SUPPLEMENT – DETAILED METHODS

Murine Model of Cisplatin-Induced Chronic Kidney Disease

Ten week old C57BL/6 mice (Harlan Sprague Dawley) were administered 2 doses of cisplatin (Aldrich 479306, 15 mg/kg IP in sterile saline) 2 weeks apart under brief isofluorane anesthesia. To alleviate the significant weight losses experienced after drug injection, saline was administered subcutaneously daily for 5 days (up to 10% body weight) following each dose. In an initial study, kidneys were harvested at 9 and 25 weeks, fixed in formalin and examined by light microscopy. In a subsequent study, GFR was measured by FITC-inulin clearance (8) under isoflurane anesthesia up to 9 weeks after the first injection of cisplatin. Urine and plasma were collected for protein, hematocrit, and creatinine determination (O'Brien Kidney Center at Yale; HPLC analysis). Non-perfused kidneys showed collapsed of proximal tubules and Bowman's space, hampering analysis of the patency of the glomerulo-tubular connections by MPM. As a result, analyzed kidneys were perfused briefly with saline to clear the kidney of blood and subsequently with neutral buffered 4% formaldehyde under manual pressure from a hand-held syringe for several minutes. Perfusion pressures were not controlle, but the detection of atubular glomeruli and the morphology of cuboidal capsule cells is unaffected by perfusion pressure variations. In addition, the relatively narrow distribution of glomerular volumes suggests that glomerular size was also relatively unaffected by perfusion pressure variations that occurred in these experiments. Kidney weight is of the fixative-filled perfused kidneys. The kidneys were divided at the mid-coronal point using a surgical blade. Portions from the mid-coronal aspect of the kidney half were paraffin embedded for routine tissue histological analysis including H&E, Sirius red, and trichrome. Two control and two treated specimens were

23 evaluated by electron microscopy, but significant differences were not noted on the limited
24 analysis.
25
26 *Immunohistochemistry/Immunofluorescence*
27 Wax-embedded histologic sections were deparaffinized, rehydrated, transferred into citrate
28 buffer, and either autoclaved or microwave treated. Sections were blocked with 3% peroxidase,
29 avidin, and biotin for 20 min each, washed, and incubated with primary antibody for 1 h at
30 room temperature. Anti-Ki67 was used for cell cycle activity, TUNEL for apoptosis, anti-alpha
31 smooth muscle actin for myofibroblasts, anti-F4/80 for macrophages, and anti-CD34 for
32 endothelial cells. A renal pathologist, masked to the identity of the study animal, reviewed
33 multiple sections from each kidney and evaluated for interstitial fibrosis and
34 immunohistochemical detection of expression. Tissue sections were scored using a previously
35 described square grid technique.[26-28] Briefly, ten independent fields on three sections each
36 were counted per kidney and the percentage of lesion area was calculated as percentage of
37 total squares counted.
38
39 *Tissue clearing*
40 One half of the perfused formalin-fixed mouse kidneys was further cut coronally into roughly 1
41 mm tissue sections with a razor blade. The sections were transferred into a methanol solution
42 containing 0.5% volume eosin Y, then stored overnight heated at 42°C. Subsequently the
43 samples were transferred to a 100% benzyl alcohol, benzyl benzoate solution in a 1:2 ratio
44 (BABB) for at least 2 hours before being imaged. Samples were placed in a glass chamber made

45 from a standard slide and covered with BABB during imaging.

46

47 *Multiphoton microscopy*

48 Image data was collected on a custom-built multiphoton microscope based on a Ti-Sapphire

49 laser (Spectra-Physics, Santa Clara, CA) with GaAsP photomultiplier tube detection

50 (Hamamatsu, Bridgewater, NJ) and Scanimage (Vidrio Technologies, Arlington, VA) software

51 control. Images were collected using a 0.95 N.A. 20X objective (Leica Microsystems, Wetzlar,

52 Germany). Excitation wavelength was 800 nm. Collection time varied depending on sample but

53 was typically 18-24 hours for a 1 mm thick coronal section at high resolution. SHG signal was

54 usually collected simultaneously in transmittance using a 400/30 nm bandpass filter and a

55 Hamamatsu bi-alkali photomultiplier tube. Every effort was made to maintain constant laser

56 power, geometry, and detector gain between samples. Nominal x-y resolution was

57 approximately 0.5 µm and images were collected at either 3, 1, or 0.5 µm steps. Higher

58 resolution sections were obtained at approximately 0.3 µm x-y resolution.

59

60 *Image processing and analysis*

61 Collected image stacks were processed and visualized using macros programmed on the FIJI

62 implementation of ImageJ.[29] The principal steps were image contrast normalization and

63 stitching of stacks, performed using standard FIJI plugins, including the Grid/Collection stitching

64 plugin.[30] Analysis required a desktop with at least 32Gb of RAM and generated stitched image

65 stacks that were typically greater than 6-10 Gb. Downsampling (4-6x) was performed for the

66 low power visualization of the largest stacks as well as for SHG analysis. The final SHG three

67 dimensional reconstructions were created using Imaris (Bitplane AG, Zurich, Switzerland).

68

69 Glomeruli and tubular connections in an image stack representing a fixed volume of kidney
70 were individually identified and tagged using Fiji. Glomerular tuft diameters were determined
71 by identifying the largest cross-sectional profile in the image stack and recording the average of
72 the maximum and minimum diameters. Glomerular volume distribution was determined by
73 manual tracing of glomerular outline through all sections, extraction of area, multiplication by
74 z-step size, and addition of volumes. Total analyzed renal volume was measured by applying a
75 very low pixel value threshold and multiplying slice areas by z-step size. This approach ensured
76 that large arcuate vessel lumens (diameter > 20 um) and tubule lumens were not counted in
77 the volume, thereby minimizing the bias of variations in perfusion pressure during fixation in
78 volume calculations. Single glomerulus/tubule 3D reconstructions were achieved by tracing
79 corresponding sections in consecutive stack slices and composing the tracings into volumes
80 using ImageJ's built-in 3D volume plugin. The comparisons of the cortical component areas
81 were done by manual segmentation and manual thresholding, ensuring that they are also
82 relatively insensitive to perfusion method since only the cellular component was included, i.e.
83 lumens and vascular spaces were not counted.

84

85 *Other quantitative calculations and statistics*

86 Glomerular density was determined by manually counting the number of glomeruli in measured
87 volumes. Total glomeruli per kidney were estimated by multiplying the total number of
88 glomeruli in the coronal slices by the fractional weight of the coronal slice relative the total

89 kidney weight. Statistics for GFR, creatinine, Ki67, immunohistochemistry counts for Ki67,

90 Tunel, CD34, SMA, and F4/80, cuboidal cell volumes, glomerular volume, glomerular diameter,

91 glomeruli per volume, and cross-sectional areas were based on two-tailed t-test and performed

92 on Excel (Microscoft, Redmond, OR) or Origin (OriginLab, Northampton, MA). Histograms, box

93 and whisker plots, and Gaussian data fits were done in Origin. Categorical comparisons for

94 glomerular capsule changes were done using Pearson's chi-square test with online resource[31].

95

96

97