MicroRNA-24 Antagonism Prevents Renal Ischemia Reperfusion Injury

Johan M. Lorenzen,*† Tamas Kaucsar,*‡ Celina Schauerte,* Roland Schmitt,† Song Rong,† Anika Hübner,* Kristian Scherf,* Jan Fiedler,* Filippo Martino,* Regalla Kumarswamy,* Malte Kölling,* Inga Sörensen,† Hebke Hinz,§ Joerg Heineke,§ Eva van Rooij,¶ Hermann Haller,† and Thomas Thum*§

*Institute of Molecular and Translational Therapeutic Strategies, †Department of Nephrology, and §Department of Cardiology and Angiology, Hannover Medical School, Hannover Germany; ‡Institute of Pathophysiology, Semmelweis University, Budapest, Hungary; ¶Hubrecht Institute, Royal Netherlands Academy of Arts and Sciences, and University Medical Center Utrecht, Utrecht, The Netherlands; and ¶National Heart and Lung Institute, Imperial College London, London, United Kingdom.

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.


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Ischemia-reperfusion (I/R) injury of the kidney is one of the primary causes of AKI. It is associated with severe morbidity and mortality and thus represents a major socioeconomic health problem. It is a consequence of a variety of different injurious insults in native kidneys (e.g., during cardiac surgery). Moreover, it is commonly associated with the transplantation procedure and thus is an unavoidable phenomenon in transplanted kidneys. During ischemic AKI, a transient drop in blood flow to the kidney is followed by a reperfusion period. Reperfusion itself, although vital to restoration of kidney function, is associated with substantial additional cellular injury. In the kidney blood flow to the outer medulla is disproportionately reduced with respect to the reduction in total blood flow. Thus, epithelial cell...
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.\(^4\) 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.\(^5\) 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.\(^6\) 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.\(^6-8\) 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\(^+/\)KIM-1\(^-\) cells) and endothelial cells (CD31\(^+\) 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\(^+/\)KIM-1\(^-\) cells). At day 7 after reperfusion, the level of miR-24 in LTA\(^+/\)KIM-1\(^-\) cells (healthy tubules) changed to levels of controls. A slight, nonsignificant decrease in miR-24 expression was detected in pericytes (PDGFRb\(^+\)). 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 \(\mu\)M 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% \(O_2\)) 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.
Figure 2. 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) (D) and H2AX and CREB normalized to Gapdh (E). Luciferase activity of S1PR1 and H2AX normalized to CTL (F). TUNEL+ cells/htf (G–J)
In our subsequent in vitro analyses, we focused on sphingosine-1-phosphate receptor 1 (S1PR1); H2A histone family, member X (H2A.X); 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 H2A.X, HO-1, and S1PR1 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 S1PR1 and H2A.X 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, H2A.X, and S1PR1) 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 H2A.X, S1PR1, 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 H2A.X 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 S1PR1 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 unilateral 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 6, D and E) 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+ and 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). H2A.X was also upregulated by miR-24 inhibition, although not to a statistically significant level (P=0.08). S1PR1 was not regulated in vivo (Supplemental Figure 4, G–I, for H2A.X; Supplemental Figure 4, J–L, for S1PR1; Supplemental Figure 4, M–O, for HO-1).

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dehydrogenase (Gapdh) and nuclear H2A.X normalized to cAMP response element-binding protein after transfection with prenegative control and pre–miR-24 oligonucleotides. Results of luciferase gene reporter assays concerning H2A.X (E) and S1PR1 (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), H2A.X virus (I), S1PR1 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 α2, 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 expression changes of various miRNAs.12 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 miRNA 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 targets in our profiling approach.17,19 S1PR1 is a predicted target of miR-24 and has previously been described as an important factor in the resolution of renal I/R injury.16 Interestingly, reconstitution of H2A.X and HO-1 in miR-24 overexpressing endothelial and tubular epithelial cells underlined their critical importance in miR-24–mediated effects on apoptosis regulation. In vivo, HO-1 was significantly

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).

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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.

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**DISCLOSURES**

None.

**REFERENCES**


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Supplemental Materials and Methods

**Statistical Analysis**

Average data are presented as mean and SEM unless otherwise stated. All statistical analyses were performed with the SPSS package (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software (GraphPad Prism Software Inc. San Diego, California, USA). Two-sided p-values <0.05 were considered statistically significant for all statistical procedures used. For statistical comparison of 2 groups, we used an unpaired 2-tailed Student t test; for the comparison of 3 or more groups, we used ANOVA followed by Tukey post hoc tests. In the Figures, probability values are indicated by 1 (p<0.05), 2 (p<0.01) or 3 (p<0.001) asterisks.

**LNA-modified miRNA oligonucleotides**

The oligonucleotides were provided by miRagen Therapeutics (Boulder, CO 80301, U.S.A.). The antimiR-24 is a 16 mer oligonucleotide chemistry composed of LNA and DNA directed against base 2-17 of mature miR-24. In brackets the mature miR-24 sequence is given, underlined are the binding sites of the LNA-modified antimiR directed against miR-24 (GACAAGGACGACUUGACUGGU). The control antimiR has a comparable chemical composition but is directed against a C. elegans expressed microRNA.

**Ischemic/Reperfusion injury protocol**

Clamping of renal pedicles was applied to induce significant renal I/R-injury as described previously\(^1\). Following isoflurane anaesthesia male C57BL/6 mice were subjected to median laparotomy, thereafter renal pedicles were dissected and a vascular clamp will be applied for 27 minutes.

Mice were dosed with intraperitoneal injections (i.p.) of a locked nucleic acid (LNA) targeting miR-24 (LNA-24) as well as control mismatch LNA at a concentration of 10 mg/kg 24 hours before the operation. Animal operation and survival analyses were performed at Phenos GmbH, Hanover, Germany. For survival analyses and measurement of renal function parameters mice were subjected to bilateral renal IR injury (20 mice received mismatch LNA, 20 mice received LNA targeting miR-24).

For the survival analysis sham operated animals (n = 4) were also included. Blood samples for analysis of renal function parameters (serum-urea and –creatinine) were drawn on days 0, 1, 3 and 7 (analysed on a Beckman Analyzer, Beckman Instruments GmbH, Munich, Germany). In a second group of mice the renal pedicle was only clamped on the left side (unilateral I/R-injury). In this setting the
contralateral kidney serves as an internal control to the injured kidney (I/R-kidney). These animals were sacrificed on day 1, day 3 and day 7 after renal I/R-injury and kidneys were harvested for further examination. At each time point 7 mice were injected with LNA targeting miR-24 and 7 mice received mismatch LNA. In vivo studies conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

**Histology, Immunostaining, and TUNEL stainings**

After kidney extraction, a representative part of each kidney was fixed immediately in PBS-buffered 4% paraformaldehyde and embedded in paraffin. Certain immunostainings were also performed in cryosections. Four-micrometer sections were used for immunostaining and for hematoxylin/eosin staining to evaluate histologic damage.

The severity of morphologic renal damage was assessed in a blinded manner using an arbitrary score based on HE-stained kidney sections following a modification of a protocol developed by Broekema et al. Briefly, the extent of four typical I/R-injury–associated damage markers (i.e., dilatation, denudation, intraluminal casts, loss of brush border membrane and cell flattening) was expressed in arbitrary units (AU) in a range of 0 to 4 according to the percentage of damaged tubules: 0, no damage; 1, less than 25% damage; 2, 25%–50% damage; 3, 50%–75% damage; and 4, more than 75% damage. Immunostainings for inflammatory cell influx was performed using the following primary antibodies: monoclonal rat anti-mouse F4/80 (Serotec, Oxford, United Kingdom), monoclonal rat anti-mouse CD45 (BD Pharmingen, BD Biosciences, Santa Cruz, CA), affinity-purified rat anti-mouse Ly-6G/Gr-1 (eBioscience, San Diego, CA), purified Rat Anti-Mouse CD4 (BD Pharmingen, BD Biosciences, Santa Cruz, CA). Analyses of capillary rarefaction in outer medulla were evaluated after fluorescent immunohistochemical staining for polyclonal rabbit anti-mouse CD31 (Abcam, Cambridge, UK). Deparaffinized kidney sections were boiled in citrate buffer for antigen retrieval, blocked with 5% milk, and incubated overnight at 4°C with primary antibodies. This was followed by antibody visualization using Alexa 488/Alexa 547 secondary antibodies (Molecular Probes/Invitrogen, Carlsbad, CA). Quantification of CD45-, F4/80-, CD31-, CD4-, and Ly-6G-expressing cells was done by counting of positive cells in ten randomly chosen, non-overlapping fields in outer medulla. A fluorescein *in situ* cell death detection kit was used according to the manufacturer’s instructions for TUNEL assay (Roche Applied Science, Mannheim, Germany). TUNEL-positive tubular cells and total DAPI (4’,6-diamidino-2-
phenylindole)-positive tubular cells were counted in ten non-overlapping fields of outer medulla in each sample. Data are presented as a percent ratio of TUNEL-positive epithelial cells versus total DAPI-positive epithelial cells.

Cell Culture experiments
For in vitro analyses primary proximal tubular epithelial cells (PTEC), immortalized human proximal tubular cells (HK-2 cells), mouse microvascular endothelial cells (MMVECs) or human umbilical vein endothelial cells (HUVECs) were used. HUVECs and MMVECs were cultured in endothelial basal medium-2 (EBM2) culture medium supplemented with EGM SingleQuots (Cambrex, Verviers, Belgium), 10% FCS and 1% Penicillin/Streptomycin. HK-2 cells were maintained in Keratinocyte Growth Medium 2 with supplements, PTECs were cultured in Renal Epithelial Cell Growth Medium 2 with supplements (both Promocell).

Cells were grown to 60% to 70% confluence and used for further downstream analyses. Cells were either subjected to hypoxia (0.1% O$_2$) or to ATP depletion (chemical anoxia) using glucose-free medium containing 10 µM Antimycin and 10 µM 2-Deoxy-D-glucose (both Sigma-Aldrich, St. Louis, MO) for 1 hour with subsequent ATP repletion using DMEM containing Glucose (4.5 mM) and 10%FCS for 30 minutes. As a positive control, cells were treated with 0.2 µM staurosporin (Sigma-Aldrich, St. Louis, MO) to induce apoptosis. Apoptosis was determined by TUNEL staining (Roche Applied Science, Mannheim, Germany) or by use of the Annexin-V-Fluos kit from Roche Diagnostics (Penzberg, Germany) according to the manufacturer's instructions using a fluorescent-activated cell sorter analysis (FACS) on a guava® easyCyte™ sorter (Millipore, Germany). All assays were done according to the manufacturers instructions. Cellular oxidative stress was assessed by measuring the cellular content of 8-OHdG DNA Damage by enzyme-linked immunosorbent assay according to the manufacturer's instructions (Cell Biolabs, Inc., USA).

Ex vivo cell purification/sorting
The cellular origin of miR-24 following induction of I/R-injury was investigated by fluorescence-associated cell sorting (FACS) analysis using specific antibodies following a protocol by Chau et al. with modifications. Following clamping of the right renal pedicle for 27 minutes and a reperfusion period of 1, 3 and 7 days both kidneys were extracted, de-capsulated, homogenized, then incubated at 37ºC for 45 min with CollagenaseII (81 U/ml) in Hank’s Balanced Salt Solution (HBSS, Gibco).
After filtration (70µm) cells were centrifuged and then re-suspended in 1 ml FACS buffer (Millipore) containing 1% BSA. Cells were separated using the following specific antibodies or lectins: rat anti-mouse-CD31-PE (1:400 BD Pharmingen) for endothelial cells, Lotus teragonolobus lectin (1:200, DAKO) for proximal epithelium and anti-mouse Tim1-biotin (1:200, E Bioscience) followed by streptavidin-APC (1:1000, BD Pharmingen) for injured proximal epithelium. PDGF-Receptor beta+ pericytes were separated from kidneys following a protocol by Schrimpf et al. Cells were incubated with rabbit anti-PDGF Receptor beta antibodies (Abcam) for 15 minutes on ice. After washing, cells were incubated with goat anti-rabbit IgG microbeads (Miltenyi Biotech) (15 minutes at 4°C) and resuspended and isolated by MACS magnetic bead separation. Subsequently, RNA was isolated by Trizol method. For detection of miRNAs in samples, TaqMan primer assays (Applied Biosystems) were applied. The small RNA molecule snoRNA-202 was amplified as a control.

Protein Analysis
Downstream mechanisms were investigated by Western blot analysis using 10 to 40 µg of total protein. Tissue was homogenized, cells were pelleted. Cell lysis was performed (Cell lysis buffer, Cell Signaling, Technology, Danvers, MA, U.S.A.) and protein electrophoresis initiated. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes, blocked with 5% milk in TBS-Tween, and probed overnight at 4°C with the following primary antibodies: polyclonal rabbit anti-mouse S1P1 (S1PR1) (Sigma Aldrich, St. Louis, MO), anti-mouse monoclonal antibody to Heme Oxygenase 1 (Abcam, Cambridge, UK.), monoclonal rat anti-mouse CD8 (Abcam, Cambridge, UK), polyclonal rabbit anti-mouse H2A.X (Abcam, Cambridge, UK). Antibody binding was visualized by chemiluminescence (Super-Signal West Pico Chemiluminescent, Thermo Scientific, Rockford, IL). Rabbit anti-mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Sigma Aldrich, St. Louis, MO) was used as an internal loading control and for normalization of protein quantification. Immunoblots were scanned and quantified using ImageJ densitometry software.

Tube formation and Boyden chamber assay of HUVECs
HUVECs were cultured in EBM-2 culture medium with supplements including 10% FCS and antibiotics. For the tube formation assay HUVECs were harvested and 15,000 cells were seeded on top of Matrigel-coated chamber slides (BD). After 4–6 h, pictures were taken on a Nikon Ti 90 microscope (Germany). Migratory capacity was assessed in a modified Boyden chamber assay. After treatment, cells were
stained with DAPI and cultured in inlets (Falcon HTS Fluoro Blok insert, 8-µm pore size), which were placed in 24-well culture dishes containing EBM-2 (Clonetics) and 50 ng/ml vascular endothelial growth factor, and 100 ng/ml stromal cell-derived factor-1. Prior to use, the inserts were coated with Fibronectin. After 4-24 hours, migrated HUVECs were manually counted by fluorescence-based microscopic evaluation of the bottom side of the membrane.

_Scratch wound healing assay of HK-2 cells_
Transfected HK-2 cells were cultivated in human keratinocyte medium at 37°C, 5% CO2. The scratches in the cell monolayer were generated with a 100-µl tip, and the cells were photographed at 0, 8, and 24 hours with a Nikon Ti 90 microscope (Germany). Subsequently, the cell free area was calculated.

_miRNA/RNA Isolation, miRNA/mRNA Reverse Transcription–Polymerase Chain Reaction, and Global Transcriptome Analysis_
RNA isolation was performed with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. For detection of miRNAs in samples, different TaqMan miRNA assays (Applied Biosystems) were applied. The small RNA molecule snoRNA-202 (mouse) and RNU48 (human) was amplified as a control. Reverse transcription–polymerase chain reaction analysis was performed in an ICycler (Bio-Rad). Gene array analysis was performed with the AffymetrixGeneChip system according to the manufacturer’s instructions (Affymetrix Systems). Reverse transcription was performed with total RNA using oligoDT primers (Bio-Rad). Amplified cDNA was used as a template for quantitative PCR. Reverse transcription polymerase chain reaction analysis was performed in an ICycler (Bio-Rad) with SYBR green mastermix. The specific primers used in our study are depicted in supplemental table 1.

_MicroRNA target prediction_
The microRNA databases and target prediction tools miRBase (http://microrna.sanger.ac.uk/), PicTar (http://pictar.mdc-berlin.de/) and TargetScan (http://www.targetscan.org/index.html) were used to identify potential microRNA targets. We focused on targets predicted by at least two prediction data bases and containing a miR-24-8mer seed match in the respective 3'UTR region.
**Transfection Assays**

Transient liposomal transfection of small inhibitory RNAs (siRNAs) or miRNAs was performed according to the manufacturers’ instructions. Briefly, cells were split 1 day before transfection to reach 60% to 70% confluence on the day of transfection. Specific siRNAs/miRNAs and control siRNA/miRNA and Lipofectamine 2000 (Invitrogen) were mixed separately and incubated for 5 minutes with Opti-MEM I media (Invitrogen). Complexes were added together and incubated for 20 minutes. Media were changed to antibiotic-free media before the addition of liposomal siRNA complexes (final concentration 150 nmol/L for siRNA and 100 nmol/L for miRNAs). Cells were incubated for 4 hours before the media were changed to fresh media. Silencing of proteins or miRNA targets was monitored for 48 hours (siRNA) or 72 hours (miRNAs) after transfection by Western blot analysis.

**Luciferase Reporter Assays**

A luciferase reporter assay system was applied to validate potential miRNA targets as described previously. A putative 3’UTR miRNA binding sequence was cloned into the SpeI and HindIII cloning site of pMIR-REPORT vector (Ambion). H2A.X wild-type (2 sites):5’-CTGGACTGAGCCTC…TGTATGCTATCTGAGCCGTCT-3’; S1PR1 (Sphingosine-1-phosphate receptor 1) wild-type 5’-AGCTTTGATTTTGCACTGAGCC…CATAGCT-3’. The resulting construct was co-transfected with the miRNAs of interest and a β-galactosidase control plasmid (Promega) into HEK293 reporter cells in 48-well plates by use of Lipofectamine 2000 (Invitrogen). A total of 0.2 µg of plasmid DNA and 100 nmol/L miRNA was applied. Cells were incubated for 24 hours before luciferase and β-galactosidase activity was measured (Promega).

**Viral Transduction**

To generate recombinant adenoviruses, the truncated cDNAs of H2A.X, HO-1 and S1PR1 were subcloned into the pShuttle-CMV vector. The adenoviruses were produced following the protocol of the AdEAsy XL Adenoviral Vector system (Agilent Technologies). For infection of cells in culture, 5 MOI of virus were added for 2 hours in serum containing culture medium and subsequently removed by washing with PBS and addition of fresh medium.
Supplemental References


**Supplemental Table 1:** Primer pairs used for gene expression analysis

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Fwd: forward; Rev: reverse
Supplemental Figure legends

**Supplemental Figure 1:** Tube formation capacity in HUVECs after pre-negative control (A) and pre-miR-24 oligonucleotide (B) transfection and quantification of results (C, n=6 experiments). Migration capacity in Boyden chamber assays of HUVECs after pre-negative control (D) and pre-miR-24 oligonucleotide (E) transfection and quantification of results (F, n=6 experiments). Tunel stainings in miR-24–overexpressing endothelial cells after transduction with adenoviral constructs lacking miR-24 binding sites for control virus (CTL, G), HO-1 virus (H), H2A.X virus (I), S1PR1 virus (J) and quantification of results (K). **: p<0.01, *: p<0.05.

**Supplemental Figure 2:** Tube formation capacity in HUVECs after silencing of investigated targets under hypoxic conditions for scrambled control siRNA (A), HO-1 siRNA (B), H2A.X siRNA (C), S1PR1 siRNA (D) and quantification of results (E, n=6 experiments). Migration capacity in Boyden chamber assays of HUVECs after silencing of investigated targets under hypoxic conditions for scrambled control siRNA (F), HO-1 siRNA (G), H2A.X siRNA (H), S1PR1 siRNA (I) and quantification of results (J, n=6 experiments). ***: p<0.0001, **: p<0.01, *: p<0.05.

**Supplemental Figure 3:** TUNEL stainings in HK-2 cells after silencing of investigated targets under hypoxic conditions for scrambled control siRNA (A), HO-1 siRNA (B), H2A.X siRNA (C), S1PR1 siRNA (D) and quantification of results (E, n=6 experiments). ***: p<0.0001, **: p<0.01, *: p<0.05.

**Supplemental Figure 4:** mRNA expression of Collagen I alpha 2 at 24 (A), 72 (B) and 168 hours (C), mRNA expression of Collagen III at 24 (D), 72 (E) and 168 hours (F) and mRNA expression of alpha smooth muscle actin (aSMA) at 24 (G), 72 (H) and 168 hours (I) in kidneys of mice. CONT contra = contralateral kidney of mice with LNA-CONT, CONT IR = clamped kidney of mice with LNA-CONT, LNA contra = contralateral kidney of mice with LNA-24, LNA IR = clamped kidney of mice with LNA-24; n=7 mice in each group and time point; ***: p<0.0001, **: p<0.01, *: p<0.05.

**Supplemental Figure 5:** MiR-24 regulation in the bilateral renal I/R-injury model. MiR-24 is elevated in the kidney of mice after bilateral I/R-injury (27 minutes of bilateral ischemia and 24 hours of reperfusion) as compared to sham controls (A) (n=4 animals per group). MiR-24 antagonism is associated with a reduction in kidney...
injury markers (KIM-1 and NGAL, B – C) as well as epithelial injury in the outer medulla (D – F). MiR-24 target regulation in vivo in bilateral I/R-injury, including H2A.X (G – I), S1PR1 (J – L) and HO-1 (M – O). ***: p<0.0001, **: p<0.01, *: p<0.05, $: p=0.08.

Supplemental Figure 6: Fibrosis and inflammatory gene expression in bilateral I/R-injury. Collagen I alpha 2 (A), Collagen III (B) and alpha smooth muscle actin (aSMA) (C) as well as MCP-1 (D), IL1beta (E), MIP2 alpha (F), IL-6 (G) and TNF alpha (H) is decreased in the kidney of mice treated with an LNA-modified antimiR targeting miR-24 after bilateral I/R-injury (27 minutes of bilateral ischemia and 24 hours of reperfusion) as compared to mismatch LNA treated controls (A) (n=4 animals per group). aSMA = alpha-SMA; IL-1beta = interleukin 1 beta; IL-6 = interleukin 6; MCP-1 = monocyte chemoattractant protein 1; MIP2 alpha = macrophage inflammatory protein 2 alpha; TNF-alpha = tumor necrosis factor alpha; ***: p<0.0001, **: p<0.01, *: p<0.05.
Supplementary Figure 3

A  B
100μm scrambled siRNA  100μm HO-1 siRNA
+ hypoxia + hypoxia

C  D
100μm H2A.X siRNA  100μm S1PR1 siRNA
+ hypoxia + hypoxia

E

TUNEL+ cells/hpf

scrambled siRNA + hypoxia
HO-1 siRNA + hypoxia
H2A.X siRNA + hypoxia
S1PR1 siRNA + hypoxia

** ** **
Supplementary Figure 4

A, B, C: Graphs showing the expression levels of Collagen I alpha 2 and Collagen I alpha 3 genes normalized to Gapdh in different groups: CONT contra, LNA contra, CONT I/R, LNA I/R.

D, E, F: Similar graphs for Collagen III gene expression.

G, H, I: Graphs showing the expression levels of aSMA gene normalized to Gapdh in different groups: CONT contra, LNA contra, CONT I/R, LNA I/R.

Significance levels are indicated with asterisks: *** for p < 0.001, ** for p < 0.01, * for p < 0.05.
Supplemental Figure 5

miR-24/snoRNA-202
(FC of CTL)

KIM-1/Gapdh

ngAL/Gapdh

Epithelial injury (%)

H2A.X+ cells/hpf (% of control)

S1PR1+ cells/hpf (% of control)

HO-1+ cells/hpf (% of control)
Supplemental Figure 6