Supplemental Materials and Methods

Statistical Analysis

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

LNA-modified miRNA oligonucleotides

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

Ischemic/Reperfusion injury protocol

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

Mice were dosed with intraperitoneal injections (i.p.) of a locked nucleic acid (LNA) targeting miR-24 (LNA-24) as well as control mismatch LNA at a concentration of 10 mg/kg 24 hours before the operation. Animal operation and survival analyses were performed at Phenos GmbH, Hanover, Germany. For survival analyses and measurement of renal function parameters mice were subjected to bilateral renal IR injury (20 mice received mismatch LNA, 20 mice received LNA targeting miR-24). For the survival analysis sham operated animals (n = 4) were also included. Blood samples for analysis of renal function parameters (serum-urea and –creatinine) were drawn on days 0, 1, 3 and 7 (analysed on a Beckman Analyzer, Beckman Instruments GmbH, Munich, Germany). In a second group of mice the renal pedicle was only clamped on the left side (unilateral I/R-injury). In this setting the

contralateral kidney serves as an internal control to the injured kidney (I/R-kidney). These animals were sacrificed on day 1, day 3 and day 7 after renal I/R-injury and kidneys were harvested for further examination. At each time point 7 mice were injected with LNA targeting miR-24 and 7 mice received mismatch LNA. In vivo studies conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Histology, Immunostaining, and TUNEL stainings

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

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

phenylindole)-positive tubular cells were counted in ten non-overlapping fields of outer medulla in each sample. Data are presented as a percent ratio of TUNEL-positive epithelial cells *versus* total DAPI-positive epithelial cells.

Cell Culture experiments

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

Cells were grown to 60% to 70% confluence and used for further downstream analyses. Cells were either subjected to hypoxia (0.1% O_2) or to ATP depletion (chemical anoxia) using glucose-free medium containing 10 µM Antimycin and 10 µM 2-Deoxy-D-glucose (both Sigma-Aldrich, St. Louis, MO) for 1 hour with subsequent ATP repletion using DMEM containing Glucose (4.5 mM) and 10%FCS for 30 minutes. As a positive control, cells were treated with 0.2 µM staurosporin (Sigma-Aldrich, St. Louis, MO) to induce apoptosis. Apoptosis was determined by TUNEL staining (Roche Applied Science, Mannheim, Germany) or by use of the Annexin-V-Fluos kit from Roche Diagnostics (Penzberg, Germany) according to the manufacturer's instructions using a fluorescent-activated cell sorter analysis (FACS) on a guava® easyCyte™ sorter (Millipore, Germany). All assays were done according to the manufacturers instructions. Cellular oxidative stress was assessed by measuring the cellular content of 8-OHdG DNA Damage by enzyme-linked immunosorbent assay according to the manufacturer's instructions (Cell Biolabs, Inc., USA).

Ex vivo cell purification/sorting

The cellular origin of miR-24 following induction of I/R-injury was investigated by fluorescence-associated cell sorting (FACS) analysis using specific antibodies following a protocol by Chau et al. with modifications [3]. Following clamping of the right renal pedicle for 27 minutes and a reperfusion period of 1, 3 and 7 days both kidneys were extracted, de-capsulated, homogenized, then incubated at 37ºC for 45 min with CollagenaseII (81 U/ml) in Hank's Balanced Salt Solution (HBSS, Gibco).

After filtration (70µm) cells were centrifuged and then re-suspended in 1 ml FACS buffer (Millipore) containing 1% BSA.

Cells were separated using the following specific antibodies or lectins: rat anti-mouse-CD31-PE (1:400 BD Pharmingen) for endothelial cells, Lotus teragonolobus lectin (1:200, DAKO) for proximal epithelium and anti-mouse Tim1-biotin (1:200, E Bioscience) followed by streptavidin-APC (1:1000, BD Pharmingen) for injured proximal epithelium. PDGF-Receptor beta^{+} pericytes were separated from kidneys following a protocol by Schrimpf et al [4]. Cells were incubated with rabbit anti-PDGF Receptor beta antibodies (Abcam) for 15 minutes on ice. After washing, cells were incubated with goat anti-rabbit IgG microbeads (Miltenyi Biotech) (15 minutes at 4°C) and resuspended and isolated by MACS magnetic bead separation. Subsequently, RNA was isolated by Trizol method. For detection of miRNAs in samples, TaqMan primer assays (Applied Biosystems) were applied. The small RNA molecule snoRNA-202 was amplified as a control.

Protein Analysis

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

Tube formation and Boyden chamber assay of HUVECs

HUVECs were cultured in EBM-2 culture medium with supplements including 10% FCS and antibiotics. For the tube formation assay HUVECs were harvested and 15,000 cells were seeded on top of Matrigel-coated chamber slides (BD). After 4–6 h, pictures were taken on a Nikon Ti 90 microscope (Germany). Migratory capacity was assessed in a modified Boyden chamber assay. After treatment, cells were

stained with DAPI and cultured in inlets (Falcon HTS Fluoro Blok insert, 8-µm pore size), which were placed in 24-well culture dishes containing EBM-2 (Clonetics) and 50 ng/ml vascular endothelial growth factor, and 100 ng/ml stromal cell-derived factor-1. Prior to use, the inserts were coated with Fibronectin. After 4-24 hours, migrated HUVECs were manually counted by fluorescence-based microscopic evaluation of the bottom side of the membrane.

Scratch wound healing assay of HK-2 cells

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

miRNA/RNA Isolation, miRNA/mRNA Reverse Transcription–Polymerase Chain Reaction, and Global Transcriptome Analysis

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

MicroRNA target prediction

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

Transfection Assays

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

Luciferase Reporter Assays

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

Viral Transduction

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

Supplemental References

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Supplemental Table 1: Primer pairs used for gene expression analysis

Gene symbol	Gene name	Primer pairs
NGAL	neutrophil gelatinase-associated lipocalin	Fwd: TGAAGGAACGTTTCACCCGCTTTG Rev: ACAGGAAAGATGGAGTGGCAGACA
KIM-1	kidney injury molecule 1	Fwd: AAACCAGAGATTCCCACACG Rev: GTCGTGGGTCTTCCTGTAGC
TNFα	Tumor necrosis factor alpha	Fwd: TACTGAACTTCGGGGTGATTGGTCC Rev: CAGCCTTGTCCCTTGAAGAGAACC
IL-1β	interleukin 1 beta	Fwd: AGGTCCACGGGAAAGACACAGG Rev: GGGCTGCTTCCAAACCTTTGAC
MIP2α	macrophage inflammatory protein 2-alpha	Fwd: CCAAGGGTTGACTTCAAGAAC Rev: AGCGAGGCACATCAGGTACG
MCP-1	monocyte chemoattractant protein 1	Fwd: GGCTCAGCCAGATGCAGTTA Rev: ACTACAGCTTCTTTGGGACA
IL-6	interleukin 6	Fwd: GAGAAAAGAGTTGTGCAATG Rev: ATTTTCAATAGGCAAATTTC
Col-I-α2	Collagen I alpha 2	Fwd: CAGAACATCACCTACCACTGCAA Rev: TTCAACATCGTTGGAACCCTG
Col III	Collagen III	Fwd: TGACTGTCCCACGTAAGCAC Rev: GAGGGCCATAGCTGAACTGA
αSMA	alpha smooth muscle actin	Fwd: ACTACTGCCGAGCGTGAGAT Rev: AAGGTAGACAGCGAAGCCAG

Fwd: forward; Rev: reverse

Supplemental Figure legends

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

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

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

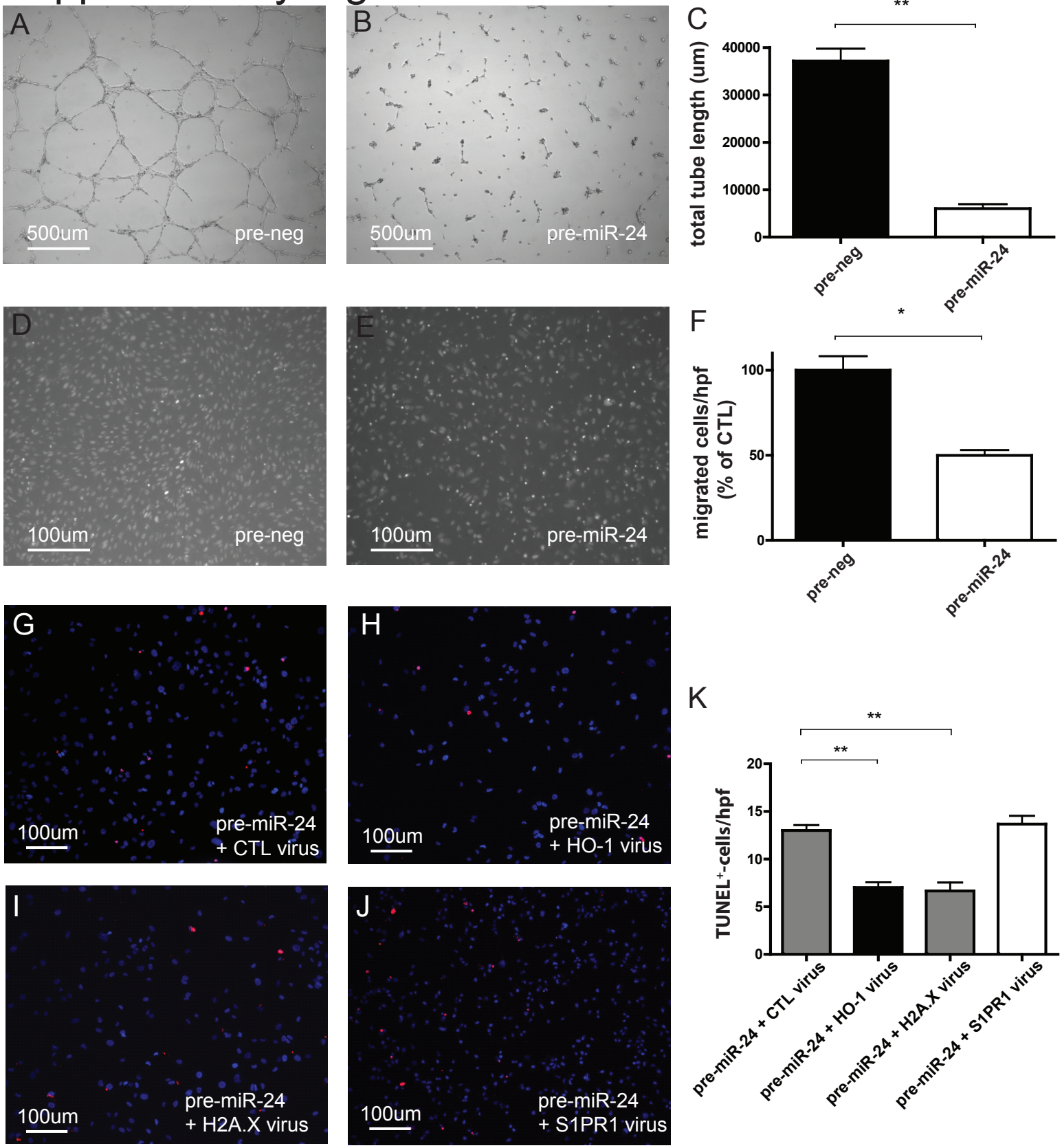
Supplemental Figure 4: mRNA expression of Collagen I alpha 2 at 24 (A), 72 (B) and 168 hours (C), mRNA expression of Collagen III at 24 (D), 72 (E) and 168 hours (F) and mRNA expression of alpha smooth muscle actin (aSMA) at 24 (G), 72 (H) and 168 hours (I) in kidneys of mice. CONT contra = contralateral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

Supplemental Figure 5: MiR-24 regulation in the bilateral renal I/R-injury model. MiR-24 is elevated in the kidney of mice after bilateral I/R-injury (27 minutes of bilateral ischemia and 24 hours of reperfusion) as compared to sham controls (**A**) (n=4 animals per group). MiR-24 antagonism is associated with a reduction in kidney

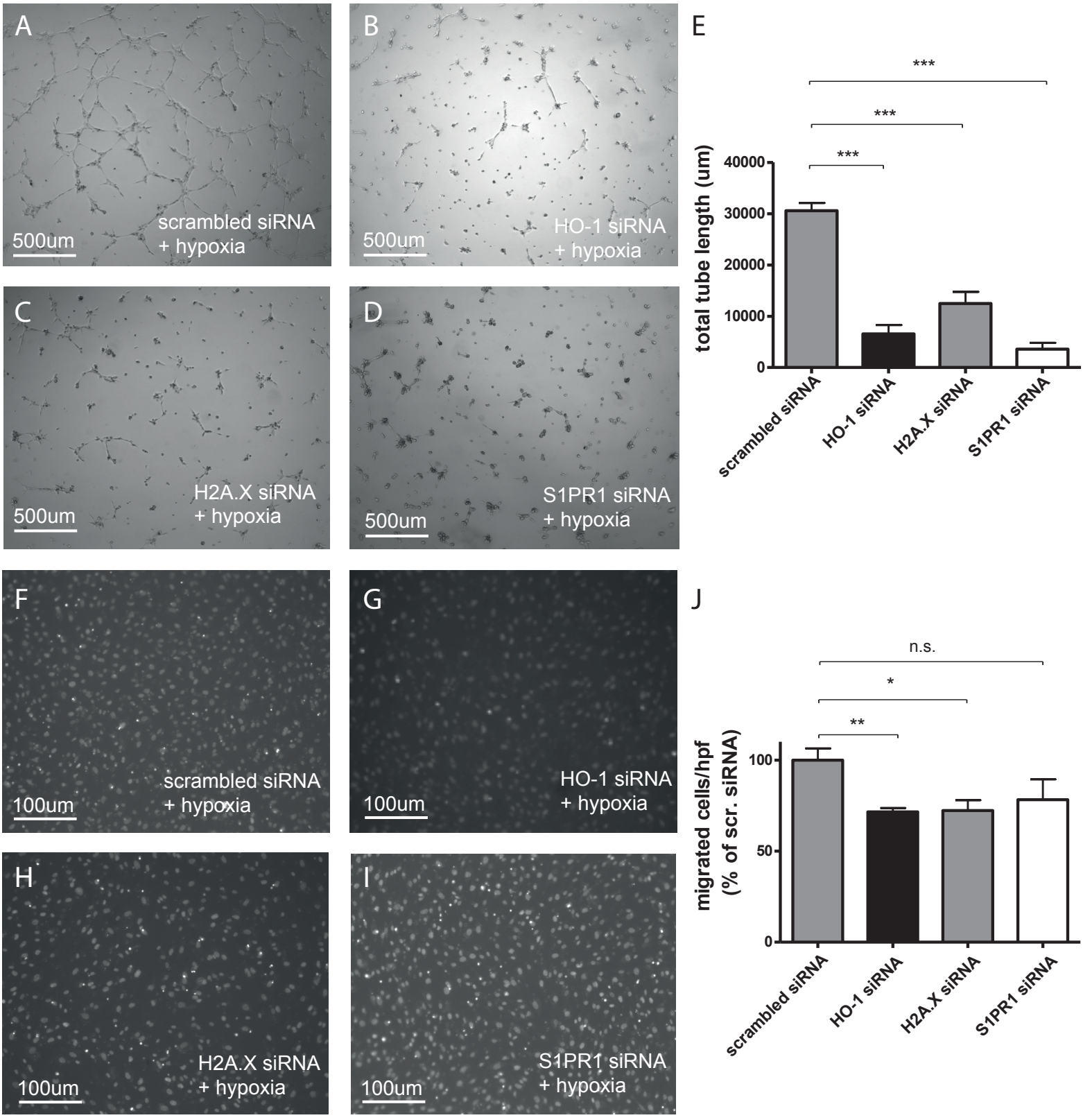
injury markers (KIM-1 and NGAL, **B – C**) as well as epithelial injury in the outer medulla (**D – F**). MiR-24 target regulation in vivo in bilateral I/R-injury, including H2A.X (**G – I**), S1PR1 (**J – L**) and HO-1 (**M – O**). ***: $p<0.0001$, **: $p<0.01$, *: $p<0.05$, $: $p=0.08$.

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

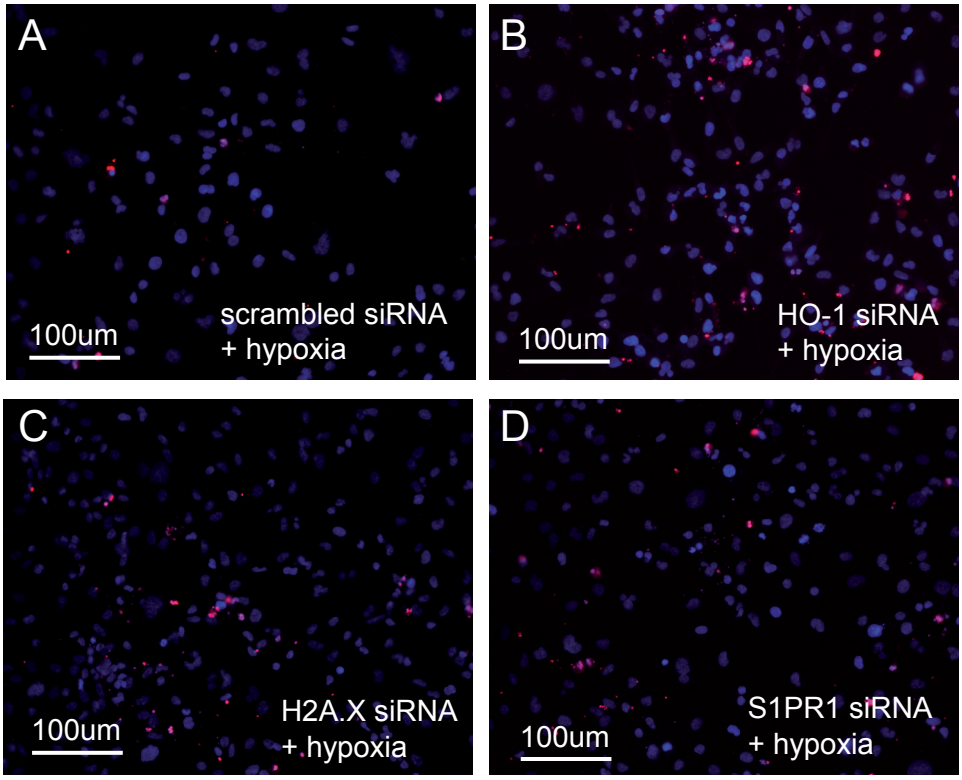
Supplementary Figure 1

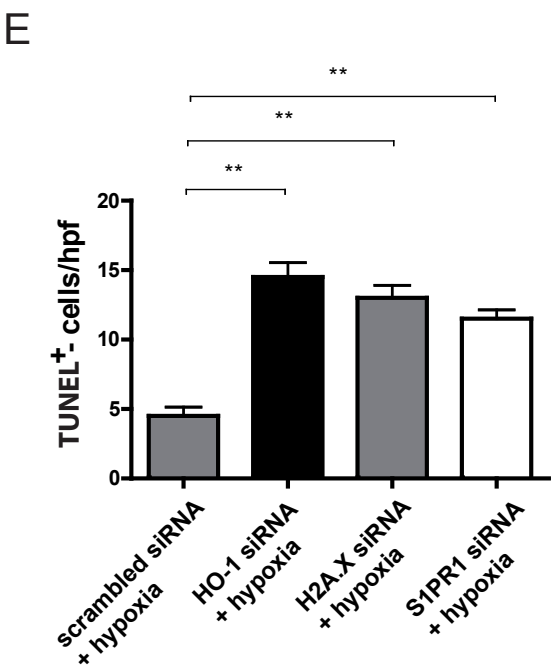


Supplementary Figure 2

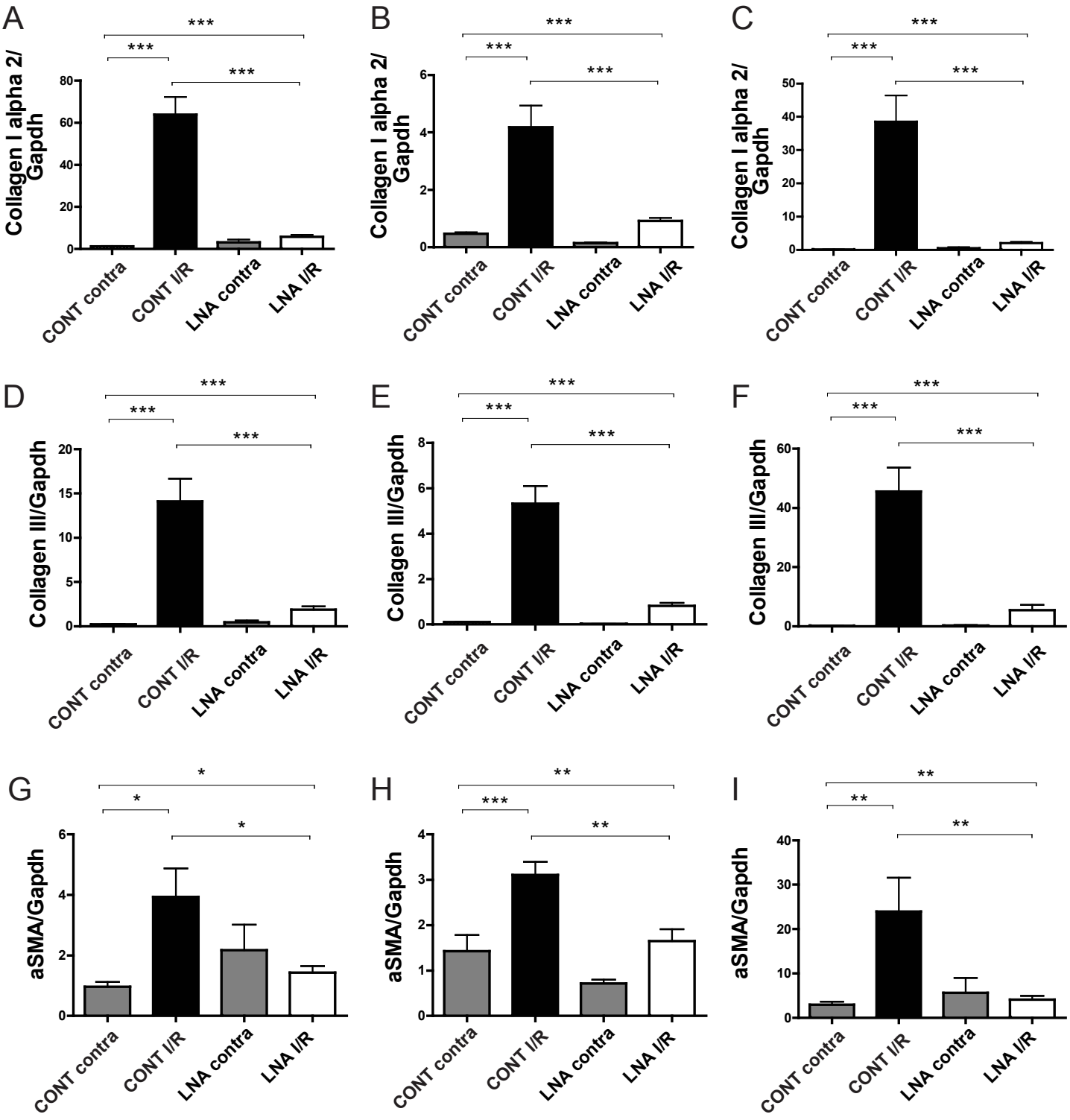


Supplementary Figure 3

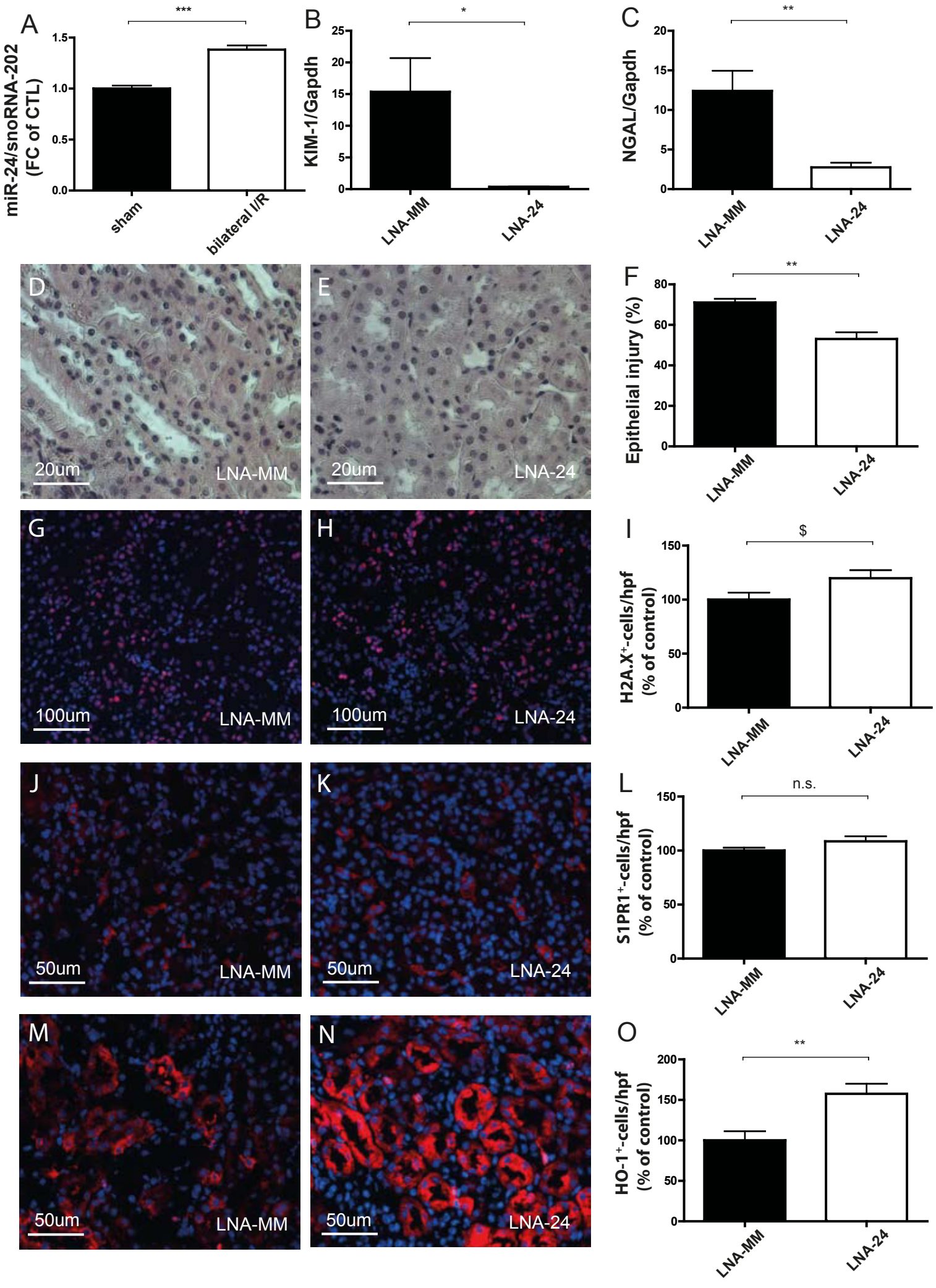




Supplementary Figure 4



Supplemental Figure 5



Supplemental Figure 6