Endothelial Progenitor Cells Restore Renal Function in Chronic Experimental Renovascular Disease

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Background—Endothelial progenitor cells (EPCs) promote neovascularization and endothelial repair. Renal artery stenosis (RAS) may impair renal function by inducing intrarenal microvascular injury and remodeling. We investigated whether replenishment with EPCs would protect the renal microcirculation in chronic experimental renovascular disease.

Methods and Results—Single-kidney hemodynamics and function were assessed with the use of multidetector computed tomography in vivo in pigs with RAS, pigs with RAS 4 weeks after intrarenal infusion of autologous EPCs, and controls. Renal microvascular remodeling and angiogenic pathways were investigated ex vivo with the use of micro–computed tomography, histology, and Western blotting. EPCs increased renal expression of angiogenic factors, stimulated proliferation and maturation of new vessels, and attenuated renal microvascular remodeling and fibrosis in RAS. Furthermore, EPCs normalized the blunted renal microvascular and filtration function.

Conclusions—The present study shows that a single intrarenal infusion of autologous EPCs preserved microvascular architecture and function and decreased microvascular remodeling in experimental chronic RAS. It is likely that restoration of the angiogenic cascade by autologous EPCs involved not only generation of new vessels but also acceleration of their maturation and stabilization. This contributed to preserving the blood supply, hemodynamics, and function of the RAS kidney, supporting EPCs as a promising therapeutic intervention for preserving the kidney in renovascular disease. (Circulation. 2009;119:547-557.)

Key Words: blood flow ■ kidney ■ progenitor cells ■ renal artery stenosis ■ hypertension, renal

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Ischemic nephropathy secondary to renal artery stenosis (RAS) represents an important cause of renovascular disease and hypertension that may induce renal injury and lead to end-stage renal disease. The presence of renovascular disease also constitutes an independent predictor for increased morbidity and mortality in cardiovascular disease and cardiac events. We have shown previously that the kidney exposed to chronic RAS shows significant functional deterioration attended by renal inflammation, fibrosis, and microvascular rarefaction and remodeling. Indeed, intrarenal microvascular disease likely aggravates the progression of renal injury in RAS and may account for the failure of renal function to improve after restoration of blood flow. However, despite
pressing clinical need, targeted interventions capable of protecting the kidney or reversing its injury in chronic renovascular disease are yet to be identified.

Recent evidence suggests potential for cell-based repair interventions in rodent models of renal injury.13,14 Nevertheless, the potential utility of progenitor cells for preserving the function and structure of the kidney in a model of chronic renovascular disease has not been investigated. Therefore, the present study was designed to test the hypothesis that replenishment of progenitor cells would improve renal function by protecting the vascular integrity of the stenotic porcine kidney.

**Methods**

The Institutional Animal Care and Use Committee approved all the procedures (A17807). Twenty-one domestic pigs (weight, 55 to 65 kg) were studied after 6 weeks and again after 10 to 12 weeks of observation. In 14 pigs (Pork Partners, Stewartville, Minn), a local-irritant coil was placed in the main renal artery at baseline and induced gradual development of unilateral RAS. We have shown previously that by 4 to 5 weeks after coil implantation, this model already exhibits a chronic decrease in renal function and that significant stenoses induce pathological renal alterations that closely resemble chronic renovascular disease in humans, including inflammation, fibrosis, and mild glomerulosclerosis.10,11,13,16 The pigs had renal angiography 6 weeks after induction of RAS and were then randomized into 2 groups that were not further treated (RAS group; n = 7) or treated with an intrarenal infusion of autologous EPCs (RAS+EPC group; n = 7). To generate EPCs, peripheral mononuclear cells were isolated from each pig 3 and 5 weeks after induction of RAS, expanded in vitro, and delivered to the same RAS+EPC pig during the 6-week renal angiography. Four weeks later, all animals underwent repeated renal angiography, as well as in vivo studies. Throughout these 10 weeks, blood pressure was monitored continuously with a telemetry system (PhysioTel, Data Sciences) implanted at baseline in the left femoral artery. Mean arterial pressure (MAP) was recorded with telemetry at 5-minute intervals and averaged for each 24-hour period10,11,17 and was also measured during in vivo studies with a side arm of the arterial catheter. The other 7 pigs were used as controls (normal group; n = 7).

By 6 weeks after induction of RAS, all of the pigs underwent renal angiography, as mentioned above. For this, all the pigs were anesthetized with intramuscular Telazol (5 mg/kg) and xylazine (2 mg/kg), intubated, and mechanically ventilated with room air. Anesthesia was maintained with a mixture of ketamine (0.2 mg/kg per minute) and xylazine (0.03 mg/kg per minute) in normal saline, administered via an ear vein cannula (0.05 mL/kg per minute). Under sterile conditions and fluoroscopic guidance, an 8F arterial catheter was advanced to the stenotic renal artery, proximal to the stenosis. Short bolus injections (4 to 6 mL) of low-osmolar nonionic contrast media (iopamidol, Isovue-370; Squibb Diagnostics, Princeton, NJ) were used to visualize the lumen of the renal artery with a fluoroscopy system (Siemens Siremobil Compact), and images were then recorded and later analyzed offline to determine the degree of RAS, as described previously.17 After angiography, in the RAS+EPC animals, EPCs (10⁶ cells/mL suspended in 10 mL of saline) were delivered into the stenotic renal artery (for details, please see the online-only Data Supplement).

Four weeks later, all the animals were again anesthetized similarly for repeated renal angiography, which was followed by in vivo functional studies. After angiography, the catheter was positioned in the superior vena cava, and in vivo helical multidetector computed tomography (CT) flow studies were performed for assessment of basal regional renal perfusion, renal blood flow (RBF), and glomerular filtration rate (GFR), as detailed previously.9,11,15,16 Briefly, this involved sequential acquisition of 160 consecutive scans after a central venous injection of iopamidol (0.5 mL/kg per 2 seconds), repeated during suprapenal infusion of acetylecholine (5 μg/kg per minute) to test endothelium-dependent responses. Blood samples were collected from the inferior vena cava and both renal veins for measurement of plasma renin activity (PRA) (radioimmunoassay) and systemic asymmetrical dimethylarginine (ADMA) levels (Euroimmun US LLC, Boonton Twp, NJ). Urine samples were collected by suprapubic bladder puncture, and protein content was measured by spectrophotometry with the Bradford method.

After completion of all studies, the pigs were allowed to recover for a few days (to allow for contrast media washout) and were then euthanized with a lethal intravenous dose of sodium pentobarbital (100 mg/kg; Sleepaway, Fort Dodge Laboratories, Inc, Fort Dodge, Iowa). Both kidneys were removed from each pig with the use of a retroperitoneal incision and immersed in 4°C Krebs’ solution containing heparin. A lobe of tissue was immersed in 10% buffered formalin (Sigma, St Louis, Mo), and a segmental artery perfusing the intact end of the stenotic kidney was cannulated and prepared for micro-CT. Other lobes were shock-frozen in liquid nitrogen and stored at −80°C or preserved in formalin.10,11,15

In vitro studies were then performed to assess renal histology and expression of angiogenic and fibrotic factors. Western blotting and immunohistochemistry were used to probe expression of the proangiogenic factors phosphorylated (p)-Akt, p-endothelial nitric oxide synthase (p-eNOS), vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF)-α, angiopoietin-1, and integrin β1. The expression of markers and mediators of renal fibrosis such as transforming growth factor (TGF)-β, tissue inhibitor of metalloproteinases (TIMP)-1, α-smooth muscle actin (α-SMA), and matrix metalloproteinases (MMP)-2 and -9 was also investigated. Furthermore, microvascular and renal tissue remodeling were assessed in 5-μm mid hilar renal paraffin-embedded slices stained with trichrome, and the presence of resident progenitor cell in the kidney was assessed by immunoreactivity of Oct-4.19 Double immunofluorescence for Dil and CD31 or cytokeratin was used to localize the EPCs in renal vessels or tubules, respectively.

**Progenitor Cells**

**Blood Collection and Cell Isolation**

Late and early EPCs were obtained as described previously.20–24 Late EPCs were cultured from peripheral mononuclear cells collected 21 days before administration, and early EPCs were obtained from cells collected and cultured 7 days before in vivo CT studies. All cells were cultured in endothelial growth medium. An equal blend of early and late EPCs (10⁶ cells) was subsequently delivered into the renal artery (see online-only Data Supplement), in agreement with their synergistic effect in promoting neovascularization20,23,24 compared with each cell type alone.

**Colony-Forming Units**

Colony-forming units (CFUs) were counted to assess the availability of circulating EPCs. EPC colonies consisting of multiple thin, flat cells emanating from a central cluster of rounded cells were counted after 7 days of culture in 10 random (>20) microscope fields per subject and expressed as CFU/cm².25,26

**Characterization of EPC Markers**

Immunofluorescence and/or Western blotting was used to determine the monocytic (CD14), progenitor (CD34, CD133), endothelial (KDR) phenotype,27 and stem cell pluripotency (Oct-4) of early and late EPCs.

**Growth Factor and Cytokine Measurement**

To determine the production and secretion of growth factors by EPCs, the culture media of late EPCs were collected for measurement of VEGF levels. The cells were then homogenized, and expression of VEGF and eNOS was evaluated.

**EPC Function**

EPC function was tested with several accepted tests28 such as acetylated low-density lipoprotein uptake, cell migration, proliferation, and tube formation.
Preparation and Delivery of EPCs

Just before delivery, all cells were labeled with both a fluorescent membrane dye (CM-DiI) and fluorescent beads.\textsuperscript{29} CM-DiI (5 μL/mL) was added to the culture medium and incubated for 30 minutes at 37°C. Fluoresbrite plain 2-μm YG (yellow-green) polymeric beads (Polysciences, Warrington, Pa) were added at a 1:25 cell-to-microspheres ratio and incubated 75 minutes at 37°C.

EPC Localization and Retention

EPC localization and retention were estimated from cells observed in kidney sections from the stenotic and contralateral kidneys.\textsuperscript{30} Labelled cells were counted manually under fluorescence microscopy in frozen 5-μm renal cross sections, the total area of each cross section was calculated, and the number of cells per square millimeter was averaged and multiplied by the section thickness and then by the total renal volume.

EPC Engraftment

Double fluorescence of CM-DiI and CD31 or cytokeratin was examined to investigate the location and phenotypical changes of EPCs into endothelial or tubular cells, respectively (see the online-only Data Supplement).

Micro-CT

A side branch of the renal artery in the dissected kidney was cannulated and infused under physiological perfusion pressure with heparinized (10 U/mL) saline, followed by the radio-opaque silicone polymer Microfil, until it filled the intrarenal vessels. For details, see the online-only Data Supplement.

Renal Protein Expression, Western Blotting, and Apoptosis

Immunohistochemistry

Staining was performed in 5-μm frozen or unstained midhilal renal cross sections to assess the expression of integrin β₃, α-SMA, and Oct-4. For details, see the online-only Data Supplement.

Western Blotting

Standard blotting protocols were followed, as described previously,\textsuperscript{9} with the use of specific polyclonal antibodies against p-Akt, p-eNOS, VEGF, HIF-1α, angiopoietin-1, TGF-β, MMP-2 and -9, TIMP-3, CD133, KDR, and Oct-4. β-Actins or GADPH was used as loading control. Protein expression (1 band per animal) was quantified by densitometry and averaged in each group. For details, see the online-only Data Supplement.

Apoptosis

For quantification of apoptotic cells, DeadEnd Fluorometric TUNEL System (Promega) was used in 5-μm renal midhilal cross sections, as shown before.\textsuperscript{11}

Data Analysis

Renal Angiography

The degree of RAS was measured by quantitative renal angiography, as described previously,\textsuperscript{9,10,15,16,31} and assessed as the decrease in luminal diameter of the renal artery at the most stenotic point compared with a proximal stenosis-free segment.

Multidetector CT Analysis

Manually traced regions of interest were selected in multidetector CT images in the aorta, renal cortex, and medulla, and time-enhancement curves were generated and analyzed to calculate RBF and GFR.

Micro-CT Analysis

Images were digitized for reconstruction of 3-dimensional volume images and analyzed with the Analyze software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, Minn), as described previously.\textsuperscript{11,32} Renal cortical microvascular density, vascular volume fraction, and single-microvascular tortuosity were calculated. For more details, see the online-only Data Supplement.

Histology

Midhilal 5-μm trichrome-stained cross sections of each kidney (1 per animal) were examined to quantify renal fibrosis, glomerulosclerosis, and Oct-4 immunoreactivity, as described previously,\textsuperscript{10,11} and peritubular capillary density was similarly quantified in CD31-stained slides. For details, see the online-only Data Supplement. For quantification of angiogenic vessels, 10 fields were randomly selected from each integrin β₃-stained slide (1 per animal). The stained vessels were counted manually in each field and averaged, and the results were expressed as number of integrin β₃-positive vessels per field. For apoptosis, the fraction of apoptotic cells was calculated in 10 randomly selected fields in each slide, as we described previously.\textsuperscript{11}

Statistical Analysis

Results are expressed as mean±SEM. Comparisons within groups were performed with the paired Student t test and among groups with 1-way ANOVA, with Student-Newman-Keuls post hoc tests for correction for multiple comparisons. Statistical significance was accepted for \( P \leq 0.05 \). For data measured over time (blood pressure), a 2-way repeated-measures ANOVA was used, and statistical significance was accepted for \( P \leq 0.05 \).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Characterization of EPCs

Both CD14 and CD133 were initially expressed in early cells, but after 21 days the expression of KDR increased as CD14 and CD133 diminished in late cells (see the online-only Data Supplement), suggesting that late EPCs acquired endothelial characteristics. The cultured EPCs were also Oct-4 positive (see the online-only Data Supplement). The number of CFUs and EPC migration were similar in RAS compared with normal pigs, but, interestingly, cells from RAS pigs showed increased proliferation, tube formation, and secretion of angiogenic factors compared with EPCs obtained from normal pigs (see the online-only Data Supplement).

Renal Function

MAP and the angiographic degree of stenosis were similarly and significantly greater in RAS and RAS+EPC animals compared with normal animals, whereas systemic PRA and ADMA levels were similar among the groups, as was renal vein PRA (Table and Figure 1, top). Basal RBF, cortical perfusion, and GFR were all diminished in RAS, but RBF and GFR significantly improved after EPC treatment (ANOVA \( P<0.03 \) for RBF and GFR, ANOVA \( P=\text{NS} \) for perfusion). Similarly, RBF and GFR responses to the endothelium-dependent vasodilator acetylcholine that were blunted in RAS were restored in RAS+EPC, suggesting improved renovascular endothelial function (Figure 1, bottom). These were accompanied by a significant increase in renal expression of p-eNOS in RAS+EPC, implying greater potential for NO availability.

Angiogenic Factors

The blunted expression of proangiogenic HIF-1α and VEGF in RAS was increased after EPC treatment (ANOVA...
Table. Characteristics and Basal Stenotic Kidney Function in Normal, RAS, and RAS Pigs Treated With Autologous EPCs (RAS+EPC)

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=7)</th>
<th>RAS (n=7)</th>
<th>RAS+EPC (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>47.3±2.3</td>
<td>50.7±1.9</td>
<td>51.7±2.0</td>
</tr>
<tr>
<td>Degree of stenosis, %</td>
<td>0.0±0.0</td>
<td>70.4±5.2*</td>
<td>69.3±6.0*</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>104.7±2.2</td>
<td>140.2±7.1*</td>
<td>130.3±6.9*</td>
</tr>
<tr>
<td>PRA, ng/(mL·h)</td>
<td></td>
<td></td>
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<tr>
<td>Inferior vena cava</td>
<td>0.22±0.04</td>
<td>0.19±0.03</td>
<td>0.21±0.04</td>
</tr>
<tr>
<td>Stenotic kidney</td>
<td>...</td>
<td>0.20±0.04</td>
<td>0.31±0.13</td>
</tr>
<tr>
<td>Contralateral kidney</td>
<td>...</td>
<td>0.19±0.02</td>
<td>0.17±0.06</td>
</tr>
<tr>
<td>Plasma ADMA, μmol/L</td>
<td>1.47±0.1</td>
<td>1.46±0.08</td>
<td>1.57±0.15</td>
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<td>Renal volume, mL</td>
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<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>98.6±7.1</td>
<td>47.0±13.4*</td>
<td>99.2±5.0</td>
</tr>
<tr>
<td>Medulla</td>
<td>43.9±2.4</td>
<td>21.9±6.4*</td>
<td>20.5±2.5*</td>
</tr>
<tr>
<td>RBF, mL/min</td>
<td>566.5±46.9</td>
<td>301.90±6.92*</td>
<td>452.93±24.7†</td>
</tr>
<tr>
<td>GFR, mL/min</td>
<td>70.8±4.3</td>
<td>47.9±10.1*</td>
<td>62.7±1.5</td>
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<tr>
<td>Perfusion, mL/(min · mL)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>4.1±0.3</td>
<td>2.7±0.6</td>
<td>4.1±0.2</td>
</tr>
<tr>
<td>Medulla</td>
<td>2.7±0.4</td>
<td>2.7±0.2</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>Proteinuria, μg/mL</td>
<td>14.3±3.7</td>
<td>21.6±4.8</td>
<td>16.8±4.1</td>
</tr>
</tbody>
</table>

Values are mean±SEM. MAP was measured during CT studies. *P<0.05 vs normal, †P=0.06 vs normal.

P=0.004 and P=0.01, respectively), suggesting a proangiogenic milieu in the treated RAS kidney. Furthermore, renal expression of p-Akt and p-eNOS, key mediators of VEGF, were significantly augmented in RAS+EPC compared with untreated RAS. This was accompanied in RAS+EPC by increased renal expression of integrin β₁ (19.7±3.3 versus 10.6±2.8 and 12.9±1.5 positive vessels per field in RAS and normal, respectively; ANOVA P=0.02) and angiopoietin-1 (ANOVA P=0.03; Figure 2), suggesting that EPCs not only promoted angiogenesis but also favored the maturation of the new vessels.

Microvascular 3-Dimensional Architecture
Microvascular density was diminished in RAS across the renal cortex (inner, middle, and outer cortex). Notably, RAS+EPC substantially increased cortical microvascular density in all the cortical regions (although it remained lower than normal), resulting in improved vascular volume fraction (ANOVA P=0.0002; Figure 3). Microvascular tortuosity was significantly increased in RAS+EPC kidneys compared with both RAS and normal controls (1.87±0.09 versus 1.30±0.02 and 1.39±0.09, respectively; ANOVA P=0.0007), supporting the notion of abundant angiogenic vessels. Furthermore, CD31 expression on capillaries was significantly reduced in RAS compared with normal (0.29±0.04% versus 1.91±0.6%; P=0.01) but improved in RAS+EPC (1.13±0.6%; P=0.3 versus normal and P=0.05 versus RAS), suggesting augmented capillary proliferation in the treated kidney.

Renal EPCs and Morphology
An average of 13.3±0.8% of the total injected EPCs was detected 4 weeks later; the cells were evident at the tubular and vascular compartments of the treated stenotic kidneys. Some of the injected EPCs were detected incorporated in very small (capillaries and vasa vasorum) microvessels (CD31⁺) and in tubules (cytokeratin⁺) and costained with these markers, suggesting that they assumed endothelial and tubular characteristics (see Figure V in the online-only Data Supplement). Interestingly, the number of Oct-4⁺ cells in renal tubules was significantly increased after EPC treatment, many of which contained DiI fluorescence, indicating that they originated from injected EPCs. Nevertheless, only EPC-treated stenotic kidneys also showed Oct-4⁺ cells unlabeled with DiI (Figure 4A), suggesting activation and mobilization of circulating or resident stem cells (Figure 4B).

RAS kidneys showed increased expression of TGF-β and α-SMA and decreased MMP-2 (Figure 5A), which were accompanied by increased glomerulosclerosis and perivascular and tubulointerstitial fibrosis compared with normal controls (Figure 5B), overall suggesting renal remodeling. Importantly, EPCs improved the expression of those factors and decreased fibrosis in the stenotic kidney, without fully normalizing it (ANOVA P=0.001). Conversely, EPCs did not affect apoptosis, as the fraction of apoptotic cells was significantly and similarly elevated in RAS and RAS+EPC compared with normal (0.59±0.1%, 0.52±0.2%, and 0.09±0.01%, respectively; P<0.04).

Contralateral Kidney
The presence of labeled cells and changes in morphology in the contralateral kidney were determined. Interestingly, although few EPCs were observed in the contralateral kidney of RAS+EPC animals (1 to 2 cells per slide), VEGF expression was increased compared with both normal and untreated RAS pigs. However, unlike the RAS+EPC kidney, the contralateral kidney of RAS and RAS+EPC animals did not show any changes in tubulointerstitial fibrosis (2.09±0.1% and 1.83±0.05%, respectively; P<0.01 versus normal), blunted number of capillaries (0.64±0.04% and 0.70±0.04%, respectively; P<0.05 versus normal), or renal expression of TGF-β (elevated compared with normal), MMP-2 and -9 (unchanged), and TIMP-1 (attenuated compared with normal), implying lack of effect of these cells in remodeling of the contralateral kidney (Figure 6). Apoptosis also remained evident (1.22±0.47 and 1.07±0.30, respectively; P<0.05 versus normal).

Discussion
The present study shows, for the first time, the feasibility of a cell-based approach to treat the ischemic kidney distal to the stenosis. A single intrarenal infusion of autologous EPCs during the evolution of RAS restored the hemodynamics and function of the ischemic kidney, preserved microvascular architecture, and attenuated renal remodeling. The capability of EPCs to restore vascular integrity and preserve the stenotic kidney may therefore enable development of novel...
therapeutic renoprotective strategies in chronic renovascular disease.

Renovascular disease is often associated with atherosclerosis but constitutes a strong predictor for increasing morbidity and mortality independent of other cardiovascular risk factors, as is a decrease in GFR. Renal disease is often characterized by decreased microvascular density as well as tubulointerstitial fibrosis, which are determinants of renal outcomes. Regression of intrarenal microvasculature accompanies many forms of renal disease such as diabetes and aging, and microvascular remodeling correlates with development of renal scarring. Indeed, we have previously shown that renal injury is evident by 1 month after development of the RAS and that deterioration of renal hemodynamics and function is paralleled by significant fibrosis, microvascular rarefaction, and remodeling. Microvascular and parenchymal damage distal to the obstruction likely contributes to renal dysfunction observed in humans with chronic renovascular disease despite revascularization, but few therapeutic options are available to restore renal viability and vascular integrity.

In many ischemic and injured organs, mobilization, homing, and transdifferentiation of EPCs play an important role in augmenting neovascularization and endothelial replacement after vascular injury. EPCs augment angiogenesis both by stimulating the secretion of angiogenic growth factors and by providing a source of progenitor cells that can differentiate into mature vascular endothelial cells. They appear to confer their beneficial effect not only by their long-term engraftment and local retention in the injured tissue but also by transient secretion of vascular growth factors within this region. Yet, despite the promise of EPC delivery in treating diseases associated with blood vessel disorders, the potential of this strategy to salvage the ischemic kidney in RAS has not been explored. The present study indicates that this strategy is feasible and effective. In the present study, we delivered into the stenotic kidney a combination of early EPCs, which retain monocytic characteristics and avid angiogenic activity, and late EPCs, which exhibit more mature endothelial-like features. Previous studies have demonstrated that these cell types synergistically enhance angiogenesis more effectively than each cell type alone. This intervention elicited new vessel formation and reversed most of the functional and structural deterioration of the stenotic kidney. We observed that some of the injected cells assumed endothelial and tubular phenotypes and incorporated into renal structures. However, when the relatively small number of EPCs retained in the tissue is considered, their autocrine and paracrine activities were

![Figure 1.](http://circ.ahajournals.org/)

**Figure 1.** Top, Changes in blood pressure over time (n=3 to 4 animals per group). Bottom, RBF (A), GFR (B), and cortical perfusion (C) at baseline and in response to acetylcholine in normal, RAS, and RAS treated with autologous EPCs (RAS+EPC) animals. EPC infusion did not decrease blood pressure in RAS. The blunted basal and challenged renal hemodynamics and function in RAS were normalized after EPC treatment, suggesting restoration of renovascular endothelial function. *P<0.05 vs normal, †P<0.05 vs baseline, **P<0.05 vs normal (2-way repeated-measures ANOVA).
likely key for the increase in protein expression and improvement in renal function.

Angiogenesis involves a sequence of events regulated by numerous factors that results in development of new vessels. Among those factors, VEGF is crucial for preservation of the microvasculature and, in concert with other factors, stimulates processes responsible for cell division, migration, and survival, extracellular matrix degradation, and tube formation that generate, repair, and maintain microvascular networks. We have previously shown both HIF-1α and VEGF to be paradoxically downregulated in chronic RAS, attended by microvascular rarefaction and renal fibrosis. HIF-1α is the most important transcription factor driving VEGF mRNA expression and production and is considered a crucial primary defense mechanism for the adaptive response to ischemia in the kidney. Remarkably, intrarenal administration of EPCs in the stenotic kidney restored HIF-1α, VEGF, eNOS, Akt, and angiopoietin-1, all of which stimulate or mediate angiogenesis. Angiopoietin-1 is an endothelial cell survival factor that in concert with VEGF can promote angiogenesis, maturation of the new vessels, and vascular repair, and the concurrent improvement of both may have had an additive effect on vascular proliferation and maturation in RAS+EPC. Augmented neovascularization was also reflected by the in-

Figure 2. Representative immunoblots (top) and densitometric quantification (bottom) demonstrating renal protein expression of p-Akt, p-eNOS, VEGF, HIF-1α, and angiopoietin-1 in the following groups: normal, RAS, and RAS treated with autologous EPCs (RAS+EPC). Replenishment of EPC in RAS augmented the expression of vascular growth factors in the stenotic kidney, suggesting a proangiogenic stimulus. *P<0.05 vs normal, †P<0.05 vs RAS, #P=0.079 vs normal.

Figure 3. Representative 3-dimensional tomographic images of renal cortex and medulla (top) and quantification (bottom) from normal, RAS, and RAS treated with autologous EPCs (RAS+EPC) kidneys. EPCs in RAS augmented intrarenal microvascular density throughout the cortex, which consequently restored renal vascular volume fraction. *P<0.05 vs normal, †P<0.05 vs RAS.
creases in renal expression of integrin β₃, peritubular CD31⁺ capillaries, microvascular density, and tortuosity, which are all indices of angiogenic vessels. Therefore, EPCs not only attenuated microvascular dysfunction and rarefaction in RAS but also improved maturation and stabilization of the new vessels, thereby improving the overall hemodynamics and function of the stenotic kidney. Although apoptosis remained elevated in RAS+EPC, this therapeutic approach significantly decreased the scarring in the stenotic kidney, possibly as a result of increased blood supply and NO availability after EPC treatment. Furthermore, it may have also been related to a decreased fibrogenic activity and improved matrix turnover, as suggested by the improved expression of TGF-β and MMP-2 and decreased fibrosis and glomerulosclerosis in RAS+EPC, which in turn may have facilitated restoration of the microvascular network as well.

The number of CFUs that we found is comparable to previous studies in which similar techniques were used.²⁶ We observed that CFUs were similar in normal and RAS animals, suggesting that circulating EPCs were not depleted in RAS, yet RAS EPCs showed increased proliferation potential and better angiogenic function (tube formation) compared with controls. These characteristics differ from EPC function in humans with chronic refractory hypertension⁴¹ but are more consistent with other clinical studies on EPCs in essential or pregnancy-induced hypertension⁴²,⁴³ or with recent studies in comparable models.⁴⁴ It is possible that duration and nature of the disease, as well as species differences, may have contributed to these differences. For example, a preferential increase in PRA at the early stage (4 to 5 weeks) of the disease³¹ might favor angiotensin II–mediated angiogenic activity ⁴⁵ that would gradually dissipate as the disease progresses and oxidative stress increases.³¹ Interestingly, the present study shows that delivery of autologous EPCs into the stenotic kidney also increased number of cells positive for Oct-4, a stem cell transcription factor and marker suggestive of pluripotency.¹⁹ Although many of these were likely the Oct-4⁺–injected EPCs, at least some of them were not labeled with the EPC markers, suggesting that this intervention also promoted mobilization of resident or homing of endogenous circulating progenitor cells. Either by incorporating and differentiating in the tissue host and/or by autocrine and paracrine activity, EPCs are capable of stimulating the function and proliferation of surrounding progenitor and mature cells.²⁰ Importantly, some of the injected EPCs were...
detected engrafted into blood vessels and tubules and assumed at least some endothelial and tubular features (CD31 and cytokeratin expression), although the level of functionality that the engrafted cells achieved remains to be determined. Interestingly, EPCs were observed incorporated only in very small microvascular vessels, likely peritubular capillaries and vasa vasorum, but not in larger vessels. Presumably, longer transit times and greater surface area facilitate their contact, adherence, retention, and migration into tissue structures.

Importantly, our study showed that EPCs restored the blunted RBF and GFR of the RAS kidney, which might be a result of the preserved microvasculature but also is likely due to augmented availability of eNOS-derived NO, as implied by the increased renal expression of activated eNOS. In turn, NO is indispensable for microvascular sprouting by maintaining vasodilation during the early steps of angiogenesis and by promoting VEGF-induced capillary proliferation. Furthermore, eNOS-derived NO might have contributed to enhance Oct-4 expression in resident progenitor cells and promoted endothelial differentiation in the RAS + EPC kidney. Hence, upregulated eNOS not only favored renal microvascular endothelial function but may have also contributed to angiogenesis in the RAS + EPC kidney. Moreover, VEGF is also a potent vasodilator that may have contributed to recovering endothelial function in RAS + EPC. It is interesting that although the degree of stenosis and hypertension in RAS remained unchanged by EPCs, renal function improved with intrarenal EPC therapy. Although the remaining obstruction of the renal artery and decrease in renal perfusion pressure might have been sufficient to activate the intrarenal renin-angiotensin system, renal vein PRA did not lateralize to the stenotic side. Alternatively, an increase in oxidative stress may also sustain hypertension at the chronic phase of untreated RAS when PRA declines. In addition, we cannot rule out the possibility that disease in the contralateral kidney results in volume retention and thereby mediates hypertension. Indeed, the sustained fibrosis and decreased capillary density in both the stenotic and contralateral kidneys indicate residual injury that might have also contributed to the persistent hypertension.

In summary, the present study shows the renoprotective effects of EPCs in a model of chronic RAS. A targeted intervention using autologous EPCs during the evolution of
the disease reversed most of the functional and structural deterioration of the stenotic kidney in this otherwise progressive disease. It is likely that restoration of the angiogenic cascade by autologous EPCs involved not only generation of new vessels but also acceleration of their maturation and stabilization. This contributed to preserving the blood supply, hemodynamics, and function of the RAS kidney and thereby decreased renal remodeling. Future studies are needed to examine the feasibility of this approach after a longer duration of chronic renal ischemia in humans and in the presence of additional cardiovascular risk factors.

Acknowledgments
The authors are grateful to Mark E. Rosenberg, MD, University of Minnesota, for his advice on the Oct-4 staining technique.

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References

Disclosures
None.


CLINICAL PERSPECTIVE

Significant attention has been directed to the biologic and therapeutic capabilities of progenitor cells. Endothelial progenitor cells mobilized endogenously in response to ischemia play a crucial role in augmenting neovascularization of ischemic tissues and repair of the vessel wall after endothelial cell denudation. A large body of experimental and clinical evidence accumulated over the last 10 years demonstrating that administration of endothelial progenitor cells could improve the function of the ischemic tissues. The present study tested the feasibility of using progenitor cells to treat the obstructed kidney in a model of chronic renovascular disease. This disease is difficult to treat and may induce hypertension and renal injury, leading to end-stage renal disease. In this model, a single intrarenal administration of endothelial progenitor cells restored renal hemodynamics and function, preserved renal microvascular architecture, and attenuated fibrosis of the ischemic kidney. The demonstrated capability of endothelial progenitor cells to restore vascular integrity and preserve the stenotic kidney may constitute an important step for designing novel therapeutic measures for management of patients with renovascular disease.
SUPPLEMENTAL MATERIAL

Progenitor cells

Blood collection and isolation of cells. Mononuclear cells were obtained from peripheral blood (100 mL), as described previously. Late EPC were obtained from cells collected 3 weeks after the baseline procedure (induction of RAS), and subsequently cultured for 3 weeks, while early EPC were obtained from cells collected and cultured 7 days before the in vivo CT studies. To obtain the cells, animals were sedated with IM telazol (5 mg/kg) and xylazine (2 mg/kg), blood drawn from the femoral vein (100 ml) under sterile conditions, and then divided in heparinized Falcon tubes placed on ice. Mononuclear cells were isolated from the blood by the density gradient method using Histopaque 1077 (Sigma, St. Louis) at 1600 rpm for 25 min, washed with PBS, treated with ammonium chloride (Stem Cell Technologies, Vancouver, BC) to eliminate red blood cells. Cells were re-suspended in EGM-2 plus EGM-2 Singlequot medium (Cambrex, Walkersville, MD). This medium contains multiple growth factors including human VEGF A, fibroblast growth factor-2, endothelial growth factor, insulin-like growth factor-1, and ascorbic acid, as well as gentamycin and amphotericin B. Cells were plated on 6-well plates (Corning Incorporated, Corning, NY) coated with fibronectin (1µg/cm², Sigma, St. Louis, MO), with initial seeding density standardized at 4x10⁶ cells per well. After 2 days of culture, non-adherent cells were removed and the media changed daily for 1 week, and every other day for the remaining time. The attached cell population at days 4-10 is usually mixed with both round and spindle cells that exhibit many characteristics of EPC, but little proliferative capacity. Late EPC colonies typically sprout around days 11-18, and are comprised of larger cells with mature endothelial cell culture characteristics (cobblestone monolayer, contact inhibition, and rapid proliferation). Cultures were examined by light microscopy and samples assayed for number,
function, and phenotyped before delivery.

**Colony forming unit counting.** Colonies consisting of multiple thin, flat cells emanating from a central cluster of rounded (and some spindle-shaped) cells were counted after 7 days of culture in 10 field under (x20) microscope, and expressed as colonies per cm$^2$ (Figure 1s-top right).

**Characterization of EPC marker expression.** Immuno-fluorescence and Western blotting (see below) were used to determine cellular phenotype. For immuno-fluorescence, cells were incubated with primary antibodies (all 1:50) against CD34 (R&D Systems, MN, Cat# AF3890, NS0-derived rpCD34), CD14 (Serotec, Oxford, UK Cat#MCA1218, Clone: MIL-2), CD133 (GeneTex, TX, Cat# GTX16518, Clone: RB1784), KDR (Santa Cruz, CA, Cat# sc-504, Clone: C-1158), and Oct-4 (1:50, Lifespan Biosciences, Seattle, WA, Cat# LS-B85, Clone: Octamer-binding Transcription Factor 3A (POU5F1)) for 1h at room temperature. FITC-goat anti-mouse or anti-rabbit IgG (or Texas red conjugated) secondary antibodies (ZYMED Laboratories, South San Francisco, CA) were incubated for 30 min at room temperature. To enable visualization of nuclei, DAPI mount media (Santa Cruz, CA) was used. Isotype identical IgG antibodies served as negative controls. Slides were examined under fluorescence microscopy (Figure 1s-left).

**EPC function** were tested using several accepted tests.

**Acetylated LDL uptake:** Cells were incubated with Dil-labeled ac-LDL (Molecular Probes, Eugene, Ore), samples examined with an inverted fluorescence microscope (Nikon), and fluorescent cells identified as EPC (Figure 2s-top).

**EPC migratory function:** VEGF-induced cell migration was examined using a modified Boyden chamber. A 24-well Transwell apparatus (Coster, Corning Inc., Corning, NY) was used, with each well containing a 6.5-mm polycarbonate membrane with 8-µm pores. Cells (4x10$^4$) were placed on the membrane, and the chamber immersed in a plate filled with growth factor-free
EBM-2 culture media, with addition of 2000 pg/ml human VEGF165 (R&D System, Minneapolis, MN). After incubation for 24-h, the membrane was washed briefly with PBS, and its upper side wiped gently with a cotton ball. The membrane was then removed and mounted with DAPI fluorescence medium (Santa Cruz, CA). EPC migration was evaluated by counting the migrated cells in 4 random high-power (x40) fluorescence microscope fields (Figure 2s-bottom-left).

*Proliferation Assay:* Late EPC were seeded at $3 \times 10^3$/well in 96-well flat-bottomed plates in EGM-2 medium containing 2% FCS and allowed to adhere for 2 h. After 24h culture with or without concurrent exposure to different concentration of vitamins, the proliferative activity was determined by MTS assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI, Figure 2s bottom-right), which monitors the number of viable cells, according to vendor instructions. Briefly, MTS solution was added at 20 µl/well and, after 4 h culture, the conversion of MTS to formazan was measured in a plate reader at 490 nm.

*Matrigel Tube Formation Assay:* assessed the ability of EPC to incorporate into endothelial cells and form vascular structures. Matrigel (BD Biosciences, Bedford, MA) was spread onto 24-well plates (Coster, Corning Inc., Corning, NY)) and allowed to polymerize for 15 min at 37°C. For the assay, early and late EPC were marked with DiI (Molecular Probes) and mixed with human umbilical endothelial cells (HUVEC, PromoCell, Heidelberg, Germany. DiI-labeled EPC ($1 \times 10^4$) and HUVEC ($4 \times 10^4$) were plated together and incubated at 37°C for 24h with EGM-2 culture medium. Tube length, and number, as well as the cells incorporated into HUVEC tubes, were counted in 4 random (x20) fields per subject and measured using MetaMorph® image analysis software (Meta Imaging Series 6.3.2, Allentown, PA). Experiments were done in triplicate and observers blinded to cell type and group (Figure 3s).
Growth factor and cytokine measurement

To determine the ability of EPC generating and secreting growth factor and cytokine, at day 20, growth factor free media was used and cultured for 24h. Media was collected for measurement of VEGF level by ELISA kit (Figure 4s-left, R & D System, MN). Cells were then homogenized, and expression of VEGF and eNOS were evaluated by Western Blotting, as previously published (Figure 4s-right).

Preparation of cells for delivery

Cells were labeled with both a fluorescent membrane dye (CM-Dil) as well as fluorescent beads. CM-Dil (5µl/ml) was added to the culture medium and incubated for 30 min at 37°C. Fluoresbrite plain 2 µm YG (yellow-green) polymeric beads (Polysciences, Warrington, PA) were added at a 1:25 cell-to-microspheres ratio, and incubated 75 min at 37°C. These beads exhibit exceptionally high brightness combined with photostability and high signal to noise ratio, thereby allowing prolonged detection. Cells were detached by incubation with 0.25% trypsin/1 mmol/L EDTA, followed by forceful pipetting and washing with PBS x3, and were kept in 10 ml PBS on ice until delivery (10⁶ cells/ml).

EPC delivery

A mixture of early and late EPC were prepared and infused distal to the stenosis after 6 weeks of RAS, based on previous reports demonstrating a synergistic effect in promoting neovascularization when both cell types are administered. Briefly, under anesthesia, sterile conditions, and fluoroscopic guidance, a 6 mm PTCA balloon catheter was advanced into the renal artery over a 0.014” guide wire, and engaged proximal to the stenosis. The balloon was inflated and expanded to full diameter, and 10 mL labeled cells (10⁶ cells/mL) suspended in saline manually injected slowly through the balloon. After injection, the catheter was immediately flushed with 10 mL of saline, the balloon kept inflated for another 5-10 min to prevent cell washout, and then removed. RAS remained unchanged.
**EPC localization and retention** was estimated from the engrafted cells observed in the kidney section\(^{11}\). Labeled cells were manually counted under fluorescence microscopy in 20 frozen 5µm renal cross-sections counterstained with hematoxylin (Figure 5s). The total area of each cross-section was obtained using an image-analysis program (MetaMorph, Meta Imaging Series 6.3.2, Allentown, PA), and the number of cells per mm\(^2\) averaged and multiplied by the section thickness. This (average number of cells/mm\(^3\)) was in turn multiplied by MDCT-derived renal volume (mm\(^3\)) to obtain the total number of EPC in the kidney, and divided by the number of injected cells (i.e., retention rate).

**EPC engraftment**: Double fluorescence of CM-DiI (labeled EPC before delivery) and immunofluorescences of CD31, cytokeratin, or Oct-4 were determined to investigate the engraftment of EPC into endothelial or tubular structures, their phenotype changes, and their relationship with endogenous stem cells. For this purpose, frozen slices (5 µm) from the stenotic kidney were incubated with CD31 (AbD Serotec, Cat#MCA1747, Clone: LC1-9), cytokeratin (AbD Serotec, Cat# MCA1907, Clone: AE1/AE3), or Oct-4 (1:50, Lifespan Biosciences, Seattle, WA) antibodies for 1h at room temperature. FITC-goat anti-mouse or rabbit IgG secondary antibodies (Zymed Laboratories, South San Francisco, CA) were incubated for 30 min at room temperature. To enable visualization of nuclei, DAPI mount media (Santa Cruz, CA) was used. Isotype identical IgG antibodies served as negative controls. Slides were examined under fluorescence microscopy. Peritubular capillary density was also evaluated using CD31 stained slides.

**Micro-CT**: A saline-filled cannula was ligated in a segmental artery perfusing the intact end of the stenotic kidney, and infusion of 0.9% saline (containing 10 units/ml heparin) was perfused under physiological perfusion pressure (Syringe Infusion Pump 22, Harvard Apparatus,
Holliston, MA). After 10-15 minutes the saline infusion was replaced with infusion (0.8 ml/min) of an intravascular contrast agent, which was a freshly mixed radio-opaque silicone polymer, containing lead chromate (Microfil MV122, Flow Tech, Inc., Carver, MA) until the polymer drained freely from the segmental vein. Then, a lobe of the polymer-filled tissue was trimmed from the kidney, placed in 10% buffered formalin, glycerinated, and encased in paraffin. The kidney samples were scanned at 0.5° increments using a micro-CT scanner, as previously described12-15 and the 3D volume images were reconstructed. The images consisted of cubic voxels of 20 µm on a side, and were displayed at 20 µm cubic voxels for subsequent analysis.

**Renal protein expression and Western Blotting**

**Immunohistochemistry:** staining was performed in 5 µm frozen or unstained mid-hilar renal cross-sections to assess the expression of integrin β3 (Chemicon International, CA, 1:80, Clone BV4), and α-Smooth muscle actin (α-SMA, Dako, CA,1:50, cat#M0851, clone 1A4). The secondary antibody, IgG Envision Plus (Dako), was followed by staining with the Vector NovaRED substrate kit (Vector Laboratories, Burlingame, CA), following vendor’s instructions. In addition, Oct-4 (1:50, Lifespan Biosciences, Seattle, WA, Clone: POU5F1) staining was performed in paraffin fixed slides incubated with primary antibody overnight at 4°C, washed by PBS, incubated with the secondary antibody, VECTASTAIN® Elite ABC kit (Vector Laboratories, Burlingame, CA), and followed by staining with the Vector DAB substrate kit (Vector Laboratories, Burlingame, CA). Nuclear counter staining was achieved using nuclear fast red (Vector Laboratories, Burlingame, CA).

**TUNEL:** Renal sections were stained using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI), following manufacturer’s instructions. To quantify the frequencies of apoptotic cells, twenty random glomeruli or tubular fields were captured from the kidneys using
a confocal microscope (Zeiss LSM 510, Germany), and apoptotic index defined as the number of positive cells divided by the total number of cells in each field.

**Western blotting:** standard blotting protocols were followed, as previously described\textsuperscript{16}, using specific polyclonal antibodies against p-Akt (Clone: Ser 473), p-eNOS, VEGF (Clone 147), HIF-1α (Clone H-206), TGF-β (Clone V), MMP-2 (Clone H-76), TIMP-1 (Clone H-150) (Santa Cruz, CA, 1:200 for all), angiopoietin-1 (Novus Biologicals, CO, 1:500), MMP-9 (Millipore, MA, 1:2,000), and Oct-4 (1:500, Lifespan Biosciences, Seattle, WA Clone: POU5F1). β-actins (Sigma, Saint Louis, MO, 1:500) or GAD(P)H (1:5000, Covance, Emeryville, CA) were used as loading controls. Protein expression was determined in each kidney, and the intensities of the protein bands (one per animal) were quantified using densitometry and averaged in each group.

**Data analysis**

**MDCT analysis:** Manually-traced regions of interest were selected in MDCT images in the aorta, renal cortex, medulla, and papilla, and their densities sampled. Time-density curves were generated and fitted with extended gamma-variate curve-fits, and the area enclosed under each segment of the curve and its first moment calculated using the curve-fitting parameters\textsuperscript{17}. These were used to calculate renal regional perfusion (ml/minute/g tissue), single-kidney GFR, and RBF, using previously-validated methods\textsuperscript{16-21}.

**Micro-CT analysis:** Images were digitized for reconstruction of 3-D volume images, and analyzed with the Analyze\textsuperscript{®} software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN). For analysis of the cortex, the three-dimensional tomographic images were oriented so that the z-axis was parallel to the radial vessels. Based on the number of cortical sections, the cortex was tomographically divided into 10 levels obtained at equal intervals, starting at the juxtamedullary cortex. For analysis, levels 1-3 were considered as inner third,
levels 4-7 as middle third, and levels 8-10 as the outer third of the cortex\textsuperscript{14}. The spatial density, average diameter, and vascular volume fraction (sum of cross-sectional areas of all vessel /area of the region of interest) of cortical microvessels (diameters <500\textmu m) were calculated in each level. Tortuosity index factor was calculated as we have recently described\textsuperscript{12, 13}. Briefly, 1-3 intra-cortical arterioles and their branches were tomographically “dissected” in each pig, the 3D path distance (total length) and linear distance (shortest distance between endpoints) of the main branches were calculated, and the tortuosity index calculated by dividing path distance by linear distance\textsuperscript{13}.

**Histology:** Mid-hilar 5 \textmu m cross sections of each kidney (1 per animal) were examined using a computer-aided image-analysis program (MetaMorph\textsuperscript{®}, Meta Imaging Series 6.3.2). In each representative slide, trichrome staining was semi-automatically quantified in 15-20 fields by the computer program, expressed as percentage of staining of total surface area, and the results from all fields averaged\textsuperscript{15, 19-22}. Glomerular score (percentage of sclerotic glomeruli) was assessed by recording the number of sclerotic glomeruli out of 100 counted glomeruli\textsuperscript{16, 19-21}.
References


**Figure legends**

**Figure 1s:** Antigenic and functional characterization of cultured autologous EPC.

Top right: bar graph showing similar quantity of colony forming units in normal and RAS animals.

Left and bottom right: Expression of monocytic (CD14), progenitor (CD34, CD133), endothelial (KDR), and stem (Oct-4) cell markers. The expression of CD14 and CD133 decreased with cellular maturation, while the expression of KDR increased, suggesting the EPC acquired endothelial phenotype. The expression of Oct-4 was evident in both early and late cells as well, suggesting pluripotency of EPC.

**Figure 2s:** Top: Uptake of acetylated LDL by EPC compared to renal tubular cells (used as controls), showing a significant uptake by EPC.

Bottom-left: Migratory function of endothelial progenitor cell (EPC) of normal and RAS pigs.

Bottom-right: The MTS assay shows increased late EPC proliferation in RAS. *p<0.05 vs. Normal

**Figure 3s:** Angiogenic ability of endothelial progenitor cells of normal and RAS pigs.

Top: DiI-labeled EPC (red) incorporate into tubes formed by HUVEC (grey). Bottom: quantitative tube number (tubes per field) and length. *p<0.05 vs. normal.

**Figure 4s:** Left: vascular endothelial growth factor (VEGF) levels in culture media of endothelial progenitor cells isolated from normal and RAS pigs. Right: densitometry and representative immunoblots of VEGF and eNOS in endothelial progenitor cells isolated from normal and RAS pigs, showing increased VEGF and eNOS expression in RAS pigs. *p<0.05 vs. normal
**Figure 5s:** Top and middle: representative perivascular (top) and peritubular (middle) double fluorescence of CM-DiI (red) and immunoreactivity of CD31 (green, x40) in frozen RAS+EPC kidney sections 4 weeks after renal injection. EPC were integrated into small vessels or capillaries (dash arrows), but not in larger vessels (white arrow). Bottom: RAS+EPC kidney sections stained with cytokeratin (green) and CM-DiI labeled EPC (red). Some injected EPC showed expression of CD31 or cytokeratin, suggesting that they assumed endothelial and tubular characteristics.
Figure 1-supplement

**CD34**

7 days

21 days

**CD14**

**CD-133**

**KDR**

**Oct-4**

**CFU/cm²**

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**Protein expression relative to GAPDH**

- **CD133**: 120 kDa
- **KDR**: 200 kDa
- **GAPDH**: 38 kDa

**Early cells**

**Late cells**

- **CD133**
- **KDR**

**Early cells**

**Late cells**

- **CD133**
- **KDR**
Figure 2-supplement

Acetylated LDL uptake

Controls

Number of migrated cells

EPC proliferation (absorbance 490nm)
Figure 3-supplement

Normal RAS

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* *
Figure 4-supplement

VEGF in culture media (pg/ml)

Normal | RAS

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<th>eNOS (140kDa)</th>
<th>Actin (42kDa)</th>
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<td>RAS</td>
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Protein expression (relative to actin)

VEGF | eNOS

* Indicates statistical significance.
Figure 5-supplement

CD31 perivascular

CD31 peritubular

Cytokeratin

Dil-labeled EPC  CD31/Cytokeratin  Merged