DOI:10.14753/SE.2015.1771

THE ROLE OF MICRORNAS IN RENAL ISCHEMIA REPERFUSION INJURY THERAPEUTIC APPLICATION OF RNA INTERFERENCE

PhD thesis

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Budapest, 2014

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List of Abbreviations

2'-OM	2'-O-methylated (CH_3 is added to the 2' OH)
3' UTR	3' untranslated region
A	adenine
ADP	adenosine diphosphate
AGO	Argonaut protein
AKI	Acute kidney injury
Akt	serine/threonine-protein kinase (Ak (mouse strain) transforming protein)
AMI	Acute myocardial infarction
AMO	Anti-miRNA Oligonucleotides
AMP	adenosine monophosphate
ANOVA	analysis of variance
APC	allophycocyanin
ASO	antisense oligonucleotides
ATF3	activating transcription factor 3
ATP	adenosine triphosphate
AU	arbitrary units
αSMA	alpha smooth muscle actin
BCL-2	B cell leukemia/lymphoma 2
bp	base pair
BSA	bovine serum albumin
BUN	blood urea nitrogen
C	cytosine
CD(no.)	cluster of differentiation (number)
cDNA	complementary (copy) DNA
CIT	cold ischemia time
CKD	chronic kidney disease
CL	contralateral
Col III	collagen, type III
Col1α2	collagen type I, alpha 2 chain
CTR	control
DAB	3,3' diaminobenzidine
DAMP	damage(danger)-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DGCR-8	DiGeorge syndrome critical region
DN	Diabetic nephropathy
DNA	Deoxyribonucleic acid
DNase	deoxyribonuclease
dsRBD	dsRNA-binding domain
dsRNA	double-stranded RNA
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminetetraacetic acid
eIF	eukaryotic translation initiation factor
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase (NOS3)
ESRD	end stage renal disease
Exp5/Xpo5	Exportin-5
FACS	fluprescence-associated cell sorting
FC	fold change
FFPE	formalin fixed paraffin embedded
fwd	forward primer

G	guanine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFR	glomerular filtration rate
H2A.X	histone 2A family, member X (H2AFX)
HAVCR1	hepatitis A virus cellular receptor 1 (KIM1, TIM1)
HE	hematoxylin eosin
HIF1α	hypoxia inducible factor 1, alpha subunit
HK-2	Human kidney 2 (proximal tubular cell line)
HO-1	heme oxygenase 1
hpf	high power field
HRP	horseradish peroxidase
hsa	homo sapiens
HSF-1	heat shock transcription factor 1
HSP	Heat shock protein
i.p.	intraperitoneal
I/R	ischemia reperfusion
ICAM-1	Intercellular Adhesion Molecule 1
IgG	immunoglobulin G
IHC	immunohistochemistry
IL	interleukin
IPC	ischemic preconditioning
KIM1	kidney injury molecule 1 (HAVCR1)
LCN2	lipocalin-2 (NGAL)
LNA	locked nucleic acid
LTA	Lotus tetragonolobus agglutinin
Ly-6G	lymphocyte antigen 6 complex, locus G
MACS	magnetic-activated cell sorting
MCL-2	myeloid cell leukemia sequence 2
MCP-1	monocyte chemoattractant protein 1
MEM	Minimal Essential Medium
MFI	median fluorescence intensity
mghv	mouse gammaherpesvirus
MIP2α	macrophage inflammatory protein 2-alpha
miRNA	micro RNA
MMP-2	matrix metallopeptidase
mmu	mus musculus
mRNA	messenger RNA
mTAL	medullary thick ascending limb
NCBI	National Center for Biotechnology Information
NGAL	neutrophil gelatinase associated lipocalin (LCN2)
NO	nitric oxide
ODN	Oligodeoxyribonucleotides
P bodies	processing bodies
PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PDCD4	programmed cell death protein 4
PDGFRβ	platelet-derived growth factor receptor beta

pDNA	plasmid DNA
PE	Phycoerythrin
PEG	poly-ethylene-glycol
PEI	poly-ethilene-imine
PIC	polyion complex
pNGAL	plasma NGAL
Pol II	RNA polymerase II
PON	peroxynitrite
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PTEN	phosphatase and tensin homolog
qPCR	quantitative (real-time) PCR
RanGTP	GTP-binding RAs-related Nuclear protein
RCC	Renal cell carcinoma
rev	reverse primer
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
ROS	reactive oxygen species
RT	reverse transcription
S1PR1	sphingosine-1-phosphate receptor 1
SEM	standard error of the mean
shRNA	short hairpin RNA
siRNA	short interfering RNA
Sirt1	sirtuin 1
snoRNA	Small nucleolar RNA
snRNA	small nuclear RNA
SPF	specific pathogen-free
STAT	signal transducer and activator of transcription
STZ	Streptozotocin
T	thymine
TAR	trans-activation response
TBS	tris-buffered saline
TIM1	T cell-immunoglobulin-mucin (HAVCR1)
TLR	Toll-like receptor
TMA	tissue microarray
TMB	3,3',5,5'-tetramethylbenzidine
TNFα	tumor necrosis factor alpha
TRBP2	TAR RNA binding protein 2
TUNEL	transferase dUTP nick end labeling
VEGF	vascular endothelial growth factor
VEGFR2	VEGF Receptor 2
VHL	von Hippel-Lindau tumor suppressor

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Introduction

In the postgenomic era, investigation of the human transcriptome has revealed that the genome encodes many thousands of functional RNAs not transcribed into proteins (non-coding RNAs) (1). Micro RNAs (miRNAs) compose a large family: about 1% of the genes encoded in the genome belong to the miRNA family (2). First described in 1993 (3) miRNAs are short, being composed of only 18-25 nucleotides (nt). Presently, it is hypothesized, that miRNA sequences play an important role in gene-expression regulation, through RNA interference (RNAi), controlling protein synthesis from most human genes at the posttranscriptional level (post-transcriptional regulation of gene-expression) (4). Emerging knowledge surrounding the role of miRNAs in the regulation of post-transcriptional protein expression has dramatically altered the view of how target genes are regulated.

The regulatory functions of our body comprise networks. The hypothesis, that miRNAs exert their regulatory function in networks is supported by the high number of non-coding RNAs which are functionally active, e.g. miRNAs that have been shown to target signaling molecules (5). Furthermore, some genes encoding miRNAs are closely located (clustered) in the genome and in some cases different miRNAs control a single messenger RNA (mRNA) target or vice versa a single miRNA may influence expression of multiple different target proteins**.** MiRNA expression profiles during different disease states can be determined by microarray studies. Such systemic approaches together with individual analysis of different miRNAs provide insight into an exciting new regulatory network of the miRNAome.

MicroRNAs (miRNAs): generation and mechanism of action (Figure 1)

MiRNAs are generated from endogenous hairpin structured transcripts throughout the genome (2). MiRNA encoding genes are transcribed by RNA polymerase II (pol II) providing long precursor transcripts, known as primary miRNAs (pri-miRNAs) (6). After transcription, still inside the nucleus, Drosha Ribonuclease (RNase): a type III nuclear RNase cleaves nucleotides from the pri-miRNA, processing it into shorter pre-miRNAs and defining their 3’ end. Efficient cleavage requires a double-stranded RNA-binding domain (dsRBD) containing cofactor: DiGeorge syndrome critical region (DGCR)-8. The

stem-loop (hairpin) structured pre-miRNA has a characteristic 5’ phosphate and 3’ hydroxy termini with a two nucleotide 3’ single-stranded overhanging end (7). This end structure is recognized by the nuclear export factor Exportin-5 (Exp5/Xpo5), which uses Ran-GTP as a co-factor (8) and transports the pre-miRNA to the cytoplasm (9). Further cytoplasmic processing by Dicer (another type III ribonuclease in the cytoplasm) performs a second cleavage at the hairpin structure, and defines the 5’ end of the mature miRNA. The Dicer also uses a double stranded (ds)RNA-binding domain (dsRBD) containing cofactor: trans activation response (TAR) RNA binding protein 2 (TRBP2). As a result of the cleavage by Dicer a double-stranded 18- to 25-nucleotide-long miRNA is generated (10). The mature miRNA is one of the strands of the dsRNA (miRNA/miRNA* duplex). One of the two strands is loaded on an Argonaut family protein (AGO1): the catalytic site of the RNA induced silencing complex (RISC), thus assembling the RISC-ribonucleoprotein complex. Unlike short interfering (si)RNAs which bind to AGO2, miRNAs bind to AGO1.

The guide strand of the miRNA is incorporated into the RNA-induced silencing complex (RISC) (11), and remains stably associated with RISC, becoming the mature miRNA. The opposite (passenger) strand is disposed. The miRNA guides RISC to the target messenger (m)RNA with complementary sequence.

Translation of the target mRNA is silenced in case of incompletely complementary sequence, and the mRNA is spliced up (cleaved) by the RISC in case of fully complementary sequence. As endogenous miRNAs often contain mismatches, the more common (primary) mechanism is translational repression: AGO1 does not cleave the mRNA, but binds to it and allosterically inhibits translation. Unlike RNAi induced by siRNA, cleavage (degradation) of the mRNA occurs more seldom, only by complete match between the miRNA and the mRNA (12).

It is interesting, that many distinct ways exist to obtain post transcriptional gene silencing by miRNA interference. Protein translation can be inhibited at translation initiation by inhibiting different eukaryotic translation initiation factors (eIF)s or at translation elongation. Furthermore, instead of translation inhibition, co-translational degradation of the nascent polypeptide chain or without interfering with the translation machinery by sequestering and processing mRNAs in discrete cytoplasmic foci: P bodies are possible ways of post-transcriptional gene silencing by miRNAs (12), (13).

DOI:10.14753/SE.2015.1771

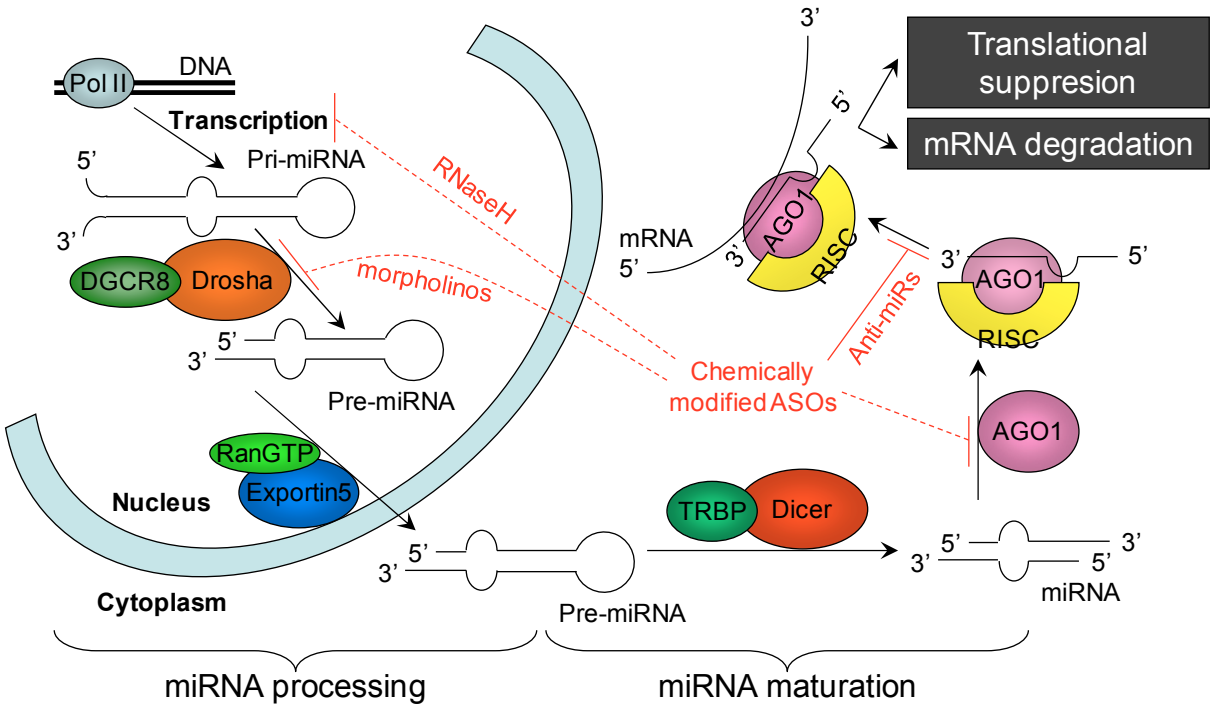


Figure 1. The miRNA machinery and sites of intervention. Micro RNA biogenesis and function (based on (14), (15), (16), (17), (18)).

Pol II: RNA polymerase II. DGCR-8: DiGeorge syndrome critical region (cofactor of Drosha RNase III). RanGTP: cofactor of Exportin-5. TRBP2: TAR RNA binding protein 2 (cofactor of Dicer (a cytoplasmic RNase III, which cuts the hairpin of the pre-miRNA. RISC: RNA-Induced Silencing Complex. AGO1: Argonaut protein (the catalytic site of the RISC). mRNA cleavage or translational suppression depends on the level of complementarity. Possible interventions (red): chemically modified AntiSense Oligonucleotides (ASOs) can block the RISC active site or inhibit mature miRNA binding to the RISC, but can interfere with miRNA processing early steps as well.

MiRNA nomenclature

The continuous discovery of new miRNAs necessitates a consistent gene naming scheme. Therefore, every mature miRNA has a "miR" prefix (precursor miRNAs are denoted with "mir") and a unique identifying number, which are assigned sequentially, in order of discovery. Identical miRNAs have the same identifying number, even between different organisms. The host organism can be designated by an abbreviated 3 or 4 letter prefix (e.g., hsa-miR for Homo sapiens, mmu-miR for Mus musculus, mghv-mir-M1-2

for mouse gammaherpesvirus etc.). Furthermore, identical miRNAs encoded in different chromosomal locations (in case of multiple copies) have numbered suffixes (ascending in order of discovery, (not chromosome number), e.g.: hsa-miR-194-1 and hsa-miR-194-2 are located on chromosome 1 and 11, respectively). Paralogous miRNA sequences, which differ only by one or two nucleotides have lettered suffixes (e.g., hsa-miR-200a, hsa-miR-200b, hsa-miR-200c). Where two different mature miRNAs are processed from the same hairpin precursor, the ending (3' or 5') of the arm of provenance has to be specified (e.g., miR-17-5p, miR-17-3p) or an asterisk can be applied to the less predominantly expressed transcript (strand) (e.g., miR-199*). The miRNA encoding genes are named using the same three-letter prefix, which can be modified according to the conventions of the host organism (capitalization, hyphenation or italics). Nevertheless, online databases also exist (e.g., http://mirbase.org/, http://rfam.janelia.org/) to prevent accidental overlap when naming newly discovered miRNAs. The new identifying number will be assigned just after the paper describing the miRNA has been accepted for publication (19), (20).

MiRNA function

A single microRNA may alter the expression of a large number of target genes, thus influencing a specific pathology by regulating whole disease-specific pathways and signaling cascades rather than a single gene (21). This unique function underlines the immense importance of these small molecules.

MiRNAs are involved in gene regulation in different processes such as embryonic (22) or hematopoetic (23) development, apoptosis (24), or tumor initiation and progression (miR-17–92 cluster, miR-21, miR-372) (25). MiRNAs are also involved in many physiological (2) and pathophysiological processes (26). The most investigated role of miRNAs is in oncogenesis. In nephrology, the involvement of miRNAs in many renal diseases is also under intense investigation, including diabetic nephropathy, immunologic renal diseases such as allograft rejection and autoimmune renal diseases, and genetically determined renal diseases such as polycystic kidney disease (27).

Influencing miRNA expression in vivo

Members of the miRNAome are explored in different disease states by genome-wide search tools such as microarrays, and substantial data has been accumulated already in several disease states and organ systems, including the kidney. Data obtained with microarray analysis has to be validated by quantitative qPCR (28). A new tool: next generation sequencing can be also applied to detect multiple miRNAs from an experimental sample (29). In many pathological processes, miRNA levels have been found to be up- or down-regulated. A functional investigation of selected miRNAs is ongoing. Presently, experimental strategies aimed at interfering with miRNAome are based on transfection of small, pre-determined nucleic acid sequences into target cells.

Nucleic acid therapy – problems and solutions of delivery

The major problem of *in vivo* therapies with nucleic acids such as short interfering RNA (siRNA), micro RNA (miRNA), antisense oligonucleotide (ASO) or plasmid DNA (pDNA) (nucleic acid therapy or nucleic acid-based next generation biopharmaceuticals (30)) is delivery itself into target organs and target cells (31). Instability of nucleotides in the extra cellular surrounding and high sensitivity to degradation by nucleases, can lead to inactivation of the applied nucleic acid (32). Small oligonucleotide molecules are rapidly cleared from the bloodstream by nucleases (33) and by the kidney (34). Thus, injected small nucleic acids disappear by enzymatic digestion and renal clearance. Finally, nucleic acids are negatively charged, hence they do not likely penetrate cell membranes and enter cells (35). Several approaches have been developed to enhance *in vivo* half-life of therapeutic nucleic acids and to promote their delivery to target organs and cellular uptake.

1. Physical forces: Naked, unmodified as well as chemically modified nucleic acid delivery can be amplified by enhanced pressure injections (local or systemic: hydrodynamic tail vein injection) – first applied for RNA interference in the kidney by Hamar et al. (36): the solvent bolus protects from nuclease degradation, and the hydrodynamic pressure forces the nucleic acid into the interstitium of parenchymal organs, and induces pore openings on parenchymal cell membranes. Such pore openings can be enhanced by local application of ultrasound (sonoporation (37)) or electric field, similarly to *in vitro* electroporation (38), (39).

2. Chemical modifications of the therapeutically applied nucleic acids include modifications of the ribose-phosphate backbone (40), or terminal modifications: addition of functional groups such as methyl, alkyl (41) or cholesteryl groups (34). Furthermore, more fundamental chemical modifications have been investigated such as morpholinos (nonionic DNA analogs) (42) or locked nucleic acid analogues (LNA) (34), (43). Chemical modifications aim to enhance resistance to nuclease enzymatic breakdown, but preserve function. In some cases chemical modifications may lead to loss or reduction of nucleic acid function (unpublished observations).

3. Delivery can be enhanced by packaging the therapeutic nucleic acids into vectors or instead of delivery of the therapeutic nucleic acids themselves, plasmid DNA (pDNA) encoding the therapeutic nucleic acid is applied widely. Nucleic acids or encoding plasmids can be delivered by viral (adenovirus, adeno associated virus, lentivirus) or non-viral: chemical complex delivery systems. Chemical complexes are formed between positively charged polyion complexes (PIC) and negatively charged nucleic acids. Such carriers (transfection reagents) include cationic liposomes (for eg.: Lipofectamine™ RNAiMax® /Invitrogen/) (44) in which nucleic acids are encapsulated in lipid vesicles, lipoplexes (self-assembling multi-lamellar lipid complexes) (45), (46), or cationic polymers: polyplexes (47) (for eg.: ciclodextrin (48), poly-ethilene-imine (PEI) (34), poly-ethylene-glycol (PEG), polyamines: such as poly-L-lysine or siPORT® /Ambion/). More recently, “nanocarriers” such as carbon nanotubes (49), iron nanoparticles combined with a magnetic field (50) or gold nanorods (51) have also been developed. These vectors or nanocarriers protect the nucleic acids from renal filtration, enzymatic degradation and enhance cellular uptake. Electrostatic surface coating of delivery particles can enhance or target their delivery (52).

4. Conjugation of the nucleic acid or the nucleic acid-delivery particle with cell surface receptor ligands can enhance cell specificity (targeting ligands) and cellular uptake (32), (53).

5. Therapeutic nucleic acid delivery can be enhanced by depo-products (carriers) with prolonged deliberation of the therapeutic nucleic acid or complex particle such as gelatin (54), hydrogels (55), athelocollagen (56), chitosan (34) or cyclodextrin (53).

Functional investigations of miRNAs include miRNA expression blockade with AntiSense Oligonucleotides (ASOs) or enhancement with different nucleic acid structures designed to target any miRNA of interest (17).

Inhibition of miRNA function

MiRNAs can be blocked at multiple levels (Figure 1). A non-sequence specific, direct method to reduce miRNA activity is to interrupt its synthesis by targeting components of the miRNA biogenesis machinery. However, this method might lead to global reduction of all miRNAs and related side-effects.

More specifically, targeted degradation of the pri-miRNA transcript in the nucleus can be achieved with antisense OligoDeoxyriboNucleotides (ODN). RNaseH recognizes RNA–DNA duplexes, cleaving the RNA strand: the pri-miRNA with ODN complementary sequence (57), (58). However, whether this is an effective approach to target miRNAs requires further study (17). Targeting the hairpin structure by siRNA, or RNaseH-ODN in the pri-miRNA/pre-miRNA state is not likely to be effective due to difficulties in accessing the loop structure with a short nucleotide sequence and which may be protected by pre-miRNA binding factors (59).

Morpholino modified antisense oligonucleotides (morpholinos) were also used to target miRNA precursors in zebra fish embryos to inhibit miRNA maturation at Drosha or Dicer processing (60).

The most effective miRNA inhibitors act on the mature miRNA (Figure 1) (17). Anti-miRNA Oligonucleotides (AMOs) are actually AntiSense Oligonucleotides, a class of ASOs that are chemically engineered short RNAs, which effectively and specifically silence miRNAs. Unlike RNaseH-ODNs, AMOs target mature miRNA in the cytosol, more specifically in the RISC.

Chemical modification of AMOs is usually applied to stabilize the AMOs against nuclease degradation, improve affinity for target miRNA and to promote tissue uptake for *in vivo* delivery. Prolongation of *in vivo* half – life of small RNAs is a crucial problem. Furthermore, improving hybridization affinity for the target RNA is necessary, as RISC-bound miRNA has a strong binding capacity for the target mRNA. Possible chemical modifications include 2′sugar modifications, locked nucleic acid (LNA) as well as phosphorothioate backbone modifications of the AMO (17). All of the 2′ modifications

improve affinity to target RNA. The phosphorothioate backbone, reduces target affinity, however provides resistance to nuclease degradation. Krüztfeldt and colleagues used 2'-O-methylated (2'-OM) sugar, phosphorothioate backbone and a cholesterol moiety containing a single – stranded RNA (also called antagomir) (61). Three low volume (end volume = 0.2 ml) tail vein injections significantly reduced miR-16, miR-122, miR-192 and miR-194 expression *in vivo* in many target organs: lung, liver, heart, intestine, bone marrow, ovaries and adrenals including the kidney. Furthermore, they also characterized the properties and function of AMOs in mice. They demonstrated that AMOs require a length >19-nt for highest efficiency to discriminate between a single nucleotide mismatch of the target miR (61).

Locked nucleic acids (LNAs) are a class of nucleic acid analogues, with high binding affinity to complementary mRNA targets leading to mRNA inhibition. Strong RNA binding ability (62) of LNA enable their utilization to inhibit also miRNAs (63). Similar to the 2'-OM AMO approach, LNA AMOs prevent miR – RISC interaction (64). LNA AMOs enable specific miRNA detection by northern blot analysis (65) and in situ hybridization (66). LNA AMOs have already been successfully used for inhibition of miRNA function *in vitro* (67) and might be utilized in cancer diagnostics and therapeutics (68). LNA AMOs, injected intravenously, effectively antagonized miR-122 in mouse liver (69) and non-human primates (70). Depletion of miR-122 by tail vein injection of unconjugated and phosphorothioated AMOs into mice reduced plasma cholesterol without toxicity. Furthermore, intraperitoneal injection of phosphorothioate backbone LNA AMO was also efficient (69). Inhibition of miR-21 was similarly effective with 2'-OM, LNA AMO or cationic liposomes (71). The 2'sugar, phosphorothioate backbone and LNA AMOs are commercially available.

AMOs function: According to one hypothesis, AMOs bind to the single stranded sense miRNA loaded into the RISC, hence preventing miRNA-RISC binding to the complementary mRNA (72). Another hypothesis is that they interfere with miRNAs (complementary pairing) before loading into the RISC (57). Recently, miRNA-AMO duplexes were demonstrated to degrade in a distinct cytosolic compartment from P-bodies, thus antagomir induced miRNA degradation is probably independent of previously described RNAi pathways (61). However, further research is necessary to elucidate the acting mechanisms of these molecules and to discover further methods of

miRNA regulation (15). The formation of stable heteroduplexes between LNA AMO and miRNA can be detected by northern analysis (69).

Enhancement of miRNA function

Besides inhibition, enhancement of miRNA function is also possible by enhancing endogenous miRNA function or by inserting short, double stranded RNA sequences (mimics) into cells with an identical nt sequence to the target miRNA.

Restoring miRNA function is important if pathologic processes are coupled with miRNA loss of function or reduced expression. Based on structural-functional homologies, exogenous siRNAs introduced into target cells may function as regulatory miRNAs.

To experimentally induce a miRNA function, cells or organs are transfected with miRNA encoding short hairpin RNAs (shRNAs: pre-miRNA hairpin sequences) that mimic natural miRNA molecules. Following intracellular delivery, pre-miRNA hairpin sequences are processed into mature miRNAs by Dicer. Short hairpin RNA coding vectors provide a powerful method for miRNA expression (73). Thus, transfection with pre-miRNA hairpin sequences mimic or increase the desired miRNA effects.

Besides delivery, another road-block to nucleic acid therapy is the incompletely mapped side-effect spectrum. Possible side-effects can be off-target effects including the induction of the antiviral interferon response, or sequence mismatched silencing of other miRNAs or mRNA-protein expression. Furthermore, it has been reported, that overloading the endogenous miRNA machinery may be harmful, even lethal (74). However, optimal dosing may circumvent these problems (75). Regarding clinical applications, presently, lethal diseases such as cancer or diseases of compartmentalized organs such as the eye or lung are the primary targets of nucleic acid therapy. These compartmentalized organs have the advantage, that they can be accessed directly (i.e. nose, eye) and not only through the systemic circulation, thus systemic side effects such as the interferon response or off-target silencing in non-targeted organs can be avoided. Direct access may also enable more efficient delivery, and protection from RNase degradation in the blood.

Kidney specific miRNAome, renal disease specific alterations, and functional investigations of miRNAs in the kidney

The role of miRNAs is currently under intense investigation in many disease areas. After detecting expression profiles, research is now trying to influence expression of miRNA in different disease states. The kidney seems to have its own miRNA network, and disease-specific alterations may provide future diagnostic tools and therapeutic targets.

Human and murine kidney – specific miRNA expression profiles have been already reported. The initial studies on miRNA expression in the kidney involved the isolation, detection, and validation of miRNAs from the whole kidney. Sun et al. compared miRNA expression in six different human organs, including the kidney, and found a highly kidney specific miRNA cluster (76). Another miRNA cluster related to the kidney was found by Sawera et al. They demonstrated that all the precursors and most of the mature miRNAs of the porcine miR-17-92 cluster were expressed in the kidney. The mir-17-92 micro RNA cluster (represented by miR-17, 18, 19a/b, 20, 25, 92, 93 and 106a/b) is of particular interest, because of its evolutionary conservation (77). In a study where a homology search was conducted using human miRNAs to query the pig genome, two of the miRNAs previously associated with kidney (miR-92 and miR-194) and two other (miR-31 and miR-210) were expressed in porcine kidney (78). Another study, this time on mice, demonstrated that miR-10b and miR-200b were expressed exclusively in kidney, and miR-192, together with miR-194, was expressed both in kidney and liver (79). It is important to mention, that though several studies suggested that most miRNAs are conserved among related species, other studies provided evidence that many miRNAs are species specific (80).

Further research has been conducted in order to identify local miRNA expression profiles. This has shown that some miRNA are only present or are predominant in the cortex while others preferentially localize to the medulla, suggesting functional differences (Table 1) (28). Based on simultaneous proteomic expression profile changes cortical and medullar miRNA-target protein pairs were established by computational algorithms suggesting a role of cortical miRNAs in oxidative stress related processes (28).

Table 1. Localization of some renal miRNAs

miR	Kidney expression pattern	References
Let-7a/b/c	whole kidney	(28)
miR-10a/b	whole kidney	(79)
miR-23	whole kidney	(80)
miR-26a	whole kidney	(28)
miR-30	whole kidney	(80)
miR-31	whole kidney	(78)
miR-99	whole kidney	(80)
miR-204	whole kidney	(76)
miR-210	whole kidney	(78)
miR-215	whole kidney	(76)
miR-216	whole kidney	(76)
miR-18	Cortex	(80), (81)
miR-19	Cortex > medulla	(77), (81)
miR-20	Cortex > medulla	(77), (81)
miR-92	Cortex > medulla	(77), (78), (81)
miR-192	Cortex > medulla	(28), (76)
miR-194	Cortex > medulla	(76), (78), (28)
miR-203	Cortex	(28)
miR-27a/b	Medulla	(28)
miR-125a/b	Medulla	(28)
miR-17	medulla > cortex	(77), (81)
miR-200	medulla > cortex	(28), (79) , (80)

Mapping the renal miRNAome with expression array studies was the first step. Next, miRNA expression patterns typical of kidney diseases were explored, to provide information about which miRNAs could be deregulated in the injured kidney. The summary of these miRNAs can be found in our review published in 2010 (82). The microarray based studies generally identify large numbers of deregulated miRNAs in different pathologies. Therefore just those miRNAs will be mentioned which were further

studied by the authors of the respective studies, or those which expression level had the greatest fold change value.

Probably, the most investigated renal miRNA expression profile changes are those, characteristic of diabetic nephropathy (DN) (14). Many miRNAs have been described in diabetic nephropathy. From these we have identified miR-21 and miR-17 during our experiments to be major regulators of ischemia-reperfusion injury of the kidney. MiR-21 was downregulated in early DN, and upregulation of miR-21 inhibited mesangial cell proliferation (83). Furthermore, transgenic over expression of miR-17 repressed fibronectin expression in mice, suggesting a possible therapeutic approach (84).

Renal fibrosis is the final common pathway of end stage renal disease leading to renal failure in many different renal diseases such as diabetic, hypertensive or chronic allograft nephropathy. Renal fibrosis is usually initiated with glomerular damage, with podocyte detachment and focal sclerosis marked by albuminuria. Albuminuria may induce subsequent tubular damage. Several podocyte associated miRNAs are involved in the regulation of glomerular ultrafiltration. Podocytes are highly differentiated cells which are implicated in many progressive renal diseases and are responsible for maintaining the glomerular architecture and synthesis and composition of the slit diaphragm and the glomerular basement membrane, a major part of the glomerular filtration barrier. Podocyte specific deletion of Dicer resulted in podocyte apoptosis, with consequent glomerular damage and proteinuria (85), (86). MiR-23b, 24, 26a, and 30 seem to be responsible for podocyte homeostasis (87), (88).

One of the earliest associations between miRNAs and disease was made in the field of oncology. However, many aspects of this research can be applied to renal diseases due to similar pathomechanisms such as hypoxia and fibrotic processes. Many miRNAs have been identified in renal tumors. From these, relevance to our studies include miR-17 and miR-106a. Kort and colleagues found that miR-17-92 cluster (oncomiR-1) were upregulated in Wilm's tumor (89) and miR-17 and miR-221 were upregulated in renal cell carcinoma (RCC) (90). Some of the most commonly deregulated miRNAs (miR-20a, 21 and 106a) can modulate von Hippel-Lindau tumor suppressor (VHL) gene. Moreover, some RCC associated miRNAs (miR-21, 26a, 27a, 106a and 210) can be induced also by hypoxia. These data highlight the importance of miRNA regulation in cancer angiogenesis (91) and renal fibrosis.

Another important process which involves both immunological response and ischemia is kidney transplantation. Microarray analysis in allograft biopsy specimens sustained the argument that miRNA expression patterns could be valuable biomarkers in clinical transplantation by reflecting the allograft status (92). Sui et al. identified 20 miRNAs differently expressed in acute rejection after renal transplantation (93). These data may also help to better understand the pathophysiologic background of kidney graft rejection.

Ischemic acute kidney injury

Acute kidney injury (AKI) is a frequent complaint in clinical nephrology and it may develop in about 30% of patients at the intensive care unit (94) resulting in a mortality rate of up to 50% (95), thus represents a major socioeconomic health problem (96). The most frequent cause of AKI has prerenal etiology (e.g. hypoperfusion in circulatory shock or during cardiac surgery) often leading to intrinsic parenchymal lesions through ischemia-reperfusion (I/R) injury of the kidney (97). Moreover, it is commonly associated with the transplantation procedure and thus an unavoidable phenomenon in transplanted kidneys (98). AKI is also increasingly recognized as a cause of chronic kidney disease (99). Currently, a targeted and specific therapy for this important clinical disorder is not available (100).

Pathophysiology

In case of decreased blood flow to the kidney, renal autoregulation tries to preserve the glomerular filtration rate (GFR). Therefore blood volume is diverted to the urinary space and less blood supply will reach the medulla (101). However the renal microvascular system due to the countercurrent exchange of oxygen leads to an a priori gradient of decreasing oxygen tension from cortex to medulla. The tubular epithelial cells of the medulla have a high energy demand established by their high reabsorptive capacity. Therefore, when oxygen supply further decreases tubular cells, mainly in the S3 segment of the proximal tubules, located in the outer medulla will be injured and acute tubular necrosis develops (102). Since the tubular epithelial cells of the distal nephron convert much easier from aerobic (oxidative) to anaerobic (glycolytic) metabolism, are more resistant to hypoxia (102). Moreover, the tubular cells from the medullary thick ascending

limb (mTAL) portion of the distal tubules are spatially close to proximal tubules. Therefore, several studies indicate a paracrine interaction between these cells: distal tubular cells might reduce injury and/or improve regeneration of the proximal tubular cells (103). Endothelial cell injury also occurs. On one hand this leads to endothelial dysfunction which impairs the autoregulatory mechanisms and enhances vasoconstriction (104). On the other hand a variety of adhesion molecules are up-regulated (ICAM-1, P-selectin, E-selectin) inducing endothelial-leukocyte interactions (105), which together with endothelial cell swelling and coagulation disorders contribute to the development of *no reflow* phenomena in the reperfusion phase (106). In this way renal blood flow might remain compromised even after the ischemic event.

Reperfusion itself, though vital to the restoration of kidney function, is associated with significant additional cellular injury (107). Inflammation is activated partly by the injured epithelial cells where first leukocytes adhere. Microvascular permeability is also increased due to alterations in contacts between endothelial cells and breakdown of perivascular matrix, allowing immune cells to infiltrate the renal parenchyma (104). Injured tubular cells also contribute to inflammations by activation of Toll-like receptors (TLR4, TLR2) by damage/danger-associated molecular patterns (DAMPs) (108) and by the generation of several pro-inflammatory cytokines such as TNFα, IL-6, IL-8, IL-1β, MCP-1, TGFβ (104). First, neutrophil-infiltration occurs, however macrophages and later also lymphocytes play an important role in the cellular immune response and oxidative stress during reperfusion (105).

Cellular injuries

Oxygen distribution impairment during ischemia interferes with oxidative phosphorylation. The lack of oxygen, which serves as an electron acceptor in the electron transport chain leads to decreased ATP production (109). In addition, the accumulated ADP and AMP is degraded to hypoxanthine which is further processed by xanthine dehydrogenase. However, due to low ATP, Ca^{2+} will not only leak out from the endoplasmic reticulum into the cytoplasm, but the externalization of cytosolic Ca^{2+} is also diminished (105). The high intracellular Ca^{2+} activates numerous enzymes (110), thus xanthine dehydrogenase will be converted by proteolysis or by sulfhydryl oxidation to xanthine oxidase which enables the generation of reactive oxygen species (111). Furthermore, cytoskeletal structures are also degraded by the activated proteases leading

to loss of epithelial and endothelial cell polarity and function (102). Activated phospholipases result in instable membrane structures, hence necrosis frequently develops during the ischemic phase (104). Ion permeability of the membranes are also disturbed and further increase in intracellular Ca^{2+}, Na^{+} and water content causes cellular swelling/edema (112). The mitochondrial membranes are also affected. However, the anaerobic glycolysis results in intracellular acidosis which keeps the mitochondrial permeability transition pores closed (113). During reperfusion, intracellular acidosis is ameliorated to the cost of mitochondrial damage whereupon reactive oxygen species (ROS) and cytochrome C is released into the cytoplasm (114). This triggers apoptosis through caspases (105), (104) but necrosis or even necroptosis in case of insufficient ATP provisions might also occur (115). Reactive oxygen radicals may interact with the increased NO to form peroxynitrite (PON), which further aggravates oxidative damage by protein nitrosylation (116). Sublethal ischemic injury also induces hypoxia inducible factors (HIFs) together with signal transducer and activator of transcription (STAT) 3 which mediate among other anti-oxidant (e.g. heme-oxygenase (HO)-1) and angiogenic (VEGF) genes (116). Stress response (heat shock) proteins (HSPs) are also activated and could have a protective effect in renal ischemia-reperfusion injury (117).

miRNAs in ischemic AKI

The first studies regarding the role of miRNAs in ischemia-reperfrusion (I/R) injury were carried out mainly on acute myocardial infarction (AMI) models and initially there were no information about the kidneys. These AMI associated miRNAs are presented in Table 2, and they served as a guide for our preliminary experiments. Meanwhile, evidence to the importance of miR-24 in I/R injury also grew (118). It has been shown that miR-24 was critically involved in endothelial apoptosis during cardiac I/R-injury as well as apoptosis of cancer and T cells (119), (120), (121).

Table 2. List of the first identified miRNAs in relation to ischemia-reperfusion (of the heart, in acute myocardial infarction).

miRNA	Expression	Role	Model	Target/Effect	Ref.
miR-1	up	apoptosis	rat I/R, H_2O_2	Bcl-2 (HSP60, HSP70)	(122)
		reduced infarct size	mouse IPC	eNOS, HSP70, HSF-1	(123), (124)
		biomarker	rat AMI		(125)
miR-21	up	reduced infarct size	mouse heat-shock/IPC	eNOS, HSP70, HSF-1	(123), (124)
		apoptosis	rat H_2O_2 treatment	PDCD4	(126)
			mouse IR	PTEN↓ (Akt-P↑) → MMP-2↑	(127)
			rat, ischemic preconditioning	PDCD4	(128)
miR-24	up	reduced infarct size	mouse heat-shock/IPC	eNOS, HSP70, HSF-1	(123), (124)
miR-29a miR-29c	down	reduced infarct size, apoptosis	rat	↓miR-29 → ↑Mcl-2	(129)
miR-199a	down	apoptosis	rat hypoxia	Hif1a, Sirt1	(130)
miR-320	down	apoptosis	murine I/R	Hsp20	(131)

The role of miRNAs in the I/R-induced AKI was first reported in a proximal tubular cell targeted Dicer knockout mouse model, in which microRNA depletion attenuated renal ischemic damage (132). Unfortunately in the first two microarray studies performed on post-ischemic kidney no overlap in miRNA expression pattern could be found (132), (133). Later, two other microarray studies were published, which confirmed miR-21, miR-362, miR-685 and miR-1894-3p upregulation, and miR-805 downregulation (132), (133), (134), (135). Godwin et al. reported the course of changes in the expression of miRNAs differentially expressed after I/R injury (133), however only the histological damage and immune infiltration were assessed without measuring kidney function in this study. This same group also emphasized a prominent role for miR-21 in the prevention of tubular cell death after hypoxia in vitro (133), and further investigated the data with principal component analysis (PCA) combined with spherical geometry and found that after I/R injury there is a distinct miRNA expression pattern compared to sham

controls (136). MiR-21 also contributed to the beneficial effects of the delayed renal ischemic- preconditioning (137), (138). Others found, that miR-494 promoted inflammation and apoptosis and thus aggravated kidney injury in AKI by targeting ATF3, a stress response molecule (139). The HIF1α regulated miR-127 was also shown to contribute to renal I/R-injury in rats through modulation of cell trafficking, evidenced on proximal tubule cells in vitro (140). Another hypoxia inducible miRNA: miR-210 (141) was shown to be involved in the angiogenic processes during renal I/R injury, by enhancing VEGF and VEGFR2 expression (134). Vascular regeneration after ischemic AKI was also achieved with hematopoietic over-expression of miR-126, which reduced kidney injury and improved kidney function as well (142). An interesting study by Cantaluppi et al. found, that an injection of microvesicles, derived from cultured endothelial cell progenitors ameliorated kidney injury (143). However, if endothelial cells were previously transfected with miR-126 and miR-296 substantial reduction in the described protective effect could been observed (143). The beneficial paracrine effect of vesicle-transported miRNAs in AKI is further supported by the discovery of non-platelet RNA-containing particles which were found to mediate kidney regeneration (144).

Besides their involvement in the pathological processes, miRNAs were also evaluated as biomarkers in renal I/R induced AKI. MiR-10a and miR-30d not only increased in the urine, but also correlated with the injury severity in ischemia and STZ induced AKI mouse model (145). In AKI patients urine miR-21 and miR-155 just changed slightly (146), but miR-494 showed a more prominent increase compared to healthy controls (139). Furthermore, miR-21 might also serve as a prognostic marker after cardiac surgery-induced AKI (147).

DOI:10.14753/SE.2015.1771

Objectives

The objective of the thesis was to gain insight and understanding into the involvement of miRNAs in renal ischemia-reperfusion injury. Therefore our aims were (Figure 1):

1. to estimate which miRNA could be changed in renal ischemia-reperfusion injury through a comprehensive literature investigation;
2. to identify and validate selected miRNAs in ischemic acute kidney injury;
3. to analyze the function of the validated miRNAs, by:
    a. time-course study, to investigate in which phase of AKI are the miRNAs upregulated;
    b. evaluating the efficiency of a miRNA based therapy.

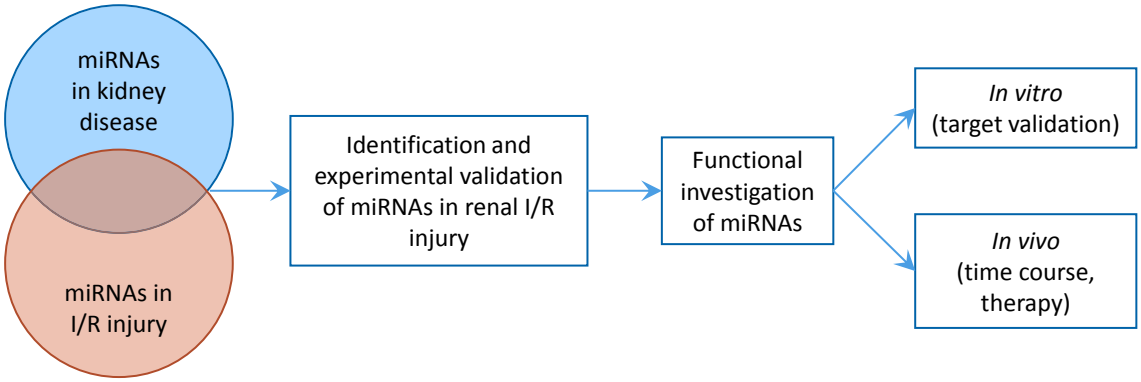


Figure 1. Objectives and work-plan.

The thesis has three major parts based on the published articles. First, the comprehensive literature investigation, partly presented in the introduction was published as a review article about the involvement of miRNA networks in kidney diseases. Second, the identification of the miR-17 family in a murine renal I/R injury model and the time-course study of several miRNAs, carried out in the laboratory of Dr. Péter Hamar, Semmelweis University resulted in an original research article (further referred to as: *miR-17 study*). The investigation of miR-24 in ischemic AKI, containing target validation and in vivo evaluation of a miR-24 based therapeutic strategy, was carried out in the laboratory of Prof. Dr. Thum Thomas, Hannover Medical School and lead to another research article (further referred to as: *miR-24 study*).

DOI:10.14753/SE.2015.1771

Methods

Patients

Renal biopsies from kidney transplant patients were obtained from the Interdisciplinary Transplant Center for Clinical Research, Hannover Medical School. From the available biopsies with prolonged (12 ± 1 hours, n=5) and short (3.5 ± 1 hours, n=5) cold ischemia time (CIT), clinical and demographic data were collected. The patient study was confirmed by the Ethics Committee of the Hannover Medical School and all patients gave their written informed consent (approval number: 2765). Patient characteristics are shown in Table 3.

Table 3: Demographics of transplant patients with long and short cold ischemia time (CIT)

	Short CIT	**Long CIT**
Recipient gender (male / female)	1 / 4	2 / 3
Recipient age (years)	46.2 ± 3.9	47.0 ±4.7
Cause of ESRD		
Glomerulonephritis	3	2
Hypertensive/diabetic nephropathy	1	2
Unknown	1	1
Cold ischemia time (minutes)	208 ± 56	707 ± 73

Animals

Male C57BL/6 mice (Charles River, Germany) were maintained under specific pathogen-free (SPF) conditions with access to standard rodent chow (Altromin standard diet, Germany) and tap water ad libitum. Mice weighing 29.4±2.9 g were used in the *miR-17 study*, and approval from the Semmelweis University Animal Research Ethical Committee has been obtained under the registration no.: XIV-I-001/2103-4/2012. Mice that were 10 to 12 weeks old weighing 20 to 30 g were used for experiments carried out in the *miR-24 study*, and the animal experimental procedures were in agreement with institutional and legislational regulations and were approved by the local authorities (approval number: 08/1434). 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).

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 (GACAAGGACGACUUGACUCGGU). The control antimiR has a comparable chemical composition but is directed against a microRNA expressed in Caenorhabditis elegans.

Kidney ischemia-reperfusion injury

Renal ischemia-reperfusion (I/R) injury was performed as established in our lab and described previously (36), (148). Briefly, the experiments were carried out at room temperature 24±0.5 °C using standard operating procedures. The intra-abdominal temperature was maintained using a heating pad (HK-3, DOPS, Czech Republic). For surgeries in the *miR-17 study*, the animals were narcotized with an intraperitoneal (i.p.) injection of 80 mg/kg ketamine and 4 mg/kg xylazin cocktail (CP-Pharma Handelsgesellschaft mbH, Germany). After median laparotomy, the left renal pedicle was prepared and clamped, to obtain sublethal (20 min) and lethal (30 min) ischemia as determined in previous studies (36), (149). After removing the clamps, we observed if the kidneys are properly reperfused. The right kidney was then removed. Sham operated control mice were also prepared in the same manner as those subjected to I/R except that the renal pedicle was not clamped. Sterile physiological salt solution was used for moistening the exposed viscera. The abdominal muscles and the skin were sutured separately with 3-0 vicryl suture (Ethicon Inc., Mexico).

In the *miR-24 study* the surgical interventions were carried out following isoflurane anesthesia. As in the miR-17 study, following median laparotomy, the left renal pedicle was prepared and the vascular clamp was 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 (LNA-CTR) at a concentration of 10 mg/kg 24 hours before the operation (n=20/group). Survival analyses and a part of the animal operation were performed at Phenos GmbH, Hanover, Germany. For the survival analysis, bilateral renal I/R or sham (n = 4) operations were performed. During the bilateral renal I/R first the left, then the right renal pedicle were prepared. The vascular clamps were placed within 15 seconds on the left and the right renal pedicle. After 27 minutes ischemia the clamps were removed, in the same order as positioned. Proper reperfusion of the kidneys was then observed. In the sorting experiment the renal pedicle was only clamped on the left side (unilateral I/R-injury). In this setting the contralateral kidney served as control to the injured kidney (I/R-kidney). The animals were sacrificed on day 1, day 3 and day 7 after renal I/R injury (n=7/group/timepoint).

Sacrifice, blood and organ collection

In the *miR-17 study*, animals were sacrificed at the specified time-points under ether anesthesia. Right before sacrifice, 500 U/mouse heparin (Merckle GmbH., Germany) was injected i.p., and blood was collected from the thoracic cavity after cross-sectioning the vena cava. After blood urea measurements blood was centrifuged at 1500 g for 8 min at 4 °C to obtain plasma for later analysis. The kidney was removed after a slow transcardiac perfusion with 20 ml physiological salt solution, pre-cooled to 4 °C. Plasma and renal tissue samples were snap frozen in liquid nitrogen and kept at -80 °C until use. In the *miR-24 study* kidney tissue samples and blood were harvested on days 0, 1, 3 and 7.

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 (150). 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 antimouse-CD31-PE (1:400 BD Pharmingen) for endothelial cells, Lotus tetragonolobus agglutinin (LTA) (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 (151). 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.

Plasma Urea and NGAL ELISA

Renal function was evaluated by determination of blood urea nitrogen (BUN) retention. Blood urea levels were measured from 32 μl whole blood obtained during organ collection with Reflotron® Urea test strips (Roche Diagnostics GmbH, Mannheim, Germany) on Reflotron® Plus device (Roche Diagnostics GmbH, Mannheim, Germany) as described in the manufacturer's protocol. Urea values were divided by 2.14 to obtain BUN levels. In the *miR-24 study,* renal function parameters (serum-urea and –creatinine) were analyzed on a Beckman Analyzer (Beckman Instruments GmbH, Munich, Germany).

Neutrophil gelatinase associated lipocalin (NGAL, Lipocalin-2) has been demonstrated to be a sensitive marker of tubular epithelial damage (152). The plasma NGAL level was determined with a mouse Lipocalin-2/NGAL DuoSet ELISA Development kit (R&D Systems, USA) as described by the manufacturer. Shortly, the 96 well plates (Nunc™ GmbH & Co. KG, Langenselbold, Germany) were coated with the capture antibody, and the non-specific binding sites were blocked with reagent diluent (1% BSA in PBS, pH 7.2 - 7.4). Adequately diluted (10^3 fold for sham- and 10^5 fold for ischemic-) samples were incubated on the plate in duplicates for 2 hours, and then the detection antibody was added. Next, Streptavidin-HRP was linked to the biotinylated detection antibody, followed by a short incubation with TMB Substrate (Sigma-Aldrich Chemie GmbH, Germany). A washing session (5 times with 300 μl of washing buffer)

was performed after each step until the addition of the substrate solution. The enzymatic reaction was terminated by stop solution containing H_2SO_4. The optical density was measured with Victor3™ 1420 Multilabel Counter (PerkinElmer, WALLAC Oy, Finland) at 450 nm with wavelength correction set to 544 nm. The NGAL concentrations were calculated with WorkOut software (Dazdaq Ltd., England), using a four parameter logistic curve-fit.

Histology and immunohistochemistry

Renal tissue samples fixed in 4% buffered formaldehyde were dehydrated and embedded in paraffin wax (FFPE) for histology and immunohistochemistry. Kidney samples from the *miR-17 study* were evaluated morphologically by tissue microarray (TMA) as described previously (153). Briefly, blocks of 70-sample TMAs contained duplicates of 2 mm diameter cylinders cut by the computer-controlled puncher of the TMA Master Device (3DHISTECH Kft, Budapest, Hungary) from the I/R sensible cortico-medullary junction of each FFPE kidney. For morphology and immunohistochemistry, 4 μm thick sections were cut from the TMA blocks. Renal tubular necrosis and regeneration were evaluated in Periodic acid-Schiff (PAS) stained TMA sections. A histological score of 0 to 4 was given by a pathologist blinded to the origin of the tissue as follows: 0= no lesion; 1= minimal or focal changes affecting less than 20% of the field; 2= mild changes or the extension of the lesion/regeneration to approx. 25% of the field; 3= moderate changes or the extension of the lesion/regeneration to less than 50% of the field; 4=severe changes or the extension of the lesion/regeneration to more than 50% of the field).

Renal tubular cell damage was evaluated by neutrophil gelatinase-associated lipocalin (NGAL, Lipocalin-2) immunostaining. Dewaxed and rehydrated TMA sections were cooked at 100 °C for 25 min in a 0.01M Tris-HCl and 0.1 EDTA buffer (TBS; pH 9.0) for antigen retrieval. The immunostaining involved consecutive incubations of TMA sections in 1% bovine serum albumin (BSA) in TBS (pH 7.4) for 15 min, rabbit anti-human NGAL IgG (1:100; R&D Systems, Minneapolis, USA) for 16 h and in goat anti-rabbit IgG EnVision-peroxidase polymer kit (Dako, Glostrup, Denmark) for 40 min, all at room temperature. Tissue-bond peroxidase activity was developed with a DAB/H_2O_2 chromogen/substrate kit (Dako). Immunostained TMA slides were digitalized using a

Pannoramic Scan instrument, and the results were analyzed with the Pannoramic Viewer software (3DHISTECH).

In the *miR-24 study* 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 (154). 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 within a high power field of view: 0: no damage; 1: less than 25% damage; 2: 25%–50% damage; 3: 50%–75% damage; and 4: more than 75% damage.

In the *miR-24 study* immunostainings were performed in cryosections. For inflammatory cell influx the following primary antibodies were used: monoclonal rat anti-mouse F4/80 for macrophages (Serotec, Oxford, United Kingdom), monoclonal rat anti-mouse CD45 for leucocytes (BD Pharmingen, BD Biosciences, Santa Cruz, CA), affinity-purified rat anti-mouse Ly-6G/Gr-1 for neutrophils (eBioscience, San Diego, CA), purified Rat Anti-Mouse CD4 for T helper lymphocytes (BD Pharmingen, BD Biosciences, Santa Cruz, CA). Capillary rarefaction in the outer medulla was evaluated after fluorescent immunohistochemical staining for polyclonal rabbit anti-mouse CD31 for endothelial cells (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 the outer medulla. A fluorescein in situ cell death detection kit was used according to the manufacturer's instructions for Terminal deoxynucleotidyl transferase dUTP nick end labeling (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 immortalized **h**uman **k**idney proximal tubular epithelial cells (HK-2) were used. HK-2 cells were maintained in Keratinocyte Growth Medium 2 with supplements.

Cells were grown to 60% to 70% confluence and used for further analyses. Apoptosis was determined by TUNEL staining (Roche Applied Science, Mannheim, Germany). All assays were done according to the manufacturer's instructions.

Transfection Assays

Transient liposomal transfection of 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 pre-miRNAs and control miRNA (pre-neg) 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 miRNA complexes (final miRNA concentration: 100 nmol/L). Cells were incubated for 4 hours before the media were changed to fresh media. Silencing miRNA targets was monitored for 72 hours after transfection by Western blot analysis.

Scratch wound healing assay of HK-2 cells

Transfected HK-2 cells were cultivated in human keratinocyte medium at 37°C, 5% CO_2. The scratches in the cell monolayer were generated with a 100-µl pipett-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.

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.), 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.

Luciferase Reporter Assays

A luciferase reporter assay system was applied to validate potential miRNA targets. A putative 3'UTR miRNA binding sequence was cloned into the SpeI and HindIII cloning site of pMIR-REPORT vector (Ambion). H2A.X wildtype (2 sites): 5'-CTGGA**CTGAGCC**TC…TGTATGCTAT**CTGAGCC**GTCT-3'; S1PR1 wild-type 5'- AGCTTTGATTTTG**CACTGAGCC**A…CATAGCT-3'. The resulting construct was cotransfected 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).

RNA preparation

Total RNA was extracted from the upper third of the kidney with TRIzol® Reagent (Invitrogen™, UK) according to the protocol provided by the manufacturer (155). In brief, the frozen renal tissues were homogenized by an IKA® DI 18 basic grinder (IKA® Works do Brasil Ltd., Brazil). Chloroform (Sigma-Aldrich, Inc., USA) was added to each sample and mixed by vortex. The aqueous phase was separated from the organic phase by centrifugation. RNA was precipitated from the transferred aqueous phase with an equal quantity of isopropyl alcohol by incubation for 30 min at room temperature. The RNA pellet was washed twice with 75% ethyl alcohol, and dissolved in 100 μl RNase free water. The RNA pellet was treated with RNase-free DNase I

(Fermentas, EU) to eliminate possible DNA contamination. The DNase was inactivated by phenol/chloroform extraction (Fluka, Sigma-Aldrich, Switzerland). The RNA concentration and purity was inspected with NanoDrop 2000c Spectrophotometer (Thermo Fisher, USA). All RNA samples had an absorbance ratio (260 nm / 280 nm) above 1.8. To investigate RNA integrity, samples were electrophoresed on 1% agarose gel (Invitrogen Ltd., Paisley, UK) in BioRad Wide mini-sub® cell GT system with BioRad PowerPac™ HC power supply, and the 28S and 18S ribosomal RNA fraction integrity was examined. The RNA solutions were kept at -80°C until further procedures.

Multiplex analysis of the microRNA profile

The miRNA expression pattern of the kidney samples was analyzed after 24 hours of reperfusion following 30 min I/R (n=9) or sham operation (n=8). Based on the review of available literature about miRNA networks in renal diseases and ischemia reperfusion injury, the largest, most suitable, commercially available Luminex miRNA panel was selected. The expression of 46 microRNAs (Table 4) was determined with the Vantage™ microRNA Detection Kit (Marligen Biosciences, Inc., USA) on the Luminex® 200™ System (Luminex Corporation, USA) as described in the protocol. First, 1.5 µg of the extracted RNA was poly (A) tailed and biotinylated and then sample RNA hybridization with the bead mix and microRNA detection was performed. All samples were tested in duplicates and the background median fluorescence intensity (MFI) was subtracted before further calculations. MicroRNAs with low MFI (<100, ≈ 3 times the background MFI) were excluded from the statistical analysis.

Table 4. List of miRNAs included in Luminex multiplex miRNA panel.

let-7a-5p	miR-17-5p	miR-29b-2-5p	miR-100-5p	miR-138-5p	miR-205-5p
let-7c-5p	miR-20a-5p	miR-30d-5p	miR-106a-5p	miR-141-5p	miR-210-3p
let-7g-5p	miR-21-5p	miR-34a-5p	miR-125a-5p	miR-181b-5p	miR-212-3p
let-7i-5p	miR-23b-5p	miR-34b-5p	miR-125b-5p	miR-182-3p	miR-218-5p
miR-9-5p	miR-24-2-5p	miR-93-5p	miR-132-5p	miR-185-5p	miR-221-3p
miR-10a-5p	miR-27a-5p	miR-95-3p	miR-135a-5p	miR-195a-5p	miR-372-3p
miR-10b-5p	miR-29a-5p	miR-96-5p	miR-136-5p	miR-199a-5p	
miR-16-5p	miR-29c-5p	miR-99a-5p	miR-137-3p	miR-200b-5p	

Quantitative real-time PCR analysis of miRNAs and gene expression in renal tissue

MicroRNA expressions were evaluated with TaqMan probes (156). First, cDNA was reverse-transcribed from 5 ng RNA sample using a miRNA-specific, stem-loop RT primer from the TaqMan® Small RNA Assays and reagents from the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems™, USA), as described in the manufacturer's protocol. Second, in the real-time PCR step, PCR products were amplified from the cDNA samples using the TaqMan® Small RNA Assay together with the TaqMan® Universal PCR Master Mix II. All measurements were done in duplicates, and the miRNA expressions were normalized to the U6 small nuclear RNA (snRNA) applied as an endogenous reference (157), (158) in the *miR-17 study*; and the small RNA molecule snoRNA-202 (mouse) and RNU48 (human) in the *miR-24 study*. The miRNA expressions were calculated with the relative quantification (ΔΔCq) method, and the efficiency of the qPCR reaction was verified with standard curves.

Gene mRNA levels were measured by dsDNA dye based real-time PCR. Reverse transcription into cDNA was carried out by the High-Capacity cDNA Archive Kit (miR-17 study: Applied Biosystem™, USA) or iScript Select c-DNA Synthesis Kit (miR-24 study: BioRad, Singapore) according to the manufacturer's protocol. In brief, 1 μg of total renal RNA was denaturated at 70°C for 5 min. After the annealing of the random hexamer or oligo(dT) primers on the RNA template at 25°C for 10 min, cDNA was synthesized at 37°C for 2 hours. The reaction was terminated by heat

inactivation (85°C for 2 min). The expression level was evaluated on the Bio-Rad C1000™ Thermal Cycler with CFX96™ or CFX384™ Optics Module real-time PCR system (Bio-Rad Laboratories, Inc., Singapore). The PCR reaction was performed with Maxima™ SYBR Green qPCR Master Mix (miR-17 study: Fermentas, EU), or iQ SYBR Green Supermix (miR-24 study: BioRad Laboratories, Singapore) according to the manufacturer's protocol. The specific primers are depicted in Table 5. Primers used in the miR-17 study were designed by NCBI/ Primer-BLAST online software and synthesized by Integrated DNA Technologies (IDT, Inc., USA). The endogenous reference gene was GAPDH. Primer annealing was set to 58°C or 60°C and the melting curve was analyzed to detect any abnormality of the PCR product. All samples were measured in duplicates and expressions were calculated using the relative quantification (ΔΔCq) method. The qPCR reaction efficiency was also verified with standard curves.

Gene array analysis was performed with Affymetrix GeneChip® Human Transcriptome Array according to the manufacturer's instructions (Affymetrix Systems). Reverse transcription, second-strand synthesis, and cleanup of double-stranded cDNA were performed according to the Affymetrix protocols (One-Cycle cDNA synthesis Kit, Affymetrix) starting from 2 μg of total RNA. Synthesis of biotin-labeled cRNA was performed with the use of the IVT Labeling Kit (Affymetrix). cRNA concentration was determined and the distribution of cRNA fragment sizes was checked by gel electrophoresis. For hybridization 15 μg of fragmented cRNA was used. For normalization and further data analysis, the XRAY Excel software tool (Biotique) was used.

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. Targets predicted by at least two prediction data bases and containing a miR-24-8mer seed match in the respective 3'UTR region were considered.

Statistical analysis

Results are presented as mean +/- standard error of the mean (SEM) unless otherwise indicated. 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). Continuous variables were compared using unpaired T-test or one-way analysis of variance (ANOVA), followed by the Dunnett's multiple comparison post hoc test versus the (sham) control group or Tukey's multiple comparisons test. Linear correlation was assessed with Pearson product-moment correlation coefficient. To compare two regression lines to each other, the online version of StatTools.net was used (159). The null-hypothesis was rejected if the two-sided p-value reached statistical significance (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$).

Table 5. Primer pairs used for gene expression analysis

Gene symbol	**Gene name**	**Primer pairs**	
GAPDH (*#17*)	Glyceraldehyde 3-phosphate dehydrogenase	Fwd	CCAGAATGAGGATCCCAGAA
		Rev	ACCACCTGAAACATGCAACA
NGAL (*#17*)	neutrophil gelatinase associated lipocalin	Fwd	ACGGACTACAACCAGTTCGC
		Rev	AATGCATTGGTCGGTGGGG
GAPDH (*#24*)	Glyceraldehyde 3-phosphate dehydrogenase	Fwd	TTCACCACCATGGAGAAGGC
		Rev	GGCATGGACTGTGGTCATGA
NGAL (*#24*)	neutrophil gelatinase associated lipocalin	Fwd	TGAAGGAACGTTTCACCCGCTTTG
		Rev	ACAGGAAAGATGGAGTGGCAGACA
KIM-1 (*#24*)	kidney injury molecule 1	Fwd	AAACCAGAGATTCCCACACG
		Rev	GTCGTGGGTCTTCCTGTAGC
TNFα (*#24*)	tumor necrosis factor alpha	Fwd	TACTGAACTTCGGGGTGATTGGTCC
		Rev	CAGCCTTGTCCCTTGAAGAGAACC
IL-1ß (*#24*)	interleukin 1 beta	Fwd	AGGTCCACGGGAAAGACACAGG
		Rev	GGGCTGCTTCCAAACCTTTGAC
MIP2α (*#24*)	macrophage inflammatory protein 2-alpha	Fwd	CCAAGGGTTGACTTCAAGAAC
		Rev	AGCGAGGCACATCAGGTACG
MCP-1 (*#24*)	monocyte chemoattractant protein 1	Fwd	GGCTCAGCCAGATGCAGTTA
		Rev	ACTACAGCTTCTTTGGGACA
IL-6 (*#24*)	interleukin 6	Fwd	GAGAAAAGAGTTGTGCAATG
		Rev	ATTTTCAATAGGCAAATTTC
Col-I-α2 (*#24*)	collagen I alpha 2	Fwd	CAGAACATCACCTACCACTGCAA
		Rev	TTCAACATCGTTGGAACCCTG
Col III (*#24*)	collagen III	Fwd	TGACTGTCCCACGTAAGCAC
		Rev	GAGGGCCATAGCTGAACTGA
αSMA (*#24*)	alpha smooth muscle actin	Fwd	ACTACTGCCGAGCGTGAGAT
		Rev	AAGGTAGACAGCGAAGCCAG

Fwd: forward; Rev: reverse; (*#17*): *miR-17 study*; (*#24*): *miR-24 study*

DOI:10.14753/SE.2015.1771

Results

miR-17 study

Lethal renal ischemia-reperfusion injury markers

In the first series of experiments, C57BL/6J mice were subjected to 30-min unilateral renal ischemia or sham operation followed by removal of the right kidney and 24 hours of reperfusion. The main histological lesions were found in the outer stripe of the outer medulla, and to a lesser degree in the cortical region (Figure 3). The renal cortex presented tubular cell cytoplasmic vacuolization, brush border loss, pyknosis, tubular dilatation and flattening of epithelial cells (Figure 3C). The outer stripe depicted massive tubular cell necrosis, nuclear loss, tubular cast formation and immune cell infiltration (Figure 3G). Quantitative histologic analysis confirmed massive tubular cell necrosis after 30 min (lethal) ischemia (Figure 3D).

Immunohistochemistry of the renal tubular damage marker NGAL demonstrated a low level of staining in the proximal tubule, and mild staining in the medulla in the kidneys of sham operated mice (Figure 3B, F, J). However, there was a strong NGAL specific tubular staining after I/R injury in the outer stripe (Figure 3H) and cortex (Figure 3D). Furthermore, whole kidney NGAL mRNA increased 149-fold ($p<0.001$) (Figure 4A), and plasma NGAL level increased 30-fold ($p=0.05$) (Figure 4B) compared to the sham operated group. In concordance with the morphological results, BUN increased 6-fold 24 hours after 30-min ischemia compared to the sham operated group ($p<0.001$) (Figure 4C).

DOI:10.14753/SE.2015.1771

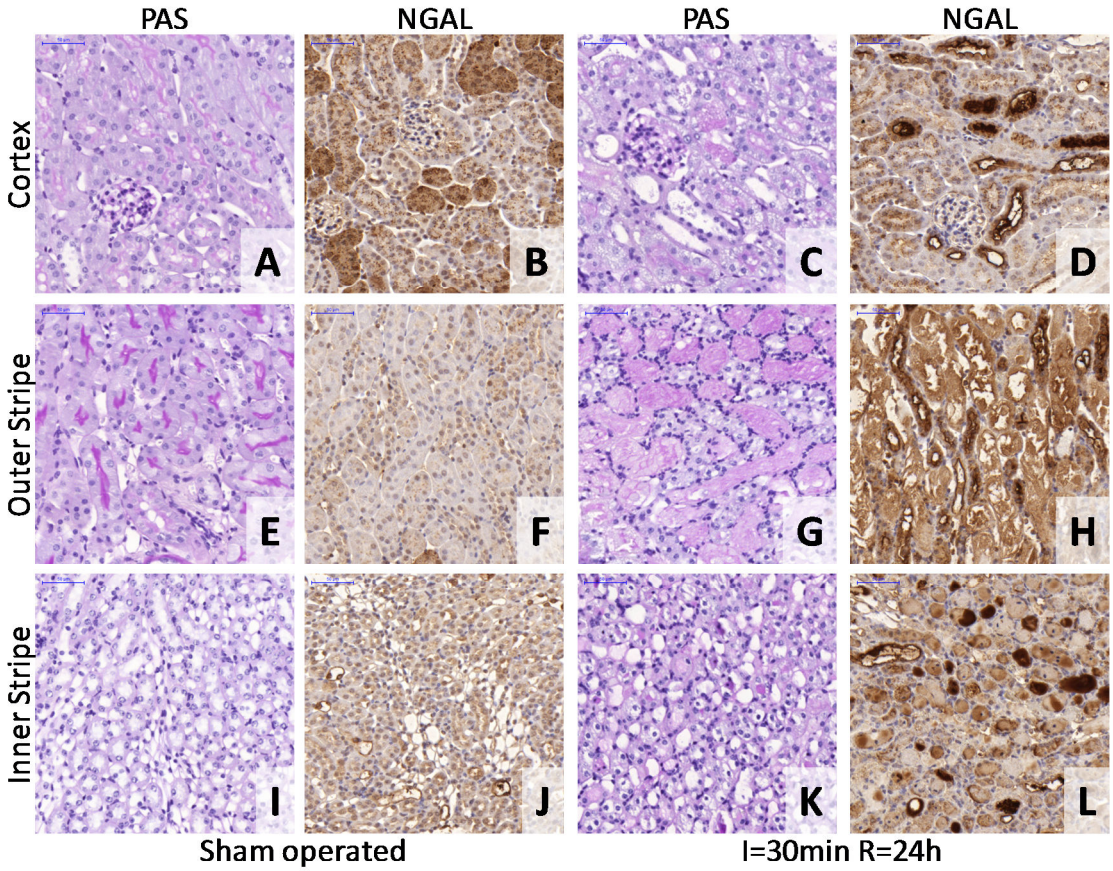


Figure 3. Histopathology and NGAL immunohistochemistry (IHC) after a 30-min renal ischemia-reperfusion (I/R) injury. Representative images scanned at x30 magnification from the cortical (A-D), outer stripe (E-H) and inner stripe (I-L) regions of the kidney from the sham-operated (PAS stain: A, E, I and NGAL IHC: B, F, J) and I/R groups (PAS stain: C, G, K and NGAL IHC: D, H, L). Bar = 50 μm.

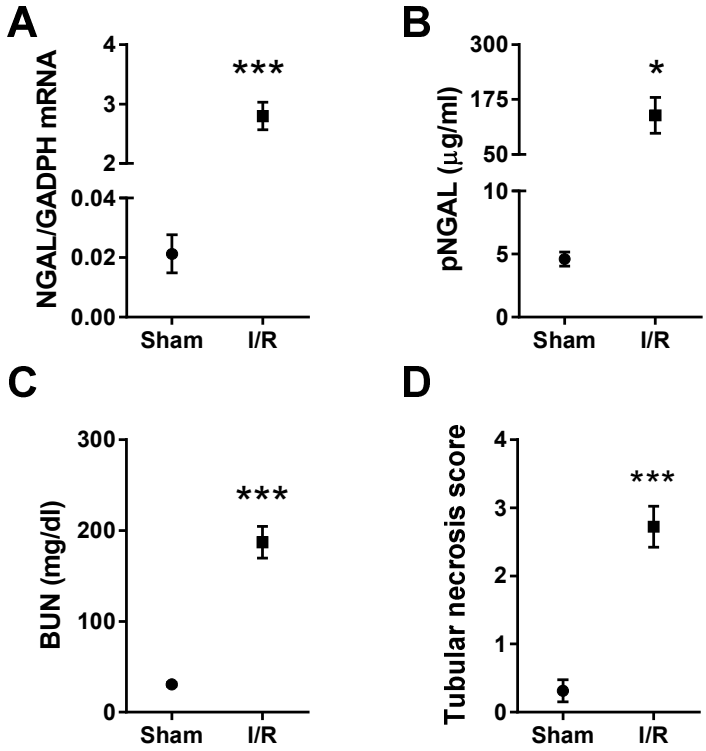


Figure 4. Kidney damage markers after 30 min ischemia-reperfusion (I/R) injury. Sham (n=8; filled dot: ●) and I/R (n=9; filled square: ■). *: $p<0.05$, ***: $p<0.001$.

Kinetics of sublethal renal ischemia-reperfusion injury markers

To measure the time-course of markers after 20 min (sublethal) I/R injury, mice were subjected to 20-min unilateral renal ischemia or sham operation with various times of reperfusion. After 24-hours of reperfusion, all kidney damage markers increased significantly. Renal NGAL mRNA expression (Figure 5A) and plasma NGAL protein levels (Figure 5B) were elevated more than 100-fold ($p<0.001$) and 16-fold ($p<0.001$), respectively. Renal function also deteriorated, as reflected by the 4-fold increase ($p<0.05$) in BUN levels compared to the sham-operated group (Figure 5C). Renal tubular necrosis score was also significantly elevated 24 hours after 20 min (sublethal) I/R injury (Figure 5D). Thereafter, histologic signs of tubular regeneration appeared (Figure 5E) and renal damage markers started to decrease. The increase in renal NGAL mRNA expression diminished to 66-fold ($p<0.05$) on day 3, and became non-significant after 4 days of

reperfusion. Plasma NGAL decreased to the control level already on day 3. On the 4th day of reperfusion all damage markers returned close to sham values.

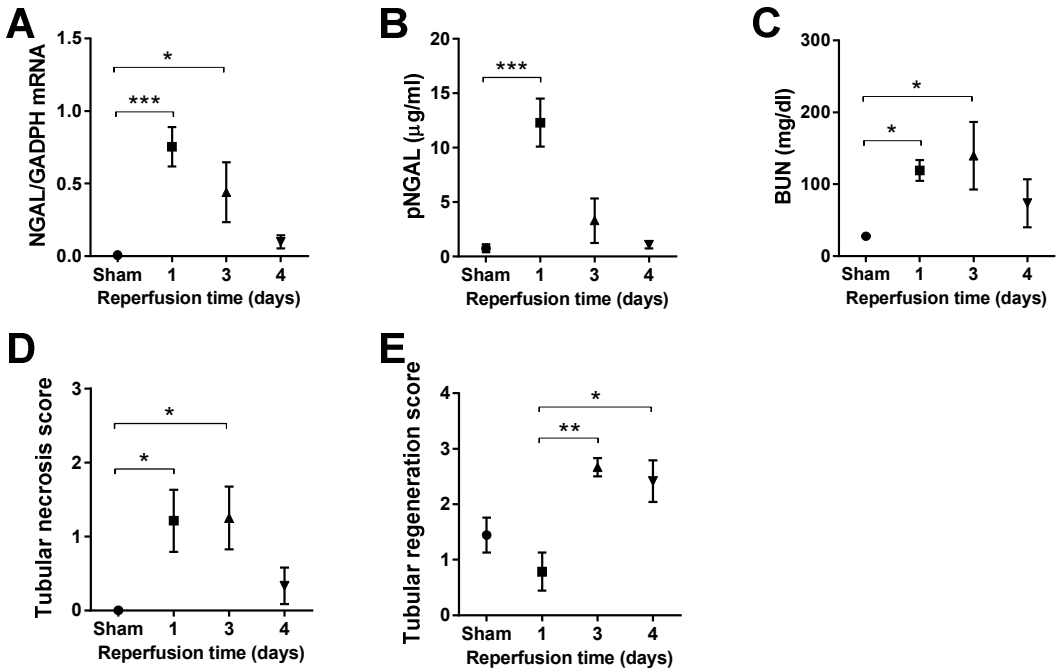


Figure 5. Time-course of kidney damage markers after a 20-min renal ischemia with various times of reperfusion. (A) Relative renal NGAL mRNA expressions (B), plasma NGAL protein, (C) blood urea levels, (D) tubular necrosis score and (E) tubular regeneration score are plotted for the sham-operated group (n=10) and I/R groups on the first (n=7), third (n=6) and the fourth days (n=6) of reperfusion. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

Micro RNA expression changes and time-course of renal miR-17-5p, miR-106a and miR-21 expressions after renal ischemia-reperfusion injury

The miRNA profile was assessed by the Luminex platform, a novel microbead-based technology. After the exclusion of miRNAs with low fluorescent signal (<100 MFI), 22 miRNAs had been included in the analysis. Five (miR-21, miR-17-5p, miR-106a, let-7c and miR-99a) out of 22 miRNAs had a significantly different expression relative to the sham operated group. However, only miR-21, miR-17-5p, and miR-106a changed more than 30% after I/R injury (Figure 6). These 3 miRNAs were further

analyzed and validated with TaqMan MicroRNA Assays. The qPCR analysis confirmed the significance of the results regarding miR-17, miR-21 and miR-106a (Table 6).

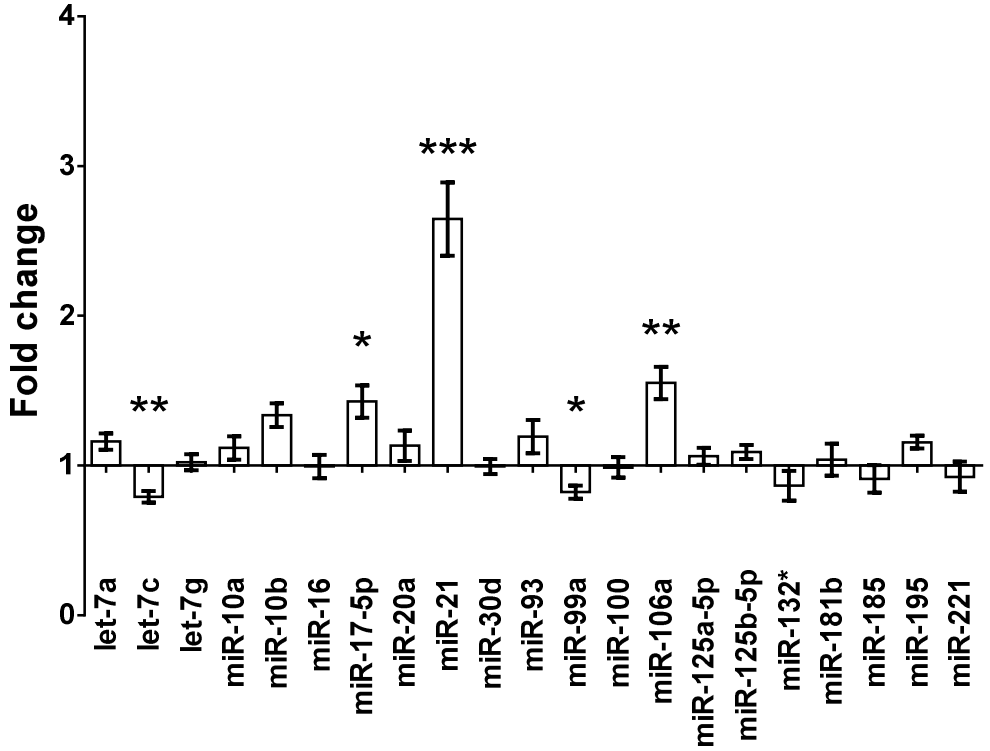


Figure 6. The miRNA expression profile of renal I/R injury, measured on the Luminex multiplex platform (fold changes observed after 24 hours of reperfusion following 30 min ischemia (n=9), compared to the sham-operated group (n=8)). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

Table 6. Real-time, quantitative PCR (qPCR) validation of the results obtained with Luminex multiplex platform (p-level: *$p<0.05$, **$p<0.01$ and ***$p<0.001$).

miRNA	**Luminex**			**qPCR**		
	Fold change	**SEM**	**P-level**	**Fold change**	**SEM**	**P-level**
miR-21	2.38	±0.22	***	1.55	±0.13	**
miR-17-5p	1.30	±0.10	*	1.44	±0.07	***
miR-106a	1.42	±0.10	**	1.40	±0.07	***

To determine if the identified miRNAs are upregulated during early or late reperfusion, their expression time-course was evaluated. MiR-21, miR-17-5p and miR-106a levels increased before any other damage markers, 24 hours after 30 min (lethal) ischemia (Figure 7). MiR-17-5p and miR-106a have a similar nucleotide sequence, and thus belong to the same miR17-family. As miR-17-5p and miR-106a also had similar expression patterns, miR-17-5p was studied in detail, as its expression was higher than that of miR-106a (Figure 7A, B). Twenty min (sublethal) ischemia was performed to identify the phases of acute kidney injury in which the expressions of miR-17-5p and miR-21 increase. First, miR-17-5p increased significantly (1.6-fold, $p<0.05$) after one day of reperfusion, and remained significantly elevated (1.7-fold, $p<0.01$) until the third day (Figure 8A). On the other hand, renal miR-21 expression was first elevated (3-fold, $p<0.001$) after three days of reperfusion, and remained upregulated (2-fold, $p<0.05$) on the fourth day (Figure 8B), when miR-17-5p expression already returned to the sham-operated level.

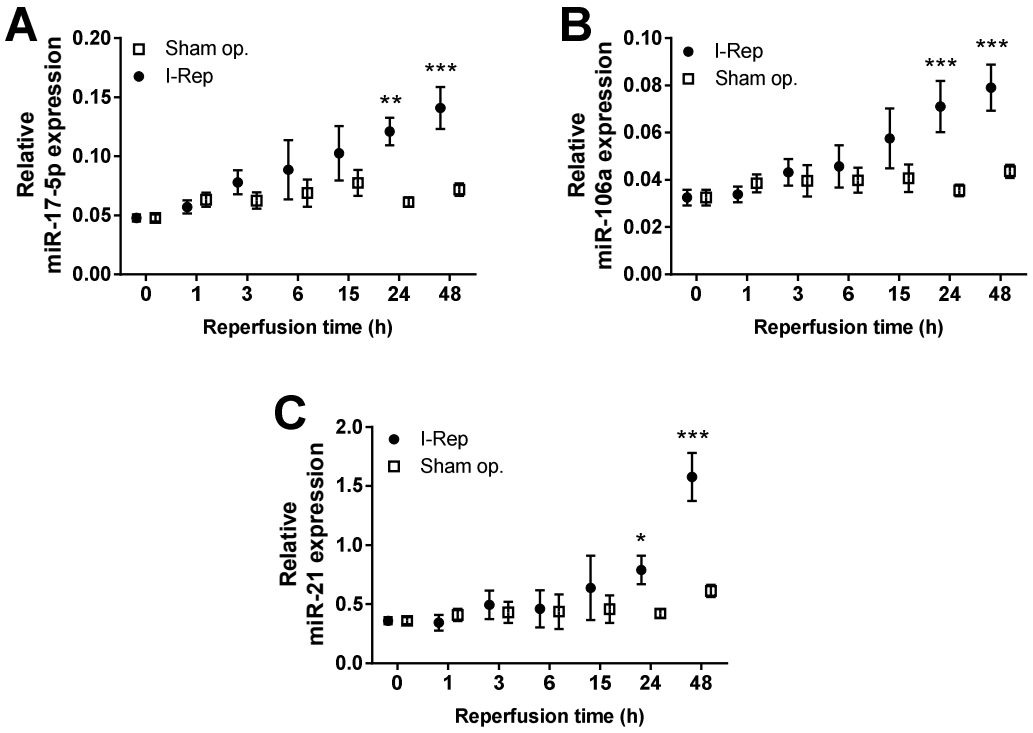


Figure 7. Time-course of (A) miR-17-5p, (B) miR-106a and (C) miR-21 expression after a 30-min ischemia with various reperfusion times (n=5 each time-point and each group). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

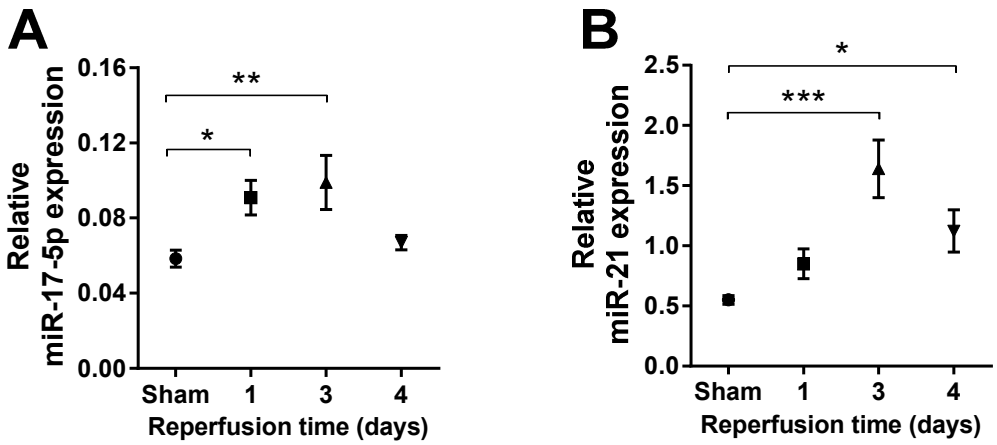


Figure 8. Time-course of (A) miR-17-5p and (B) miR-21 expressions after a 20-min ischemia with various times of reperfusion (sham n=10; day 1 n=7; day 3 n=6; day 4 n=6) * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Correlation between the two renal miRNA expressions and other markers of renal I/R injury

Significant correlations were detected between renal miR-17-5p and miR-21 expressions at every studied time point in both the ischemia-reperfusion and sham-operated groups (Figure 9). However, the slopes of the regression lines were significantly different between the ischemia-reperfusion and sham-operated groups on the third and fourth days of reperfusion ($p=0.019$ and $p=0.001$). Moreover, the relationship between the expression of the 2 miRNAs changed with the duration of reperfusion: the slopes became steeper as the reperfusion time increased (sham: $m = 6.3$, $p=0.011$; day 1: $m = 12.2$, $p=0.004$; day 3: $m = 15.8$, $p=0.003$; day 4: $m = 39.4$, $p=0.024$, Figure 9).

Nevertheless, miR-17-5p positively correlated with both renal tubular cell injury markers: NGAL mRNA ($r^2=0.34$, $p<0.001$) and blood urea ($r^2=0.38$, $p<0.001$). However, miR-21 correlated only with blood urea retention ($r^2=0.20$, $p<0.05$).

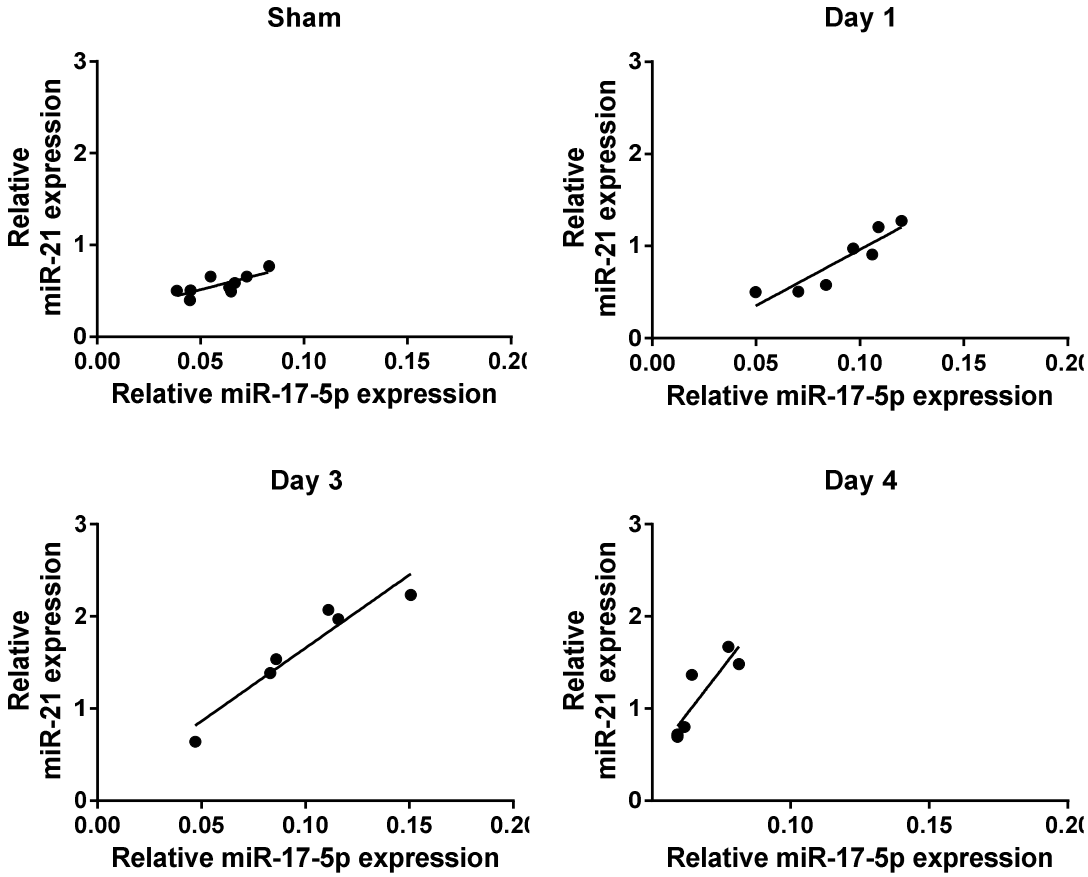


Figure 9. Correlation of miR-17-5p with miR-21 in the naive, sham operated and I/R groups after different reperfusion times. Sham (n=9): r^2=0.56, p=0.012; Day 1 (n=7): r^2=0.83, p=0.004; Day 3 (n=6): r^2=0.90, p=0.003; and Day 4 (n=6): r^2=0.76, p=0.024.

miR-24 study

miR-24 in renal I/R-injury

Levels of miR-24 are increased in mouse kidneys at 1 and 7 days after induction of I/R injury compared to contralateral control kidneys (Figure 10A). 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 10B) at 1 day of reperfusion. At a reperfusion time of 7 days after I/R-injury miR-24 was up-regulated 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, non-significant decrease in miR-24 expression was detected

in pericytes (PDGFRb+). In kidney transplant biopsies of patients with prolonged cold ischemia time (CIT; n=5 per group) an increase in miR-24 was detected, indicating a distinct pathophysiological role in this setting (Figure 10C).

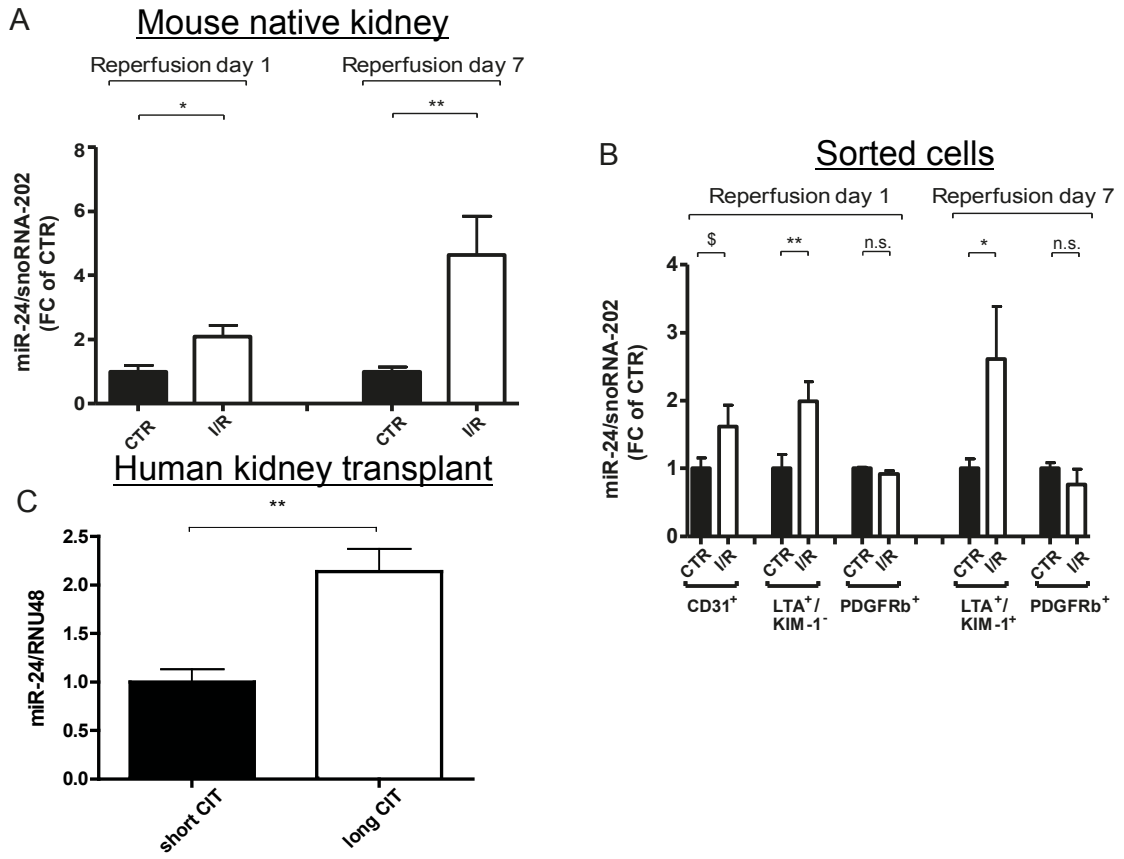


Figure 10. Expression and function of miR-24 in the kidney and distinct renal cell populations. The expression of miR-24 in mouse kidneys is depicted at day 1 and 7 (A) following unilateral I/R-injury (n=7 each). Expression of miR-24 in sorted cells after digestion of post-ischemic mouse kidney at reperfusion for 1 and 7 days is shown (B). CD31+= endothelial cells; LTA+/KIM-1-= uninjured proximal tubular epithelial cells; LTA+/KIM-1+ = injured proximal tubular epithelial cells; PDGFRb+ = pericytes; levels of miR-24 were compared to snoRNA-202 as control. MiR-24 expression normalized to RNU-48 in biopsies of kidney transplant biopsies of patients with long compared to short cold ischemia time (CIT; n=5 in each group) (C). $: p=0.08; **: p<0.01, *: p<0.05. CTR = contralateral control kidney; I/R = ischemia/reperfusion-injury.

DOI:10.14753/SE.2015.1771

Functional role of miR-24 in tubular epithelial cells

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 11A – C) . Scratch migration analysis following miR-24 enrichment indicated a defect in tubular epithelial migratory capacity (Figure 11D – F).

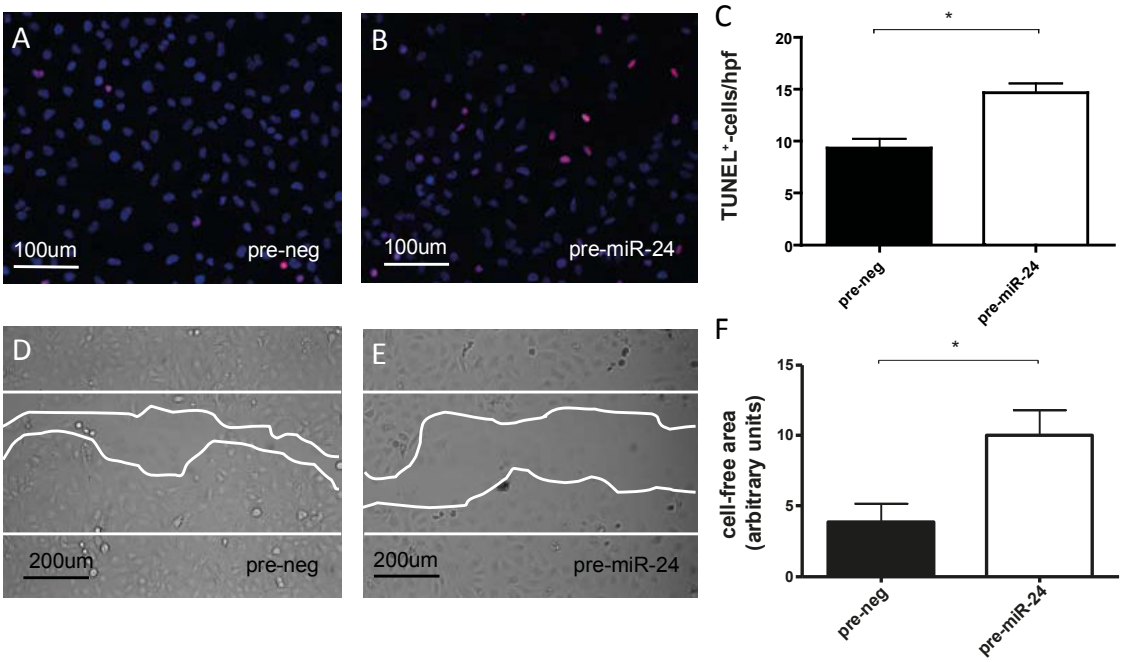


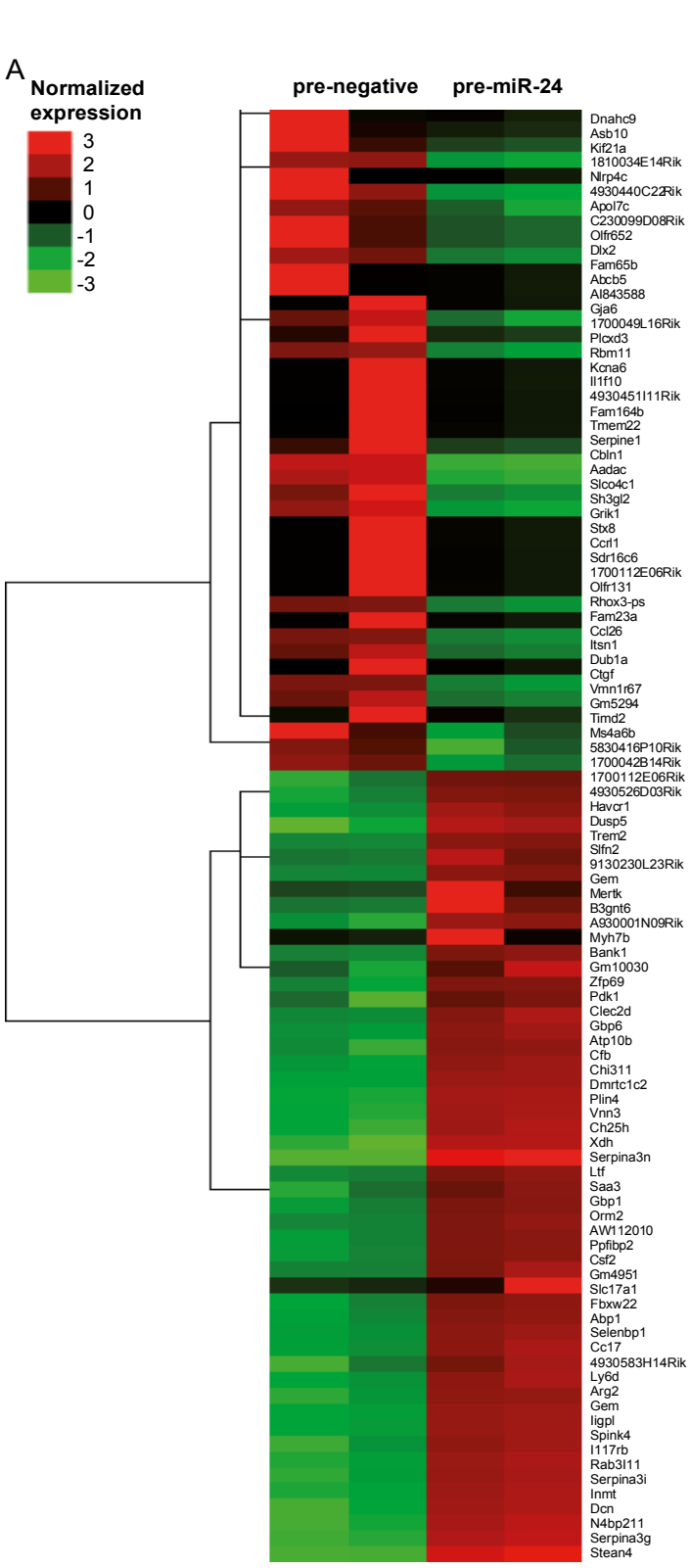
Figure 11. Tubular cell apoptosis and migration after miR-24 transfection *in vitro*. TUNEL staining in cultured HK-2 cells after pre-negative control (A) and pre-miR-24 oligonucleotide (B) transfection and quantification of results (C, n=6 experiments). Scratch migration analysis in normoxia in HK-2 cells after pre-negative control (D) and pre-miR-24 oligonucleotide (E) transfection and quantification of results (F, n=6 experiments). *: $p<0.05$; hpf = high power field.

Sphingosine-1-phosphate receptor 1 (S1PR1), H2A histone family, member X (H2A.X) and Heme Oxygenase-1 (HO-1) are direct targets of miR-24 in vitro

In order 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 a large number of 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 12A). In total, 1822 genes were down-regulated in cells overexpressing miR-24 compared to cells transfected with a pre-negative control oligonucleotide. Cluster analysis of the top 50 up- and down-regulated genes identified a number of genes involved in apoptosis regulation (Figure 12A). Down-regulated genes of the array were subsequently merged with predicted targets of miR-24 (Targetscan). These are shown in Figure 12B. In our subsequent 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, first 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 tubular epithelial cells (Figure 13A). Furthermore transfection with synthetic miR-24 precursors significantly repressed luciferase activity in cells, where the respective 3'UTR regions of S1PR1 and H2A.X were fused to a luciferase reporter gene (Figure 13B and C). We thus identified S1PR1 and H2A.X as novel direct targets of miR-24. HO-1 was previously confirmed to be a bona fide target of miR-24 (160).

DOI:10.14753/SE.2015.1771



B

Gene name	Downregulation
EYA4	2.0
DYRK2	2.0
IGF2BP1	2.0
HEG1	2.0
UCK2	2.0
GRIP1	2.0
IFFO2	2.1
ANKRD52	2.1
SAMD4B	2.1
TGIF2	2.1
MIDN	2.1
AMMECR1	2.2
PLK3	2.2
PVRL2	2.2
NOS1	2.3
FURIN	2.3
SPCS3	2.3
KLHL1	2.3
AMOTL2	2,3
GRIA3	2.3
ODZ1	2.3
DUSP8	2.3
SEMA5A	2.4
WNT4	2.5
H2A.X	2.5
AXL	2.6
CACHD1	2.6
PRKCA	2.6
SEMA6A	2.6
FST	2.6
HO-1	2.7
PCDH10	2.7
TCF7	2.8
ENTPD7	2.9
VGLL3	3.0
CDX2	3.0
FZD5	3.0
ENTPD6	3.1
TGFBI	3.2
NUP210	3.4
ITGA3	3.6
NPSR1	3.9
DLC1	4.3
CHL1	5.3
MMP14	5.3
KCNA6	8.9

Figure 12. Affymetrix gene array and cluster analysis in tubular epithelial cells transfected with pre-negative 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). Down-regulated genes (fold regulation) of the array, subsequently merged with predicted targets of miR-24 (Targetscan), are also shown. Targets further analyzed (H2A.X and HO-1) are highlighted in red (B).

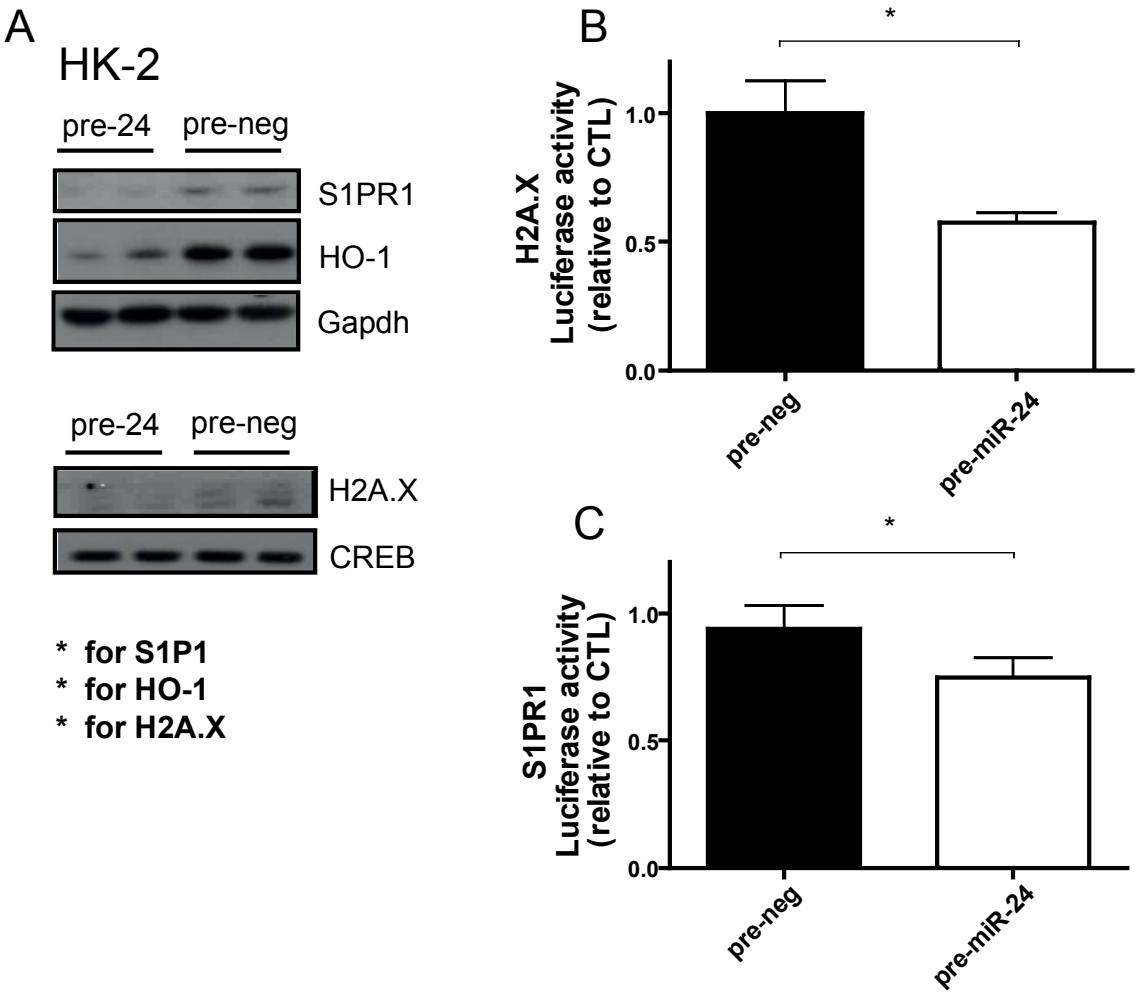


Figure 13. Western blot analysis in HUVECs and HK-2 cells of cytosolic S1PR1 and HO-1 normalized to glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and nuclear H2A.3 normalized to cAMP response element-binding protein after transfection with prenegative control and pre–miR-24 oligonucleotides (A). Results of luciferase gene reporter assays concerning H2A.X (B) and S1PR1 (C). * $p<0.05$; ** $p<0.01$.

Markers of kidney damage and endothelial activation in I/R-injury after miR-24 silencing

Treatment of mice with an LNA-modified antimiR targeting miR-24 before unilateral I/R injury (Figure 14G – J) resulted in a marked reduction of kidney injury

DOI:10.14753/SE.2015.1771

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 antimiR targeting miR-24 (Figure 15D – F).

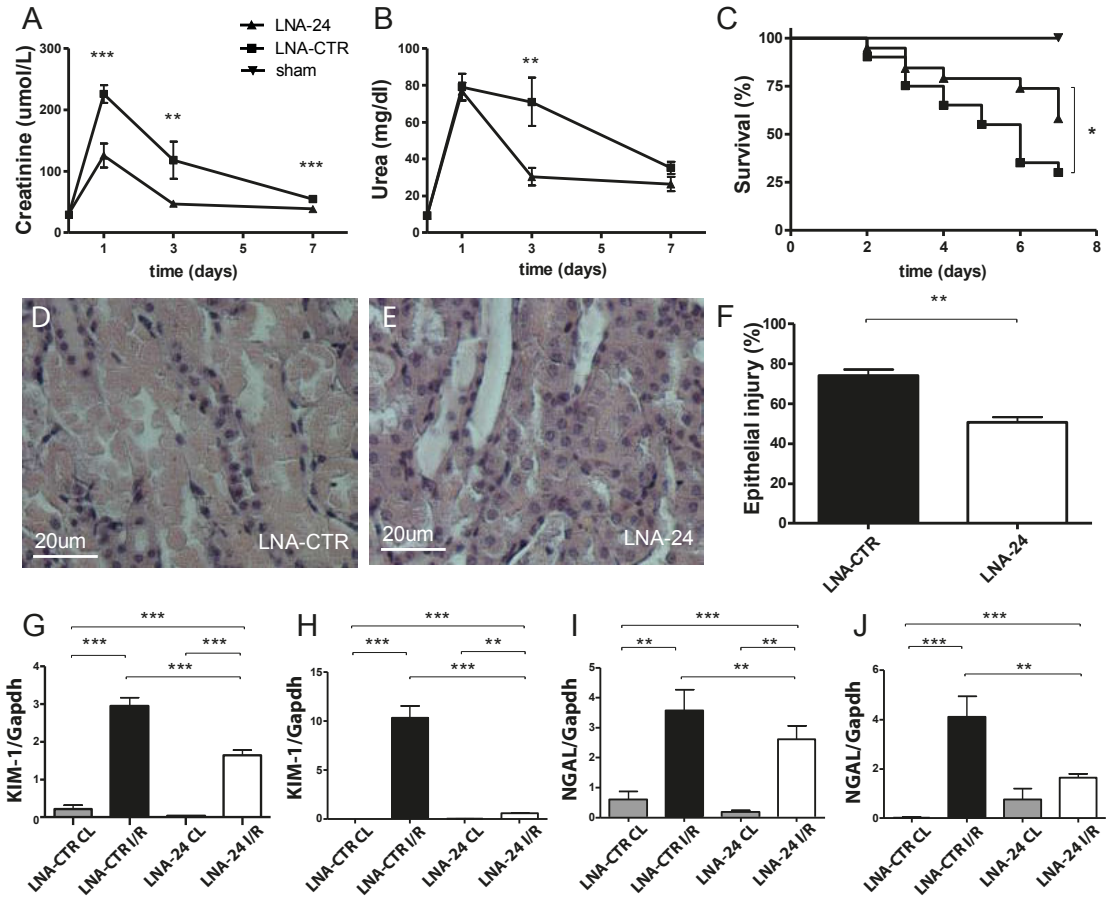


Figure 14. Protective rescue of renal I/R-injury following anti-miR-24 therapy. Renal function parameters (serum-creatinine (A) and –urea (B)) as well Kaplan Meier curve survival analysis (C) in mice treated with an LNA-modified antimiR targeting miR-24 (LNA-24) and a control LNA (LNA-CTR) 24 hours before induction of I/R-injury as well as sham operated animals. Bilateral renal I/R-injury was performed for 27 minutes. Observation period from day 0 - 7; n=20 per treatment group, n=4 in the sham group. Differences in urea levels at day 7 are underestimated due to loss of uremic mice in the control group. Histological degree of epithelial injury after ischemia and reperfusion for 24 hours in mice receiving LNA-CTR (D) and LNA-24 (E) and unilateral clamping of renal pedicles as well as quantification of results (F, n=7 each).

KIM-1 as well as NGAL mRNA levels in post-ischemic unilaterally clamped kidneys after 1 day (G, I) and 7 days (H, J) of reperfusion; LNA-CTR CL = contralateral kidney

of mice with LNA-CTR, LNA-CTR I/R = clamped kidney of mice with LNA-CTR, LNA-24 CL = contralateral kidney of mice with LNA-24, LNA-24 I/R = clamped kidney of mice with LNA-24; n=7 mice in each group and time point; * $p<0.05$, ** $p<0.01$, *** $p<0.0001$.

Kidney morphology, infiltration of immune cells, 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 14D – E) as well as bilateral I/R-injury (Figure 16D – E) and a reduction in epithelial injury in mice (Figure 14F for unilateral and Figure 16F for bilateral I/R-injury). Infiltration of CD45+-, F4/80+ macrophages, CD4+- and CD8+T-cells, Ly6g+-neutrophils significantly decreased following LNA-24 treatment as assessed by immunofluorescence at all investigated time points (Figure 17A – O, data at 7 days of reperfusion in unilateral I/R is shown). Tubular cell apoptosis as assessed by TUNEL staining was significantly lower in LNA-24 treated animals at 1 day of reperfusion (Figure 15A – C).

DOI:10.14753/SE.2015.1771

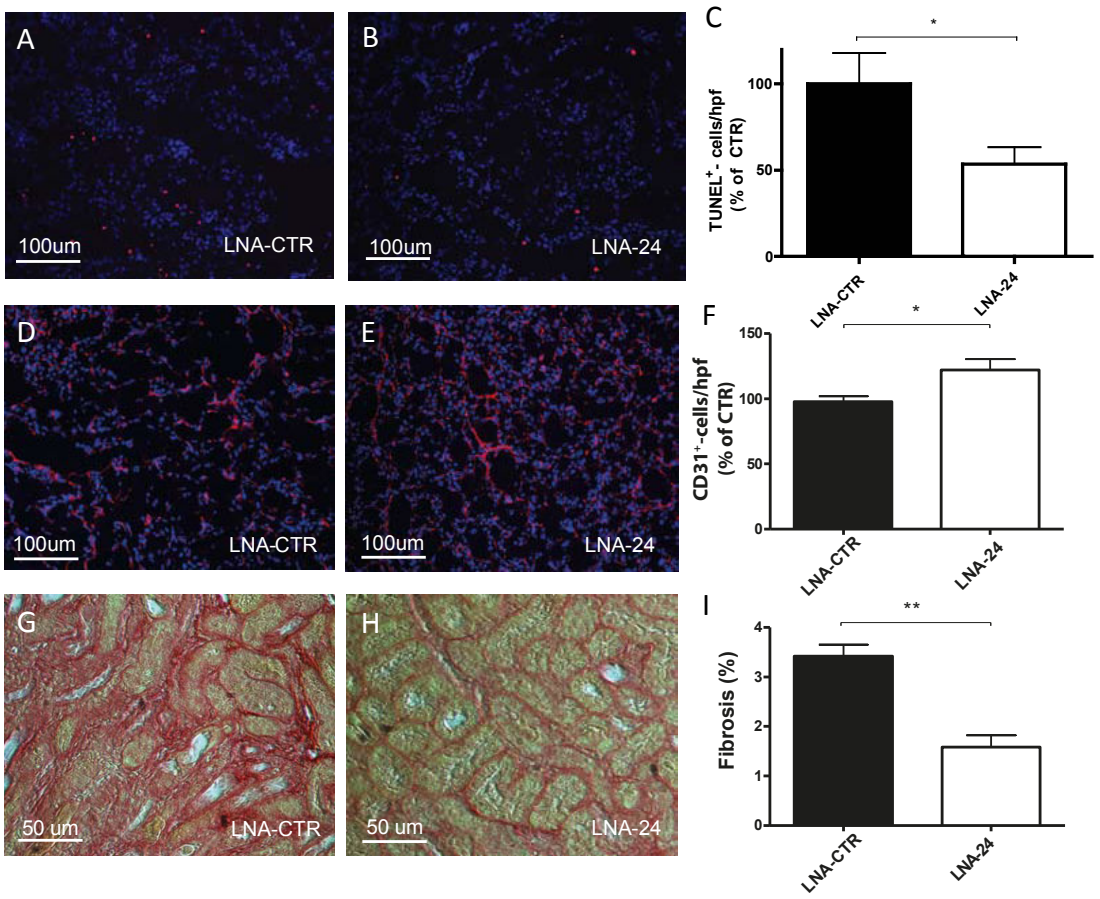


Figure 15. Apoptosis, endothelial cell activation and fibrosis in renal unilateral I/R injury following miR-24 LNA pretreatment. TUNEL+-cells in outer medulla in mice after unilateral ischemia and 1 day of reperfusion, treated with control LNA (LNA-CTR, A) and LNA-24 (B) and quantification of results (C). Capillary rarefaction (CD31-staining) analysis in mice treated with control LNA (LNA-CTR) (D) and LNA-24 (E) and quantification of results (F, n=7 per group) at reperfusion day 1 post clamping. Fibrosis development (sirius red-staining) analysis in mice treated with control LNA (LNA-CTR) (G) and LNA-24 (H) and quantification of results at reperfusion for 7 days (I, n=7 each). * $p<0.05$, ** $p<0.01$, *** $p<0.0001$

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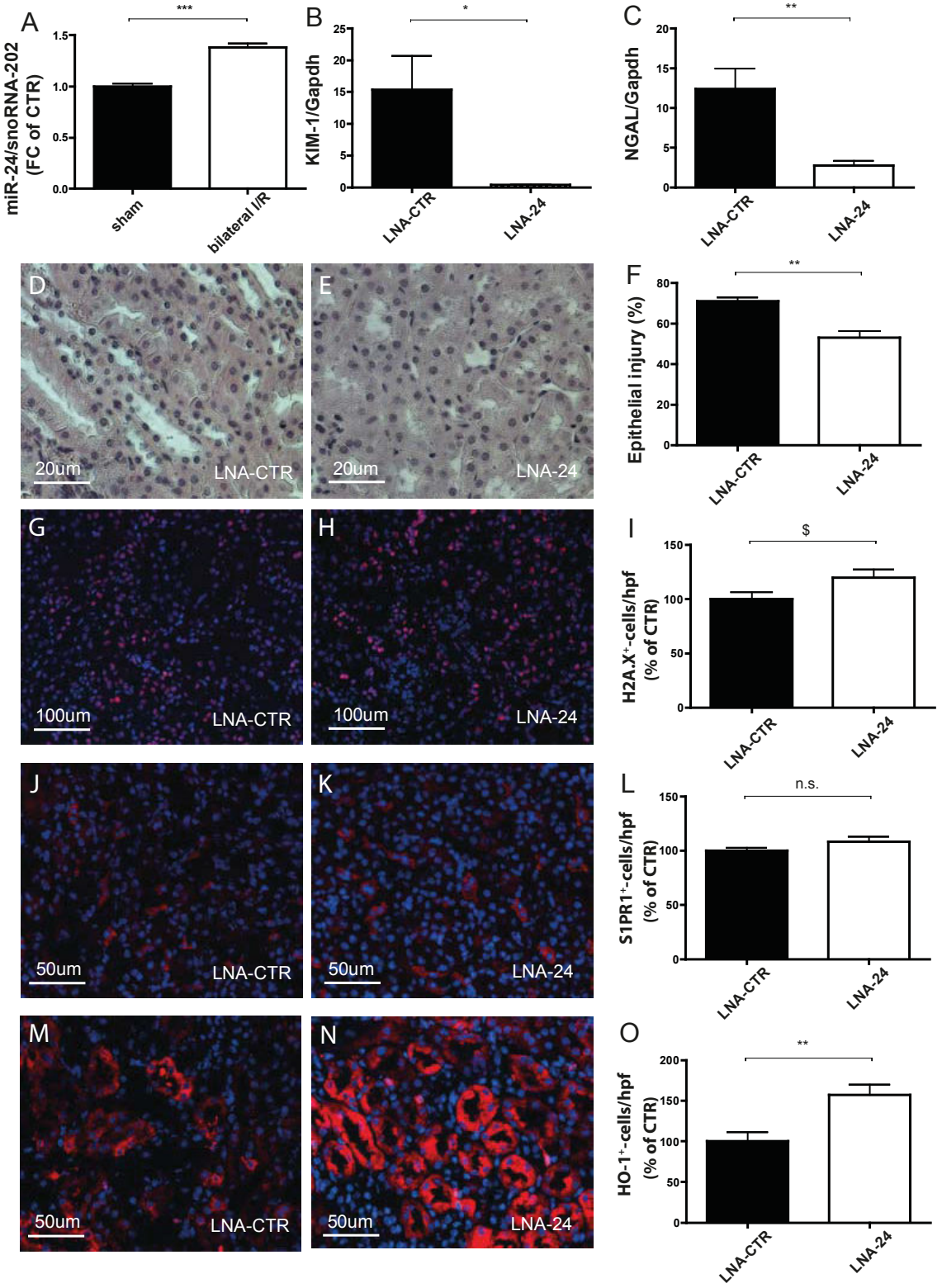

Figure 16. MiR-24 regulation in the bilateral renal I/R-injury model. MiR-24 is elevated in the kidney of mice after bilateral I/R-injury (27 minutes of bilateral ischemia and 24 hours of reperfusion) as compared to sham controls (A) (n=4 animals per group). MiR-24 antagonism is associated with a reduction 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). ***: $p<0.0001$, **: $p<0.01$, *: $p<0.05$, $: $p=0.08$.

Regulation of miR-24, survival, kidney function as well as markers of kidney damage and inflammation in bilateral I/R-injury in vivo

Treatment of mice with an LNA-modified antimiR targeting miR-24 (anit-miR-24 LNA) prior to induction of I/R-injury (24 hours) resulted in a significant improvement of survival as compared to control LNA treated animals (Figure 14C). This was accompanied by preserved kidney function (lower levels of serum-creatinine and –urea levels; Figure 14A and B). On day 7 after induction of bilateral I/R-injury levels of renal function parameters decreased further. Moreover, treatment of mice with an anit-miR-24 LNA in bilateral renal I/R-injury (Figure 16B – C) resulted in a marked reduction of kidney injury marker gene expression (NGAL and KIM-1). In addition, expression of inflammatory gene expression (interleukin 1 beta, interleukin 6, monocyte chemoattractant protein 1, macrophage inflammatory protein 2 alpha, tumor necrosis factor alpha) in bilateral I/R-injury was significantly lower in animals treated with LNA-modified antimiR-24 after I/R-injury (Figure 18D – H). In vivo, in a model of bilateral I/R-injury we found HO-1 to be significantly up-regulated in the outer medulla after anit-miR-24 LNA as compared to control LNA treated animals ($p<0.01$). H2A.X was also found to be up-regulated by miR-24 inhibition, though not to a statistically significant level ($p=0.08$). S1PR1 was not regulated in vivo (Figure 16G – I for H2A.X, Figure 16J – L for S1PR1 and Figure 16M – O for HO-1).

DOI:10.14753/SE.2015.1771

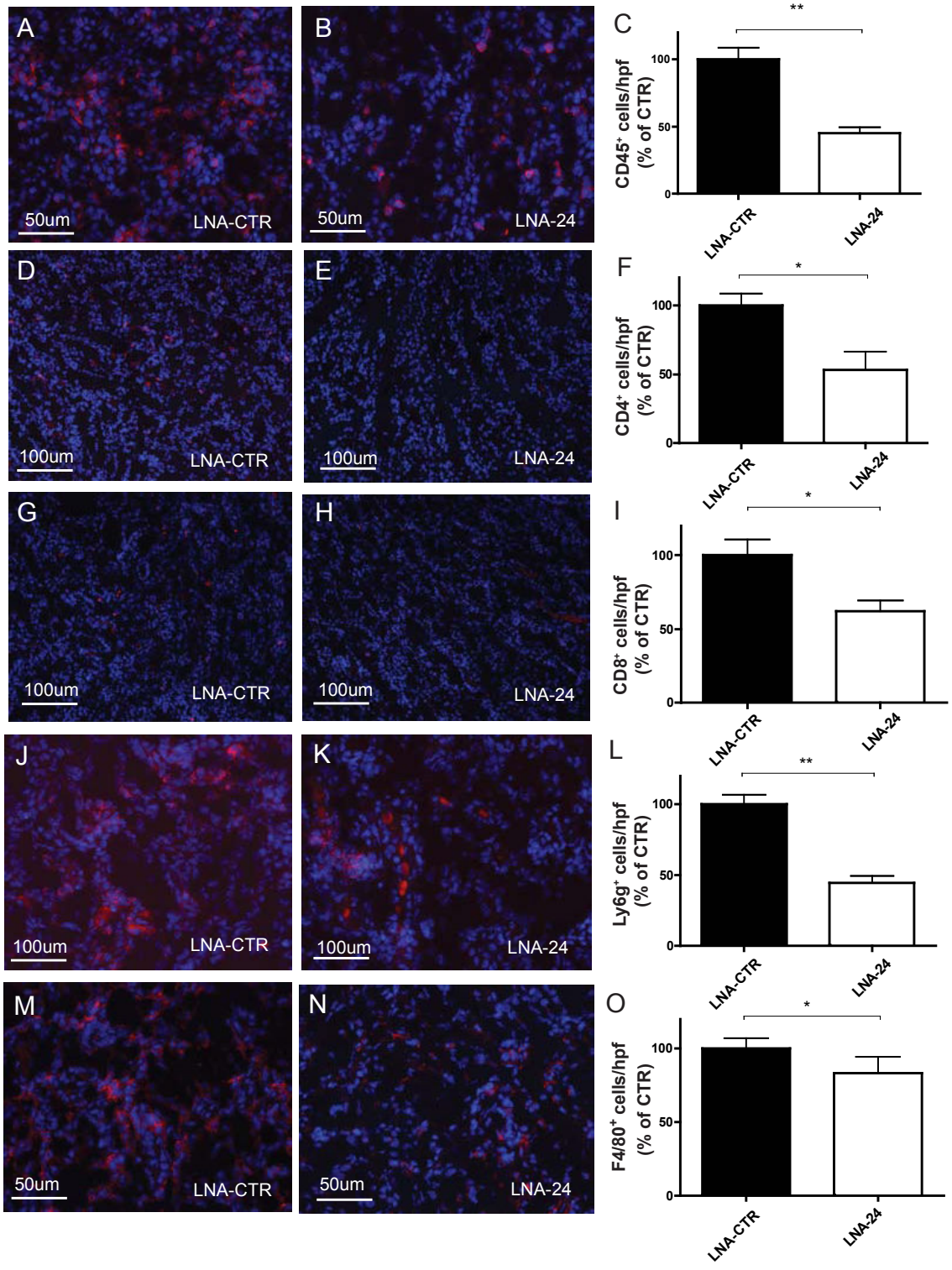

Figure 17. Immunofluorescence stainings as well as quantification of representative cryosections (4 μm) in outer medulla of mice treated with control LNA (LNA-CTR) and LNA-24 concerning CD45+- (A – C), CD4+- (D – F), CD8+- (G – I), Ly6g+- neutrophils (J – L) and F4/80+-macrophages (M – O) at reperfusion time of 7 days after unilateral

ischemia. Specific immunofluorescence stainings in red and Dapi in blue. **: p<0.01, *: p<0.05.

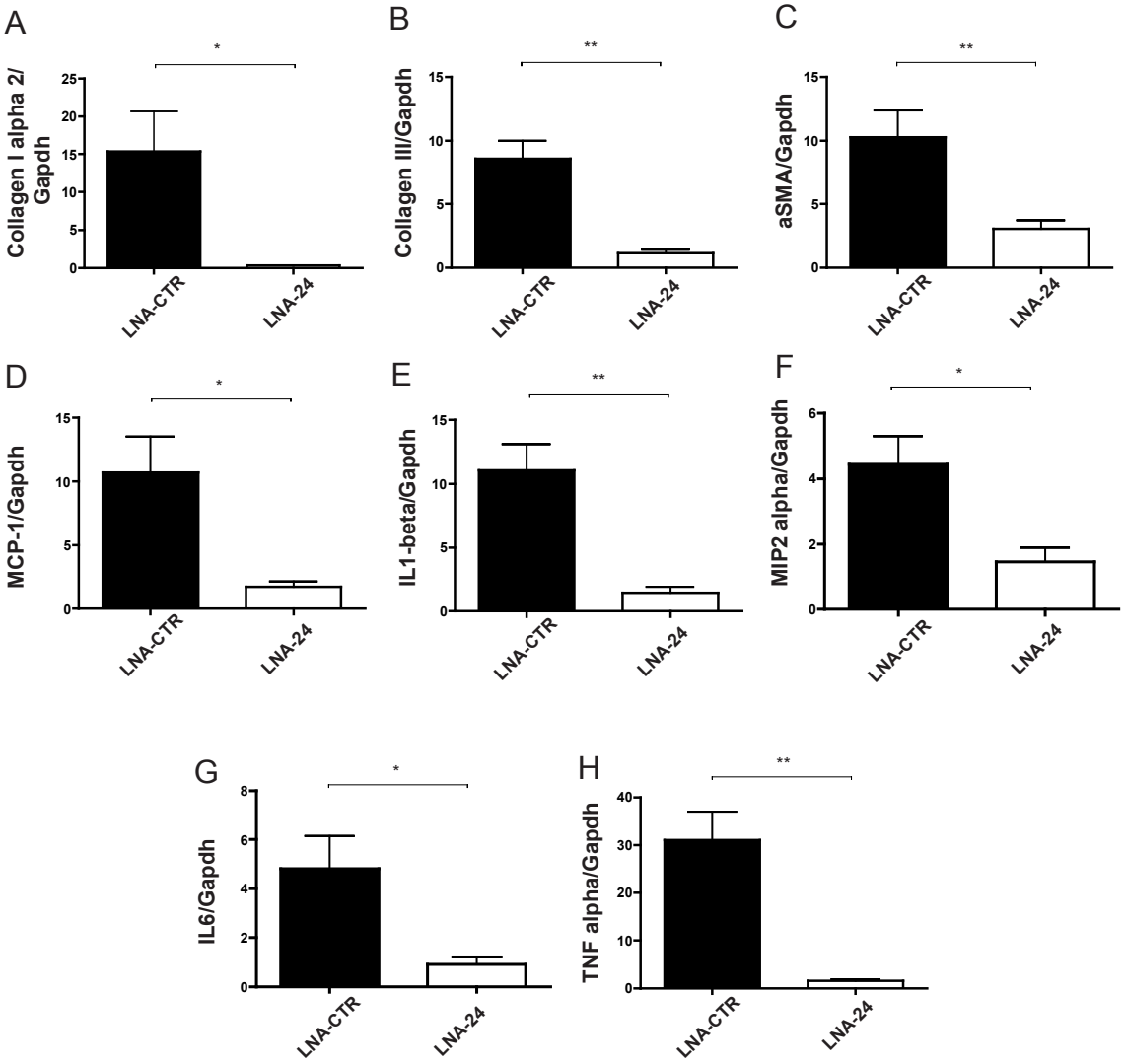

Figure 18. Fibrosis and inflammatory gene expression in bilateral I/R injury. Collagen I alpha 2 (A), Collagen III (B) and alpha smooth muscle actin (αSMA) (C) as well as MCP-1 (D), IL1beta (E), MIP2 alpha (F), IL-6 (G) and TNF alpha (H) is decreased in the kidney of mice treated with an LNA-modified antimiR targeting miR-24 after bilateral I/R-injury (27 minutes of bilateral ischemia and 1 day of reperfusion) as compared to control LNA treated mice (A) (n=4 animals per group). αSMA = 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.

MiR-24 in the progression from acute kidney injury to chronic kidney disease

At a reperfusion time of 7 days the level of developing fibrosis was highly attenuated in mice treated with an anit-miR-24 LNA (Figure 15G – I) compared to control LNA treated animals subjected to unilateral I/R-injury. Additionally, the expression of fibrosis-associated genes including Collagen I alpha 2, Collagen III, alpha SMA, was blunted in LNA-24 treated mice at all 3 investigated time points (Figure 19A – I for unilateral I/R and Figure 18A – C for bilateral I/R).

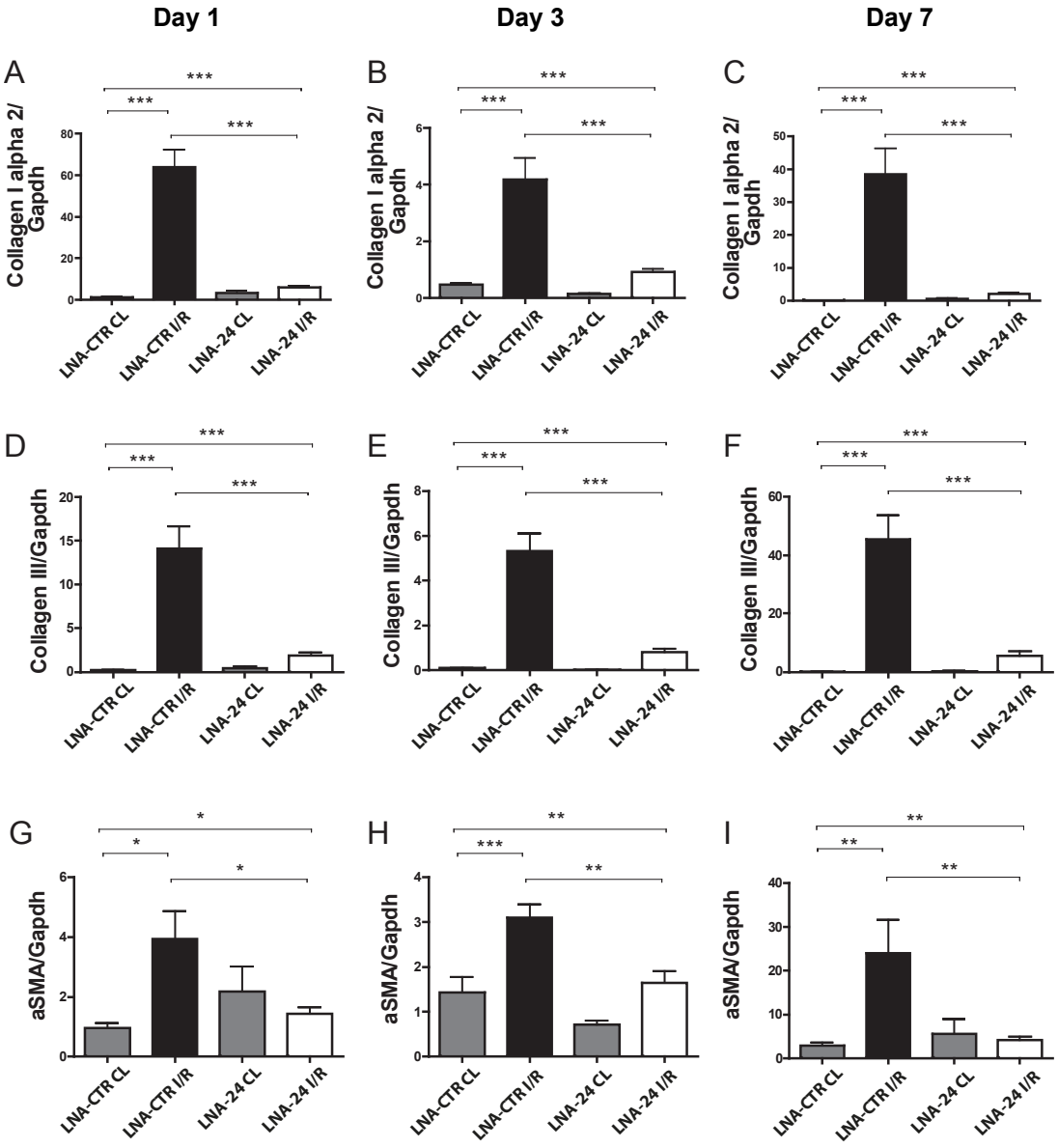

Figure 19. mRNA expression of Collagen I alpha 2 at 1 (A), 3 (B) and 7 days (C), mRNA expression of Collagen III at 1 (D), 3 (E) and 7 days (F) and mRNA expression of alpha

smooth muscle actin (αSMA) at 1 (G), 3 (H) and 7 days (I) in kidneys of mice. LNA-CTR CL = contralateral kidney of mice with LNA-CTR, LNA-CTR I/R = clamped kidney of mice with LNA-CTR, LNA-24 CL = contralateral kidney of mice with LNA-24, LNA-24 I/R = clamped kidney of mice with LNA-24; n=7 mice in each group and time point; ***: $p<0.0001$, **: $p<0.01$, *: $p<0.05$.

Discussion

The *miR-17 study* provides further evidence for a functional role of miRNAs in the recovery from renal I/R injury. We found for the first time that miR-17-5p and miR-106a expression increased in the I/R-induced acute kidney injury similarly to that of the previously described miR-21 (133), (137), (146), (134). Furthermore, elevation of miR-17-5p and miR-106a expression preceded that of miR-21. Based on the correlation between miR-21 and miR-17-5p we speculate that these miRNAs may be part of a regulatory network that can contribute to determine the outcome of I/R injury.

High BUN levels indicated that 30-min ischemia caused severe renal dysfunction one day after ischemia in C57BL/6 mice similarly to the results of Burne et al. (161). Histology indicated severe tubular damage mainly in the outer stripe of the outer medulla, where the S3 segment of the proximal tubule and the medullary thick ascending limb (mTAL) are located, which are both very sensitive to ischemia (162). In accordance with previous reports (163), tubular origin of renal dysfunction was also verified by NGAL, a sensitive marker of tubular epithelial damage (164), as whole kidney NGAL mRNA and plasma NGAL levels both increased by orders of magnitudes one day after ischemia. Similarly to previous studies by us (36), (149) and others (115), 30 min renal ischemia was lethal in C57BL/6 mice (165). To follow the time-course of miRNA expression during recovery from the ischemia-induced AKI, mice were subjected to 20 min ischemia leading to reversible injury (36, 149).

The sublethal, 20-min ischemia-induced acute renal failure was also severe. BUN, renal NGAL mRNA expression and plasma NGAL level significantly increased on the first day after ischemia though the changes were not as great as after 30 min of ischemia. Furthermore, massive necrosis was also present in the outer stripe of the outer medulla. Post-ischemic AKI has been divided into 3 phases: extension, maintenance and regeneration. The extension phase is characterized by continued ischemic injury. During the maintenance phase, both tubular cell death and regeneration occur simultaneously, while renal function is still at its nadir. As the kidney enters the recovery phase, cell recovery and proliferation balance shifts from cell death to regeneration. Regeneration is associated with improvement of renal function (105, 166). Our data support the above stages of AKI time-course. Histologically, tubular regeneration was observed from day 3. Less tubular necrosis, improvement of BUN and plasma NGAL demonstrated

functional and histological recovery on day 4 after ischemia similarly to previous studies (167, 168). Therefore, in our sublethal AKI model, the extension and maintenance phases took place during the first three days and the recovery phase was initiated on the third day of reperfusion (169). The changes in the above kidney damage markers became non-significant after 4 days of reperfusion, a period that covered the extension, maintenance and recovery phases of AKI.

Though miR-17-5p is encoded by the miR-17~92 cluster on the mouse chromosome 14, and miR-106a is encoded by the miR-106a~363 cluster on the X chromosome, miR-17-5p and miR-106 share the same seed sequence. Therefore they are both members of the miRNA-17 family (170). Since both miR-17-5p and 106a had a similar expression pattern after lethal ischemia, and miR-17-5p levels were higher, we focused on miR-17-5p. However, miR-106a also has functional relevance in I/R injury as miR-106a was upregulated in an experimental model of ischemic retinopathy in mice (171). One day after reperfusion, renal miR-17-5p was similarly over-expressed both after lethal and sublethal ischemia. The high renal miR-17-5p expression was present until the third day of reperfusion, i.e. miR-17-5p expression was upregulated during the maintenance phase. During the recovery phase on day four, the expression of miR-17-5p positively correlated with the renal expression and plasma levels of the tubular epithelial damage marker: NGAL. However, a causative relationship between miR-17-5p and NGAL expression could be established only by further studies.

Recently, the overexpression miR-17~92 cluster was associated with renal cyst formation and its inactivation lead to improved kidney function and survival in a mouse model of polycystic kidney disease (172). Expression of miR-17 family members may be activated by pro-inflammatory cytokines (173, 174) (175). MiR-17 influences immune cell function (176) and regulates lymphocyte (177), monocyte (178), (179) and B-cell development (180). These findings point out that the miR-17 family could be involved in the regulation of immune processes during I/R injury. Overexpression of miR-17-5p inhibits hypoxia-induced apoptosis (181) in both tumors and renal tubules (182), and in the heart (183). On the other hand, inhibition of miR-17-5p with antisense oligonucleotides induced apoptosis in lung cancer cells (184). A microarray study has demonstrated that cardiac miR-17 family members were over-expressed on the second post-ischemic day in a mouse model of myocardial infarction (127). These studies suggest

that miR-17-5p can be involved in the regulation of recovery not only from renal but from myocardial I/R injury as well.

We observed high miR-21 expression after both 20 and 30 min renal ischemia, although after sublethal (20 min) ischemia miR-21 expression started to increase later. The duration and severity of the maintenance phase is determined by the balance between cell survival and death (105). After sublethal I/R miR-21 overexpression was demonstrated mainly in the late maintenance and recovery phases, therefore miR-21 could have a role in determining the outcome of cell survival/death balance. Similarly to our results significant up-regulation of miR-21 was found in post-ischemic kidneys in mice *in vivo*, and in proliferating renal tubular epithelial cells, *in vitro* (133). However cell death following ischemia could not be prevented by miR-21 transfection in this latter study.

The correlation between miR-21 and miR-17-5p is an intriguing finding of this study. MiR-17-5p and miR-21 were differentially expressed over the course of functional recovery from the renal ischemic insult in our study. There were significant linear correlations between their renal expression on all days of reperfusion also in the sham-operated mice. Co-overexpression of these two miRNAs was also reported in malignant diseases (185), (186), (187), (188) and in the aging heart (189), though the correlation between them was not analyzed. We found not only that miR-21 and miR-17-5p expressions strongly correlated with each other after I/R and in the sham control group but the slopes of correlations increased from time-to-time in the maintenance phase of AKI. These results suggest that renal miR-21 and miR-17-5p expression may influence each other.

Research aimed to determine the changes in the expression of miRNAs in response to I/R injury can lead to the development of new diagnostic, prognostic or therapeutic tools. Both the miR-21 and miR-17 families are well conserved among species (4), (190), therefore it is reasonable to surmise that similar mechanisms operate in humans as well. There are already studies which propose miR-21 as a biomarker in AKI, as its serum and urine levels were altered by renal I/R (146). The miRNAs identified in our study (miR-17 family and miR-21) were upregulated only in the later phases of AKI, suggesting that they might be connected with the clinical outcome of AKI. Indeed, there are publications to suggest that urine and plasma levels of miR-21 are potential risk

markers for AKI progression associated with poor prognosis after cardiac surgery (147). Regarding the therapeutic possibilities, two recent papers demonstrated that miR-21 could be involved in the renoprotective effect of preconditioning (137),(138). Furthermore, chemically synthesized miR-21 oligonucleotide reduced infarct size, *in vivo*, in a murine (123) and rat (128) model of cardiac I/R, suggesting that miR-21 modulation may have therapeutic potential both in the heart and the kidney. Further studies are however indispensable to investigate the possible clinical implications of miR-17 family in ischemic AKI.

In the *miR-24 study* we found miR-24 to impact on tubular epithelial and endothelial 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 a number of apoptosis-associated genes to be deregulated after miR-24 modulation. In particular, miR-24 was found to target prominent anti-apoptotic proteins including S1PR1, H2A.X and HO-1 in vitro. Additionally, HO-1 and H2A.X were demonstrated to be prominent targets of miR-24 in vivo. Finally, silencing miR-24 in vivo ameliorated renal I/R-injury, infiltration of various immune cells and survival as well as kidney function in mice. Silencing miR-24 in vivo culminated in a repression of renal fibrosis following I/R-injury.

As mentioned in the introduction, the first I/R injury studies in the heart proposed an important role for miR-24 (118). Furthermore, it has been demonstrated that miR-24 was critically involved in endothelial apoptosis during cardiac I/R-injury as well as apoptosis of cancer and T cells (119), (120), (121). Therefore, we hypothesized that miR-24 could be also involved in the I/R injury of the kidneys.

The initial finding of our study, that miR-24 is highly enriched in kidneys of transplant patients with prolonged cold ischemia time 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 1 day of reperfusion through sorting of cells based on distinct surface receptors. Interestingly, at a reperfusion time of 7 days after I/R-injury miR-24 was also up-regulated 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 see a robust up-regulation of miR-24 in injured tubular epithelial cells at day 7 (LTA+/KIM-1+-cells).

In order to identify the downstream mechanism of miR-24-regulated protection we employed a global messenger-RNA array analysis, which revealed a number of 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 (191), (192), (193). 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 (192), (194). The sphingosine-1-phosphate receptor 1 (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 (191). In vivo, HO-1 was found to be significantly up-regulated by miR-24 antagonism, underscoring its in vivo significance as a downstream effector of miR-24. H2A.X showed a trend for regulation, while S1PR1 was not regulated. We thus propose HO-1 as the major factor in miR-24 mediated ischemic acute kidney injury.

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

DOI:10.14753/SE.2015.1771

Conclusions

The presented results demonstrated that miR-21, miR-17-5p, miR-106a and miR-24 are involved in the pathophysiologic processes of the I/R-induced AKI.

The *miR-17 study* showed that miR-17 upregulation occurred during the maintenance phase and was followed later by miR-21 upregulation, which in turn lasted until the recovery phase. The expression of these miRNAs correlated with each other, which finding should be further investigated to get a deeper understanding of a possible relationship between miR-21 and miR-17. The timing of miRNA up-regulations suggests that miR-17 and miR-21 could play a role in the recovery phase of the I/R-induced AKI. Considering the progresses made in miRNA research in the kidney (82) and miRNA based therapeutic approaches (195) miR-17-5p, miR-106a and miR-21 could represent novel targets in the treatment of the ischemia-induced AKI.

The *miR-24* study revealed that miR-24 contributes to renal I/R-injury by influencing tubular epithelial and endothelial apoptosis through regulation of anti-apoptotic HO-1 and H2A.X. MiR-24 has been also shown previously to have an anti-apoptotic function in cancer cells and cardiomyocytes (196), (197). However, here we provide clear evidence of the pro-apoptotic role of miR-24 in renal I/R-injury. Silencing miR-24 ameliorates the apoptotic response in vivo leading to suppressed tubular epithelial and endothelial apoptosis, which is associated in turn with enhanced capillary density and reduced tubulo-interstitial fibrosis and the potential of blunting the AKI (acute kidney injury) to CKD (chronic kidney disease) continuum. Of note, this is the first evidence demonstrating that pharmacological 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 transplant kidneys of patients with prolonged cold ischemia time, 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 of patients with AKI.

DOI:10.14753/SE.2015.1771

Summary

Micro RNAs (miRNAs) are a class of small, non-coding RNAs with the function of posttranscriptional gene expression regulation. MiRNAs may function in networks, forming a complex relationship with diseases. Ischemia-reperfusion (I/R) is the main cause of acute kidney injury (AKI). Our aim was to investigate the involvement of miRNAs in renal I/R injury and the potential benefit of a miRNA based therapeutic approach. First, renal microRNA (miRNA) expression profiles and the time course of changes in selected miRNA expressions after murine renal I/R were evaluated. The role of apoptosis-associated miR-24 in renal I/R-injury was further investigated.

On the first day after renal I/R miR-21, miR-17-5p, and miR-106a were elevated out of the 21 miRNAs successfully profiled on the Luminex multiplex assay. After 20-minute, sublethal I/R, renal miR-17-5p and miR-106a expressions were elevated on the first and second days of reperfusion, while miR-21 expression increased later and lasted longer. Renal miR-17-5p and miR-21 expressions correlated with each other. Furthermore, following murine I/R-injury and after human kidney transplantation miR-24 was also up-regulated in the kidney. Cell sorting experiments revealed a specific miR-24 enrichment in renal endothelial and tubular epithelial cells after I/R-induction. Enrichment of miR-24 induced apoptosis, in vitro. In vivo, silencing of miR-24 in mice following I/R-injury resulted in a significant improvement of survival and kidney function, a reduction of apoptosis, improved histological tubular epithelial injury and less infiltration of inflammatory cells. HO-1 and H2A.X were also found to be regulated by miR-24 in vivo.

Therefore, miR-24 inhibition is a promising future therapeutic option in the treatment of patients with ischemic acute kidney injury. Furthermore, the correlation between renal miR-17-5p and miR-21 expressions warrants further investigation of how they may influence each other and the outcome of renal ischemia-reperfusion injury.

DOI:10.14753/SE.2015.1771

Összefoglalás

A mikroRNS-ek (miRNS) a kis, nem-kódoló RNS-ek osztályába tartoznak és a poszt-transzkripcionális génexpresszió jelentős szabályozói. A miRNS alapú szabályozó-hálózatoknak fontos szerepe lehet a betegségek kialakulásában. Az iszkémia-reperfúzió (I/R) az akut vesekárosodás leggyakoribb oka. Célunk ezért a vese iszkémia-reperfúziós károsodásában részt vevő miRNS tanulmányozása és egy miRNS alapú terápia hatásának vizsgálata volt. Első lépésben a vese miRNS expressziós profilját illetve a jelentősebb miRNS-ek expressziójának időkinetikáját határoztuk meg. Ezt követően részletesebben megvizsgáltuk az apoptózis szabályzásáról már ismert miR-24 szerepét.

Egy nappal vese I/R után, a Luminex multiplexes módszerrel mérhető 21 miRNS közül a miR-21, miR-17-5p és miR-106a expressziós szintje növekedett szignifikánsan. Szublethális iszkémiát követően a miR-17-5p és miR-106a expresszió az első két napon, míg a miR-21 expresszió csak később, a vesefunkció javulásakor emelkedett meg. A vese miR-17-5p és miR-21 expressziója szignifikánsan korrelált egymással. Továbbá, egér vese-iszkémiát és humán vese-transzplantációt követően a miR-24 renális expressziója is fokozódott. Sejtszortírozással kimutattuk, hogy az iszkémia hatására a tubuláris és endothel sejt-frakciókban is emelkedett a miR-24 szintje. A miR-24 kezelés indukálta az apoptózist, in vitro. A miR-24 in vivo csendesítése egér vese I/R károsodásban javította a túlélést és a vesefunkciót, csökkentette az apoptózist, a tubuláris károsodást és a gyulladásos sejtek infiltrációját. A HO-1 és a H2A.X a miR-24 szabályozása alatt állt, in vivo.

Eredményeink értelmében a miR-24 egy ígéretes terápiás target lehet iszkémiás akut vesekárosodásban szenvedő betegek esetében. A miR-21 és miR-17-5p közötti korreláció további vizsgálatával megismerhetjük az egymásra kifejtett hatásukat, illetve hogy hogyan befolyásolják az vese iszkémia-reperfúzió károsodásának kimenetelét.

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DOI:10.14753/SE.2015.1771

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DOI:10.14753/SE.2015.1771

Acknowledgements

First I would like to thank my supervisor, **Dr. Péter Hamar** for his efforts in teaching and supporting me during my PhD studies, pointing out the importance of the *in vivo* experiments.

I am also grateful to all my colleagues in the Institute of Pathophysiology: **Prof. Dr. László Rosivall**, Head of the Basic Medicine Doctoral School for the opportunity to carry out my PhD studies; **Dr. Gábor Szénási** for his indispensable advices and for sharing me his broad research experience; **Mária Godó**, **Csaba Révész**, **Ágnes Cser**, **Dr. Csaba Szalay**, **Dr. Zsuzsanna Rácz** and **Csaba Bodor** for their kind help in the experiments and for teaching me numerous methods. **Dr. Gábor Kökény** for his valuable suggestions serving to the improvement of the present thesis.

Special thanks to **Prof. Dr. Thomas Thum**, **Dr. Johan Lorenzen** and **Dr. Sándor Bátkai** and all the members of the Institute of Molecular and Translational Therapeutic Strategies, Hannover Medical School for their support during my KAAD Scholarship and for the possibility to be involved in and learn from their innovative research.

I would also like to thank my previous supervisiors: **Dr. Ioana Berindan Neagoe** and **Dr. Maria Dronca** for initiating me in biomedical research and for supporting my application for PhD studies.

Many thanks to the community of the **ReNaissance House** (and to **Ulrich Kiss SJ**) for being my "second family" during my PhD studies in Budapest.

Nevertheless I am very grateful to my **parents**, my **sister** and my **grandparents** for trusting me and for the unconditional support and encouragement during my professional trainings.

Research in this thesis was supported by U.S. National Institutes of Health Research grant #R03 TW07069 (Fogarty International Center, National Institute of Diabetes and Digestive and Kidney Diseases); Hungarian Scientific Research Fund (OTKA: K81972, NF69278); Health Science Council (ETT: 011-07/2009); Else-Kröner-Fresenius Foundation (J.L. and T.T); Braukmann-Wittenberg-Herz-Stiftung and Deutsche Forschungsgemeinschaft (Cell Sorting Core Facility, Hannover Medical School). My research in Germany was supported by the scholarship of the Catholic Academic Exchange Service (KAAD).