MicroRNA-24 Antagonism Prevents Renal Ischemia Reperfusion Injury

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ABSTRACT

Ischemia-reperfusion (I/R) injury of the kidney is a major cause of AKI. MicroRNAs (miRs) are powerful regulators of various diseases. We investigated the role of apoptosis-associated miR-24 in renal I/R injury. miR-24 was upregulated in the kidney after I/R injury of mice and in patients after kidney transplantation. Cell-sorting experiments revealed a specific miR-24 enrichment in renal endothelial and tubular epithelial cells after I/R induction. In vitro, anoxia/hypoxia induced an enrichment of miR-24 in endothelial and tubular epithelial cells. Transient overexpression of miR-24 alone induced apoptosis and altered functional parameters in these cells, whereas silencing of miR-24 ameliorated apoptotic responses and rescued functional parameters in hypoxic conditions. miR-24 effects were mediated through regulation of H2A histone family, member X, and heme oxygenase 1, which were experimentally validated as direct miR-24 targets through luciferase reporter assays. In vitro, adenoviral overexpression of miR-24 targets lacking miR-24 binding sites along with miR-24 precursors rescued various functional parameters in endothelial and tubular epithelial cells. In vivo, silencing of miR-24 in mice before I/R injury resulted in a significant improvement in survival and kidney function, a reduction of apoptosis, improved histologic tubular epithelial injury, and less infiltration of inflammatory cells. miR-24 also regulated heme oxygenase 1 and H2A histone family, member X, in vivo. Overall, these results indicate miR-24 promotes renal ischemic injury by stimulating apoptosis in endothelial and tubular epithelial cell. Therefore, miR-24 inhibition may be a promising future therapeutic option in the treatment of patients with ischemic AKI.

Figure 1. Expression and function of miR-24 in the kidney and distinct renal cell populations. The expression of miR-24 in mouse kidneys is depicted at 24 and 168 hours (A) after unilateral I/R injury (n=7 each). Expression of miR-24 in sorted cells after digestion of post-ischemic mouse kidney at reperfusion for 24 and 168 hours is shown (B). Levels of miR-24 were compared with snoRNA-202 as control. MiR-24 expression normalized to RNU-48 in biopsy specimens from kidney transplant recipients with long compared with short CIT (n=5 in each group) CD31+, endothelial cells; LTA+/KIM-1−, uninjured proximal tubular epithelial cells; LTA+/KIM-1+, injured proximal tubular epithelial cells; LTA+/KIM-1−, uninjured proximal tubular epithelial cells; LTA+/KIM-1+, injured proximal tubular epithelial cells.
injury is most pronounced in the S3 segment of the proximal tubule located in the outer medulla and is particularly susceptible to hypoxia.\textsuperscript{4} Capillary rarefaction during this process is associated with chronic hypoxia, potentiating tubular injury and leading to tubulointerstitial fibrosis. Currently, a targeted therapy for this important clinical disorder is not available.

MicroRNAs (miRNAs) are under intense investigation as powerful regulators of various diseases with potential critical effect on disease initiation and/or progression, including kidney disease.\textsuperscript{5} MiRNAs represent small noncoding RNA transcripts with a length of approximately 22 nucleotides, which through post-transcriptional binding of the 3’-untranslated region (UTR) of mRNA targets lead to the repression of gene/protein expression and/or translational inhibition of protein synthesis.\textsuperscript{5} Intriguingly, a single miRNA may alter the expression of many target genes, thus influencing a specific abnormality by regulating whole disease–specific pathways and signaling cascades rather than a single gene. This unique function underlines the immense importance of these small molecules. Recently, miR-24 has been shown to be critically involved in endothelial apoptosis during cardiac I/R injury as well as apoptosis of cancer and T cells.\textsuperscript{6–8} In the present study we demonstrated miR-24 to be upregulated in kidneys of renal transplant recipients with prolonged cold ischemia time (CIT) and mice subjected to experimental I/R injury. In addition, miR-24 inhibition affected kidney function and inflammation of I/R kidneys through regulation of tubular epithelial as well as endothelial cell apoptosis. Moreover, treatment with a locked nucleic acid (LNA) targeting miR-24 resulted in improved renal outcome and survival of mice subjected to bilateral I/R injury.

**RESULTS**

**miR-24 in renal I/R injury**

Levels of miR-24 are increased in mouse kidneys at 24 and 168 hours after induction of I/R injury compared with contralateral control kidneys (Figure 1A). Cell-sorting analysis after digestion of whole kidneys after I/R injury revealed a specific enrichment of miR-24 in tubular epithelial (LTA\textsuperscript{+}/KIM-1\textsuperscript{−} cells) and endothelial cells (CD31\textsuperscript{+} cells) (Figure 1B) at 24 hours of reperfusion. At a reperfusion time of 168 hours after I/R injury, miR-24 was upregulated in injured tubular epithelial cells (LTA\textsuperscript{+}/KIM-1\textsuperscript{−} cells). At day 7 after reperfusion, the level of miR-24 in LTA\textsuperscript{+}/KIM-1\textsuperscript{−} cells (healthy tubules) changed to levels of controls. A slight, nonsignificant decrease in miR-24 expression was detected in pericytes (PDGFRb\textsuperscript{+}). In kidney transplant biopsy specimens from patients with prolonged CIT (n=5 per group), miR-24 increased, indicating a distinct pathophysiologic role in this setting (Figure 1C).

**Functional Role of miR-24 in Tubular Epithelial Cells**

Chemical anoxia/ATP depletion for 1 hour followed by ATP repletion for 30 minutes led to an enrichment of miR-24 in HK-2 cells (Figure 2B). ATP depletion as well as treatment of cells with 0.2 μM staurosporin (to induce apoptosis) resulted in a significant increase in apoptosis, as assessed by terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining (data not shown). Intriguingly, transfection of cells with miR-24 precursors without any additional cellular stressors culminated in an increase in apoptosis as assessed by TUNEL staining (Figure 1, D–F) and FACS analysis following Annexin V–Cy657/7-AAD staining of cells (Figure 2D). Analysis of 8-OHdG formation in HK-2 cells following miR-24 precursor transfection indicated a significant increase in reactive oxygen production (data not shown). Scratch migration analysis following miR-24 enrichment indicated a defect in tubular epithelial migratory capacity (Figure 1, G–I).

**Functional Role of miR-24 in Endothelial Cells**

Incubation of human umbilical vein endothelial cells in a hypoxic environment for 24 hours (0.1% O\textsubscript{2}) followed by reoxygenation for 3 hours resulted in a significant enhancement of apoptosis (TUNEL and Annexin V-Cy657/7-AAD staining of cells) (Figure 2, C–F) and upregulation of miR-24 (Figure 2A) in vitro. Transient enrichment of miR-24 suppressed tube formation capacity as well as migration capacity (Supplemental Figure 1, A–F).

**Sphingosine-1-Phosphate Receptor 1; H2A Histone Family, Member X; and Heme Oxygenase-1 Are Direct Targets of miR-24 In Vitro**

To identify miR-24 targets, which potentially induce tubular as well as endothelial cell apoptosis, we first used bioinformatic miRNA target prediction tools and observed many genes with putative 3’UTR binding sites for miR-24 that had previously been described to have important functional roles in apoptosis development. In addition, we performed a global messenger RNA expression analysis in proximal tubular epithelial cells following overexpression of miR-24 precursors (Figure 3A). In total, 1822 genes were downregulated in cells overexpressing miR-24 compared with cells transfected with a prenegative control oligonucleotide. Cluster analysis of the top 50 up- and downregulated genes identified several genes involved in apoptosis regulation (Figure 3A). Downregulated genes of the array were subsequently merged with predicted targets of miR-24 (Targetscan). These are shown in Figure 3B.
Expression of miR-24 in distinct kidney cells and role in apoptosis regulation. Expression of miR-24 normalized to RNU-48 in hypoxic compared with normoxic control human umbilical vein endothelial cells (HUVECs) (A) and HK-2 cells exposed to ATP depletion (B) is shown. Percentage of early apoptotic (Annexin+/7AAD−) as well as late apoptotic (Annexin+/7AAD+) HUVECs (C) and proximal tubular epithelial cells (D) in FACS analysis is shown. TUNEL staining in cultured endothelial cells after prenegative control (E) and pre-miR-24 oligonucleotide (F) transfection and quantification of results (G) (n=6 experiments). TUNEL+ cells in outer medulla in mice after ischemia and reperfusion for 24 hours treated with control LNA (LNA-CONT; H) and LNA-24 (I) and quantification of results (J). ***P<0.0001; **P<0.01; *P<0.05. CTL = control.
Figure 3. Cluster analysis of miR-24 targets in tubular epithelial cells overexpressing miR-24. Affymetrix gene array and cluster analysis in tubular epithelial cells transfected with prenegative control and pre-miR-24 oligonucleotide (A) is shown. Bioinformatically predicted targets of miR-24 (as obtained from Targetscan) were cross-checked with the results of the array (B). Downregulated genes (fold regulation) of the array, subsequently merged with predicted targets of miR-24 (Targetscan), are shown in Figure 3B. Targets further analyzed (H2A.X and HO-1) are highlighted in red (B).
Figure 4. Validation of miR-24 targets and adenoviral rescue assays. Protective role of anti–miR-24 treatment in tubular epithelial cells and miR-24 targets involved in miR-24s action in renal epithelial cells and endothelial cells. Scratch migration analysis in hypoxic HK-2 cells after antinegative control (A) and anti–miR-24 oligonucleotide (B) transfection and quantification of results (C) (n=6 experiments). Western blot analysis in HUVECs and HK-2 cells of cytosolic S1PR1 and HO-1 normalized to glyceraldehyde 3-phosphate dehydrogenase (Gapdh) expression (D–F). TUNEL staining in HK-2 cells infected with H2A.X virus (G–I) and S1PR1 virus (J) and quantification of TUNEL-positive cells (K) (n=3 experiments).
In our subsequent in vitro analyses, we focused on sphingosine-1-phosphate receptor 1 (SIP1R); H2A histone family, member X (H2AX); and heme oxygenase-1 (HO-1). To validate these targets, proximal tubular and endothelial cells were transfected with miR-24 precursors. This resulted in the repression of H2AX, HO-1, and SIP1 protein expression in endothelial and tubular epithelial cells (Figure 4D).

When we fused the respective 3′ UTR regions to a luciferase reporter gene and determined luciferase activity in cells transfected with synthetic miR-24 precursors, miR-24 significantly repressed luciferase activity (Figure 4, E and F). We thus identified SIP1R and H2AX as novel direct targets of miR-24. We previously confirmed HO-1 to be a bona fide target of miR-24.9

Functionally, small interfering RNA–mediated silencing of these miR-24 targets (HO-1, H2AX, and SIP1R) in cells subjected to hypoxia (0.1% O2 for 24 hours with subsequent reoxygenation for 3 hours) mimicked the miR-24 enrichment results and induced an impairment of endothelial tube formation (Supplemental Figure 2, A–E) and migration capacity (Supplemental Figure 2, F–J) as well as tubular (Supplemental Figure, 3A–E) and endothelial cell apoptosis (data not shown).

Protective Therapeutic Effect of miR-24 Antagonism In Vitro and Adenoviral Rescue Assays

Silencing of miR-24 in tubular epithelial cells subjected to hypoxia significantly improved migratory capacity in scratch migration assays (Figure 4, A–C). In hypoxic endothelial cells, tube formation capacity was significantly improved through silencing of miR-24 (Figure 5, A–C). To assess whether the miR-24 effects are mainly mediated by its targets H2AX, SIP1R, and HO-1, we reconstituted those targets in miR-24–overexpressing endothelial and tubular epithelial cells by transduction with adenoviral constructs lacking miR-24 binding sites. Reconstitution of miR-24–resistant H2AX and HO-1 rescued miR-24–mediated endothelial apoptosis (data not shown), impaired tube formation capacity (Figure 5, D–D), and migration capacity (Figure 5, I–M), as well as tubular epithelial cell apoptosis (Figure 4, G–K), while SIP1R had no effect on any of the investigated end points. Adenoviral constructs alone (without pre-miR transfection) do not induce apoptosis.

Markers of Kidney Damage, Endothelial Activation, and Inflammation in Unilateral I/R Injury after miR-24 Silencing

Treatment of mice with an LNA-modified anti-miR targeting miR-24 before induction of I/R injury (Figure 6, G–J) resulted in a marked reduction of kidney injury marker gene expression (NGAL and KIM-1). Capillary rarefaction on day 1 after I/R injury was significantly improved in animals treated with an LNA-modified anti-miR targeting miR-24 (Figure 6, K–M). In addition, markers of endothelial activation (vascular cellular adhesion molecule-1) decreased significantly (data not shown).

Kidney Morphology, Infiltration of Immune Cells, and Level of Apoptosis after miR-24 Silencing

LNA-24 treatment resulted in a significant improvement of kidney morphology on day 1 after I/R injury in unilateral (Figure 6D, E and F) as well as bilateral I/R injury (Supplemental Figure 4, D and E) and a reduction in epithelial injury in mice (see Figure 6F for unilateral, Supplemental Figure 4F for bilateral I/R injury). Infiltration of CD45+, F4/80+ macrophages, CD4+ and CD8+ T cells, and Ly6g+ neutrophils significantly decreased after LNA-24 treatment as assessed by immunofluorescence at all investigated time points (Figure 7A–O, data for reperfusion of 168 hours in unilateral I/R are shown). Tubular cell apoptosis as assessed by TUNEL staining was significantly lower in LNA-24–treated animals at 24 hours of reperfusion (Figure 2, G–I).

Regulation of miR-24, Survival, Kidney Function, and Markers of Kidney Damage and Inflammation in Bilateral I/R Injury In Vivo

Treatment of mice with an LNA-modified anti-miR targeting miR-24 before induction of I/R injury (24 hours) resulted in significant improvement of survival compared with mismatch control–treated animals (Figure 6C). This was accompanied by preserved kidney function (lower levels of serum creatinine and urea levels; see Figure 6, A and B). On day 7 after induction of bilateral I/R injury, levels of renal function decreased further and normalized to levels in sham-operated animals on day 14 (data not shown). Moreover, treatment of mice with an LNA-modified anti-miR targeting miR-24 in bilateral renal I/R injury (Supplemental Figure 4, B and C) resulted in a marked reduction of kidney injury marker gene expression (NGAL and KIM-1). In addition, expression of inflammatory gene expression (IL-1β, IL-6, monocyte chemoattractant protein 1, macrophage inflammatory protein-2α, TNF-α) in bilateral I/R injury was significantly lower in animals treated with LNA-modified anti-miR-24 after I/R injury (Supplemental Figure 5, D–H). In vivo, in a model of bilateral I/R injury, we found HO-1 to be significantly upregulated after miR-24 antagonism compared with LNA-mismatch treated animals in the outer medulla (P<0.01). H2AX was also upregulated by miR-24 inhibition, although not to a statistically significant level (P=0.08). SIP1R was not regulated in vivo (Supplemental Figure 4, G–I, for H2AX; Supplemental Figure 4, J–L, for SIP1R; Supplemental Figure 4, M–O, for HO-1).

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dehydrogenase (Gapdh) and nuclear H2AX normalized to cAMP response element-binding protein after transfection with prenegative control and pre–miR-24 oligonucleotides. Results of luciferase gene reporter assays concerning H2AX (E) and SIP1R (F). TUNEL stainings in miR-24–overexpressing tubular epithelial cells after transduction with adenoviral constructs lacking miR-24 binding sites for control virus (CTL; G), HO-1 virus (H), H2AX virus (I), SIP1R virus (J), and quantification of results (K). **P<0.01; *P<0.05. hpf, high power field.
Figure 5. Adenoviral rescue assays in miR-24 overexpressing endothelial cells. Tube formation capacity (total tube length in micrometers) in hypoxic HUVECs transfected with antinegative control oligonucleotides (A), anti-miR-24 oligonucleotides (B), and quantification of results (C). Tube formation capacity in miR-24-overexpressing endothelial cells after transduction with adenoviral constructs lacking miR-24 binding sites for control virus (CTL; D), HO-1 virus (E), H2A.X virus (F), S1PR1 virus (G), and quantification of results (H). Migration capacity in Boyden chamber assays in miR-24-overexpressing endothelial cells after transduction with adenoviral constructs lacking miR-24 binding sites for control virus (CTL; I), HO-1 virus (J), H2A.X virus (K), S1PR1 virus (L), and quantification of results (M). **P<0.01; *P<0.05.
Figure 6. *In vivo* effect of miR-24 antagonism in murine I/R injury. Protective rescue of renal I/R injury following anti-miR-24 therapy. Renal function parameters (serum creatinine [A] and urea [B]) as well as Kaplan–Meier curve survival analysis (C) in mice treated with an LNA-modified anti-miR targeting miR-24 (LNA-24) and a mismatch control LNA (CONT) 24 hours before induction of I/R injury, as well as sham-operated animals. Bilateral renal I/R injury was performed for 27 minutes. Observation period from days 0 to 7; n=20 per treatment group, n=4 in the sham group. Differences in urea levels at day 7 are underestimated because of loss of uremic mice in the
MiR-24 in the Progression from AKI to CKD
At a reperfusion time of 168 hours, the level of developing fibrosis was highly attenuated in mice treated with an LNA-modified anti-miR targeting miR-24 (Figure 6, N–P) compared with mismatch treated animals subjected to unilateral I/R injury. In addition, the expression of fibrosis-associated genes, including those for collagen I, αI, collagen III, and α-smooth muscle actin, was blunted in LNA-24 treated mice at all three investigated time points (see Supplemental Figure 6, A–I, for unilateral I/R; Supplemental Figure 5, A–C, for bilateral I/R).

DISCUSSION

In the present study, we found that miR-24 affected endothelial and tubular epithelial cell apoptosis in murine renal I/R injury as well human kidney transplant–associated renal I/R injury. A global messenger RNA expression analysis in proximal tubular epithelial cells revealed many apoptosis-associated genes to be deregulated after miR-24 modulation. In particular, miR-24 was found to target prominent antiapoptotic proteins, including S1PR1, H2A.X, and HO-1 in vitro. Additionally, HO-1 and H2A.X were prominent targets of miR-24 in vivo. Viral reconstitution of these targets in miR-24–overexpressing endothelial and tubular epithelial cells ameliorated various functional parameters in vitro. Finally, silencing of miR-24 in vivo ameliorated renal I/R injury, infiltration of various immune cells and survival as well as kidney function in mice. Silencing of miR-24 in vivo culminated in a repression of renal fibrosis following I/R injury.

Several studies have recently examined the role of specific miRNAs in the pathogenesis of renal I/R injury. For instance, Godwin and coworkers elegantly identified miR-21 as one of the most highly induced miRNAs during renal I/R injury.10 MiR-127 contributed to the severity of renal I/R injury through a modulation of cell trafficking.11 However, none of the studies presented so far evaluated the protective potential of specific pharmacologic miRNA inhibition during renal I/R injury. We provide evidence of the in vivo relevance of miR-24 in human and murine renal I/R injury. Moreover, we identify the underlying molecular mechanisms by analyzing specific apoptosis–associated targets of miR-24. Most important, we demonstrate the feasibility and efficacy of in vivo miR-24 inhibition as a protective therapeutic option during renal I/R injury.

A recent study showed that targeted deletion of DICER from the proximal tubular epithelium protects against I/R-induced renal injury through expression changes of various miRNAs.12 Interestingly, DICER knockdown was associated with reduced expression of miR-27a. This miRNA belongs to an miRNA cluster, which is simultaneously transcribed. The miR-23/27/24 gene cluster is composed of two separate gene clusters located at different genomic loci.13 The intergenic miR-24–2 cluster is encoded on human chromosome 19p13.13, expressing miR-23a, miR-27a, and miR-24–2.13,14 This cluster is involved in angiogenesis and endothelial apoptosis in cardiac ischemia and retinal vascular development.13 Moreover, miR-24 regulates apoptosis of cancer and T cells.7,8 Overexpression of the miR-23a, miR-27a, and miR–24–2 cluster induced apoptosis of human embryonic kidney cells.15 The initial finding of our study, that miR-24 is highly enriched in kidneys of transplant recipients with prolonged CIT, prompted us to identify the underlying mechanisms in a mouse model of renal I/R injury. Most strikingly, miR-24 inhibition ameliorated kidney injury and function as well as overall survival of mice. We demonstrated specific enrichment of miR-24 in endothelial (CD31+ cells) and tubular epithelial cells (LTA+/KIM-1− cells) at 24 hours of reperfusion through sorting of cells based on distinct surface receptors. Interestingly, at a reperfusion time of 168 hours after I/R injury, miR-24 was also upregulated in injured tubular epithelial cells (LTA+/KIM-1− cells). We believe that miR-24 is elevated soon after induction of I/R injury in tubular epithelial cells and drives the subsequent injurious events in these cells. In line with this hypothesis, we saw robust upregulation of miR-24 in injured tubular epithelial cells at day 7 (LTA+/KIM-1− cells).

To identify the downstream mechanism of miR-24–regulated protection, we used a global mRNA array analysis that revealed many deregulated apoptosis–associated genes. We focused on H2A.X, HO-1, and S1PR1, all of which have established roles in recovery of renal I/R injury and/or apoptosis.16–18 H2A.X and HO-1 are protective concerning DNA-damage and oxidative stress and were among the strongest down-regulated events in these cells. In line with this hypothesis, we saw robust upregulation of miR-24 in injured tubular epithelial cells at day 7 (LTA+/KIM-1− cells).

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Figure 7. Infiltration of inflammatory cells following miR-24 antagonism in vivo. Immunofluorescence stainings and quantification of representative cryosections (4 μm) in outer medulla of mice treated with control LNA (LNA-CONT) and LNA-24 concerning CD45⁺ (A–C), CD4⁺ (D–F), CD8⁺ (G–I), Ly6g⁺ - neutrophils (J–L), and F4/80⁺ macrophages (M–O) at reperfusion time of 168 hours. Specific immunofluorescence stainings in red and DAPI in blue. **P<0.01; *P<0.05.
upregulated by miR-24 antagonism, underscoring its in vivo significance as a downstream effector of miR-24. H2A.X showed a trend for regulation, while SIPRI was not regulated. We thus propose HO-1 as the major factor in miR-24-mediated ischemic AKI.

MiR-24 inhibition in renal I/R injury primarily resulted in protection against endothelial and tubular epithelial apoptosis. Strikingly, posts ischemic fibrosis development was also highly attenuated in mice treated with an LNA-modified anti-miR targeting miR-24. These effects can be attributed to enhanced capillary density and tubular epithelial cell survival after miR-24 inhibition.

In conclusion, we show that miR-24 contributes to renal I/R injury by influencing endothelial and tubular epithelial apoptosis through regulation of antiapoptotic HO-1 and H2A.X. MiR-24 has also previously been shown to have an antiapoptotic function in cancer cells and cardiomyocytes.20,21 However, here we provide clear evidence of the proapoptotic role of miR-24 in renal I/R injury. Silencing of miR-24 ameliorates the apoptotic response in vivo, leading to suppressed tubular epithelial and endothelial apoptosis; this, in turn, is associated with enhanced capillary density and reduced tubulointerstitial fibrosis and potentially blunting of the AKI-to-CKD continuum. Of note, this is the first report showing that pharmacologic miRNA inhibition might be a viable therapeutic option in the treatment of patients with this life-threatening clinical disorder. Intriguingly, miR-24 is also enriched in transplanted kidneys of patients with prolonged CIT; indicating its potential role in human renal I/R-injury. This study highlights that miR-24 modulation might ultimately lead to the first targeted clinically applicable therapy for AKI.

CONCISE METHODS

Patients
In kidney transplant recipients, who presented with prolonged (707 ± 73 minutes, n=5) and short (208 ± 56 minutes, n=5) CIT, renal biopsy specimens and clinical and demographic data were collected. RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. For detection of miRNAs in samples, TaqMan miR-24 primer assays (Applied Biosystems) were applied. The small RNA molecule RNU-48 was amplified as a control. The Ethics Committee of the Hannover Medical School approved the study, and all patients gave their written informed consent (approval number 2765). Patient characteristics are shown in Table 1.

Animal Studies
Male C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany) and were housed under standard conditions. Mice 10–12 weeks old weighing between 20 and 30 g were used for all experiments.

All animal experimental procedures agreed with institutional and legislative regulations and were approved by the local authorities (approval number 08/1434).

Other methodologic details are given in the Supplemental Material.

ACKNOWLEDGMENTS

We would like to acknowledge the support of the Else-Kröner-Fresenius Foundation awarded to J.L. and T.T. and the assistance of the Cell Sorting Core Facility of the Hannover Medical School, supported in part by Braukmann-Wittenberg-Herz-Stiftung and Deutsche Forschungsgemeinschaft. The technical assistance of Annette Just is greatly appreciated.

DISCLOSURES

None.

REFERENCES


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2013121329/-/DCSupplemental.