Hematopoietic MicroRNA-126 Protects against Renal Ischemia/Reperfusion Injury by Promoting Vascular Integrity


*Department of Nephrology, †Einthoven Laboratory for Experimental Vascular Medicine, ‡Department of Cardiology, §Department of Immunohematology and Blood Transfusion, and ‖Department of Thrombosis and Haemostasis, Leiden University Medical Center, Leiden, The Netherlands

ABSTRACT
Ischemia/reperfusion injury (IRI) is a central phenomenon in kidney transplantation and AKI. Integrity of the renal peritubular capillary network is an important limiting factor in the recovery from IRI. MicroRNA-126 (miR-126) facilitates vascular regeneration by functioning as an angiomiR and by modulating mobilization of hematopoietic stem/progenitor cells. We hypothesized that overexpression of miR-126 in the hematopoietic compartment could protect the kidney against IRI via preservation of microvascular integrity. Here, we demonstrate that hematopoietic overexpression of miR-126 increases neovascularization of subcutaneously implanted Matrigel plugs in mice. After renal IRI, mice overexpressing miR-126 displayed a marked decrease in urea levels, weight loss, fibrotic markers, and injury markers (such as kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin). This protective effect was associated with a higher density of the peritubular capillary network in the corticomedullary junction and increased numbers of bone marrow–derived endothelial cells. Hematopoietic overexpression of miR-126 increased the number of circulating Lin−/Sca-1+/cKit+ hematopoietic stem and progenitor cells. Additionally, miR-126 overexpression attenuated expression of the chemokine receptor CXCR4 on Lin−/Sca-1+/cKit+ cells in the bone marrow and increased renal expression of its ligand stromal cell-derived factor 1, thus favoring mobilization of Lin−/Sca-1+/cKit+ cells toward the kidney. Taken together, these results suggest overexpression of miR-126 in the hematopoietic compartment is associated with stromal cell–derived factor 1/CXCR4-dependent vasculogenic progenitor cell mobilization and promotes vascular integrity and supports recovery of the kidney after IRI.


Received June 20, 2013. Accepted December 3, 2013.
R.B. and C.V.S. contributed equally to this work.

Correspondence: Prof. Anton Jan van Zonneveld, Department of Nephrology, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands. Email: a.j.vanzonneveld@lumc.nl

Copyright © 2014 by the American Society of Nephrology

Ischemia/reperfusion injury (IRI) is a central event in such clinical conditions as AKI and in organ transplantation, and it is strongly associated with delayed graft function and long-term graft survival.1–3 Emerging evidence indicates that the renal microvascular endothelium of the outer medullary peritubular network is the primary site of injury in the pathogenesis of ischemia-induced renal dysfunction.4 Following ischemia, perfusion of the...
peritubular capillary network is rapidly impaired as a consequence of endothelial cell (EC) swelling,5 impaired vasorelaxation,6 and increased leukocyte adhesion.7 In addition, microvascular destabilization initiated by the loss of EC–EC interaction8 and EC–pericyte interactions can lead to significant reductions in peritubular capillary density due to microvascular rarefaction.8,9 The resulting loss in renal perfusion can further exacerbate medullary ischemia and drive the development of interstitial fibrosis by stimulation of profibrogenic factors, such as TGF-β.10 As a consequence, integrity of the peritubular capillary network is a key determinant for the preservation of renal function. Indeed, clinical biopsy studies have shown an association between loss of tubular structure and function on the one hand and capillary rarefaction on the other.11,12

Because of their limited replicative capacity, renal ECs are thought to be insufficiently capable to completely repair the injured endothelium of the peritubular capillary plexus after IRI.13,14 Therefore, current therapeutic strategies to prevent microvascular loss have focused on the prevention of pericyte perturbation to reduce capillary rarefaction.15–18 However, once rarefaction has occurred, these strategies may fail to induce sufficient revascularization required to reverse renal dysfunction.19 In search of ways to augment neovascularization in the injured kidney, many laboratories have investigated the biology and therapeutic use of circulating vascular progenitor cells originating from the bone marrow (BM) compartment.20 These progenitor cells were shown to incorporate into the injured microvasculature in experimental models for GN,21 ischemic nephropathy,22,23 and interstitial fibrosis.24 This phenomenon has been particularly observed when extensive or repetitive endothelial injury occurs, for example in kidney transplantation.25 Microvascular incorporation of BM-derived progenitor cells has been linked to preservation of the vasculature because they may serve as an alternative cellular source to facilitate re-endothelialization.26 In addition, the CXCR4+ fraction of progenitor cells is mobilized to the ischemic kidney by local secretion of the chemokine stromal cell–derived factor-1 (SDF-1)23,27 and has been shown to exert renoprotective effects in a paracrine fashion.

Phosphoinositide 3-kinase (PI3K)/AKT signaling in progenitor cells plays a critical role in mobilization of progenitors from the BM via the SDF-1/CXCR4 axis28 and their subsequent differentiation toward vascular cells.29 MicroRNA-126 (miR-126) is a key regulator of PI3K/AKT signaling by direct targeting of the negative regulator PI3K regulatory subunit 2 (PI3KR2/p85-β) and targets genes that play key roles in angiogenesis and inflammation.29–31 In addition, miR-126 was shown to coregulate the expansion and mobilization of hematopoietic stem cells and progenitor cells.32,33 We hypothesized that miR-126 overexpression in the hematopoietic compartment of mice can enhance the vasoprotective potential of these progenitors and that this will translate to decreased renal injury.

RESULTS

Generation of Mice Overexpressing miR-126 in the Hematopoietic Compartment

To investigate the provasculogenic effect of miR-126, we generated mice overexpressing miR-126 in the hematopoietic compartment. To that end, mice were transplanted with lineage-negative enriched CD45.1+ BM cells transduced with a lentiviral vector (LV) driving the expression of miR-126 and dsRed under control of a constitutive human U6 and a phosphoglycerate kinase 1 (PGK1) promoter, respectively (Supplemental Figure 1). Eight weeks after transplantation, mice were euthanized and blood and BM samples were obtained. Smears of peripheral blood (PB) and BM of transplanted animals showed dsRed positivity, confirming the successful transduction of the transplanted BM (Figure 1A). FACS analysis for the expression ratios between congenic markers CD45.1 (donor) and CD45.2 (acceptor) in PB and BM of the transplanted mice demonstrated high levels of chimerrism (Figure 1B), as CD45.1 expression in the control group (LV-C) and miR-126 overexpression group (LV-126) was similar to CD45.1 expression in donor mice. Quantitative PCR confirmed a 5.1-fold (Figure 1C; P<0.001) and 6.0-fold (Figure 1D; P<0.05) higher expression level of miR-126 in BM and PB cells, respectively, in the LV-126 group compared with the LV-C group. Finally, confirming downstream effects of miR-126 overexpression, we observed that mRNA levels of seven of nine established miR-126 targets in total BM were reduced in the LV-126 group compared with LV-C controls (Supplemental Figure 2).

Hematopoietic Overexpression of miR-126 Increases Neovascularization of Subcutaneously Implanted Angiogenic Matrigel Plugs in Mice

To assess the effect of hematopoietic overexpression of miR-126 on vasculogenesis, we performed angiogenic matrigel plug assays in vivo. Therefore, 7 weeks after BM transplantation, Matrigel plugs enriched with recombinant proangiogenic factors SDF-1 and vascular endothelial growth factor, were subcutaneously implanted in the flanks of LV-126 and LV-C mice. After 7 days, the skin was opened to visualize the neovascularization of the implants by a sidestream dark field camera. Movies (Figure 2B, Supplemental Video 1) confirmed the ingrowth of functional neovascularity with active flow of red blood cells. To quantify the extent of neovascularization, microscopic images were taken from both sides of the angiogenic plugs. Figure 2A shows a panel of representative microphotographs, with arrowheads indicating the blood-filled microvascular structures that had formed in the Matrigel plugs following implantation. Using these photographs, we counted the number of vessels per Matrigel implant and observed a nearly significant increase in the number of vessels in the LV-126 mice compared with the LV-C animals (Figure 2C; n=10, P=0.05). Moreover, quantification of the total microvascular surface area revealed a significant increase (40%) in vascularization in

Basic Research

www.jasn.org
the LV-126 mice (Figure 2D; $P<0.02$), which correlated directly with the levels of miR-126 expression in the BM of the mice (Figure 2E; $r^2=0.20$; $P=0.05$).

Immunohistological analyses of the vascularized Matrigel plugs (Figure 2, F and G) demonstrated stabilized, maturing vessels and a profound infiltration of BM-derived, dsRed$^+$ cells that overlap with staining for murine EC antigen-32 (MECA32) or von Willebrand factor (Figure 2F, Supplementary Figure 3A). Around the newly formed blood vessels, cells positive for PDGF receptor-$\beta$ (PDGFR$\beta$) and neural/glial antigen-2 (Figure 2G, Supplementary Figure 3B) were detected, which were also positive for dsRed, suggesting a BM origin.

Our data indicate that overexpression of miR-126 in the hematopoietic compartment stimulates neovasculogenesis of implanted Matrigel plugs that possibly involves both BM-derived EC as well as BM-derived perivascular cells.

**MiR-126 Overexpression in the Hematopoietic Compartment Protects against Renal IRI**

Given the observed provasculogenic effects in mice overexpressing miR-126 in the hematopoietic compartment, we hypothesized that these mice would be less susceptible to IRI because of an improved capacity to maintain renal microvascular integrity. To investigate this hypothesis, 8 weeks after BM transplantation, LV-126 and LV-C mice were subjected to bilateral renal ischemia and were euthanized 3 days or 3 weeks after the procedure. Renal IRI resulted in extensive renal dysfunction in the LV-C mice, as shown by increased blood levels of urea (Figure 3A). In contrast, mice overexpressing
miR-126 were protected against renal dysfunction, with a moderate elevation in urea levels compared with control mice. At 72 hours after reperfusion, this deterioration of renal function in the LV-C mice was accompanied by a significantly lower weight than the LV-126 mice (Figure 3B) and significantly lower mRNA levels of the tubular injury markers kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin, proinflammatory markers IL-6 and chemokine (C-C motif) ligand 2 , and upregulation of anti-inflammatory cytokine IL-10 (Figure 3C). In addition, analyses of post-IRI, hematoxylin and eosin–stained kidney sections revealed that mice in the LV-126 group displayed significantly less acute tubular necrosis and tubular cast formation (rating 0–3) compared with the LV-C mice (Figure 3C). Direct immunohistochemical staining confirmed a markedly lower expression of tubular injury marker KIM-1 (2.6-fold) in the LV-126 group (Figure 3F; P<0.001). Tubular injury after reperfusion was accompanied by extensive infiltration of CD45+ leukocytes (Figure 3G). However, overexpression of miR-126 did not reduce the total number of infiltrating leukocytes, nor did it affect the total number of F4/80+ macrophages (Figure 3H). In contrast, we did find a decrease in the presence of Gr1+ granulocyte count as a result of overexpression of miR-126 (Figure 3I). Because miR-126 has been described as positively regulating mast cell proliferation and cytokine production,34 we stained the sections with toluidine blue to determine the mast cell content of the kidneys, but we could not observe any positive mast cell staining in the kidneys of either group (data not shown).

To determine whether the early protective effects observed in the LV-126 group also have beneficial effects on the longer-term fibrotic complications of IRI,35 we measured gene expression levels of fibrosis markers collagen-1α1 (Col1α1), collagen-3α1 (Col3α1), and TGF-β, and of the myofibroblast marker α-smooth muscle actin in mice that were euthanized 3 weeks after reperfusion. Indeed, we confirmed that expression of each of these markers was significantly reduced in the kidneys from the LV-126 group compared with those of the LV-C mice (Figure 3D).
**Figure 3.** miR-126 protects against renal IRI. (A) miR-126 overexpression results in decreased blood urea levels after IRI. (B) miR-126 overexpression results in less weight loss 3 days after IRI. Quantitative RT-PCR analysis normalized on glyceraldehyde 3-phosphate dehydrogenase mRNA levels of renal gene expression in LV-126 and LV-C mice of (C) KIM-1, neutrophil gelatinase-associated lipocalin, chemokine (C-C motif) ligand 2, IL-6, and IL-10 3 days after injury and (D) α-smooth muscle actin, Col1α1, Col3α1, and TGF-β 3 weeks post IRI.
Hematopoietic Overexpression of miR-126 Preserves Capillary Density after IRI and Is Associated with Increased Numbers of BM-Derived Peritubular Capillary EC

To determine the effect of hematopoietic miR-126 overexpression on renal vascular integrity after IRI, we assessed the density of the peritubular capillary network by staining for MECA32 before and after IR. We focused on the corticomedullary junction since it is known that this region is disproportionately damaged by IRI. As shown in Figure 4, A and E, we observed that the density of MECA32+ peritubular capillaries in the LV-C mice was markedly decreased 3 days after IR, while microvascular density was nearly maintained in the LV-126 mice (Figure 4G). Because both the control and miR-126 viral vectors carried the dsRed reporter gene, BM-derived cells could be identified on the basis of their dsRed positivity. Notably, a 2.2-fold increase was observed in the number of dsRed+ BM-derived cells in the kidneys of the LV-126 mice compared with the LV-C group (Figure 4, B and H). Importantly, this increase was IR dependent, as the number of dsRed+ cells in the LV-126 mice in the non-IR group was similar to that of the LV-C mice (Figure 4, F–H). Because no differences were observed in the numbers of infiltrated CD45 leukocytes or the macrophage content between the two experimental IR groups, we sought to determine the cell type that could be responsible for the increase in dsRed+ cells. On the basis of the interstitial localization of the dsRed+ cells, we could exclude the possibility that they were tubular epithelial cells. Double staining of kidneys for dsRed and the endothelial marker CD31 demonstrated that a major fraction of the BM-derived dsRed+ cells were ECs (Figure 4C). Quantification of these BM-derived capillary EC (CD31+/dsRed+) showed a significant 2.0-fold increase (P=0.04) in the LV-126 group compared with the control group (Figure 4D). Confocal imaging confirmed the presence of BM-derived ECs as we readily detected numerous dsRed+ cells costaining for MECA32 integrated into networks of MECA32+/dsRed− ECs (Figure 4, I–K). In concordance with the results of the matrigel plug assay, also in the kidney we observed colocalization of dsRed and PDGFRβ (Supplemental Figure 4). DsRed+CD31+ cells were also found in the endothelium of the larger vessels (Supplemental Figure 5A) and at a lower rate in the glomeruli of the LV-126 mice (Supplemental Figure 5B). We conclude that overexpression of miR-126 in the hematopoietic compartment helps to preserve the integrity of the renal microvasculature following IRI and is associated with an increased incorporation of BM-derived cells into the vasculature.

LV-126 Mice Display Increased Hematopoietic Stem Cell and Progenitor Cell Numbers in the Circulation Following IRI

After having shown the provasculogenic effects of BM miR-126 overexpression in the Matrigel plug assay and in the kidney after IRI, we assessed the effect of miR-126 overexpression on the hematopoietic system itself. Therefore, a detailed comparison was performed of the composition of cells in the BM and in the circulation in LV-126 and LV-C mice (Supplemental Tables 1 and 2). Little to no differences were observed in the number of white blood cells, red blood cells, platelets, hemoglobin, or hematocrit content between the two experimental groups in blood samples collected before and 4 and 8 weeks after BM transplantation. In addition, no substantial differences were found in absolute numbers of T cells, B cells, natural killer cells, plasmacytoid dendritic cells, neutrophils, or eosinophils. Because circulating myeloid cells are known to display proangiogenic properties and we previously demonstrated that proangiogenic cells could be cultured from a BM-derived immature, CD31+/Ly6Ch+ myeloid progenitor cell fraction, the monocytic cells were further subdivided into Ly6Ch, Ly6Cmed, and Ly6Clo fractions. Again, however, no significant differences could be observed between the experimental groups. Next, we assessed the effect of overexpression of miR-126 on the number of Lin−/Sca-1−/cKit+ (LSK) and Lin−/Sca-1−/Flk+ (LSF) hematopoietic stem/progenitor cells in PB and BM, before (pre-IRI) and 3 days after kidney IRI. Although the absolute numbers of circulating LSK (Figure 5A) or LSF cells (Figure 5B) before kidney IRI did not differ significantly between the experimental groups, 3 days after IRI, the LSK and LSF cells in PB were induced 2.5- and 1.5-fold, respectively (P<0.05 for both). Concomitant with their elevation in the circulation, BM levels of the LSK and LSF cells were decreased, supporting enhanced mobilization of these cells from BM to the periphery after overexpression of miR-126.

Correlation analysis (Table 1) revealed a strong positive correlation between the expression level of miR-126 in the BM and LSK cell number in PB. The number of LSK cells correlated positively with capillary density (MECA staining) in the kidney, while strong negative correlations were observed with renal injury markers (urea, KIM-1, neutrophil gelatinase-associated lipocalin), further supporting that miR-126 is the driver of the observed protective effects.

CXCR4/SDF-1 Signaling Is Affected in LV-126 Mice after IRI

Because the mobilization of LSK and LSF cells was markedly increased in the LV-126 mice, we sought to determine whether...
Figure 4. miR-126 preserves capillary density by increasing incorporation of BM-derived EC. (A) Representative microscopic images (×100) and (G) quantification of MECA32 staining in corticomedullary junctions show higher capillary density in mice that overexpress miR-126 3 days after IRI. (B) Representative microscopic images (×100) and (H) quantification of dsRed staining in the kidney show increased dsRed signal in LV-126 mice. (C) Representative images (×400) showing CD31+/dsRed+ cells as indicated by arrows and (D)
overexpression of miR-126 in the hematopoietic compartment was also associated with a shift in the balance between BM and peripheral SDF-1/CXCR4 signaling. As CXCR4 expression retains hematopoietic stem and progenitor cells in the BM and its inhibition can be used to mobilize these cells from the BM, we measured CXCR4 expression on BM-LSK cells using flow cytometry. Indeed, CXCR4 expression was selectively reduced on BM-LSK cells in the LV-126 group, while Lin<sup>+</sup> cells showed an elevated expression of CXCR4 (Figure 5, C and D). This relative loss of CXCR4 expression on LSK cells in miR-126 overexpressing BM (Figure 5D) may cause the selective mobilization of these cells to the periphery (Figure 5A). On the other hand, recruitment of hematopoietic stem and progenitor cells to ischemic tissue depends on SDF-1 expression at the site of injury. As shown in Figure 5, in the tubuli of post-ischemic kidneys of LV-126 mice, both SDF-1 mRNA (Figure 5E) and protein expression (Figure 5, F and G) were increased compared with the LV-C mice both 3 days and 3 weeks after IRI, while SDF-1 mRNA expression levels were not changed in nonischemic kidneys.

**DISCUSSION**

Here we describe that overexpression of miR-126 in the hematopoietic compartment augments neovascularization in subcutaneously implanted Matrigel plugs and protects the kidney from IRI. Protection of the kidney and vascular integrity correlated significantly with the levels of circulating vascular progenitor cells. In its turn, mobilization of LSK cells and the integration of dsRed<sup>+</sup> cells in the renal microvascular network in the LV-126 mice were directly related to BM miR-126 expression levels. Therefore, our data support a direct causal role for miR-126 augmented vasculogenesis leading to the preservation of renal function following IRI.

One mechanism by which miR-126 augments vasculogenesis is by selectively enhancing the mobilization of potentially vasculogenic stem and progenitor cells. Elevated SDF-1 expression by the kidney following IRI is known to be a main driver of the homing of BM-derived progenitor cells to the injured kidney by altering the balance of SDF-1 expression from the BM to the periphery. However, it has been demonstrated that plasma elevation of SDF-1 also mobilizes Lin<sup>+</sup> leukocytes. In our study, detailed flow cytometric analyses of the circulating hematopoietic cells in the LV-126 mice demonstrated selective mobilization of LSK and LSFL cells in response to elevated SDF-1 expression by the ischemic kidney. A possible explanation for this observation could be that, in the BM, only the LSK cells displayed lower CXCR4 expression while the lineage-positive leukocytes expressed increased levels of CXCR4 and therefore would have a higher propensity to be retained in the BM.

Several studies have described a regulatory role for miR-126 in SDF-1/CXCR4 signaling. MiR-126 was shown to target SDF-1 directly but also indirectly via targeting regulator of G protein signaling 16 (RGS16), a negative regulator of CXCR4 function. Silencing of RGS16 is thought to stimulate an auto-regulatory feedback loop that increases the production of SDF-1. Silencing of RGS16 by miR-126 could provide a mechanism for the elevated renal epithelial SDF-1 expression by LV-126 mice. Although this is speculative, miR-126 could be increased in the tubular epithelial cells by lateral transfer through the fusion of miR-126 rich, blood cell derived microvesicles as it was recently described that injection of endothelial progenitor cell (EPC)–derived miR-126–rich microparticles exerted a protective effect in IRI. Alternatively, the increased number of BM-derived EC that line the microvascular network of the kidney of the LV-126 mice could serve as a source of miR-126 via the production of microparticles; it was recently shown that, under certain conditions, such particles can cross the tubular basement membrane. In addition, activated platelets may be involved because miR-126 is among the most abundant microRNAs in platelets, and they could serve as a transporter of miR-126 to the site of injury. Because EPCs can take up platelets and their molecular content, platelets could also add to EPC function through miR-126 transfer. Furthermore, platelets constitute a rich source of local SDF-1 deposition themselves.

We propose that preservation of the capillary density in the LV-126 kidneys following IRI is a major contributor to the protected kidney function in the LV-126 mice. As this maintenance of vascular integrity after IRI is associated with an enhanced incorporation of BM-derived dsRed<sup>+</sup> endothelial cells and pericytes, we propose that overexpression of miR-126 in the hematopoietic compartment improves microvascular repair as compensation for the loss of capillaries. In addition, BM-derived EC and/or perivascular cells that had already been incorporated into the kidneys before IRI could be more resistant to injury as result of miR-126–dependent enhancement of pro-survival AKT signaling and therefore add to the protection after IRI.

Next to the effects on their mobilization, hematopoietic overexpression of miR-126 could also have direct beneficial effects on the function of vasculogenic stem and progenitor cells via its actions on AKT signaling. For instance, it was recently shown in preeclampsia that miR-126 modulates the proangiogenic properties of EPC through targeting PI3KR2, which also appears to be affected in our study.

---

quantification showing higher numbers of BM-derived EC in the miR-126 group. (E and F) Representative microscopic images (×100) and (G and H) quantification of MECA32 and dsRed staining in corticomedullary junctions of nonischemic kidney shows no differences as a result of miR-126 overexpression. (I) Confocal images (×400) confirm dsRed<sup>+</sup> ECs by MECA staining. (J) Zoomed image of dsRed<sup>+</sup> EC and corresponding crosssectional profile of fluorescent labels (K, colors correspond to images) show overlap of MECA32 and dsRed.
Augmenting AKT signaling in vascular progenitor cells could be particularly relevant in kidney disease because most patients are characterized by a state of EPC dysfunction. In addition, kidney graft function and the use of immunosuppressants directly affected EPC number and survival. In addition to BM-derived EC, we observed dsRed+ cells expressing the pericyte marker PDGFRβ in the Matrigel plug. Furthermore, a subpopulation of dsRed+ cells in the kidney were shown to be most likely pericytes. This suggests that pericytes also originated from BM. Support for this notion is emerging in the literature, where the role of BM-derived pericytes is increasingly discussed.

Taken together, the current findings demonstrate that enhancing the vasculogenic potential by overexpression of miR-126 in the hematopoietic compartment protects the kidney against injury.
kidney from IRI, further confirming the critical role of EC integrity in the progression of kidney disease. Therefore, strategies aimed at improvement of the endogenous vasculogenic potential such as described in this study not only would enhance the mobilization of the vasculogenic progenitors to the injured kidney but also may correct EPC dysfunction in renal disease.

CONCISE METHODS

Mice

C57BL/6J wild-type (CD45.2⁺) and B6.SJL-Ptprca Pepcb/BoyCrl (CD45.1⁺) mice were obtained from Charles River Laboratories (Maastricht, The Netherlands). The animal welfare committee of the Leiden University Medical Center approved all animal experimental protocols.

LVs

Stable expression of miR-126 in BM-derived Lin⁻ cells was accomplished using the vesicular stomatitis virus G protein–pseudotyped self-inactivating HIV–1–based vector LV.hU6.miRNA-126.hPGK1.DsRed.T4. In the shuttle plasmid for generating this vector, the coding sequence of murine miRNA-126 is preceded by the human U6 promoter and the coding sequence of a rapidly maturing variant of the red fluorescent protein of Discosoma species (Supplemental Figure 1A). The shuttle plasmid encoding the control vector LV.hPGK1.DsRed.T4 has the same genetic makeup as LV.hU6.miRNA-126.hPGK1.DsRed.T4 except for the absence of the human U6 gene promoter and the coding sequence of the hPGK1 gene promoter and the coding sequence of a rapidly maturing variant of the red fluorescent protein of Discosoma species (Supplemental Figure 1A). The shuttle plasmid for generating this vector, the coding sequence of murine miRNA-126 is preceded by the human U6 promoter and the coding sequence of a rapidly maturing variant of the red fluorescent protein of Discosoma species (Supplemental Figure 1A). The shuttle plasmid encoding the control vector LV.hPGK1.DsRed.T4 has the same genetic makeup as LV.hU6.miRNA-126.hPGK1.DsRed.T4 except for the absence of the human U6 gene promoter and the coding sequence of the hPGK1 gene promoter and the coding sequence of a rapidly maturing variant of the red fluorescent protein of Discosoma species (Supplemental Figure 1A).

Transduction and Transplantation of BM Cells

BM cells were isolated from the femora and tibia of CD45.1⁺ mice and were enriched for Lin⁻ cells using a Lineage Cell Depletion Kit (Miltenyi Biotec, Bergish Gladbach, Germany). Following isolation, Lin⁻ cells (80%–90% purity; Supplemental Figure 1B) were grown in StemSpan-SFEM (Stemcell Technologies, Inc., Vancouver, BC, Canada) supplemented with 50 ng/ml recombinant mouse stem cell factor, 10 ng/ml recombinant mouse thrombopoietin, and 50 ng/ml recombinant mouse fms-related tyrosine kinase 3 ligand (all from R&D Systems, Minneapolis, MN). After 24 hours, the cells were transduced with LV.hU6.miRNA-126.hPGK1.DsRed.T4 or LV.hPGK1.DsRed.T4 by spin occlusion in the presence of 4 µg/ml proteamine sulfate (Sigma-Aldrich) and maintained for another 24 hours in supplemented StemSpan-SFEM. Transduced CD45.1 donor cells (300,000/mouse) were combined with supportive spleen cells (500,000/mouse) and injected into the tail vein of lethally irradiated (8 Gy) male C57BL/6J, CD45.2⁺ acceptor mice.

Matrigel Plug Assay

Seven weeks after transplantation, mice received subcutaneous flank injections of 0.5 ml ice-cold Matrigel (BD Biosciences, Breda, The Netherlands) supplemented with 100 ng/ml recombinant mouse SDF-1 (Invitrogen) and 50 ng/ml recombinant mouse vascular endothelial growth factor (Invitrogen). After 7 days, the skin was opened to allow monitoring of the vasculature of the implants with a sidestream dark field camera. Subsequently, implants were extracted, imaged with a Leica DMi6000 microscope (Leica Microsystems, Rijswijk, The Netherlands), fixed in 4% paraformaldehyde, and snap-frozen at −80°C. From all microscopic images of the Matrigel implants, the number of visual vessels were counted on both sides of the implants. To obtain the total length of the vessels, pictures were

Table 1. Statistical analysis among different research indices

<table>
<thead>
<tr>
<th>Variable</th>
<th>Correlation</th>
<th>miR-126</th>
<th>LSK</th>
<th>LSF</th>
<th>Urea</th>
<th>MECA</th>
<th>KIM-1</th>
<th>KIM-1</th>
<th>NGAL</th>
<th>dsRed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material</td>
<td></td>
<td>BM</td>
<td>PB</td>
<td>PB</td>
<td>Serum</td>
<td>Kidney</td>
<td>Kidney</td>
<td>Kidney</td>
<td>Kidney</td>
<td>Kidney</td>
</tr>
<tr>
<td>Method</td>
<td></td>
<td>PCR</td>
<td>FACS</td>
<td>FACS</td>
<td>IHC</td>
<td>PCR</td>
<td>IHC</td>
<td>IHC</td>
<td>IHC</td>
<td>IHC</td>
</tr>
<tr>
<td>miR-126</td>
<td></td>
<td>P</td>
<td>0.007⁺</td>
<td>0.34</td>
<td>0.46</td>
<td>0.53</td>
<td>0.65</td>
<td>0.15</td>
<td>0.36</td>
<td>0.02⁺</td>
</tr>
<tr>
<td>LSK PB</td>
<td></td>
<td>r</td>
<td>0.65⁺</td>
<td>0.26</td>
<td>−0.20</td>
<td>0.20</td>
<td>−0.12</td>
<td>−0.38</td>
<td>−0.24</td>
<td>0.58⁺</td>
</tr>
<tr>
<td>LSF PB</td>
<td></td>
<td>P</td>
<td>0.007⁺</td>
<td>0.004⁺</td>
<td>0.020⁺</td>
<td>0.008⁺</td>
<td>0.03⁺</td>
<td>0.04⁺</td>
<td>0.04⁺</td>
<td>0.03⁺</td>
</tr>
<tr>
<td>r</td>
<td></td>
<td>0.65⁺</td>
<td>0.64⁺</td>
<td>−0.54⁺</td>
<td>0.68⁺</td>
<td>−0.52⁺</td>
<td>−0.50⁺</td>
<td>−0.50⁺</td>
<td>0.51⁺</td>
<td></td>
</tr>
<tr>
<td>MECA</td>
<td></td>
<td>P</td>
<td>0.34</td>
<td>0.004⁺</td>
<td>0.003⁺</td>
<td>0.001⁺</td>
<td>0.0005⁺</td>
<td>0.03⁺</td>
<td>0.001⁺</td>
<td>0.76</td>
</tr>
<tr>
<td>r</td>
<td></td>
<td>0.26</td>
<td>0.64⁺</td>
<td>−0.66⁺</td>
<td>0.77⁺</td>
<td>−0.74⁺</td>
<td>−0.51⁺</td>
<td>−0.73⁺</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>0.53</td>
<td>0.008⁺</td>
<td>0.001⁺</td>
<td>0.009⁺</td>
<td>0.002⁺</td>
<td>0.02⁺</td>
<td>0.001⁺</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r</td>
<td>0.20</td>
<td>0.68⁺</td>
<td>0.77⁺</td>
<td>−0.67⁺</td>
<td>−0.75⁺</td>
<td>−0.61⁺</td>
<td>−0.77⁺</td>
<td>−0.02</td>
</tr>
</tbody>
</table>

r indicates Spearman correlation coefficient. NGAL, neutrophil gelatinase-associated lipocalin; IHC, immunohistochemistry.

⁺Positively correlated.
⁻Negatively correlated.
digitalized and the total pixel area of the vessels was calculated using ImageJ software (National Institutes of Health, Bethesda, MD).

**Mouse Model for IRI**

Eight weeks after transplantation the renal artery and vein of mice were clamped bilaterally for 15 minutes using surgical clamps (SRT AG, Neuhausen, Switzerland) followed by reperfusion, as described previously. Kidney function was determined by measuring urea in serum samples using standard auto-analyzer methods by our hospital research services. Kidneys were recovered 72 hours or 3 weeks after reperfusion for immunohistologic analyses and assessment of gene expression.

**Immunohistology**

Sections (4 μm) of snap-frozen kidneys were air-dried and acetone-fixed. Twenty-micrometer sections of Matrigel plugs were fixed with methanol on a glass slide and subsequently blocked with 2% FCS (Bio Whittaker/Cambrex, Verviers, Belgium) and 3% BSA (Sigma-Aldrich) in PBS. For stainings involving horseradish peroxidase–conjugated secondary antibodies, endogenous peroxidase was blocked with H2O2. Sections were then incubated with specific antibodies directed against murine KIM-1 (R&D Systems), CD54 (BD-Pharmingen), neural/glial antigen-2 (Abcam, Cambridge, UK), and von Willebrand factor (Dako, Glostrup, Denmark), followed by secondary antibody conjugated to Alexa-488 or Alexa-568 (Molecular Probes). As negative control, isotype-matched IgGs were used. Horseradish peroxidase–conjugated antibodies directed against murine KIM-1 (R&D Systems), CD45.1-PE-Cy7 (BD-Pharmingen), and a cocktail against lineage-positive cells (APC-conjugated; BD-Pharmingen). Likewise, samples were prepared for these markers in combination with staining for CXCR4 (APC-conjugated; BD-Pharmingen). In a separate tube, the sample was incubated with an appropriate cocktail of isotype controls. Data were analyzed using FACS-Diva software (BD Biosciences). The gating strategy for PB subpopulations is described in detail in Supplemental Figure 6.

**RNA Isolation and Quantitative RT-PCR Analysis**

Total RNA was isolated from kidney sections or BM using Trizol reagent (Invitrogen). Reverse transcription was performed using a 5-minute 65°C incubation of 250 ng total RNA with deoxyribonucleotide triphosphates (Invitrogen) and oligo(dT) (Invitrogen) or using specific Taqman microRNA probes (miR-126; Applied Biosystems, Bleiswijk, The Netherlands). For mRNA detection, cDNA was synthesized using an M-MLV First-Strand Synthesis system (Invitrogen), and validation was carried out using SYBR Green Master Mix (Applied Biosystems). Primer sequences can be found in Supplemental Table 3. Levels of expression were determined by normalizing to glyceraldehyde 3-phosphate dehydrogenase.

The miR-levels were validated using Taqman miR assays (Applied Biosystems). These levels were normalized on RNU6B levels obtained from the same RNA. Results were normalized using Gene Expression Analysis for iCycler IQ RT-PCR Detection System (Bio-Rad Laboratories, Veenendaal, The Netherlands).

**Flow Cytometry and Blood and BM Analysis**

Whole blood was collected by incision of the tail vein or heart puncture and analyzed using a semi-automatic hematology analyzer (Sysmex F-280; Sysmex, Corp., Etten-Leur, The Netherlands), microscope (LSM 700; Leica), and flow cytometry (FACS, LSR II; BD Biosciences). Hematologic values obtained included white blood cell counts (number of cells×106/ml), red blood cell counts (number of cells×109/ml), platelets (number of cells×109/ml), hematocrit (%/%), and hemoglobin (mmol/L). For microscopic images, a smear was made from PB or BM on a glass cover, and images were made using a fluorescence microscope. For FACS analysis, we incubated 35 μl of whole blood or 106 BM cells for 30 minutes at 4°C with directly conjugated antibodies directed against CD45.1-PE-Cy7 (BD-Pharmingen), CD45.2-APC-Cy7 (Becton Dickinson), B220-APC-Cy7 (eBioscience), CD11b-APC (Biolegend, San Diego, CA), Ly6G-PE (Becton Dickinson), CD115-PerCP-Cy5.5 (R&D Systems), and Ly6C-FITC (Bioconnect, Huisken, The Netherlands). A separate sample was prepared for incubation with Sca-1- FITC (BD Biosciences), CD117-PerCP-Cy5.5 (BD-Pharmingen), and a cocktail against lineage-positive cells (APC-conjugated; BD-Pharmingen). Likewise, samples were prepared for these markers in combination with staining for CXCR4 (MBL International, Woburn, MA). After labeling, cell suspensions were washed with PBS containing 1% BSA (Sigma-Aldrich) and 0.01% sodium azide. Erythrocytes were removed by addition of lysis buffer (0.155 M NH4Cl, 0.01 M KHCO3, 0.1 mM EDTA), and, finally, cells were fixed with 1% paraformaldehyde. In a separate tube the sample was incubated with an appropriate cocktail of isotype controls. Data were analyzed using FACS-Diva software (BD Biosciences). The gating strategy for PB subpopulations is described in detail in Supplemental Figure 6.

**Statistical Analyses**

Results are expressed as mean±SEM. Statistical analysis was performed using t tests. For correlation analysis, Spearman rank correlation coefficient was used. P<0.05 was considered to represent statistically significant differences. Asterisks and hashtags indicate levels of significance as follows: *P<0.05, **P<0.01, ***P<0.001, and ##P<0.10.

**ACKNOWLEDGMENTS**

We would like to thank Martijn H. Brugman and Kim J.M. Janssen for technical support. This work was supported in part by the Dutch Heart Foundation (NHS grant 2006B145), the Dutch Kidney Foundation (grants C07.2227 and C09.2329), a grant from the Genzyme Renal Innovations Program, and a grant from The Netherlands Institute of Regenerative Medicine.
DISCLOSURES

None.

REFERENCES

40. Hattori K, Heissig B, Ra

This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2013060640/-/DCSupplemental.