Supplementary Methods

Humanization of hSFRP2 monoclonal antibody

V region genes encoding the murine SFRP2 monoclonal antibody 80.8.6 [13] were initially cloned, and used to construct chimeric antibodies comprising the murine V regions combined with human IgG1 heavy chain constant regions, and κ light chain constant regions. The chimeric antibodies and combinations of composite heavy and light chains (14 antibodies in total) were expressed in NS0 or HEK293 cells. Their purity was verified by SDS Page on 4-12% gradient gels stained with colloidal blue. They were then tested for binding to SFRP2 peptide in a competition Enzyme-linked immunosorbent assay (ELISA) assay (data not shown) as well as function in inhibition of endothelial tube formation in SVR angiosarcoma (see endothelial tube formation assay method). The lead antibody was then selected based on binding and function. Endotoxin testing of the lead hSFRP2 mAb was performed with Endosafe-PTS kit, Charles River Inc., Margate, UK.

Immunogenicity testing

The lead fully-humanized anti-SFRP2 antibody and the reference chimeric anti-SFRP2 antibody were assessed for immunogenic potential against a cohort of 22 healthy donors using EpiScreen™ time course T-cell assay in order to determine the relative risk of non-specific immunogenicity. Bulk cultures were established using $CD8^+$ depleted PBMCs according to EpiScreen™ optimized protocol (Antitope, Cambridge UK) and T-cell proliferation was measured at various time points by incorporation of [^{3}H]-Thymidine after the addition of the samples. The samples were tested at a final concentration of 50 μg/ml. Cell viability was calculated using trypan blue dye exclusion of PBMC, 7 days after culture with the test samples.

Antibodies and Proteins

For ELISA, an HRP conjugated goat anti-human IgG from Abcam (#ab6858, Cambridge, MA, USA) was used. A control IgG1, omalizumab, was purchased from Novartis (Basel, Switzerland). Human SFRP2 protein (SFRP2) was prepared as previously described [23] and provided by the Protein Expression and Purification Core Lab at University of North Carolina at Chapel Hill. For immunohistochemistry Ki67 antibody was used in a 1:40 dilution (PA1-21520, ThermoFisher Scientific, Waltham, MA). Proteinase K used for the apoptosis TUNEL assay was from Millipore, Burlington, MA, USA (#21627).

Determination of hSFRP2 mAb binding affinity, EC_{50}, and Kd

A microplate solid phase protein binding (ELISA) assay was used to determine the half maximal effective concentration (EC_{50}) for hSFRP2 mAb and its binding affinity for SFRP2. Flat-bottom Ni^{2+} coated 96-well microplates (#15442, Thermo Fisher Scientific, Waltham, MA, USA) were blocked with 0.05% bovine serum albumin (BSA, #001-000-162, Jackson ImmunoResearch, West Grove, PA, USA) in phosphate buffered saline (PBS, #BP399-1, Fisher Scientific) overnight at 4°C. 1µM his-tagged SFRP2 diluted in PBS (pH 7.4) was incubated, shaking, on the blocked plate overnight at 37°C. The plates were washed 3 times with 250µl/well of PBS. Increasing doses of hSFRP2 mAb in PBS (0pM (PBS only), 100pM, 200pM, 400pM, 800pM, 1.6nM, 3.15nM, 6.3nM, 12.5nM, 25nM, 50nM, 100nM) were incubated on the plate with SFRP2 at 37°C overnight, shaking. Plates were washed 3 times, blocked for 1 hour at room temp in 0.1% BSA in PBS, and subsequently incubated with 100µl/well of secondary antibody (HRP conjugated goat anti-human IgG), diluted 1:40,000 in PBS, for 1 hour at 37°C, shaking. After plates were washed 5 times, each well was incubated with 100µl K-Blue TMB substrate (#308176, Neogen, Lexington, KY, USA) for 5 minutes in the dark. The reaction was stopped with 100ul 2N H_2SO_4. Absorbance was read at 450 nm using an EPOCH plate reader (BioTek,

Winoosky, VT, USA). EC_{50} calculations were determined via non-linear regression analysis with variable slope using GraphPad Prism log (inhibitor) vs. normalized response – variable slope function with top constrained to 100%. EC_{50} was converted to a dissociation constant (*Kd*) using the Cheng-Prusoff equation where agonist concentration and EC_{50} were equal[24]. Results are expressed as the means ± standard error of the mean. Each data point is the result of 8 independent measurements (n=8). Hill coefficient was determined utilizing the Hill equation to quantify degree of binding between hSFRP2 and it'.

Cell Culture

2H11 mouse endothelial cells (#CRL-2163, ATCC®, Manassas, VA, USA) were cultured in Opti-MEM (#22600134, Thermo Fisher Scientific, Waltham, MA, USA) with 5% heat-inactivated fetal bovine serum (FBS, #FB-12, Omega Scientific, Biel/Bienne, Switzerland) and 1% penicillin/streptomycin (v/v). Hs578T human breast carcinoma triple negative cells (#HTB-126, ATCC®, Manassas, VA, USA) and MDA-MB-231 human breast carcinoma triple negative cells (#HTB-26, ATCC®, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM, #30-202, ATCC®) with 10% FBS, 0.01 mg/ml bovine insulin (#I0516,Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin (#MT30009C, Thermo Fisher Scientific). SVR angiosarcoma cells were obtained from American Type Culture Collection (#CRL-2280, ATCC®) and cultured in Opti-MEM (Thermo Fisher Scientific) with 8% FBS and 1% penicillin/streptomycin (v/v). All cell lines were cultured at 37°C in a humidified 5% CO_2-95% room air atmosphere. All cell lines were authenticated by ATCC®, and mouse cells tested by Charles River Research Animal (Wilmington, MA, USA) for rodent pathogens, including mycoplasma whenever they were used *in vivo*.

Endothelial tube formation assay

2H11 endothelial cells were plated in Opti-MEM with 5% FBS and allowed to settle for 24 hrs. Quiescence was induced by maintaining the cells in Opti-MEM with 2.5% FBS overnight. Matrigel™ (#ECM625, Millipore, Bedford, MA, USA), was polymerized in the wells of a 96-well plate according to the In Vitro Angiogenesis Assay protocol (#ECM625 Millipore). In this assay, nine treatment conditions were prepared: IgG1 alone (5µM; omalizumab); SFRP2 protein (30nM) with IgG1 (5µM); or SFRP2 (30nM) combined with increasing concentrations of hSFRP2 mAb (0.5, 1, 5, 10 or 20µM). Treatments resuspended in Opti-MEM with 2.5% FBS were pre-incubated on a rocker at 37°C, 5% CO2, for 90 minutes prior to adding them to the cells. $1.9x10^4$ cells were resuspended in 150µl of pre-incubated treatments, and then incubated for an additional 30 min on a rocker at 37°C, 5% CO2. The cell suspension was added to each well pre-coated with polymerized Matrigel™. Each experiment was repeated 4 independent times, with n=4 per condition. Control cells were given fresh Opti-MEM with 2.5% FBS and 5µM IgG1. For each treatment condition, after 4h of incubation at 37°C, 5% CO_2, images were acquired using the 4X objective lens of the EVOS FL Digital Imaging System (Thermo Fisher Scientific, Waltham, MA, USA). Branch points were counted using ImageJ Angiogenesis Analysis software (National Institutes of Health, Bethesda, MD, USA). Data was analyzed to determine IC_{50} using non-linear regression and the Dose-response – Inhibition equations In the GraphPad Prism software.

Proliferation Assay

Hs578T breast carcinoma triple negative, MDA-MB-231 triple negative and SVR angiosarcoma cells were plated in a 96 well plate at 3,000 cells/well. After 4 hours, hSFRP2 mAb (1, 5, or 10µM) was added to the growth medium at the indicated concentrations. Cells were allowed to incubate for 72 hours at 37ºC, 5% CO_2. Proliferation was assessed using the Cyquant Direct Cell

Proliferation Assay Kit (#C35011, Thermo Fisher Scientific, Waltham, MA, USA). Control wells contained untreated (UT) growth medium and medium with 5µM Omalizumab. Each data point was the result of 3 independent experimental repeats, each containing 4 separate wells (total n=12). Images were acquired using the EVOS FLc Digital Imaging System (Life Technologies Inc., Waltham, MA, USA) at 20X magnification. Cells were counted using the FIJI cell counting software.

Apoptosis/Necrosis

Hs578T breast carcinoma triple negative, MDA-MB-231 triple negative and SVR angiosarcoma cells were plated in 16 well chamber slides (#178599, Thermo Fisher Scientific, Waltham, MA, USA) at $2x10^4$ and $3x10^4$ cells/well, respectively. The next day, cells were incubated at 37°C, 5% CO_2 with 1, 5 or 10 µM of hSFRP2 mAb or 5 µM of IgG1 (Omalizumab) control in suspension with growth medium for 2 hours. Necrosis and apoptosis were determined following the protocol of the Apoptotic/Necrotic Detection kit (#PK-CA707-30017, PromoCell, GmbH, Heidelberg, Germany) where apoptotic cells were positive for FITC and necrotic cells were positive for Texas Red. Images were acquired using the 20X objective lens of the EVOS FLc Digital Imaging System (Thermo Fisher Scientific, Waltham, MA, USA). Cells were counted using ImageJ cell counting software. Each data point was the result of 3 independent experimental repeats, each containing 4 separate wells (total n=12).

Microplate Solid Phase Protein Binding (ELISA) Assay for Pharmacokinetics (PK) of hSFRP2 mAb

A microplate solid phase protein binding assay was used to determine the *Kd* for SFRP2 (UNC, Chapel Hill) and hSFRP2 mAb (Catalent). Flat-bottom Ni+2 coated 96-well microplates (Thermo Fisher Scientific, Waltham, MA, USA) were blocked with 0.05% bovine serum

albumen (BSA) in phosphate buffered saline (PBS) overnight at 4°C. 1µM his-tagged SFRP2 diluted in PBS (pH 7.4) was incubated overnight at 37°C. The plates were washed 3 times with 250µl/well of PBS. Then, a 1:50 dilution of mouse serum isolated from the pharmacokinetic study (described below) was added to the plate and incubated and shaken gently at 37°C overnight. Plates were washed 3 times, blocked for 1 hour at room temp in 0.1% BSA in PBS, and subsequently incubated with 100 µl/well of secondary antibody (HRP conjugated goat anti-human IgG from Abcam, Cambridge, MA cat no. ab6858), diluted 1:40,000 in PBS for 1 hour at 37°C. After plates were washed 5 times, each well was incubated with 100µl K-Blue TMB substrate (Neogen, Lexington, KY, USA) for 5 minutes in the dark. The reaction was stopped with 100 µl 2N H_2SO_4. Absorbance was read at 450 nm with an EPOCH plate reader (BioTek, Winooski, VT, USA). For PK estimates of AUC, t1/2, CL, Vd, T_{max} and C_{max} were determined using EXCEL and non-compartmental analysis (NCA) [25]. NCA uses the linear trapezoidal rule to determine the area under the plasma concentration-versus-time curve (AUC). T1/2 represents the terminal half-life. For AUC calculations, nM concentrations were converted to mg/L. PK parameters were calculated individually per mouse for six mice to determine mean values and error.

In vivo studies

Pharmacokinetic study: Male and female C57BL/6 mice were injected with 4 mg/kg of hSFRP2 mAb at different time points (0, 5 min, 1, 2, 7, 14, 21, 28, 35, and 42 days). Three mice were used for each time point (n=3). At each point, blood samples were taken through the portal vein and placed in separator tubes (#367981, Becton Dickinson, Franklin Lakes, NJ, USA. Samples were centrifuged at 1300xg for 15 min.

Maximum Tolerated Dose (MTD) of hSFRP2 mAb in vivo: Animal experiment protocols were consistent with NIH guidelines for the care and use of laboratory animals. All studies were approved by the Animal Care and Use Committee at the Medical University of South Carolina. 10^6 SVR angiosarcoma cells were injected subcutaneously into the right flank of 6 week old nude male and female mice obtained from Charles River (Wilmington, MA, USA). The following day, mice (n=5 per group) were treated intravenously (i.v.) with IgG1 control 4 mg/kg iv with various concentrations of hSFRP2 mAb (2, 4, 10, and 20 mg/kg) injected via the tail vein every 3 days. Animal were treated and tumor volumes were measured every three days until control tumors reached an average diameter of 2 cm, which was defined as the end-point. After euthanasia, lungs and livers were harvested and fixed in 10% formalin, sectioned, and examined by H&E by a board certified pathologist (LS). A blind analysis of tissues from animals selected randomly was performed using the presence of karyomegaly, apoptosis, infiltrating lymphocytes, mitoses and inflammation as criteria for treatment-induced abnormalities.

Angiosarcoma allografts and Hs578T breast carcinoma- triple negative xenografts: 10^6 SVR angiosarcoma cells were injected subcutaneously into the right flank of 6 week old nude male and female mice obtained from Charles River (Wilmington, MA, USA) and treatment started the following day; or 10^6 Hs578T cells were inoculated in the mammary fat pad in 5- to 6-week-old nude female mice from Charles River (Wilmington, MA, USA) and treatment began on day 30 after tumor injection, when the average tumor size was approximately 100 mm^3. Mice (n=10 animals/group) were injected i.v. with hSFRP2 mAb (4 mg/kg) or IgG1 control (omalizumab 4 mg/kg) via the tail vein, and were treated every 3 days. Serial caliper measurements of perpendicular diameters performed twice a week were used to calculate tumor

volume using the following formula: [(L (mm) x W (mm) x H (mm)) x 0.5]. Mice were monitored daily for body conditioning score and weight. Mice were sacrificed when the controls reached 2 cm diameter, and tumors were resected and fixed in formalin and embedded in paraffin.

Immunohistochemistry

Formalin fixed, paraffin embedded tumor sections were deparaffinized twice for ten minutes in Xylene and hydrated twice in absolute ethanol, twice in 95% ethanol, and then tap water. Slides were incubated in 3% hydrogen peroxide for ten minutes at room temperature followed by two washes in PBS 1X. A citrate buffer antigen retrieval step was performed in a vegetable steamer using the kit Vector Antigen Retrieval Citrate Buffer pH6 (H-3300) for 40 minutes with 10 minutes to cool. Slides were incubated in blocking serum provided in the Vector Rabbit IMPRESS HRP Kit (MP-4100) in a humidified slide chamber at room temperature for 1 hour. The blocking serum was then drained off, and the slides were incubated overnight at 4ºC with the Ki67 antibody 1:40 dilution (PA1-21520, ThermoFisher Scientific, Waltham, MA). The next day, the slides were rinsed 3 times in PBS for 5 min/wash. The secondary antibody from the Vector Rabbit IMPRESS HRP Kit was added and the slides were incubated for 30 min RT, and then rinsed 3 times in PBS for 5 min/wash. DAB solution was prepared and added to the slides as instructed in the Vector DAB kit (SK-4100) for 5 min, rinsed in PBS, and counterstained with hematoxylin for 30 seconds. Slides were then washed in distilled water, followed by ammonia alcohol, dehydrated twice in 95% ethanol, twice in 100% ethanol, twice in xylene, and then mounted with a coverslip. Tumor proliferation was quantified as the number of positively stained

cells/unit area, using the average of 3 fields per slice. Images were taken with the 20X objective lens on the EVOS FLc imaging system (Thermo Fisher Scientific, Waltham, MA, USA).

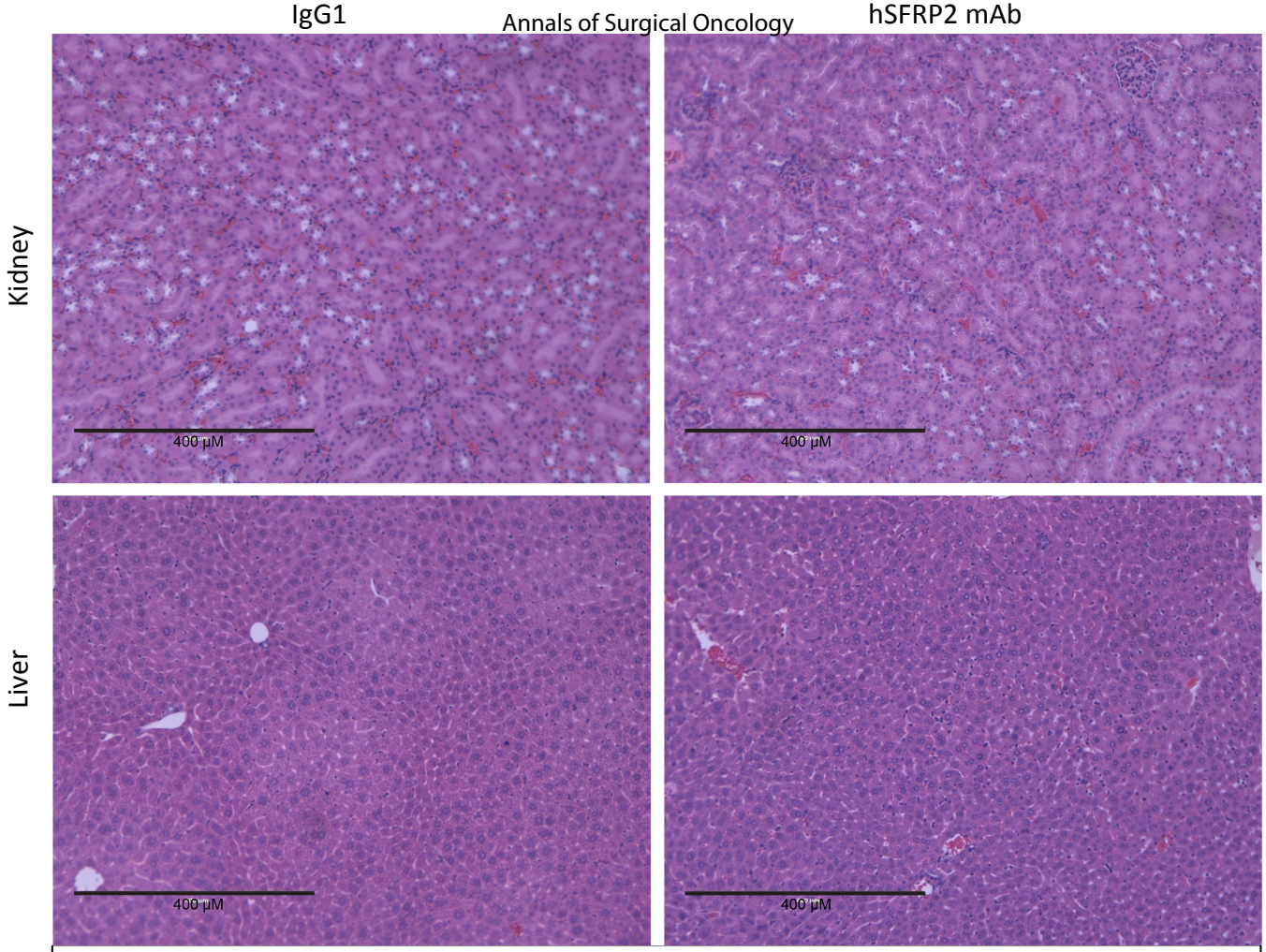
TUNEL assay

Sections from resected Hs578T and SVR tumors were stained for apoptotic cells following the manufacturer protocol for the Apoptag® Peroxidase In Situ Apoptosis Detection Kit (#S7100). All sections were deparaffinized with Histoclear (#HS-200, National Diagnostics, Atlanta, GA, USA). The following materials were purchased separately: 30% Hydrogen peroxide (#5155-01, J.T. Baker, Phillipsburg, NJ, USA), Proteinase K (#21627, Millipore, Burlington, MA, USA), Metal enhanced DAB substrate kit (#34065, Thermoscientific, Waltham, MA, USA), stable peroxidase substrate buffer 1X (#1855910, Thermoscientific, Waltham, MA, USA) and 1-Butanol (#B7908, Sigma-Aldrich, St. Louis, MO, USA). Five fields were randomly selected in each sample and photographed using the 20X objective lens of the EVOS FLc microscope. In each field, tumor apoptosis was quantified as the number of apoptotic nuclei/HPF, and the mean value was calculated. The number of apoptotic cells was compared between control and hSFRP2 mAb treated tumors.

Statistics

For *in vitro* assays, statistical differences between IgG1 and hSFRP2 mAb treatments were calculated using a two-tailed student's t-test, with $p \leq 0.05$ considered significant. For *in vivo* tumor studies in angiosarcoma (where treatment was started day after tumor inoculation), a two-tailed student's t-test was used. For Hs578T, where treatment was started on day 30 when tumors were palpable, the data was normalized to adjust for differences in baseline tumor volumes, by dividing tumor volume from day 34 to 82 with each group's baseline (day 30) tumor volume. A two-sample t-test for each time point was used and compared the tumor volume between treated

and control animals. To satisfy normality assumption for t-test, normalized tumor volume is log-transformed.



Supplementary Figure 1. Treatment with hSFRP2 mAb has no toxic effect on histologic analysis. H&E staining of sections at 10X magnification of normal kidney (top) or liver (bottom) following 21 days of treatment with IgG1 control (left) or hSFRP2 mAb (right).