Supplemental file

Methods

In vitro studies

Generation of Constructs and Purification of Polypeptides: The coding sequence for human $VEGF_{121}$ was fused in frame with the ELP coding sequence, and the chimeric protein was recombinantly expressed and purified, as recently described[1]. For *in vitro* comparison studies, recombinant human $VEGF_{121}$ was used (ProSpec, East Brunswick, NJ).

Cell Culture: Primary HGME cells were purchased from Cell Systems (Kirkland, WA) and sub-cultured according to the manufacturer's recommendations using Attachment Factor™ and complete classic medium supplemented with Culture Boost™ (Cell Systems). The cells were maintained in a 37°C humidified incubator at 5% CO_2. Cells in passage 4-13 were used for all performed experiments.

HGME Proliferation Assay: HGME cells were seeded at 10,000 cells / well in 96 well plates and incubated at 37°C in humidified incubator with 5% CO_2 overnight. The cells were serum and growth factor starved for 2-3 hours before treatment. After starvation, the proteins (ELP, VEGF, and ELP-VEGF) were added at 100 µl volume in 10% complete media to final concentrations of 1, 10, and 100 nM and incubated for an additional 72 hours. Viable cells were detected using the MTS cell proliferation assay (Promega, Madison, WI). The data shown represent the mean ± SEM. of three independent experiments in octuplicate.

HGME Tube Formation Assay: A 24well plate, sterile and non-tissue culture treated was coated with growth factor reduced Matrigel (BD Biosciences). HGME cells were

serum and growth factor starved for 2-3 hours before seeding them over Matrigel coated wells at 50,000 cells / well in 5% complete media containing 0.1 mg/mL of heparin in the absence or presence of 100 nM final concentration of ELP, VEGF, or ELP-VEGF. The cells were incubated at 37°C in a humidified incubator with 5% CO_2 for 5 hours. At the end of the incubation, the cells were imaged with an inverted microscope using bright field illumination and 10x magnification. Five non-overlapping fields per well were imaged, and the tubes between two cell nodes were counted for each field, averaged for each well, and expressed relative to untreated wells. The data represent the mean ± SEM of three independent experiments.

HGME Migration Assay: Corning BioCoat growth factor reduced Matrigel Invasion Chambers (Corning Biocoat) were warmed to room temperature, and the interior of the inserts were rehydrated with basal media (Cell Systems) for 2 hours in a humidified incubator at 37°C with 5% CO_2. HGME cells at 30,000 cells / well in basal media containing 1% fetal bovine serum and 0.1 mg / mL heparin were added to the interior of the inserts in 500 µl volume. ELP, VEGF, and ELP-VEGF at 100 nM final concentration in 750 µl final volume were added in the same media in the wells of a 24 well tissue culture treated plate. The inserts were gently placed into each designated well avoiding air bubbles. The cells were incubated for 16-24 hours in a humidified incubator at 37°C with 5% CO_2. After incubation, any cell suspension left in each insert was removed, the inserts were rinsed with DPBS, and non-invading cells were scrubbed from the upper surface of the membrane using a cotton swab. The cells on the lower surface of the membrane were stained with 0.1% crystal violet in 10% ethanol at room temperature for 30 minutes. The inserts were rinsed with water and air dried for an additional 60 minutes.

Membranes were photographed using an inverted microscope and 10x magnification objective on five independent fields per membrane. The number of cells per field were counted and averaged for each well, and the data were expressed relative to untreated wells. The data represent the mean ± SEM of three independent experiments.

Labeling ELP-VEGF with fluorescent probes: ELP-VEGF was labeled with Alexa Fluor 633® succinimidyl ester (Life Technologies), as recently described[1].

In vivo **determination of pharmacokinetics and bio-distribution after intra-renal administration in the swine model:** The Institutional Animal Care and Use Committee at the University of Mississippi Medical Center approved all the procedures. Three pigs were anesthetized, a 9F vascular sheath catheter was first inserted into the carotid artery, and then a 9F J4 guide catheter containing a 7F balloon catheter was inserted through the sheath and directed to the renal artery under fluoroscopy guidance. Alexa Fluor 633® labeled ELP-VEGF was diluted immediately before injection into a final volume of 10 mL PBS at a sufficient concentration to achieve a dose of 1 mg/kg body weight. For the intra-renal injection of the construct, the balloon was inflated at the catheter tip to block blood flow into and out of the kidney, and the polypeptide was slowly injected into the kidney through the balloon's lumen. The balloon was left inflated for three minutes after the injection and then deflated to allow blood flow to resume. Blood was sampled from a previously placed venous catheter (jugular vein) at 1, 3, 5, 15, 30 minutes after injection and every 30 minutes thereafter for 4 hours, and plasma was collected and frozen after centrifugation. At the fourth hour, the pigs were euthanized by an overdose injection of sodium pentobarbital (100mg/kg), and the organs were removed for analysis. Plasma and organs from 2 non-injected pigs were used for auto-fluorescence controls.

Plasma fluorescence was directly measured using a Nanoquant® plate and fluorescence plate reader (Tecan). Raw fluorescence data were fit to a standard curve of the labeled protein that was produced from the same labeling batch as the injection to determine plasma levels in μg/mL. Data from the third minute and following time points (after deflation and removal of the balloon) were fit to a two-compartment pharmacokinetic model using GraphPad Prism as described previously [1-3]. Data represent the mean±SD of three pigs.

To determine organ distribution, whole organs were imaged *ex vivo* using an IVIS Spectrum (Caliper Life Sciences, Perkin Elmer) with 605 nm excitation, 660 nm emission, and auto exposure. Mean fluorescence radiant efficiency was determined for each organ using Living Image Software (Caliper). A standard curve was produced by performing two-fold serial dilutions of the injected protein. 100 μL of each protein standard was placed in wells of a black 96 well plate and imaged with the same settings as were used for tissue imaging. Background auto-fluorescence from tissues of non-injected animals was subtracted from each organ's fluorescence, and mean fluorescence radiant efficiency of each organ was fit to the standard curve values to determine tissue concentrations. Data represent the mean±SEM of three pigs.

In vivo renal functional studies

The Institutional Animal Care and Use Committee at the University of Mississippi Medical Center approved all the *in vivo* studies and procedures. Twenty-nine pre-juvenile domestic pigs (*sus scrofa domestica*) were used for the study, which lasted a total of 10 weeks. In 22 pigs, unilateral renal artery stenosis was induced at baseline by placing a local-irritant copper coil (on day 1 of the study) inside the main renal artery

constituting a surrogate of RVD, as previously shown[4, 5]. Blood pressure was continuously measured by telemetry (PhysioTel, Data Sciences International) and averaged for each 24-hour period, as described[4-6]. Additional animals were used as normal controls (normal, n=7).

Six weeks after induction of RVD, all pigs were anesthetized with intra-muscular telazol (5 mg/kg) and xylazine (2 mg/kg), intubated, and mechanically ventilated on room air. Anesthesia was maintained with a mixture of ketamine (0.2 mg/kg/min) and xylazine (0.03 mg/kg/min) in normal saline, and administered via an ear vein cannula (0.05 mL/kg/min). Pigs then underwent renal angiography to quantify the degree of renal artery stenosis, as described[5,37,48]. After angiography, the catheter was positioned in the superior vena cava, and *in vivo* helical MDCT flow studies were performed for quantification of single-kidney RBF, perfusion, and GFR.

Immediately after completion of the *in vivo* MDCT studies at 6 weeks, and while still under anesthesia, all RVD animals were treated with a single intra-renal (stenotic kidney) infusion of vehicle (RVD, n=7), ELP (100ug/kg, RVD+ELP, n=5) or ELP-VEGF (100ug/kg, RVD+ELP-VEGF, n=7).). In addition, a smaller group of pigs were treated with a single intra-renal administration of unbound $VEGF_{121}$ (at a dose of 18.65 μg/kg, which matches the concentration of VEGF in the ELP-VEGF construct) to serve as treated controls to determine the differences in therapeutic efficacy between ELP-VEGF and unbound VEGF (RVD+VEGF, n=3). After intra-renal infusions, catheters were removed, vascular incisions sutured, and pigs allowed to recover and observed for 4 additional weeks with no further treatments. Blood pressure was continuously monitored by telemetry, and at 10 weeks, MDCT *in vivo* studies were repeated as done at 6 weeks.

RVR was calculated at 6 and 10 weeks as recently described[7]. Blood from the inferior vena cava and renal veins (from the stenotic kidney) and urine were collected (at 6 and 10 weeks) to measure plasma creatinine (QuantiChrom Creatinine Assay Kit, BioAssay Systems, Hayward, CA), nephrin in urine (suggestive of podocyte damage, ELISA, Exocell, PA, USA) and albuminuria (ELISA, Alpha Diagnostic, San Antonio, TX), following vendors' instructions.

Upon completion of all the *in vivo* studies, the pigs were allowed 2 days to recover and then euthanized by an intravenous overdose of sodium pentobarbital (100mg/kg). Kidneys were then removed and immersed in heparinized saline (10 units/mL) before preparation for *ex vivo* studies. A kidney lobe was used for micro-CT reconstruction. Another lobe was removed, snap-frozen in liquid nitrogen and stored at -80^{o} C to investigate the expression of angiogenic and fibrogenic factors by westernblotting (see below) and the renal concentration of pro-inflammatory tumor necrosis factor (TNF)-α (R&D Systems, MN, USA), following vendor's instructions. Another portion was preserved in 10% formalin and used to investigate renal morphology in mid-hilar renal cross-sections stained with trichrome and H&E, as shown[8-11].

High-resolution CT imaging

MDCT analysis: Manually-traced regions of interest were selected in MDCT images in the aorta, renal cortex, medulla, and papilla; their densities were sampled and time-density curves generated. The area under each segment of the curve and its first moment were calculated using curve-fitting parameters and used to calculate single-kidney RBF (ml/min), GFR (mL/min), and renal perfusion (ml/minute/cc tissue), using previously-validated methods[4, 12, 13].

Micro-CT: The stenotic kidney was perfused with an intravascular contrast agent (Microfil MV122, Flow Tech, Inc., Carver, MA), samples scanned at 0.3˚ increments using a micro-CT scanner and reconstructed for subsequent analysis, as described[5]. The cortex and medulla were tomographically divided and the spatial density and distribution of microvessels (diameters <500μm) and images then analyzed with Analyze® (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN), as described[5, 14].

***Ex vivo* studies:** protein expression and renal morphology were assessed in Normal, RVD and RVD+ELP-VEGF pigs.

Western blotting: Standard blotting protocols in renal cortical tissue homogenates were followed, as previously described[11, 15], using specific polyclonal antibodies against VEGF, the specific receptors Flk-1, pro-angiogenic Ang-1 and -2, and the Tie-2 receptor, p-akt, SDF-1 and its receptor CXCR4. Furthermore, the renal expression of anti-angiogenic angiostatin; tissue-remodeling factors such as TGF-β and mediators smads-4 and -7, and MMP-2 and its inhibitor TIMP-1 (Santa Cruz Biotechnology, CA for all) were also measured. β-actin (Sigma, Saint Louis, MO, 1:500) was used as loading control.

Histology: Mid-hilar 5 μm cross sections of each kidney (1 per animal) were examined. In each slide, trichrome staining was semi-automatically quantified in 15-20 fields using a computer-aided image-analysis program (NIS Element 3.0, Nikon Instruments, Melville, NY), expressed as percentage of staining of total surface area, and the results from all fields averaged. Glomerular score (expressed as percentage) was assessed by recording the number of sclerotic glomeruli out of 100 counted glomeruli, as described[4, 5]. Media-to-lumen ratio was assessed as previously described[4].

Statistical Analysis: Results are expressed as mean ± SD or SEM as indicated.

Comparisons within groups were performed using paired student's t-test, and among groups using one-way ANOVA, with Bonferroni correction for multiple comparisons. Statistical significance was accepted for $p \leq 0.05$.

Results

***In vitro* characterization of ELP-VEGF activity:** Before beginning *in vivo* evaluation of ELP-VEGF in the swine model, we first determined whether the construct was active using *in vitro* models of glomerular microvascular endothelial cell proliferation, tube formation, and migration. Primary Human Glomerular Microvascular Endothelial (HGME) cells were used to insure the signaling properties of VEGF were retained even after fusion to the ELP carrier. As shown in **Figure 1a**, both unbound VEGF and ELP-VEGF stimulated proliferation of HGME cells, while the ELP polypeptide alone had no effect on HGME proliferation. Furthermore, no significant differences were seen in the potency of the unbound cytokine and the ELP-fused VEGF, suggesting that the ELP-fused VEGF is still able to bind its receptor. To test this further, HGME cells were used in to a tube formation assay on growth factor reduced Matrigel. As shown in **Figure 1b**, very little tube formation was observed on this matrix without additional stimulation. However, when the media was supplemented with unbound VEGF or ELP-VEGF, tube formation was significantly induced. Quantification of tubes per visual field showed that both unbound VEGF and ELP-VEGF significantly induced tube formation relative to untreated cells (**Figure 1c**). There were also more average tubes per field in the ELP control-treated samples, though the difference did not reach statistical significance. Finally, to assess the ability of ELP-VEGF to serve as a chemokine for HGME cells, a Matrigel migration assay was used. As shown in **Figure 1d** and quantified in **Figure 1e**,

both unbound VEGF and ELP-VEGF strongly induced HGME cell migration through Matrigel, while the control ELP had no effect. Again, there was no difference in potency between VEGF and ELP-VEGF.

***In vivo* pharmacokinetics and bio-distribution of ELP-VEGF following single kidney intra-renal administration:** Three pigs (average weight 49.2 ± 6.3 kg) were administered fluorescently labeled ELP-VEGF by direct intra-renal injection under fluoroscopy guidance. Blood flow into and out of the injected kidney was occluded for three minutes following the injection. Blood was sampled intermittently after release of the balloon, and direct fluorescence measurements were taken to monitor ELP-VEGF levels. As shown in **Figure 2**, plasma levels spiked immediately after release of the balloon. There was an initial rapid clearance / distribution phase followed by a slow elimination phase. The data fit well with a two-compartment pharmacokinetic model. The distribution phase half-life was 2.95 minutes and the terminal plasma half-life was 810.1minutes.

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