEGFwt (red ○), scVEGFmut (blue ■), scVEGF m27I (cyan ●), scVEGF 7H (orange ▲), scVEGF 7I (green ♦), or scVEGF 7P (black ▼). A relatively broad minimum at ~210 nm was observed, similar to that previously shown for the $VEGF_{165}$ isoform (11) and the structurally-related protein platelet derived growth factor (PDGF) (12). Similar spectra were obtained for all proteins, with scVEGF 7P having a slightly decreased ellipticity. Overall, this data demonstrates that the scVEGF proteins are composed mainly of β-sheet and random coil structures. Thermal denaturation, monitored by CD, was not observed for any of the scVEGF proteins upon heating from 20-95 °C, suggesting that they are highly stable. In addition, scVEGF variants maintained their structural integrity upon storage at 4 °C for up to 6 months as determined by CD. Collectively, these results demonstrate that introduction of substantial mutations within scVEGFmut loop 3 did not significantly affect protein secondary structure or stability.

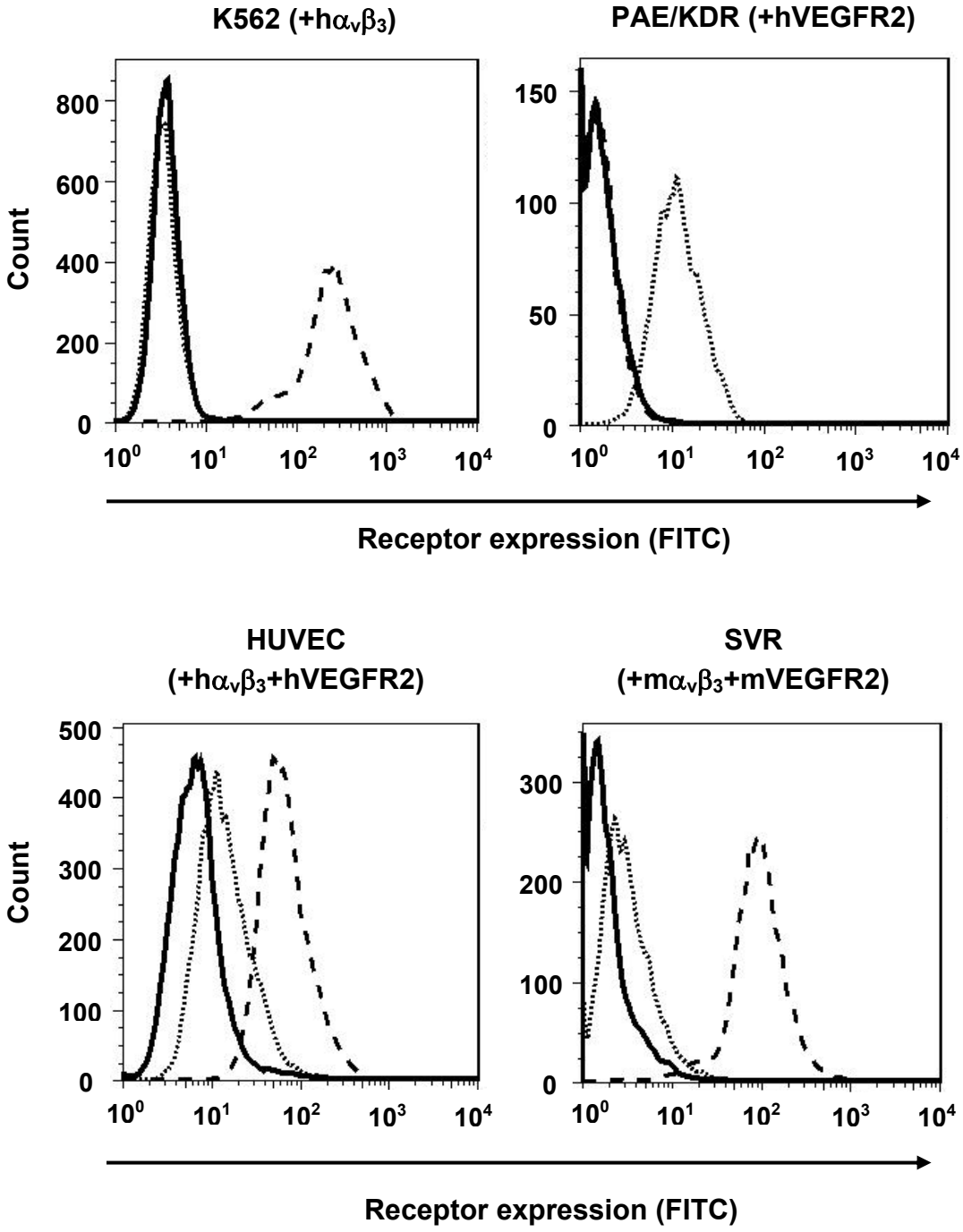

Fig. S4. Cell surface expression of VEGFR2 and α_v integrin subunit. Solid line, cells only; dotted line, VEGFR2; dashed line, α_v integrin subunit. Cells (1×10^5) were stained with fluorescein (FITC)-labeled mouse anti-human CD51 (integrin α_v) (1:25, Biolegend) or FITC-labeled anti-human VEGFR2/KDR (1:25, R&D Systems), and incubated at 4 °C for 30 min with gentle agitation. Cells were then washed with 1 ml PBSA and analyzed by flow cytometry. For mouse SVR cells, biotinylated anti-mouse CD51 (integrin α_v) and biotinylated anti-mFlk-1 (mVEGFR2) (1:50, eBioscience) were used, followed by streptavidin-FITC (1:100, eBioscience).

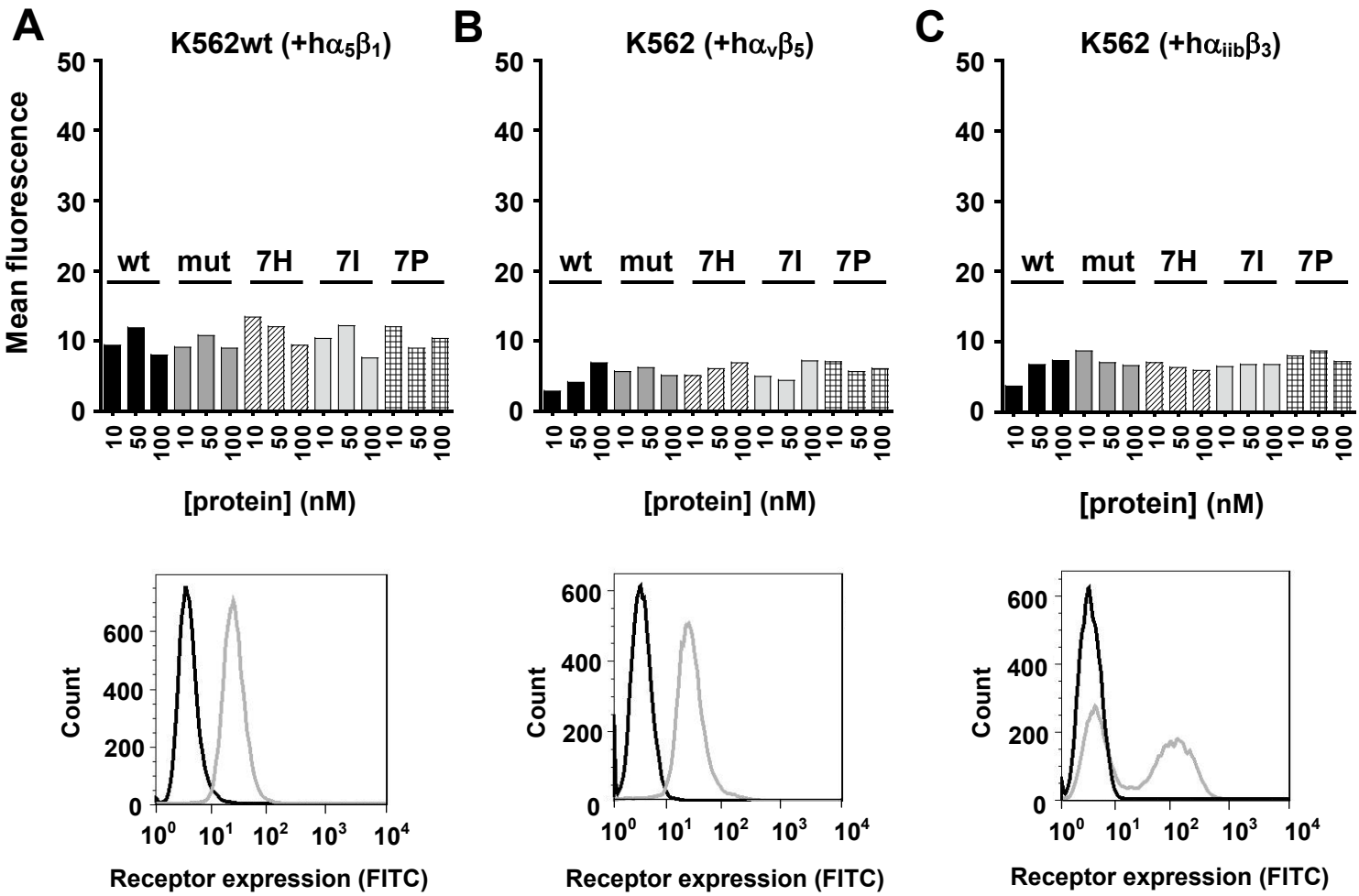

Fig. S5. Binding specificity to K562 cell lines transfected to express different integrins. (A) K562wt cells (express $\alpha_5\beta_1$). (B) K562-$\alpha_v\beta_5$ cells. (C) K562-$\alpha_{iib}\beta_3$ cells. The lower panels show receptor expression levels (grey) versus a negative control (black) in each cell line. scVEGF binding assays were performed as described in *SI Materials and Methods*. To measure receptor expression levels, cells (1×10^5) were labeled with fluorescein (FITC)-labeled mouse anti-human CD51 (integrin α_v) (1:25, Biolegend), FITC-labeled anti-human CD49 (integrin $\alpha5$) (1:25, Biolegend) or FITC-labeled anti-human CD41 (integrin α_{iib}) (1:25, Biolegend). After incubation at 4 °C for 30 min, cells were washed with 1 ml PBSA and analyzed by flow cytometry.

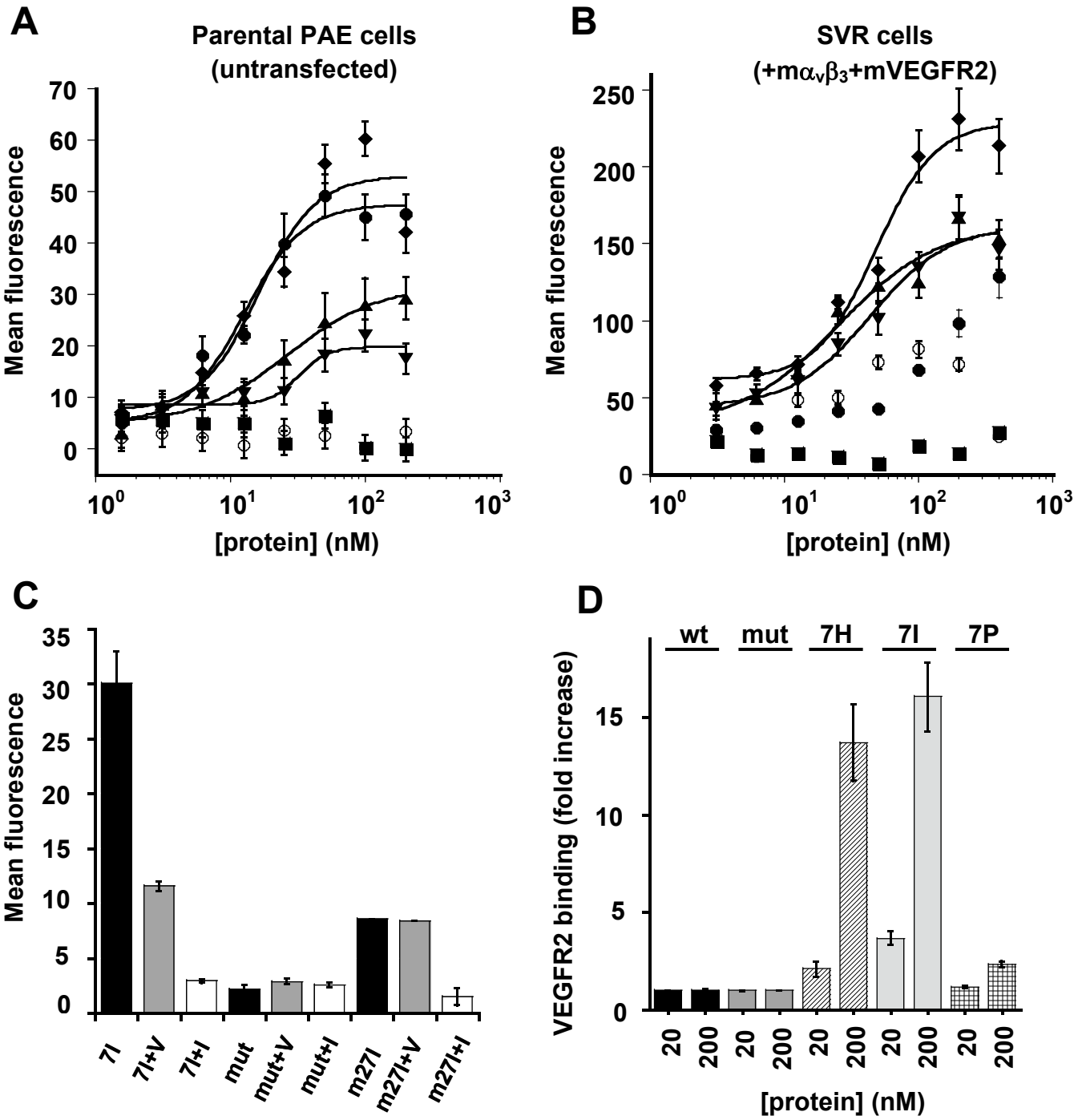

Fig. S6. Binding assays. (A-B) Binding titrations of scVEGF proteins against (A) untransfected porcine PAE cells and (B) murine SVR cells. Mean fluorescence values were determined by flow cytometry using a phycoerythrin-labeled antibody against the N-terminal FLAG epitope tag. Symbols: scVEGFwt (○), scVEGFmut (■), scVEGF m27I (●), scVEGF 7H (▲), scVEGF 7I (♦) and scVEGF 7P (▼). (C) Competition assay to measure dual receptor binding. An excess (10 μM) of a VEGFR2 blocking agent (+ V) or an integrin blocking agent (+ I) was added to SVR cells and binding of 100 nM Alexa488 labeled scVEGF 7I, scVEGFmut, or scVEGF m27I was detected by flow cytometry. (D) Dual receptor direct binding assay. A mixture of soluble human VEGFR2 (500 nM) and scVEGF proteins (20 or 200 nM) was added to K562-$\alpha_v\beta_3$ cells and binding was detected by flow cytometry using a fluorescein-conjugated VEGFR2 antibody. The fold increase in binding signal over buffer alone is reported. Data shown are the average of triplicate values and error bars represent standard deviations.

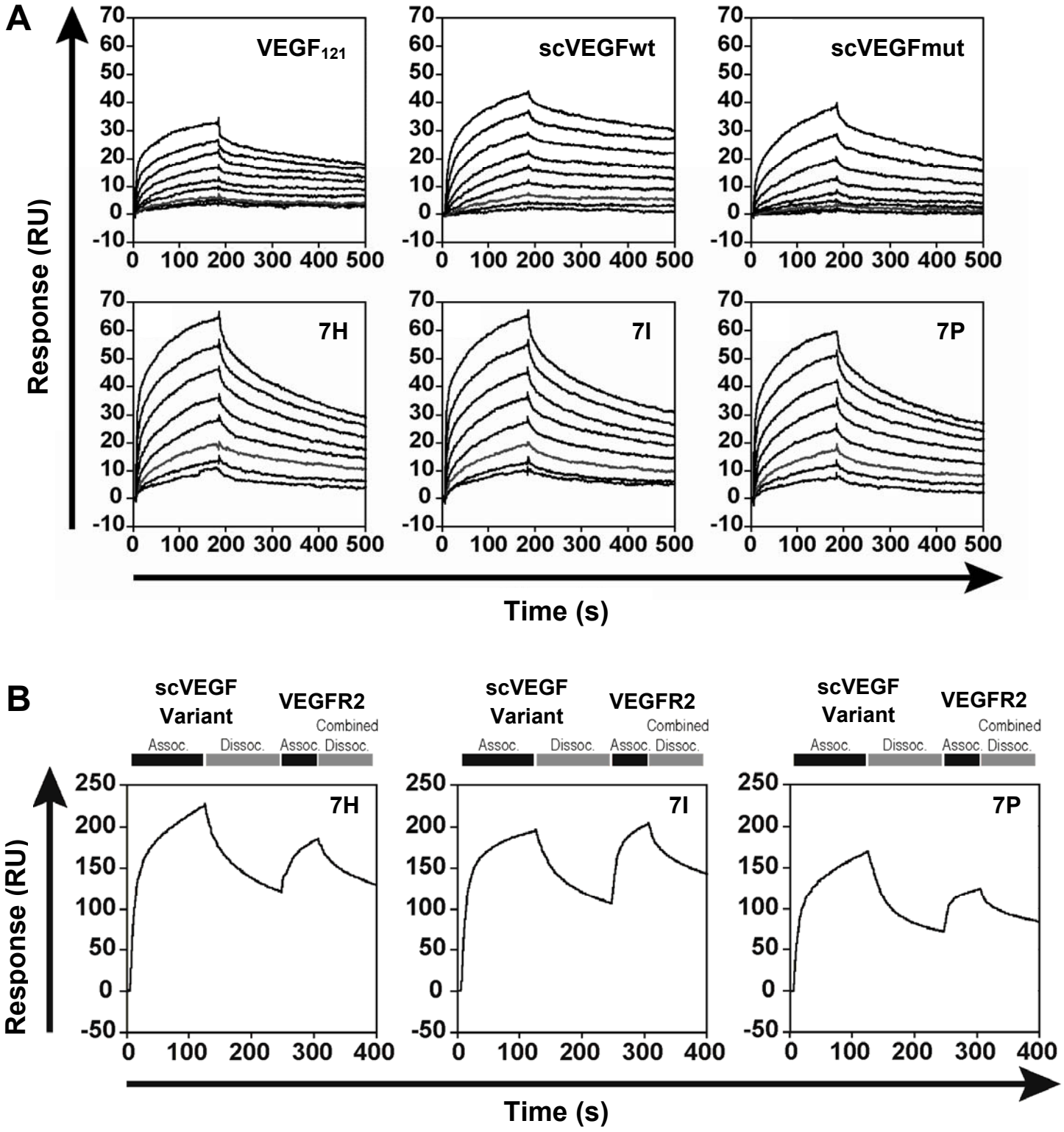

Fig. S7. SPR sensorgrams. (A) Binding of wild-type $VEGF_{121}$ and scVEGF proteins to immobilized VEGFR2. $VEGF_{121}$ (0.78 nM – 200 nM); scVEGFwt (0.78 nM – 200 nM); scVEGFmut (0.78 nM – 200 nM); scVEGF 7H (12.5 nM – 1.6 μM); scVEGF 7I (12.5 nM – 1.6 μM); scVEGF 7P (12.5 nM – 1.6 μM). The ranges of protein concentrations analyzed are indicated in parentheses. (B) Binding of scVEGF variants to immobilized $\alpha_v\beta_3$ integrin and soluble VEGFR2. Representative sensorgram traces show the response of consecutive injections of scVEGF mutants 7H, 7I, or 7P in IB running buffer, followed by a short dissociation phase and injection of soluble VEGFR2 in PBS running buffer.

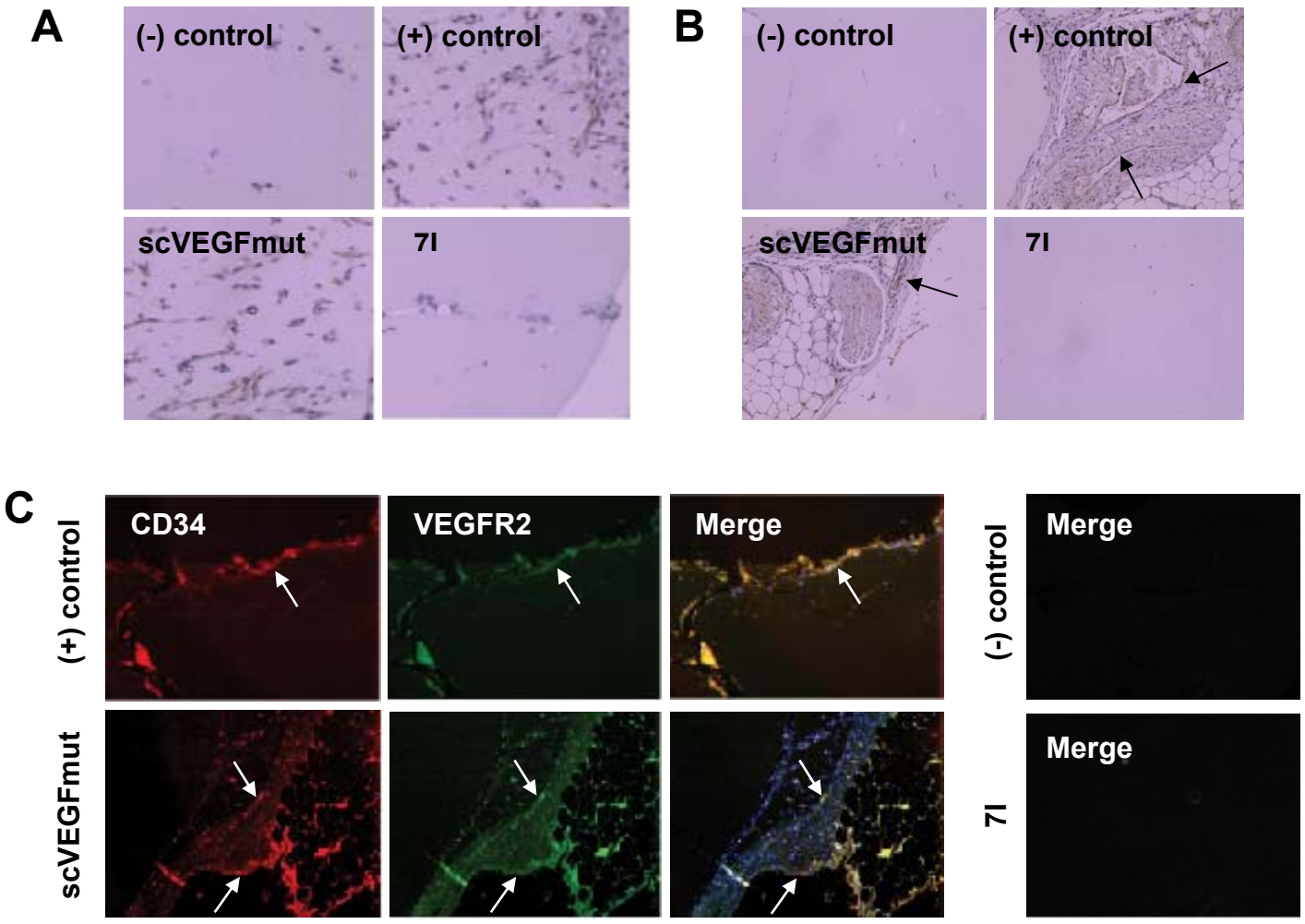

Fig. S8. Immunohistochemical analysis of Matrigel plug sections. (A-B) Haematoxylin staining (blue) and receptor expression (brown). (A) Murine integrin expression. (B) Murine VEGFR2 expression. (C) Immunofluorescent staining of Matrigel sections for murine VEGFR2 (green) and the endothelial marker CD34 (red), showing co-localization (merge). Yellow = co-expression of CD34 and VEGFR2; Blue = DAPI nuclear stain. Arrows indicate receptor expression on blood vessels. Matrigel alone (negative control), $VEGF_{165}$/heparin alone (positive control), or $VEGF_{165}$/heparin + 200 nM scVEGFmut or scVEGF 7I.

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