Renal Vein Levels of MicroRNA-26a Are Lower in the Poststenotic Kidney


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ABSTRACT

MicroRNA-26a (miR-26a) is a post-transcriptional regulator that inhibits cellular differentiation and apoptosis. Renal vascular disease (RVD) induces ischemic injury characterized by tubular cell apoptosis and interstitial fibrosis. We hypothesized that miR-26a levels are reduced in the poststenotic kidney and that kidney repair achieved by adipose tissue-derived mesenchymal stem cells (ad-MSCs) is associated with restored miR-26a levels. Renal function and renal miR-26a levels were assessed in pigs with RVD not treated (n=7) or 4 weeks after intrarenal infusion of ad-MSC (2.5×10⁵ cells/kg; n=6), patients with RVD (n=12) or essential hypertension (n=12), and healthy volunteers (n=12). In addition, the direct effect of miR-26a on apoptosis was evaluated in a renal tubular cell culture. Compared with healthy control kidneys, swine and human poststenotic kidneys had 45.5±4.3% and 90.0±3.5% lower levels of miR-26a, respectively, which in pigs, localized to the proximal tubules. In pigs, ad-MSC delivery restored tubular miR-26a expression, attenuated tubular apoptosis and interstitial fibrosis, and improved renal function and tubular oxygen-dependent function. In vitro, miR-26a inhibition induced proximal tubular cell apoptosis and up-regulated proapoptotic protein expression, which were both rescued by ad-MSC. In conclusion, decreased tubular miR-26a expression in the poststenotic kidney may be responsible for tubular cell apoptosis and renal dysfunction but can be restored using ad-MSC. Therefore, miR-26a might be a novel therapeutic target in renovascular disease.


Renal vascular disease (RVD) is a major cause of renovascular hypertension, and kidney injury in RVD may lead to ESRD and cardiovascular events.¹ We have previously shown that chronic RVD induced significant renal functional deterioration in association with renal inflammation, macrophage infiltration, and fibrosis.²–⁴ Clinically, interventions capable of protecting the kidney or reversing its injury in chronic RVD are still limited, and a better understanding of the pathophysiology of RVD is needed to design more effective therapies.

MicroRNAs (miRs) are a class of post-transcriptional regulators. They are single-stranded, noncoding RNAs ranging from 18 to 24 nucleotides in length, and they are highly conserved and ubiquitously expressed in all species.⁵ Some miRs, including miR-21, miR-27, or miR-210, are involved in cardiovascular and kidney repair.⁶ miR-26a is abundant in cardiac and other tissues and promotes vascular smooth muscle cell proliferation but inhibits cellular differentiation, apoptosis,⁷ and angiogenesis.⁸ In mice, lack of mature podocyte miRs induces marked proteinuria and glomerular and tubular injury. Furthermore, glomerular miR-26a expression implies a possible role in preservation of glomerular filtration barrier.

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function.9 Given its postulated protective properties, loss of miR-26a is likely to be associated with kidney damage. However, its role in cellular injury in the poststenotic kidney is unknown.

We have previously shown that endothelial progenitor cell or mesenchymal stem cell (MSC) infusion into the stenotic kidney improved renal function and structure.10–13 In recent years, MSCs have become a major resource for regenerative medicine, partly because of their prominent anti-inflammatory properties. Indeed, MSCs have been applied for treatment of acute kidney disease14 and CKD15,16 but the mechanisms by which MSCs prevent cell loss and promote tissue repair are incompletely understood. Notably, recent studies identified miRs in a cell line of MSCs,17 but the ability of MSCs to modulate tissue miR levels remains unknown.

Thus, we hypothesized that a fall in miR-26a levels is associated with stenotic kidney damage in RVD and that MSCs attenuate kidney injury by modulating miR-26a.

RESULTS

Swine RVD

Compared with normal pigs, 10 weeks after induction of RVD, all RVD pigs had similar moderate degrees of stenosis and elevated BPs, indicating renovascular hypertension (Table 1), whereas plasma renin activity (PRA; both systemic and renal veins), creatinine, and urine protein levels were not significantly different among the groups. Supplemental Figure 1A shows a representative angiographic image of renal artery stenosis. To ascertain the hemodynamic significance of such stenoses, we also measured intra-arterial pressures in the aorta and distal to both chronic and acute renal artery stenosis. In three pigs with chronic RVD, we found a significant pressure gradient (13±3 mmHg) between the aorta and distal to a stenosis of 77%. The ratio of distal pressure corrected for aortic pressure was 0.85. Furthermore, in three other pigs we found a significant pressure gradient (18.7±1.5 mmHg) when a 77% acute stenosis was generated by inflating a 5-mm balloon catheter within a normal renal artery (Supplemental Figure 1B). These observations suggest that our model of RVD leads to a hemodynamically meaningful stenosis. Immunohistochemistry showed that the number of renin-positive cells was significantly increased in both the stenotic and contralateral kidneys in RVD and remained elevated in MSC-treated RVD pigs compared with normal kidney (Supplemental Figure 1, C and D).

Renal miRs

Of the miRs investigated using plate-based hybridization, including miR-21, miR-26a, miR-27, miR-126, miR-192, and miR-210, only miR-26a expression was profoundly altered in the poststenotic kidney, because its levels significantly decreased in RVD pigs (Figure 1, A and B). Expression of miR-210 was slightly elevated in RVD without reaching statistical significance (Figure 1A). Fluorescent in situ hybridization staining showed, in the porcine kidney, that miR-26a was expressed predominantly in tubular cells and that its expression was significantly decreased in the stenotic kidney (Figure 1C). Furthermore, Dicer, an endoribonuclease in the RNase-III family that cleaves cytoplasmic pre-miR, was overexpressed in RVD kidneys (Figure 1D).

Renal Injury

To evaluate the effects of RVD on the renal microcirculation, excised kidneys were injected ex vivo with a contrast agent and scanned with microcomputed tomography (micro-CT). Image analysis showed a significant decrease of microvascular density in the stenotic kidney (Figure 2, A and B). The number of renal apoptotic cells was elevated in RVD (Figure 2, C and D) (P=0.005 versus RVD), and both apoptosis-inducible factor (AIF) and cleaved caspase-3, effectors of apoptosis, were upregulated (Figure 2, E and F). Kidney inflammation was enhanced in RVD, which was indicated by increased expression of the inflammatory marker TNF-α and P-NF-κB and decreased anti-inflammatory marker IL-10 (Figure 3, A and B). Renal fibrosis and glomerulosclerosis were elevated in RVD (Figure 3, C–E).

Renal Function

To evaluate the effect of RVD on kidney function, pigs were scanned in vivo with multidetector CT and magnetic resonance imaging (MRI). RVD pigs had lower CT-derived poststenotic renal blood flow (RBF) and GFR compared with normal pigs (Figure 4A). In addition, renal oxygenation was assessed as R2* (deoxyhemoglobin level) using blood oxygen-level dependent (BOLD) MRI before and after an intravenous bolus injection of furosemide. Basal R2* was similar in RVD compared with normal (22.6±1.9 and 23.5±0.8 at 1 per second, respectively), but attenuated medullary tubular oxygen-dependent response to furosemide was observed in RVD kidneys (Figure 4B).

MSC Phenotype

MSCs derived from subcutaneous fat expressed CD44, CD90, and CD105 (Figure 4C), all of which constituted >70% of

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Table 1. Systemic characteristics (mean±SEM) of normal pigs, RVD pigs, and RVD pigs 4 weeks after MSC delivery (RVD+MSC)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal (n=7)</th>
<th>RVD (n=7)</th>
<th>RVD+MSC (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of stenosis (%)</td>
<td>0.0±0.0</td>
<td>73.4±6.2a</td>
<td>70.3±6.7a</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>96.8±4.4</td>
<td>116.9±5.6a</td>
<td>117.8±3.9a</td>
</tr>
<tr>
<td>PRA inferior vena cava (ng/ml per hour)</td>
<td>0.22±0.05</td>
<td>0.20±0.04</td>
<td>0.21±0.06</td>
</tr>
<tr>
<td>PRA stenotic kidney (ng/ml per hour)</td>
<td>--</td>
<td>9.1±5.4</td>
<td>3.9±3.7</td>
</tr>
<tr>
<td>PRA contralateral kidney (ng/ml per hour)</td>
<td>--</td>
<td>7.1±4.3</td>
<td>4.0±3.8</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.3±0.2</td>
<td>1.6±0.3</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>Urinary protein (μg/ml)</td>
<td>14.4±3.7</td>
<td>21.6±4.8</td>
<td>18.8±4.1</td>
</tr>
</tbody>
</table>

P<0.05 versus normal.
Basal R2* levels were elevated in RVD+MSC pigs compared with normal or control; also attenuated renal in MSC-treated kidneys (Figure 3, A and B). Furthermore, MSCs caspase-3 (Figure 2, E and F). TNF-a expression was normalized in MSC-treated kidneys (Figure 3, A and B). Furthermore, MSCs also attenuated renal fibrosis and glomerulosclerosis (Figure 3, C–E). Functionally, MSCs did not affect BP (Table 1) but improved both RBF and GFR (Figure 4A) \( (P<0.001 \text{ versus RVD}) \). Basal R2* levels were elevated in RVD+MSC pigs compared with normal and RVD pigs (30.2±2.0 at 1 per second, \( P<0.05 \) versus normal and RVD), but MSCs improved the attenuated medullary tubular oxygen-dependent responses to furosemide observed in RVD kidneys (Figure 4B). Intrinsic miR-26a expression in porcine kidney tubular cells but not MSCs was higher than in human umbilical vein endothelial cell controls (Figure 1E), suggesting that restoration of miR-26a in tubular cells by MSCs was unlikely achieved by direct miR delivery.

**Human RVD**

Compared with healthy volunteers and patients with essential hypertension (EH), patients with RVD had similar age, sex, body mass index, total cholesterol, LDL, HDL, and medication use (Table 2). Triglyceride levels were elevated only in patients with RVD. PRA was significantly increased in both patients with EH and patients with RVD, and patients with RVD had higher PRA than patients with EH; however, there were no significant differences between venous levels in stenotic and nonstenotic kidneys. Renal function was impaired only in patients with RVD, who had higher serum creatinine and lower eGFR compared with healthy volunteers (Table 2).

Renal vein levels of miR-26a (adjusted for GFR) were significantly lower in the stenotic RVD kidney compared with the EH kidney, whereas its systemic levels remained unchanged among the groups (Figure 5A), implicating decreased miR-26a in kidney

gated cells in FACS (Figure 4E), and were negative for CD34, CD31, and CD45. Four weeks after infusion, around 12% of infused MSCs remained in the kidney and were commonly observed in the interstitium (50%) (Figure 4D), tubular structures (48%), or perivascular regions (approximately 2%).

**Effects of MSCs on RVD Kidney**

MSCs had no effect on expression of miR-21, miR-27, miR-126, and miR-192, whereas miR-210 expression was decreased by MSCs compared with RVD (Figure 1A), and miR-26 expression was normalized (Figure 1B and C). MSCs also improved microvascular density in the middle and outer cortex of the stenotic kidney (Figure 2, A and B). The number of renal apoptotic cells was markedly decreased in RVD+MSC pigs (Figure 2, C and D) and accompanied by significantly improved AIF and cleaved caspase-3 (Figure 2, E and F). TNF-a expression was normalized in MSC-treated kidneys (Figure 3, A and B). Furthermore, MSCs also attenuated renal fibrosis and glomerulosclerosis (Figure 3, C–E). Functionally, MSCs did not affect BP (Table 1) but improved both RBF and GFR (Figure 4A) \( (P<0.001 \text{ versus RVD}) \). Basal R2* levels were elevated in RVD+MSC pigs compared with damage distal to RVD independent of GFR. The stenotic human kidney showed greater hypoxia (by BOLD MRI) both under basal condition and after furosemide injection compared with the kidneys of patients with EH, although both groups increased oxygenation after furosemide (Figure 5B). To investigate whether decreased miR-26a in RVD is associated with kidney or systemic inflammation, we also measured inflammatory cytokines in both the renal vein (RVD and EH) and systemic vein (all groups) blood samples. We found that, similar to RVD pigs, decreased miR-26a in patients with RVD was also accompanied by increased circulating and renal vein TNF-a levels and decreased circulating IL-10 level (Figure 5, C and D), suggesting a possible link between miR-26a and inflammatory cytokine expression. Furthermore, systemic myeloperoxidase levels were significantly increased only in patients with RVD (Figure 5F). Inflammatory cytokines may activate renovascular endothelial cells, which was illustrated by significantly elevated renal vein E-selectin levels in patients with RVD compared with patients with EH (Figure 5E). Increased circulating levels of granulocyte colony-stimulating factor observed in RVD (Figure 5G) may represent stem cell homing signals for circulating reparative cells.
Cell Culture

To investigate the direct effects of miR-26a on kidney tubular cell apoptosis, in vitro experiments explored the effects of miR-26a inhibition on kidney tubular cell apoptosis. Compared with negative controls, miR-26a inhibition using small interfering RNA increased kidney tubular cell apoptosis, which was assessed by flow cytometry using an Annexin V-Cy318 Apoptosis Detection Kit (Figure 6). Importantly, tubular cell apoptosis induced by miR-26a inhibition was reversed after coculture with MSCs. These observations were supported by the finding that miR-26a inhibition upregulated AIF and caspase-3 expressions, which were also reversed by coculture with MSCs (Figure 6).

DISCUSSION

This study implicates a fall in miR-26a levels in stenotic kidney damage in both experimental and clinical RVD and suggests that the beneficial effects that MSCs confer on the stenotic kidney might be mediated partly by preservation of miR-26a, a novel renoprotective therapeutic target in renovascular disease.

miRs are processed from pre-miR transcribed from either miR genes or small parts of protein-coding transcripts. Pre-miRs are exported to the cytosol, where they are further processed by Dicer until finally mature miRs are assembled and bind to targeted mRNA, resulting in cleavage or translational repression of target mRNAs. Over 700 mRNAs have been identified in mammalian cells. We examined a series of miRs implicated in kidney cell apoptosis and angiogenesis. Unlike the involvements of miR-21, miR-27, miR-126, and miR-192 in other forms of kidney disease, we found no change in the expression of these miRs in RVD, possibly because of different disease and species settings. For example, miR-21 contributes to xenon-conferred amelioration of renal ischemia-reperfusion injury in mice but plays a pathogenic role in kidney fibrosis in mice with unilateral ureteral obstruction. The MSC-induced decrease in miR-210 expression in RVD might reflect the changing tissue oxygen environment after...
MSC delivery, because miR-210 is often related to cell survival under hypoxic conditions.\(^{25}\)

Consistent with observations in swine, miR-26a levels were decreased in the stenotic kidney vein in patients with RVD but not the renal vein of patients with EH, linking miR-26a to stenotic kidney damage in renovascular disease. Previous studies showed that miR-26a promotes vascular smooth muscle cell proliferation\(^7\) while inhibiting cellular apoptosis. Furthermore, loss of functional podocyte miR in mice has been identified to induce glomerular and tubular damage, aberrant apoptosis, and proliferation, and miR-26a is one of four major miRs found in glomeruli.\(^9\) Notably, in the normal and ischemic pig kidneys, we observed that miR-26a was expressed mainly in tubular cells, possibly because of species differences. Indeed, downregulation of miR-26a in porcine stenotic kidney tubules may be responsible for increased tubular cell apoptosis, impaired tubular oxygen-dependent transport activity (blunted responses to furosemide in BOLD MRI), and ultimately, kidney fibrosis. This was supported by our finding that miR-26a inhibition increased kidney tubular cell apoptosis in vitro. Proximal tubular cells are highly susceptible to hypoxia,\(^{26}\) and increased apoptosis may cause loss of tubular cells and dysregulated paracellular transport and thereby, increased back leak, which reduces GFR.\(^{27,28}\) Downregulation of miR-26a might have led to tubular cell apoptosis through a caspase-3–dependent pathway, which was upregulated in conjunction with decreased miR-26a levels in vitro and in vivo. A similar pattern was observed with TNF-\(\alpha\)-induced endothelial cell apoptosis involving miR-23a.\(^{29}\) Furthermore, miR-26a has (in its promoter region) a putative binding element for NF-\(\kappa\)B, a key downstream effector of TNF-\(\alpha\) that plays an important role in the inflammatory pathway. A recent study\(^{30}\) showed that the target genes for miR-26a include collagen I and connective tissue growth factor and that inhibition of NF-\(\kappa\)B in cardiac fibroblast restores miR-26a expression, attenuating collagen I and connective tissue growth factor expression. However, miR-26a overexpression inhibits NF-\(\kappa\)B activity. Our data show that P-NF-\(\kappa\)B expression was upregulated in RVD and normalized by MSCs in association with restored miR-26a and attenuated renal fibrosis, underscoring a potential role of MSCs in regulating miR-26a through the TNF-\(\alpha\)/NF-\(\kappa\)B pathway in RVD.

Our previous studies\(^3\) in a unilateral RVD swine model showed in the stenotic kidney impaired function, microvascular rarefaction, endothelial dysfunction, and increased release of cytokines and inflammatory mediators with subsequent apoptosis and fibrosis. We have found that MSC infusion into the stenotic kidney improved kidney function by partly restoring microvascular structure (as underscored by micro-CT in this study) and angiogenic signaling, and when delivered during revascularization, MSCs attenuated oxidative stress, microvascular rarefaction, and interstitial fibrosis in stenotic kidneys.\(^{11-13}\) Interestingly, in our study, RVD both downregulated miR-26a expression and increased tubular cell apoptosis in association with elevated TNF-\(\alpha\) levels. Importantly, these were all restored in MSC-treated kidneys. The miR-26a restoration by MSCs was

![Figure 3](image-url). MSC attenuates renal inflammation and fibrosis in RVD. (A and B) Western blotting for TNF-\(\alpha\), NF-\(\kappa\)B, and IL-10 in normal, RVD, and RVD+MSC pigs. TNF-\(\alpha\) and P-NF-\(\kappa\)B were upregulated and IL-10 was downregulated in the stenotic kidney, and MSCs improved them. MSCs attenuated renal fibrosis (trichrome staining, C and D) and glomerulosclerosis (E) in RVD. *P<0.05 versus normal; \(\dagger\)P<0.05 versus RVD. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. P-NF-\(\kappa\)B, phospho-nuclear factor kappa B.

MSC delivery, because miR-210 is often related to cell survival under hypoxic conditions.\(^{25}\)
unlikely achieved by direct miR delivery, because MSCs did not express high endogenous miR-26a levels compared with human umbilical vein endothelial cell controls but were profoundly elevated in kidney tubular cells, which was also observed ex vivo in pig kidneys. Moreover, the accompanying increased renal vein and circulating TNF-α level and decreased circulating IL-10 level suggest comparable miR-26a and inflammatory pathways in human RVD. The mechanisms by which MSCs partially ameliorated the decreases in RBF and GFR may be related to their anti-inflammatory effects, which improved endothelial function and attenuated renal fibrosis. Furthermore, renovascular endothelial activation, indicated by elevated renal vein E-selectin and systemic myeloperoxidase levels, may play an important role in the development of atherosclerosis and progression of RVD. Additional studies are needed to establish the effects of MSCs on miR-26a level in patients with RVD.

**Study Limitations**

We used relatively young animals with no chronic atherosclerotic disease, and the relatively preserved serum creatinine in RVD reflects the unilateral disease and absence of comorbidities. MSC treatment did not lower BP, mainly because the degree of the stenosis remained unaltered. We used standard MSC doses, and additional studies are needed to determine the optimal dose and timing of delivery as well as the long-term effect on miR-26a expression. We did not find a difference in PRA among the three swine groups, but the number of renin-positive cells significantly increased in RVD compared with normal pigs and was unaltered in MSC-treated RVD pigs.

We do not have biopsy samples available to examine renal apoptosis in patients. Systemic levels of miR-26a were statistically unchanged in patients with RVD, possibly because of the big variability in PCR and the relatively small cohorts. The
patients with hypertension were all treated with blockers of the renin-angiotensin system, which elevate PRA, and they may also account for the improved tubular oxygenation response to furosemide. Our data indicate that MSCs mainly repair tubular cells. In moderate RVD, a decrease in O2 consumption parallels the decrease in RBF; thus, initial loss of renal function is not necessarily accompanied by impaired oxygenation.

A recent study showed increased miR-26a expression in a mouse model of acute myocardial infarction and patients with acute coronary syndrome as well as inhibition of miR-26a upregulated angiogenesis. Therefore, the acute phase of ischemic disease may lead to compensatory upregulation of miR-26a, but at least in the kidney, chronic ischemia clearly downregulates miR-26a; additionally, increased miR-26a might sustain kidney function and structure.

Conclusion

Taken together, our studies implicate a fall in miR-26a level in poststenotic kidney injury in both humans and swine with RVD that likely involves tubular apoptosis. Furthermore, our data showed that MSCs decrease inflammatory cytokines in swine RVD in association with restored miR-26a expression and decreased apoptosis, which thereby rescues renal structure and function. Our study, using clinically relevant tools, suggests miR-26a as a therapeutic target and supports rigorous development of regenerative strategies to improve the damaged kidney.

CONCISE METHODS

Detailed methods are in Supplemental Material.

Table 2. Systemic characteristics (mean±SEM) in healthy volunteers, patients with RVD, and patients with EH

<table>
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<th>Parameters</th>
<th>Healthy Volunteers (n=12)</th>
<th>RVD (n=12)</th>
<th>EH (n=12)</th>
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<tbody>
<tr>
<td>Age (yr)</td>
<td>70.3±2.1</td>
<td>70.3±1.9</td>
<td>69.1±1.9</td>
</tr>
<tr>
<td>Sex (men/women)</td>
<td>5/7</td>
<td>7/5</td>
<td>8/4</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.7±1.3</td>
<td>27.9±0.9</td>
<td>28.3±1.0</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>120.0±2.9</td>
<td>143.2±5.6a</td>
<td>141.0±5.6a</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>70.4±2.4</td>
<td>69.7±2.5</td>
<td>69.8±4.1</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>191.8±7.3</td>
<td>184.8±9.3</td>
<td>178.6±9.7</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>107.9±5.3</td>
<td>100.7±6.2</td>
<td>103.8±6.7</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>62.2±3.4</td>
<td>51.2±7.1</td>
<td>48.8±3.9</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>107.9±8.6</td>
<td>164.7±23.3a</td>
<td>130.2±12.5</td>
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<tr>
<td>PRA systemic (ng/ml per hour)</td>
<td>0.5±0.1</td>
<td>10.8±2.5ab</td>
<td>6.4±2.3ab</td>
</tr>
<tr>
<td>PRA stenotic kidney (ng/ml per hour)</td>
<td>—</td>
<td>14.7±3.3</td>
<td>8.2±2.7</td>
</tr>
<tr>
<td>PRA contralateral kidney (ng/ml per hour)</td>
<td>—</td>
<td>14.0±3.4</td>
<td>9.4±3.3</td>
</tr>
<tr>
<td>Medications, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACEIs/ARBs</td>
<td>12 (100)</td>
<td>12 (100)</td>
<td></td>
</tr>
<tr>
<td>Statins</td>
<td>9 (75)</td>
<td>8 (67)</td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.9±0.2</td>
<td>1.5±0.3ab</td>
<td>1.0±0.3</td>
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<tr>
<td>Neutrophil gelatinase-associated lipocalin</td>
<td>66.9±8.1</td>
<td>152.7±12.1ab</td>
<td>63.1±10.8</td>
</tr>
<tr>
<td>eGFR (ml/min per 1.73 m²)</td>
<td>75.3±2.1</td>
<td>46.3±2.4ab</td>
<td>72.2±7.3</td>
</tr>
</tbody>
</table>

ACEIs/ARBs, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers.

aP<0.05 versus healthy volunteer.
bP<0.05 versus EH.

Animal Procedures

The Mayo Clinic Institutional Animal Care and Use Committee approved all procedures in 20 domestic pigs (45–55 kg) included in the study. At baseline, unilateral RVD was induced in 13 pigs, and a telemetry transducer (PhysioTel; Data Sciences) was implanted in the left femoral artery to record mean arterial pressure as previously described. Subcutaneous adipose tissue (approximately 5 g) was harvested from the femoral artery incision site of each pig, digested in collagenase H for 1 hour, filtered, and cultured in advanced MEM media supplemented with 5% PLTmax (Mill Creek Life Sciences, Rochester, MN). Phenotypic markers for MSCs were characterized following the recommendations of the International Society for Cellular Therapy, and MSCs were further confirmed by their capacity to transdifferentiate into osteocytes, chondrocytes, and adipocytes. The third passages of MSCs were collected and kept at −80°C for later use.

Six weeks after induction of RVD, pigs were randomized into two groups that were either not further treated (RVD; n=7) or subsequently treated with an intrarenal infusion of autologous adipose tissue-derived MSCs (RVD+MSC; n=6). The other seven pigs were used as sham controls (normal; n=7). Pigs were anesthetized and incubated, and renal angiography was performed to determine the degree of RVD as previously described. In the RVD+MSC animals, MSCs (2.5×10⁶ cells/kg suspended in 10 ml saline) were then infused into the stenotic renal artery over 5–7 minutes. For subsequent tracking, MSCs were prelabeled with CM-DiI dye for 30 minutes before injection.

Four weeks later, single-kidney RBF and GFR were evaluated by multiple detector computed tomography, and oxygen-dependent imaging, MSCs were prelabeled with CM-DiI dye for 30 minutes before injection.
puncture to measure the protein content by spectrophotometry using the Bradford method.

Three days after completion of all studies, the pigs were euthanized with intravenous sodium pentobarbital (100 mg/kg; Sleepaway; Fort Dodge Laboratories, Fort Dodge, IA). Kidneys were removed, and they were shock frozen in liquid nitrogen and stored at −280°C, preserved in formalin for in vitro studies, or perfused with Microfil for micro-CT scan (for microvascular density measurement). Frozen (5 μm) sections were cut from the stenotic kidneys infused with MSCs and stained with cytokeratin for MSC tracking using standard immunohistologic protocol; MSC retention rate was calculated as previously detailed.10,36 In 10–15 fields sampled in each section, MSCs were manually counted and recorded by locations (tubular, perivascular, or interstitial).13

Figure 5. Decreased renal vein miR-26a level in patients with RVD is associated with increased inflammatory cytokines. (A) miR-26a levels (adjusted for GFR) were significantly lower in the renal veins of patients with RVD compared with patients with EH. (B) In patients with RVD, the stenotic kidney showed greater hypoxia R2* level by BOLD MRI compared with patients with EH, whereas R2* responses to furosemide were significant in both patients with RVD and patients with EH. (C) In patients with RVD, systemic and renal vein TNF-α levels were significantly increased. (D) Patients with RVD had decreased systemic IL-10 level. (E) Compared with patients with EH, patients with RVD showed increased E-selectin levels in the renal vein. (F) Patients with RVD had increased systemic myeloperoxidase level compared with HVs. (G) Systemic granulocyte colony-stimulating factor level was only increased in patients with RVD. HV, healthy volunteers. *P<0.05 versus EH; †P<0.05 versus HV; ‡P<0.05 versus baseline.

Ex Vivo Studies
miRs were isolated from kidney tissue using an mirPremier MicroRNA Isolation Kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer’s instructions. MiRNA Plate Assay Kits (Signosis, Sunnyvale, CA) were used for the detection of miR-21, miR-26a, miR-27, miR-126, miR-192, and miR-210. To localize miR-26a expression in the kidney, fluorescent in situ hybridization staining was performed at the Mayo Cytogenetic Core Laboratory. Furthermore, protein expression of Dicer (1:800; Abcam, Inc., Cambridge, MA), which processes pre-miR in the cytoplasm, was evaluated using Western blotting.

Kidney injury was evaluated by glomerular score (percentage sclerotic of 100 glomeruli)37,38 and fibrosis by trichrome staining. Renal microvascular density was evaluated using micro-CT. For apoptosis, renal sections were stained using the Dead-End Fluorometric
TUNEL System (Promega, Madison, WI) following the manufacturer’s instructions. Protein expressions of AIF (1:400; BD, Franklin Lakes, NJ) and caspase-3 (1:600; Abcam, Inc.) were determined by Western blotting. The proinflammatory factor TNF-α (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), NF-κB (1:500; Abcam, Inc.), phospho–NF-κB (1:500; Abcam, Inc.), and anti-inflammatory factor IL-10 (1:500; Abcam, Inc.) were evaluated using Western blotting. Phospho–NF-κB expression was related to total NF-κB, and all other proteins were related to glyceraldehyde-3-phosphate dehydrogenase.

Procedures in Humans
The Mayo Clinic Institutional Review Board approved the study, and informed written consent was obtained from all patients with RVD or EH and matched healthy volunteers (n=12 each). Hypertension was defined as systolic BP≥140 mmHg or diastolic BP≥90 mmHg in the supine position after 20 minutes of rest on 2 separate days. Clinical and laboratory parameters and exclusion criteria are detailed in Supplemental Material.

In patients with RVD or EH, BOLD MRI examinations were performed at baseline and after a 20-mg furosemide (Lasix; Sanofi-Aventis, Bridgewater, NJ) intravenous injection with a 3.0-T system (Twin Speed Signa Excite; GE Medical Systems, Waukesha, WI). miR-26a level was evaluated using RT-PCR in renal vein and inferior vena cava samples in patients with RVD or EH and peripheral venous samples from healthy volunteers. Total RNA was isolated from 400-μl plasma samples by the mirVana PARIS Total RNA Isolation Kit (Life Technologies, Carlsbad, CA). Real-time PCR (Applied Biosystems ViiA7) used the miR-26a primer (Life Technologies).

Systemic and renal vein levels of TNF-α, IL-10, E-selectin, myeloperoxidase, and granulocyte colony-stimulating factor were detected using Luminex Assay Kits (EMD Millipore, Billerica, MA).

Cell Culture Studies
To test the direct role of miR-26a on tubular cell apoptosis, porcine kidney tubular cells (LLC-PK1; ATCC, Manassas, VA) were incubated with miR-26a inhibitor and its negative/positive controls (all designed
and produced by Ambion; Life Technologies) and transfection agent (Lipofectamine RNAiMAX; Invitrogen; Life Technologies) for 48 hours following the manufacturer’s instructions. MSCs were added into tubular cells after a 24-hour miR-26a inhibition and cocultured for 24 hours. At the end of the study, the MSCs and LLC-PK1 cells were separated using their different response to trypsin, which we previously described. Tubular cell apoptosis was evaluated using an Annexin V-Cy3 Apoptosis Detection Kit (Enzo Life Science, Farmingdale, NY) and FACS on a flow cytometer (FlowSight; Amnis, Seattle, WA). Furthermore, tubular cell expressions of AIF (1:400; AG31750, and C06-RR018898 and the Mayo Clinic Center for Regenerative Medicine contributed to renal dysfunction in swine renovascular hypertension. Results are expressed as means ± SEMs except when otherwise noted. Patient miR-26 results were calculated from absolute PCR copy numbers divided by eGFR for adjustment. Comparisons among groups used one-way ANOVA with Tukey post hoc tests for correction and t test between two groups. Comparisons within the same individuals (pre- and postfurosemide) were performed using paired t tests. Patients’ medication data analyses were performed using chi-squared tests. Statistical significance was accepted for P ≤ 0.05. Detailed methods are in Supplemental Material.

Statistical Analyses
Results are expressed as means ± SEMs except when otherwise noted. Patient miR-26 results were calculated from absolute PCR copy numbers divided by eGFR for adjustment. Comparisons among groups used one-way ANOVA with Tukey post hoc tests for correction and t test between two groups. Comparisons within the same individuals (pre- and postfurosemide) were performed using paired t tests. Patients’ medication data analyses were performed using chi-squared tests. Statistical significance was accepted for P ≤ 0.05. Detailed methods are in Supplemental Material.

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DISCLOSURES
None.

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Supplemental methods

Animal Procedures

Induction of RVD

At baseline, unilateral RVD was induced in 13 pigs by placing a local-irritant coil in the main renal artery, as previously described\(^1,2\). A telemetry transducer (PhysioTel, Data Sciences) was implanted in the left femoral artery, and mean arterial pressure (MAP) subsequently recorded continuously at 5-minute intervals and averaged for each 24-hour period.

MSC culture and characterization

Subcutaneous adipose tissue (~5g) was harvested from the femoral artery incision of each pig, digested in collagenase H for 1hr, filtered, and cultured in Advanced MEM media supplemented with 5% PLTmax (Mill Creek Life Sciences, Rochester, MN). The culture media is devoid of animal serum, and thus suitable also for clinical applications of MSC. Phenotypic markers for MSC were characterized following the recommendations\(^3\) of the International Society for Cellular Therapy using CD90, CD44, CD105, CD34, CD31, and CD45, and further quantitatively analyzed with fluorescence-activated cell sorting (FACs) for CD44, CD90, and CD105, using standard protocols. For further confirmation of their phenotype, MSC were also transdifferentiated into osteocytes, chondrocytes, and adipocytes\(^4\). The third passages of MSCs were collected and kept in -80°C for later use.

Renal angiography

Pigs were anesthetized with intra-muscular telazol (5 mg/kg) and xylazine (2 mg/kg), intubated, and mechanically ventilated with room air. Anesthesia was maintained with intravenous ketamine (0.2 mg/kg/min) and xylazine (0.03 mg/kg/min) in normal saline. Under sterile conditions and fluoroscopic guidance, renal angiography was performed to determine the
degree of RVD, as previously described\textsuperscript{1,2}, and confirmed using CT angiography. To measure pressure gradients across chronic RVD, arterial pressures were measured in the aorta and distal to the renal artery stenosis using a small catheter (5F) with a transducer. To measure pressure gradients across acute renal arterial stenosis, a 5mm balloon catheter was inflated in the renal artery of 3 normal pigs, and pressures proximal/distal to inflated balloon were then measured using a transducer. The degree of stenosis induced by balloon was subsequently quantified using CT angiography. The pressure drop was expressed as the ratio of distal pressure (Pd) to proximal aortic pressure (Pa), as previously published\textsuperscript{5}.

\textbf{In-vivo Studies}

Four weeks after MSC infusion \textit{in vivo} functional studies were performed. After angiography, the catheter was positioned in the superior vena cava, and MDCT flow studies were performed for assessment of basal regional-renal perfusion, renal blood flow (RBF), and glomerular filtration rate (GFR), as previously detailed\textsuperscript{1,6}. This involved sequential acquisition of 160 scans after a central venous injection of iopamidol (0.5 cc/kg/2 sec).

Blood Oxygen-dependent Level (BOLD) MRI is sensitive to the concentration of deoxyhemoglobin in blood. Elevated basal levels of the index R2\textsuperscript{*} are indication of hypoxia. In addition, oxygen-dependent tubular transport function can be assessed by the change in medullary oxygenation in response to a loop reabsorption inhibitor such as furosemide. For this purpose, two sets of BOLD images were collected before and 15 minutes after furosemide injection (5mg/kg IV, Hospira, Inc., Lake Forest, IL). The medullary BOLD index, R2\textsuperscript{*}, was calculated in each set, and the difference between the pre and post-furosemide values used as a measure of oxygen-dependent tubular function\textsuperscript{7}.

\textit{Micro-CT procedure}
An intravascular Microfil® silicone rubber (MV-122, Flow Tech, Inc) was perfused through the cannulated renal artery at a flow rate of 0.9 ml/min to visualize vascular structure in micro-CT scan. A portion of the kidney (≈2×1×1cm) was then sectioned, prepared, and scanned as previously described. Images were digitized for reconstruction of 3-D volume images, which consisted of cubic voxels of 20μm on-a-side for subsequent analysis. Images analysis was performed with the Analyze software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN). The kidney cortex was tomographically divided into 3 equal parts, and the data were analyzed in 10 slices obtained at equal intervals from each third. The spatial density of micro vessels (diameters <500μm) was calculated in each region by counting the vessels.

Renin expression was evaluated on kidney section from both the stenotic and contralateral kidney using standard immunohistochemistry protocol (1:500, Abcam, Cambridge, MA).

**Procedures in humans**

In all patients, clinical and laboratory parameters were collected via the electronic medical records. Dietary intake was regulated at 150 mEq of sodium with an isocaloric diet prepared on site for a 3-day period that all hypertensive patients spent at the Clinical Research Unit. Patients with RVD were identified using criteria similar to those stipulated for recruitment in the Cardiovascular Outcomes in Renal Atherosclerotic Lesions Trial with cross-sectional angiographic luminal occlusion of ≥60%. All RVD patients had a history of refractory stage II hypertension defined as a systolic blood pressure greater than 155 mmHg while taking two or more antihypertensive medications. RVD patients were well-characterized with the clinical syndrome of atherosclerotic renal artery disease and hypertension with hemodynamically significant lesions. Exclusion criteria included uncontrolled hypertension (SBP >180 mmHg, despite antihypertensive therapy), serum creatinine >1.7 mg/dL, diabetes requiring insulin or oral
hypoglycemic medications, recent cardiovascular events (myocardial infarction, stroke, congestive heart failure within 6 months), pregnancy, and kidney transplant. Estimated GFR was calculated using Modification of Diet in Renal Disease study equation. Peripheral blood samples were collected for measurements of cholesterol, triglycerides, and creatinine, levels. Renal vein samples were collected in essential and renovascular hypertensive patients through a 5F Cobra catheter (Cook, Inc) advanced from the common femoral vein to the right, left, and infrarenal inferior vena cava. In healthy volunteers blood samples were collected from an antecubital vein. Systemic and renal vein levels of PRA, TNF-α, IL-10, E-selectin, myeloperoxidase, and granulocyte colony-stimulating factor were detected using luminex assay kits (Millipore, Billerica, MA).

Patient MRI and BOLD Methods: BOLD MR imaging examinations were performed with a 3.0-T system (Twin Speed Signa Excite; GE Medical Systems, Waukesha, Wis). Parametric images of R2* were then generated by fitting signal intensity–versus–echo time data to an exponential function on a voxel-by-voxel basis and solving for R2*. After the first BOLD MR acquisition, 20 mg of furosemide (Lasix; Sanofi-Aventis, Bridgewater, NJ) was administered intravenously and flushed with 20 mL of saline. BOLD MR measurements for each kidney were repeated 15 minutes later. Gadolinium-enhanced MR angiograms were obtained after BOLD MR imaging to confirm the presence or absence of large-vessel renal arterial disease. Analysis of BOLD MR data from axial images was performed by drawing parenchymal ROIs on two to four sections through the midpole hilar region of each kidney on representative T2*-weighted images and then transferring the ROIs to the corresponding R2* parametric image by one author. Two ROIs were traced: In one, the renal cortex (large segment) was selected, and in the other, the entire kidney section, including both cortex and medulla while excluding the renal collecting
system and any incidental renal cysts was selected. The fractional tissue hypoxia was determined as we have recently developed by measuring the percentage of voxels from the whole-kidney ROI with $R_2^*$ values above 30 sec$^{-1}$, taking the average of all available sections.

Mir-26a expression was evaluated using RT-PCR in stenotic kidney vein samples in patients with RVD, renal vein samples from patients with essential hypertension, or systemic samples from volunteers. Briefly, total RNA was isolated from 400µl plasma samples by mirVana PARIS total RNA isolation kit (Life Technologies). Due to the lack of established endogenous small RNA controls for plasma samples, we spiked 25fmol of cel-mir-39 (Life Technology) into each sample after adding the 2X denaturing solution supplied by the kit. Real-time PCR (Applied Biosystems ViiA7) used miR26a primer (Life Technologies). Numbers of miR26a copies were calculated from a standard curve generated by using standard miR-26a.

Cell culture studies

The mature miR-26a sequence used for miR-26a inhibitor design was

UUCAAGUAAUCCAGGAUAGGCU, stem loop sequence: GUGGCCUCGUUCAAGUAACUCAGGAUAGGCUUACUUGCACGGGGACGC.

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Figure 1s: Renal artery angiography, pressure gradient measurement, and kidney tissue renin immunohistochemistry. A: Representative renal angiography in swine RVD. B: Pressure gradient, the ratio between pressures in the proximal aorta and distal to the stenosis, and the degree of stenosis in chronic and acute RVD. C&D: Renin immunohistochemistry images and quantification in normal, RVD, and RVD+MSC pigs, including the stenotic and contralateral kidneys.