Online Supplemental information

Methods

Sirius red for collagen staining. Sections (3 μm) from Duboscq-fixed paraffin-embedded kidney specimens were stained with Weigert's hematoxylin to show the nuclei followed by 0.1% picro-sirius red for 1 hour and washing with 0.5% acetic acid. The extent of interstitial collagen in the cortex was estimated using computer-based analysis. For each kidney section, 60 to 70 fields of cortical tissue were systematically digitized using a x20 objective, and digital images were processed with interactive tools (Image J, http://rsb.info.nih.gov/ij). Glomeruli were cropped off from the image. Sirius red-stained structures were identified based on hue, saturation and brightness (HSB) separation algorithm [1] by setting thresholds for the hue component leading to pixel selection in the red range. The area of the thresholded signal and the total area were automatically calculated. Data were expressed as V_v of tubulointerstitial collagen determined as the percent ratio of the tubulointerstitial area positive for Sirius red over the total cortical tubulointerstitial area.

In situ hybridization. The expression of individual miRNAs was confirmed by locked nucleic acid (LNA)-based in situ hybridization (ISH). 5'-Dig-labeled LNA probes with approximately 30% LNA base insertions (Exiqon) were used to detect mature miRNAs. ISH was performed on paraffin sections. Briefly, sections were deparaffinized and treated with proteinase K (10 μg/mL, Sigma-Aldrich, Milan, Italy) for 10 minutes. The hybrydization solution was composed of 2X standard sodium citrate (SSC), 10% dextran sulphate, 1X Dendhart's solution, 20 nmol/L Vanadyl Ribonucleoside Complex (Invitrogen, Life Technologies, Milan, Italy), 0.1 mol/L sodium phosphate. Hybridization was performed at 21°C below the Tm of the LNA probe overnight. Sections were blocked with blocking buffer (50 mg/mL skimmed dried milk in 150 mmol/L NaCl, 100 mmol/L Tris HCl, pH 7.8) and then incubated with anti-digoxigenin-alkaline phosphate Fab fragment (Roche Diagnostic GmbH, Mannheim, Germany) in blocking buffer for 45 minutes at 37°C. The sections were repeatedly washed in PBS and detection was performed using 1-step NBT/BCIP solution (Roche) according to manufacturer's instructions. Sections were then mounted in glycerol and viewed under light microscopy. U6 and scramble probes were used as positive and negative controls respectively.

Estimation of changes in the percentage of glomeruli with Bowman's capsules positive for miR324-3p was based on in situ staining. Seventy glomeruli on average per kidney section from untreated or lisinopril-treated MWF 60W were analyzed by the same observer, blinded to the nature of the experimental groups.

Analysis of Prep mRNA and Prep and α–SMA protein expression in miR-324-3p-transfected NRK-52E cells. Levels of the Prep mRNA in miR-324-3p-transfected NRK-52E cells were assessed by quantitative real time PCR. Briefly, cells were harvested in Trizol Reagent (Invitrogen) and total RNA was extracted according to the manufacturer's instructions. Total cDNA synthesis was achieved using the SuperScript™ II First Strand Synthesis System (Invitrogen). The transcript levels of rattus Prep (NM_002726) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were assessed with an ABI 7300 Real Time PCR System using SYBR GREEN PCR Master Mix (Applied Biosystems) with the following primers: rattus Prep, forward 5'–TAGCTCAGGTCCGCTCCCGGA–3', reverse 5'–AATCCTGTACGGCGGTCTCGTCG–3'; GAPDH, forward 5'–TCATCCCTGCATCCACTGGT–3', reverse 5'–CTGGGATGACCTTGCCCAC–3'. The ΔΔCt technique was used to calculate cDNA content in each sample using the cDNA expression in untransduced cells as calibrator.

For western blot analysis, cells were harvested in RIPA Buffer (20 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% TritonX-100, 0.1% SDS, 1% sodium deoxycholate, 1X protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined by BCA Protein Assay (Pierce Thermo Scientific, Rockford, IL, USA) and equal amounts of protein samples (5 μg) were subjected to SDS-PAGE on a 10% acrylamide gel and transblotted to polyvinylidene difluoride (PVDF) membrane and blocked overnight in 3% milk in TBS 1X (20 mmol/L Tris, 140 mmol/L NaCl, pH 7.6) at 4°C. Membranes were incubated with a primary antibody in 2% milk in TBS + 0.1% Tween for 2 hours at room temperature. Primary antibodies were as follows: rabbit anti-Prep (Ab58988, Abcam, 1:5000), mouse anti-actin (Sigma-Aldrich, 1:4000), mouse anti α-tubulin (T9026, Sigma, 1:500) and mouse anti-α Smooth Muscle Actin (A2547, Sigma, 1: 400). The signals were visualized using the corresponding secondary HRP-conjugated antibodies (goat anti-rabbit A6154, 1:10000; rabbit anti-mouse 1:5000 Sigma) and ECL Western blotting Detection Reagent (Pierce). Bands were quantified by densitometry using the Image J 1.40g software.

Immunohistochemistry. Duboscq-Brazil fixed, paraffin embedded renal sections (3 μm) were deparaffinized, hydrated and incubated for 30 minutes with 0.3% H_2O_2 in methanol to quench endogenous peroxidase. Tissues were treated with proteinase-K (20 μg/ml), for 10 minutes at 37°C. Antigen retrieval was performed by boiling sections using microwave (twice for 5 minutes in citrate buffer 10 mmol/L, pH 6.0 at operating frequency of 2450 MHz and 600W power output). Slides were incubated overnight with goat anti-Prep antibody (diluted 1:25, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with specie-specific biotinylated secondary antibodies and diaminobenzidine (DAB, Merck, Darmstadt, Germany) substrate solution, slides were counterstained with hematoxylin, dehydrated in graded alcohols, mounted with coverslips and observed by light microscopy.

The V_v of Prep-positive tubules was estimated by point counting according to Weibel [2] as follows: the entire field of the kidney section was digitized under x40 objective using Leica DM6000 motorized microscope (Leica Microsystems, Wetzlar, Germany) and digital images were processed with Gimp (Gimp, *http://www.gimp.org*). The medulla was cropped off leaving the cortical area of the whole kidney section on which an orthogonal grid of 400 $pixel^2$ area per point was digitally overlaid. The number of grid points hitting Prep-positive tubules and those hitting the whole kidney cortex were manually counted. Then, the V_v of Prep-positive tubules was expressed as percent ratio between the two measurements.

A semiquantitative analysis of Prep staining was assessed in both tubules and glomeruli by giving a score of 0 to 3, which considers both the intensity and the distribution of the signal (0, no staining; 1, weak; 2, moderate; 3, strong staining and diffusion). The final score per section was calculated as the weighted mean. An average of 80 glomeruli and 25 fields were examined for each animal. All renal sections were analyzed by the same observer, who was unaware of the nature of the experimental groups.

References

1. Foley JD, van Dam A, Feiner SK, Hughes JF: *Computer Graphics: Principles and Practice.* Reading, MA, Eddison-Wesley Publishing Co., 1990
2. Weibel ER: *Practical methods for biological morphometry. Stereological methods.* London, Academic Press, 1979

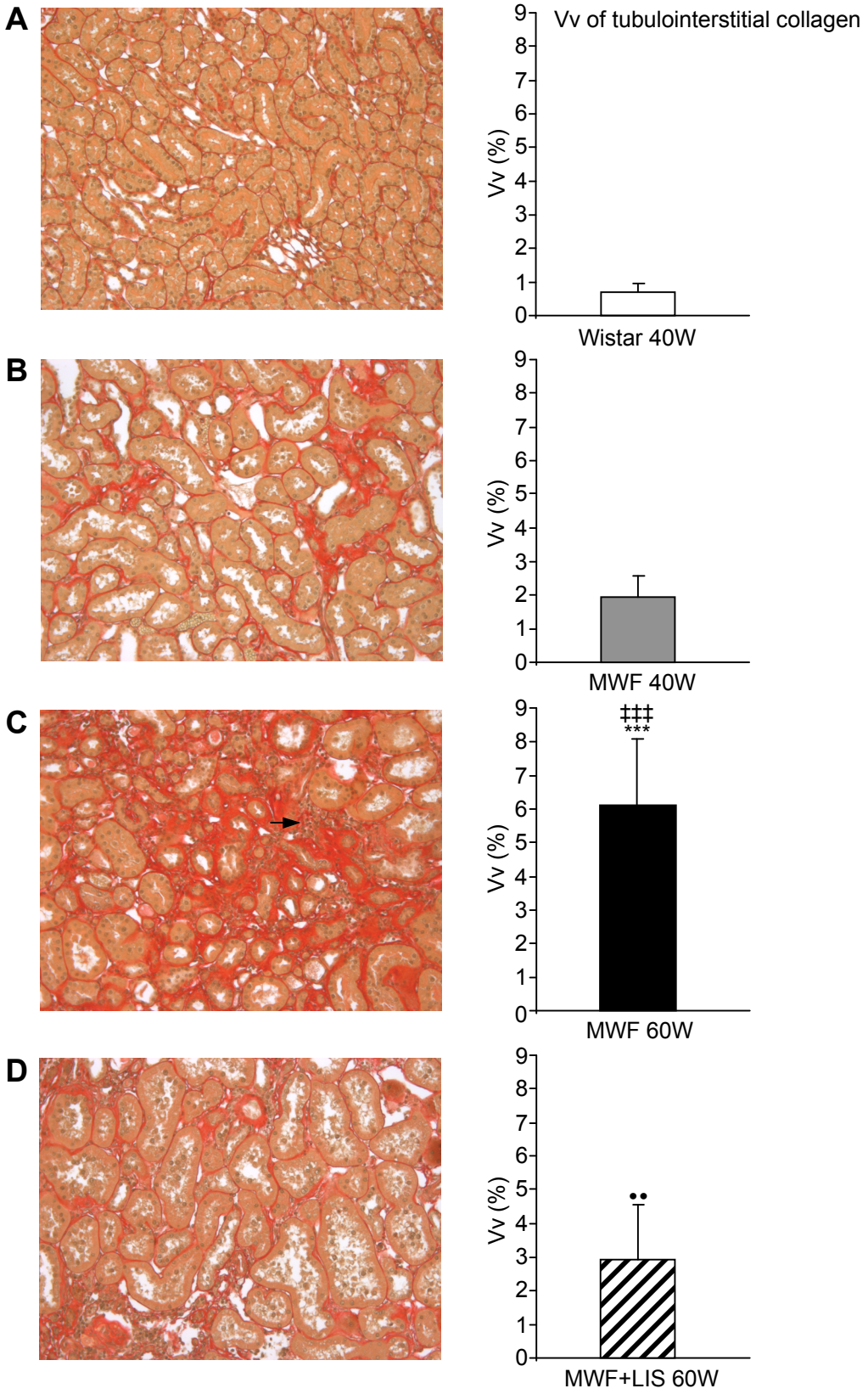

Figure S1. *Evaluation of tubulointerstitial collagen in Wistar rats and in untreated or lisinopril-treated MWF rats*. Sirius red staining for collagen markedly increased in the peritubular interstitium of MWF rats (B and C) as compared with control Wistar rats (A). (D) ACE inhibition significantly reduced interstitial collagen accumulation. The arrow in panel C marks an area of interstitial hypercellularity. Magnification, x200 (A-D). Graph bars are mean ± SD of V_v of tubulointerstitial collagen.***P < 0.001 *vs.* Wistar; ‡‡‡P < 0.001 *vs.* MWF 40W; ••P < 0.01 *vs.* MWF 60W.