Renal Therapeutic Angiogenesis Using a Bioengineered Polymer-Stabilized Vascular Endothelial Growth Factor Construct

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ABSTRACT

Renovascular disease (RVD) induces renal microvascular (MV) rarefaction that drives progressive kidney injury. In previous studies, we showed that renal vascular endothelial growth factor (VEGF) therapy attenuated MV damage, but did not resolve renal injury at practical clinical doses. To increase the bioavailability of VEGF, we developed a biopolymer-stabilized elastin-like polypeptide (ELP)-VEGF fusion protein and determined its in vivo potential for therapeutic renal angiogenesis in RVD using an established swine model of chronic RVD. We measured single-kidney blood flow (RBF) and GFR and established the degree of renal damage after 6 weeks of RVD. Pigs then received a single stenotic kidney infusion of ELP-VEGF (100 μg/kg), a matching concentration of unconjugated VEGF (18.65 μg/kg), ELP alone (100 μg/kg), or placebo. Analysis of organ distribution showed high renal binding of ELP-VEGF 4 hours after stenotic kidney infusion. Therapeutic efficacy was determined 4 weeks after infusion. ELP-VEGF therapy improved renal protein expression attenuated in RVD, restoring expression levels of VEGF, VEGF receptor Flk-1, and downstream angiogenic mediators, including phosphorylated Akt and angiopoietin-1 and -2. This effect was accompanied by restored MV density, attenuated fibrogenic activity, and improvements in RBF and GFR greater than those observed with placebo, ELP alone, or unconjugated VEGF. In summary, we demonstrated the feasibility of a novel therapy to curtail renal injury. Recovery of the stenotic kidney in RVD after ELP-VEGF therapy may be driven by restoration of renal angiogenic signaling and attenuated fibrogenic activity, which ameliorates MV rarefaction and improves renal function.


Renal vascular disease (RVD), usually caused by renal artery stenosis, can lead to CKD and ESRD. RVD increases cardiovascular morbidity and mortality, hospitalization, shortens life expectancy, and is increasing at a sustained pace in the United States. Moreover, renal function does not improve or even deteriorates in almost half of the patients with RVD despite treatment. Recent clinical studies suggest that patients undergoing current therapeutic strategies, which include drugs and renal angioplasty, do not show differences in outcomes that could demonstrate distinct benefits of one treatment over the other when compared side by side. Moreover, renal function does not improve or even deteriorates in almost half of patients with RVD despite treatment, highlighting a pressing need for novel therapeutic strategies for the growing population of patients suffering from RVD.

Using a clinically relevant swine model of chronic RVD that mimics several of the pathologic features...
and the progressive nature of human RVD,4,5 we showed that a key pathologic feature accompanying the loss of renal function and the progression of renal injury in the stenotic kidney is a progressive microvascular (MV) rarefaction. We demonstrated that the latter is paralleled by defective renal angiogenesis, which is likely driven by a progressive decrease in the renal availability of vascular endothelial growth factor (VEGF), since intrarenal VEGF therapy significantly attenuated MV damage and loss and improved renal function.6,7 However, these therapeutic effects were still insufficient to resolve renal injury. Potential reasons for the incomplete resolution of renal damage may be related to VEGF’s short $t_{1/2}$, or its susceptibility to degradation in vivo. A potential alternative to overcome these limitations is to increase the frequency of treatment. However, repeated intrarenal administrations to enhance the effects are impractical for clinical use and may increase the risk of adverse events. Therefore, we sought a means to prolong the plasma $t_{1/2}$ of exogenously administered VEGF and improve its renal targeting and efficacy.

Elastin-like polypeptides (ELP) are a class of bioengineered proteins with great potential as drug delivery vectors due to their long plasma $t_{1/2}$, low immunogenicity, and adaptability to be fused to nearly any therapeutic peptide, protein, or small molecule drug. Because ELPs are protein-based, their sequence is genetically encoded. This facilitates their modification for fusion with peptides and protein-based therapeutics prolonging their $t_{1/2}$ and tissue residence time, protecting them from proteolysis, and providing an excellent opportunity for developing tailored treatments.8–11 ELPs naturally accumulate at high levels in kidney and liver tissues. We have recently generated and characterized in vitro an ELP fusion with human VEGF. This protein construct is highly active in vitro, the ELP fusion did not adversely affect the function of VEGF, and the addition of the ELP carrier reduced the plasma clearance rate and extended the $t_{1/2}$ compared with unconjugated VEGF.12 However, whether ELP-VEGF constructs could serve as therapeutic tools to protect the kidney has not to our knowledge been previously investigated.

We aim to determine the potential therapeutic application of ELP-VEGF constructs to renal therapy. We hypothesize that stabilization and kidney accumulation achieved by fusing VEGF to the biopolymer carrier will lead to therapeutic efficacy for renal recovery in RVD.

**RESULTS**

**In vitro Characterization of ELP-VEGF Activity**

Using primary human glomerular microvascular endothelial (HGMEM) cells, proliferation, tube formation, and migration were determined. The ELP-VEGF construct shows similar potency, inducing cell proliferation, migration, and tube formation (Figure 1, A–E). For details, please see Supplemental Material.

**In vivo Pharmacokinetics and Biodistribution of Fluorescently Labeled ELP-VEGF following Single Kidney Intrarenal Administration**

Blood was sampled at fixed time-points and plasma fluorescence measurements were taken to monitor ELP-VEGF levels. Distribution phase $t_{1/2}$ was 2.95 minutes and the terminal plasma $t_{1/2}$ was 810.1 minutes (Figure 2).

Organ biodistribution was determined 4 hours after injection. Retention of ELP-VEGF in the injected kidney was a 2.4-fold higher than the contralateral kidney (Figure 3A), although some protein did reach systemic circulation and other organs (Figure 3B). These results demonstrate that most ELP-VEGF is retained in the injected kidney, suggesting that intrarenal administration is a viable route for delivery of ELP-VEGF for therapy of RVD. For details, please see Supplemental Material.

**In vivo Efficacy of ELP-VEGF**

We then sought to determine whether a single intrarenal dose of ELP-VEGF was efficacious for improving renal function in the swine RVD model.

**General Characteristics**

Body weight, blood pressure, and degree of stenosis were similar among RVD- and RVD+ELP-VEGF–treated pigs (Tables 1 and 2).

**Multidetector Computed Tomography-Derived Renal Hemodynamics and Function**

As measured by multidetector computed tomography (MDCT), renal blood flow (RBF) and GFR were similarly attenuated in all pigs with RVD after 6 weeks of observation, which correlated with a significant increase in renal vascular resistance of the stenotic kidney and were accompanied by increased plasma creatinine (Table 1). Blunted RBF and GFR remained unchanged in RVD at 10 weeks, but were dramatically improved after ELP-VEGF therapy (around 70%) compared with 6 weeks pretreatment values (Figure 4A, top). Improvements in proteinuria, renal vascular resistance, and a plateau in plasma creatinine accompanied the improvements in renal function (Table 2), suggesting slower or halted progression of renal damage after ELP-VEGF therapy.

**Micro Computed Tomography Quantification and Morphometric Analysis of the Renal Microcirculation**

As shown by micro computed tomography (CT), the stenotic kidney showed a significant reduction in cortical and medullary MV density accompanied by substantial MV remodeling compared with normal controls (Figure 4A, bottom). Notably, intrarenal ELP-VEGF significantly improved both cortical and medullary MV density and remodeling of small and large microvessels (0–500 μm in diameter), which was evident throughout the renal parenchyma (Figure 4B).

**Effects of ELP Alone on Renal Function and MV Architecture**

General characteristics were similar to untreated RVD (Table 3), and no improvements in RBF, GFR, or MV rarefaction (Figure 4A)
were observed after administration of ELP, suggesting that the carrier did not have therapeutic effects and renal improvements were due to the polymer-stabilized VEGF construct.

Effects of Intrarenal Administration of Unconjugated VEGF on Renal Function and MV Architecture
A single intrarenal administration of unconjugated (free) VEGF121 significantly improved stenotic RBF but not GFR (P<0.05 and P=NS, respectively, versus pretreatment values) and the magnitude of those changes was significantly less compared with ELP-VEGF therapy (Figure 5A). Furthermore, intrastenotic kidney infusion of acetylcholine (quantified at 10 weeks) improved RBF but not GFR in unconjugated VEGF-treated kidneys, whereas both RBF and GFR were improved after ELP-VEGF (Figure 5B). Finally, unconjugated VEGF therapy improved MV density only in those cortical microvessels under 200 μm in diameter and not in larger microvessels (200–500 μm in diameter, Figure 5C), which matches our previous work using VEGF165.6 Overall, these findings strongly support a superior efficacy of ELP-VEGF therapy over unconjugated VEGF.

Renal Protein Expression in the Stenotic Kidney
Angiogenic Factors
Expression of VEGF, the receptor Flk-1, angiopoietin-1 and -2 (Ang-1 and Ang-2), and the Tie-2 receptor were significantly reduced in RVD but largely restored and accompanied by improved expression of phosphorylated Akt (p-Akt), stromal-derived factor (SDF)-1, and the CXCR4 receptor, and attenuated expression of antiangiogenic angiostatin after ELP-VEGF therapy, suggesting a proangiogenic milieu in the stenotic kidney of ELP-VEGF–treated pigs (Figure 6).

Inflammatory and Fibrotic Factors and Podocyte Damage
ELP-VEGF therapy decreased the renal concentration of TNF-α (Table 2), and attenuated the expression of profibrotic TGF-β, smad-4, and tissue inhibitor of matrix-metalloproteinase (TIMP)-1, whereas improved smad-7 and matrix metalloproteinase-2 (MMP-2) compared with untreated RVD, suggesting a potential decrease in proinflammatory, profibrotic, and tissue remodeling activity (Figure 7A). Furthermore, ELP-VEGF therapy improved glomerular expression of podocin (Figure 7B) and reduced nephrinuria (Table 2), suggesting protection on podocytes.

Stenotic Kidney Morphology and MV Remodeling
Glomerulosclerosis and tubule-interstitial fibrosis (7.3±0.01% and 9.3±0.04%, respectively, P<0.05 versus Normal) were significantly reduced (2.3±0.04% and 3.4±0.1%, respectively, P<0.05 versus RVD and Normal) after
ELP-VEGF therapy (Figure 7C). Similarly, MV media-to-lumen ratio (0.34 ± 0.01, P<0.05 versus Normal) was improved after ELP-VEGF therapy (0.18 ± 0.005, P<0.05 versus RVD, P=NS versus Normal), suggesting attenuated MV remodeling in addition to the improvements in MV rarefaction.

DISCUSSION

The current study focused on the design, characterization, and potential application of a novel ELP-VEGF construct for renal therapy. Our study showed that the construct is active in vitro and effective in vivo, since it stimulated proliferation, migration, and tube formation of cells, it accumulated in the injected kidney, significantly reduced renal injury, and improved renal function in the stenotic kidney. Notably, this novel compound showed a distinct protective effect on the stenotic kidney microvasculature by reducing MV remodeling and rarefaction and promoting generation of new cortical and medullary microvessels, which likely led the recovery of RBF and GFR. These findings suggest an exciting new therapeutic application of a compound that has not, to our knowledge, been previously tested in renal disease. The enhancement of the therapeutic actions without decreasing the potency or inducing collateral effects of VEGF therapy may represent a breakthrough in targeted approaches for the kidney that could go beyond RVD. Thus, our study provides a first step for pharmacologic validation of a novel drug candidate that could promote the translation of this technology toward the ultimate goal of clinical application.

CKD is a progressive disorder affecting almost 14% of the general population. It is an independent risk factor for cardiovascular morbidity and mortality, as patients with diagnosed cardiovascular disease show a staggering 40.8% prevalence of CKD, a number that has doubled in less than 20 years. Chronic RVD can progressively deteriorate renal function and lead to CKD and ESRD. It affects between 9% and 11% of the general population, but this number goes up in older patients or those with diagnosed coronary artery or peripheral vascular disease. Although renal arterial stenosis and the resultant decrease in blood flow is the initial and possibly main instigator of renal injury in RVD, therapeutic strategies that aim to resolve the vascular obstruction such as renal angioplasty and stenting are effective in recovering renal function in less than half of the cases. The disparity between technical success and outcomes has served as the impetus for numerous trials to assess the efficacy of current medical therapy (that includes renin-angiotensin blockers, lipid-lowering drugs, and so on) versus renal angioplasty. Currently, the bulk of evidence shows no significant advantages of renal angioplasty compared with standard medical therapy that could justify the risk of undergoing revascularization procedures. These observations emphasize a need for development of new treatments and strategies to improve the renal recovery prospects and outcomes in RVD.

Previous studies (including ours) showed that the progressive damage of the stenotic kidney MV architecture in RVD is accompanied (and likely mediated) by decreased expression and availability of renal VEGF and downstream effectors, suggesting blunted renal angiogenesis. It is possible that disruption of VEGF signaling in the kidney initiates MV rarefaction or alternatively, the absence of VEGF exacerbates the progressive damage of the renal microvasculature. VEGF is a pivotal angiogenic cytokine that maintains MV networks everywhere in the body and is crucial for MV repair and

Figure 2. Pharmacokinetics of ELP-VEGF in pigs show a long terminal t1/2 after single intrarenal administration. Three pigs (average weight 49.2 kg) were given fluorescently labeled ELP-VEGF by direct intrarenal administration. A balloon was inflated to block blood flow in and out of the injected kidney for 3 minutes. The balloon was released, and plasma was sampled to determine ELP-VEGF levels. Plasma levels were determined by direct detection of fluorescence and fit to a two compartment pharmacokinetic model.
proliferation via increasing recruitment of progenitor cells. Furthermore, VEGF in the kidney is crucial for the health, integrity, and function of podocytes, key components of the glomerular filtration barrier. Thus, it is clear that VEGF plays an important role in the kidney that goes beyond the angiogenic effects.

Recent studies from our laboratory as well as others have shown that administration of exogenous VEGF protects the kidney. We showed that improvement of renal VEGF signaling by a single administration of recombinant-human VEGF165 into the stenotic kidney expanded the MV architecture, improved MV rarefaction and remodeling, and decreased fibrosis, accompanied by improved hemodynamics and filtration function. Our results support the feasibility of therapeutic angiogenesis by VEGF therapy to recover renal function in RVD. However, while these data were promising, there were limitations that prevent it from being practical in the clinical setting (e.g., short t1/2 of VEGF, ease of delivery, rapid degradation, and tissue targeting). Such limitations may have played a role in the persistence of some of the renal damage despite VEGF therapy being administered at an early stage of RVD. Therefore, novel strategies to enhance the capability of VEGF therapy may have a significant impact for a rapid application of this new treatment into clinical practice.

The use of ELP technologies as a therapeutic tool, to our knowledge, has not been previously tested for renal therapy. In the current study we characterized a novel ELP-VEGF construct in vitro and in vivo. The in vitro studies showed that...
**Table 1.** General characteristics in normal, RVD, and RVD pigs before treatment with ELP/ELP-VEGF

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>RVD</th>
<th>RVD+ELP-VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>50.2±3.8</td>
<td>49.5±2.2</td>
<td>51.2±3.4</td>
</tr>
<tr>
<td>Degree of stenosis (%)</td>
<td>0.0±0.0</td>
<td>73.3±5.1²</td>
<td>74.8±11.1³</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>110.0±2.0</td>
<td>143.3±8.3²</td>
<td>135±5.1³</td>
</tr>
<tr>
<td>Plasma creatinine (µmol/l)</td>
<td>78.6±7.5</td>
<td>105.4±7.2</td>
<td>111.9±8.1</td>
</tr>
<tr>
<td>Albuminuria (µg/ml)</td>
<td>7.9±0.3</td>
<td>128.0±28.5⁴</td>
<td>108.2±44.5⁴</td>
</tr>
<tr>
<td>RVR (mmHg/ml per min)</td>
<td>0.18±0.05</td>
<td>0.56±0.1</td>
<td>0.46±0.1</td>
</tr>
<tr>
<td>Cortical volume (cc)</td>
<td>114.8±8.1</td>
<td>62.5±6.7⁴</td>
<td>74.8±4.8⁴</td>
</tr>
<tr>
<td>Medullary volume (cc)</td>
<td>35.6±2.1</td>
<td>18.1±2.2⁴</td>
<td>18.1±1.9⁴</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM, n=7 per group. Parameters were obtained after 6 weeks of observation. MAP, mean arterial pressure; RVR, renal vascular resistance.

*P<0.05 versus Normal.

ELP-VEGF could stimulate proliferation, tube formation, and migration of primary human glomerular endothelial cells at doses equivalent to unconjugated VEGF121, and that fusion to ELP did not alter the potency of VEGF. These results are consistent with our recent study showing that both unconjugated VEGF and ELP-VEGF induce such effects in HUVEC cells with equal potency.¹² We also observed that injected ELP-VEGF constructs in the swine have a prolonged t½ (concurring with our recent data¹²) and are retained in the kidney in vivo, which supported the feasibility for a following testing of the therapeutic ability of the ELP-VEGF to protect the kidney in our RVD model. We observed that single intrarenal ELP-VEGF treatment improved RBF and GFR of the stenotic kidney, parameters that remained attenuated in those placebo- or ELP-control–treated kidneys. Furthermore, improvements in stenotic kidney RBF and GFR were greater than unconjugated VEGF₁₂¹ therapy, highlighting a superior efficacy of ELP-VEGF to recover renal function.

The recovered stenotic kidney RBF and GFR were accompanied by a decreased in the MV media-to-lumen ratio and a significant expansion of both cortical and medullary MV density, which was evident in all vessels with diameters under 500 µm. We showed that intrarenal VEGF₁₆₅ therapy expands the renal microvasculature, but this effect was evident only in those microvessels of smaller diameters (under 200 µm), and was also observed after intrarenal administration of unconjugated VEGF₁₂¹ in the current study. The expansion of the smaller renal microvasculature suggests MV sprouting from preexisting vessels (angiogenesis) possibly via VEGF-integrins interactions.⁷,²⁴ On the other hand, the expansion in renal microvessels of all sizes after ELP-VEGF therapy supports a potential effect on the renal microcirculation possibly driven by both angiogenesis and also by improved repair and remodeling of the preexisting vasculature. However, we believe it is unlikely that our treatment induced proliferation of larger microvessels (e.g., interlobar arteries). Speculatively, augmented MV density of larger vessels may reflect improved MV remodeling possibly driven by increased transmitted pressure from the expanded downstream microvasculature. However, further studies are needed to elucidate this possibility.

The expansion of the renal microvasculature was accompanied and likely mediated by the distinct increased expression of VEGF and the Flk-1 receptor, Ang-1 (which may have improved the maturation and accelerated the functionality of the newly generated vessels²³), Ang-2 (which is proangiogenic when tissue levels of VEGF are high²⁶), and the Tie-2 receptor, and augmented p-Akt.²⁷ These are all crucial downstream effectors of the VEGF-mediated angiogenic cascade and suggest a potential restoration of proangiogenic activity. Furthermore, administration of ELP-VEGF improved the expression of SDF-1 and CXCR4. Together with angiopoietins, these are important promoters of mobilization and homing of cell progenitors, which are crucial steps to achieve angiogenesis that have been shown to be stimulated by VEGF.²⁹ In addition to the protective effects on the renal MV architecture, intrarenal ELP-VEGF therapy improved glomerular expression of podocin, reduced the excretion of nephrin (both major podocyte slit diaphragm-associated proteins), and reduced albuminuria, implying a reduction in podocyte damage. Since podocytes are both targets and sources of VEGF in the kidney, ELP-VEGF administration may have in turn stimulated a positive feedback mechanism that could have potentiated podocyte production of VEGF and contributed to renoprotection.

Additional benefits of renal ELP-VEGF therapy include a potential attenuation in proinflammatory and fibrogenic activity. Indeed, the renal concentration of TNF-α was reduced in the stenotic kidney after ELP-VEGF treatment, suggesting a potential reduction in the renal inflammatory milieu. Furthermore, we also observed a distinct reduction

**Table 2.** General characteristics and renal concentration (ELISA, stenotic kidney) of TNF-α in normal, RVD, and RVD pigs 4 weeks after treatment with ELP/ELP-VEGF

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>RVD</th>
<th>RVD+ELP-VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>56.8±4.8</td>
<td>59.2±2.3</td>
<td>59.5±2.7</td>
</tr>
<tr>
<td>Degree of stenosis (%)</td>
<td>0.0±0.0</td>
<td>75.3±4.6⁴</td>
<td>76.8±9.2⁴</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>110.0±2.5</td>
<td>145.2±11.4⁴</td>
<td>135.8±2.0⁴</td>
</tr>
<tr>
<td>Plasma creatinine (µmol/l)</td>
<td>83.8±6.9</td>
<td>142.4±13.4⁴</td>
<td>129.4±10.2⁴</td>
</tr>
<tr>
<td>Albuminuria (µg/ml)</td>
<td>7.6±0.4</td>
<td>145.9±39.6⁴</td>
<td>57.9±6.6⁴</td>
</tr>
<tr>
<td>Nephrin (µg/ml urine)</td>
<td>0.3±0.16</td>
<td>1.5±0.3⁴</td>
<td>0.1±0.05⁵</td>
</tr>
<tr>
<td>RVR (mmHg/ml per min)</td>
<td>0.19±0.01</td>
<td>0.54±0.07</td>
<td>0.32±0.06⁶abc</td>
</tr>
<tr>
<td>Cortical volume (cc)</td>
<td>124.1±7.0</td>
<td>64.3±8.7⁴</td>
<td>96.8±5.0⁴abc</td>
</tr>
<tr>
<td>Medullary volume (cc)</td>
<td>37.2±5.3</td>
<td>18.4±2.1⁴</td>
<td>20.2±2.5⁴</td>
</tr>
<tr>
<td>TNF-α (pg/mg tissue)</td>
<td>9.8±1.4</td>
<td>19.4±0.6⁶</td>
<td>13.4±3.2⁶abc</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM, n=7 per group. Parameters were obtained at 10 weeks. MAP, mean arterial pressure; RVR, renal vascular resistance.

⁎P<0.05 versus Normal.

⁴P<0.05 versus RVD.

⁶P<0.05 versus pretreatment.
in the renal expression of TGF-β/smad-4, TIMP-1, and augmented MMP-2 after ELP-VEGF therapy, which are pivotal mediators for extracellular-matrix accumulation and turnover that may determine the expansion of the MV networks. The TGF-β pathway is an important promoter of renal fibrosis often involved in renal disease from different etiologies. In addition, TGF-β can also lead to renal fibrosis by promoting endothelial-to-mesenchymal transition and blunting angiogenesis, an effect that may have contributed to MV rarefaction and the subsequent improvement following treatment. Therefore, an improvement in MMP-2/TIMP-1 coupled with an augmented MV proliferation may explain the expansion of the renal MV architecture after ELP-VEGF therapy. Consequently, the ELP-VEGF–treated kidney showed a reduction of renal fibrosis at both the glomerular and tubule-interstitial level.

It is possible that the greater renoprotective effects of ELP-VEGF therapy were mediated by a combined increased in tissue binding and extended t½ (or alternatively a slow plasma clearance) of the construct. These properties of ELP-VEGF may have extended the time for VEGF to bind to its receptors widely distributed in vascular endothelial cells, glomerular cells, and podocytes and amplified autocrine/paracrine actions of VEGF in the kidney, as in turn may explain the superior efficacy over unconjugated VEGF therapy. However, a potential limitation of our study may be that the model may represent an early stage of RVD and that only one time-point was evaluated, since RVD in humans is a slowly progressive disease and intrinsic kidney damage develops over months and years. Future studies are needed to determine whether the protective actions of ELP-VEGF may persist for a longer term or could also be effective when

Figure 4. Intrarenal administration of ELP-VEGF improved renal function, and cortical and medullary vascular density in the stenotic kidney. Effect of intrarenal ELP-VEGF on renal function (top) and MV architecture (three-dimensional micro-CT reconstruction, bottom) and quantification in normal, RVD-, RVD+ELP-, and RVD+ELP-VEGF–treated kidneys. *P<0.05 versus Normal; †P<0.05 versus RVD/RVD+ELP; ‡P<0.05 versus 6 weeks. (B) Cortical and medullary quantification of MV density divided by MV diameter in normal, RVD-, and RVD+ELP-VEGF–treated kidneys. *P<0.05 versus Normal; †P<0.05 versus RVD.
applied at more advanced stages of RVD. Such studies may contribute for the understanding and translation of the findings to human RVD. We are also aware that the improvements in renal expression of angiogenic or fibrogenic factors after ELP-VEGF therapy are associations and a cause-effect relationship or in-depth mechanisms were not determined. However, the clear improvements in stenotic kidney outcomes after ELP-VEGF therapy are major strengths of this preclinical study that support the potential application of ELP technology to enhance the renal therapeutic potential of VEGF supplementation. Application of ELP-VEGF therapy for the kidney may help many patients who have intermediate renal artery stenosis that, if untreated, could progress to more severe RVD and subsequent development of CKD with the added increase in cardiovascular risk.42,43 Furthermore, although the scope of the current manuscript was on the renal therapeutic effects of the constructs in RVD, it is possible that the implications of our study may go beyond renal injury and open new avenues for potential application of our VEGF delivery system to target vascular injury in other tissues. Achieving high levels of the construct in renal tissue without decreasing the activity of the therapeutic factor (VEGF) may provide the basis for new studies to determine the use of ELP in other models of acute and/or chronic renal diseases.

### CONCISE METHODS

#### In vitro Studies

**Generation of Constructs and Purification of Polypeptides**

The coding sequence for human VEGF<sub>121</sub> was fused in frame with the ELP coding sequence, and the chimeric protein was recombinantly expressed and purified, as recently described.12 For in vitro comparison studies, recombinant human VEGF<sub>121</sub> was used (ProSpec, East Brunswick, NJ).

**Cell Culture**

Culture studies were performed in primary HGME cells. Cells in passage 4–13 were used for all performed experiments. For details, please see Supplemental Material.

**HGMEM Proliferation Assay**

HGMEM cells were seeded at 10,000 cells/well in 96-well plates. Viable cells were detected after 72 hours of exposure to test agents using the MTS cell proliferation assay (Promega, Madison, WI). For details, please see Supplemental Material.

### Table 3. General characteristics in RVD pigs before and 4 weeks after intrarenal administration of free ELP (not bound to VEGF)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>RVD (6 Weeks)</th>
<th>RVD+ELP (10 Weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>47.4±6.6</td>
<td>48.4±7.5</td>
</tr>
<tr>
<td>Degree of stenosis (%)</td>
<td>74.2±8.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.9±7.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>133.1±4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>133.3±6.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RVR (mmHg/ml per min)</td>
<td>0.52±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.45±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cortical volume (cc)</td>
<td>75.6±9.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.8±9.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Medullary volume (cc)</td>
<td>27.1±6.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.1±2.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Parameters were obtained at 6 and 10 weeks. MAP, mean arterial pressure; RVR, renal vascular resistance.*

<sup>a</sup> P<0.05 versus Normal.
HGME Tube Formation Assay
Cells were seeded in 24-well plates (sterile and nontissue culture treated) coated with growth factor reduced Matrigel (BD Biosciences). After a 5-hour incubation with test agents, five nonoverlapping 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. For details, please see Supplemental Material.

HGME Migration Assay
Corning BioCoat growth factor reduced Matrigel Invasion Chambers (Corning) were used to quantify HMGE migration. Membranes were photographed using an inverted microscope and ×10 magnification objective on five independent fields per membrane. The number of cells per field were counted and averaged for each well, and the data are expressed relative to untreated wells. For details, please see Supplemental Material.

Labeling ELP-VEGF with Fluorescent Probes
ELP-VEGF was labeled with Alexa Fluor 633 succinimidyl ester (Life Technologies), as recently described. In vivo determination of Pharmacokinetics and Biodistribution after Intrarenal Administration in the Swine Model
The Institutional Animal Care and Use Committee at the University of Mississippi Medical Center approved all studies, and all animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. Three pigs were anesthetized and intrarenally injected with Alexa Fluor 633-labeled ELP-VEGF to achieve a dose of 1 mg/kg body wt. Blood was sampled from a previously placed venous catheter (jugular vein) at 1, 3, 5, 15, and 30 minutes after injection and every 30 minutes thereafter for 4 hours, and plasma was collected and frozen after centrifugation. At 4 hours, the pigs were euthanized by an overdose injection of sodium pentobarbital (100 mg/kg), and the organs were removed for analysis. For details, please see Supplemental Material.

In vivo Renal Functional Studies
The Institutional Animal Care and Use Committee at the University of Mississippi Medical Center approved all the studies. Twenty-nine prejuvenile 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 and blood pressure continuously measured by telemetry, as described. Six weeks after induction of RVD, the degree of renal artery stenosis was quantified in all pigs by renal angiography, as described. Immediately after completion of the MDCT studies, and while still under anesthesia, all RVD animals were treated with a single intrarenal (stenotic kidney) infusion of vehicle (RVD, n=7), ELP (100 µg/kg, RVD+ELP, n=5), or ELP-VEGF (100 µg/kg, RVD+ELP-VEGF, n=7). In addition, a smaller group of pigs were treated with a single intrarenal administration of unconjugated

Figure 6. Intrarenal administration of ELP-VEGF improved the expression of angiogenic factors and promoters of mobilization and homing of progenitor cells in the stenotic kidney. Representative renal protein expression (two bands per group) and quantification of VEGF, its receptor Flk-1, p-Akt, Ang-1 and Ang-2, Tie-2, angiostatin (angio), SDF-1 and its receptor CXCR4, and quantification (bottom) in normal, RVD-, and RVD+ELP-VEGF-treated kidneys. *P<0.05 versus Normal; †P<0.05 versus RVD.
VEGF$_{121}$ (at a dose of 18.65 µg/kg, which is an equimolar dose that matches the concentration of VEGF in the ELP-VEGF construct) and serve as treated controls to determine the differences in therapeutic efficacy between ELP-VEGF and unconjugated VEGF (RVD+VEGF, n=3). Blood and urine were collected (at 6 and 10 weeks) to measure plasma creatinine, nephrin in urine (suggestive of podocyte damage), and albuminuria, following vendors’ instructions. Pigs were then observed for 4 additional weeks and then MDCT in vivo studies repeated. After completion of all the in vivo studies, the pigs were euthanized, kidneys removed, and ex vivo studies performed, as shown.6,45,46 For details, please see Supplemental Material.

**High-Resolution CT Imaging**

MDCT analysis was used to calculate single-kidney RBF (ml/min), GFR (ml/min), and renal perfusion (ml/minute per cc tissue), using previously validated methods.4,47,48 For details, please see Supplemental Material. Micro-CT reconstruction and quantification of renal MV density was performed as extensively described.7,16 For details, please see Supplemental Material.

**Ex vivo Studies**

Protein expression and renal morphology were assessed in Normal, RVD, and RVD+ELP-VEGF pigs.

**Western Blotting**

Standard blotting protocols were followed, as described,5,46 to determine renal expression of VEGF, the specific receptor Flk-1, proangiogenic Ang-1 and Ang-2, and the Tie-2 receptor, p-Akt, SDF-1, and its receptor CXCR4. Furthermore, antiangiogenic angiostatin, tissue-remodeling factors TGF-β and mediators smads-4 and -7, and MMP-2 and its inhibitor TIMP-1 (all obtained from Santa Cruz Biotechnology, CA) were also measured. For details, please see Supplemental Material.

**Histology**

Midhilar 5-µm cross-sections of each kidney (one per animal) were examined to quantify tubule-interstitial fibrosis, glomerulosclerosis, and media-to-lumen ratio as described.4,7 Additional cross-sections were used to determine glomerular expression of podocin. For details, please see Supplemental Material.

**Statistical Analyses**

Results are expressed as mean±SD or SEM as indicated. Comparisons within groups were performed using paired t test, and among groups using one-way ANOVA, with Bonferroni correction for multiple comparisons. Statistical significance was accepted for P<0.05.

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**DISCLOSURES**

A.R.C. serves as a consultant for Actelion Pharmaceuticals US, Inc. G.L.B. is owner of Leffore Technologies LLC, a private company working to commercialize ELP-based technologies in several disease areas.

**REFERENCES**


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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% CO₂ 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% CO₂. 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% CO₂. 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 described¹.

**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. 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 domesticus) 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\textsuperscript{4,5}. Blood pressure was continuously measured by telemetry (PhysioTel, Data Sciences International) and averaged for each 24-hour period, as described\textsuperscript{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\textsuperscript{5,37,48}. After angiography, the catheter was positioned in the superior vena cava, and \textit{in vivo} helical MDCT flow studies were performed for quantification of single-kidney RBF, perfusion, and GFR.

Immediately after completion of the \textit{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\textsubscript{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 \textit{in vivo} studies were repeated as done at 6 weeks.
RVR was calculated at 6 and 10 weeks as recently described\textsuperscript{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 \textit{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 \textit{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\textdegree C to investigate the expression of angiogenic and fibrogenic factors by western blotting (see below) and the renal concentration of pro-inflammatory tumor necrosis factor (TNF)-\textalpha{} (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\textsuperscript{8-11}.

\textbf{High-resolution CT imaging}

\textbf{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\textsuperscript{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\(^\text{®}\) (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 angiotatin; 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≤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 810.1 minutes.

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**Disclosures:** GLB is owner of Leflore Technologies LLC, a private company working to commercialize ELP-based technologies in several disease areas.
References


