RON kinase inhibition reduces renal endothelial injury in sickle cell disease mice

Alfia Khaibullina,[1] Elena A. Adjei,[1,2] Nowah Afangbedji,[1] Andrey Ivanov,[1] Namita Kumari,[1] Luis E.F. Almeida,[3] Zenaide M.N. Quezado,[3] Sergei Nekhai[1,4,5] and Marina Jerebtsova[5]

[1]Center for Sickle Cell Disease, College of Medicine, Howard University, Washington, DC; [2]Departments of Genetics and Human Genetics, College of Medicine, Howard University, Washington, DC; [3]Department of Perioperative Medicine, NIH Clinical Center, National Institutes of Health, Bethesda, MD; [4]Department of Medicine, College of Medicine, Howard University, Washington, DC and [5]Department of Microbiology, College of Medicine, Howard University, Washington, DC, USA

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Correspondence: marina.jerebtsova@howard.edu

Supplemental Methods

Mice

The animal protocol was approved by the Institutional Animal Care and Use Committee at the Children's National Health System. We examined kidneys from B6:129-*Hba*$^{tm1(HBA)Tow}$ *Hbb*$^{tm2(HBG1,HBB*)Tow}$ /*Hbb*$^{tm3(HBG1,HBB)Tow}$/J strain, here referred to as the Townes (SCD) mice (1, 2). Townes mice (homozygous HBSS, and controls, HBAA) do not express mouse hemoglobin. Homozygous Townes (hα/hα::β^S/β^S, HBSS) mice carry normal human α- (hα) and sickle hemoglobin beta (β^S) genes and express over 90% of human sickle hemoglobin. Control Townes (hα/hα::β^A/β^A) mice have hα and β^A genes and express normal human hemoglobin breeding pairs were obtained from the Jackson Laboratory (stock numbers 013071 Bar Harbor, ME) and bred in our animal facility. Genotypes for all animals were confirmed as previously described. Kidneys were collected from four-month old SCD and control mice (5 mice per group) and used for microscopic evaluation.

Immunohistochemistry

Paraffin embedded tissues sections were used for immunostaining with rat anti-mouse F4/80 (Cat# MCA497, AbD Serotec), rabbit anti-vWF (Cat# IR52761-2, Dako), rat anti- mouse ICAM (Cat# 116120, BioLegend), rat anti-mouse CD34 (Cat#CL8927AP, Cedarline), and mouse anti-MSP1 (Cat# MAB735, R&D Systems). AEC (red staining) and DAB (brown staining) kits were obtained from Vector Laboratory. PCNA kit was obtained from Invitrogen (Cat#93-1143). Images were acquired by Olympus 1x51 microscope with Olympus DP 72 camera. Quantification of positive staining was performed using ImageJ Fiji version (AEC and DAB staining) and CellSens Standard (Olympus, glomeruli and capillary size measurement) software.

Isolation of mouse renal glomeruli and glomeruli permeability assay

Mouse renal glomeruli were isolated from control mice using sieving technique (3). Glomerular permeability was measured by a determination of albumin permeability as described previously (4) with slight modification. Briefly, 50 μl of bovine serum albumin (4 g/dL BSA, isooncotic solution, Sigma-Aldrich) solution in PBS was added into 96-well plate with or without recombinant 1 μM MSP1 (R&D Systems) and 200 nM RONi (BMS-777607, Santa Cruz Scientific). Glomeruli were collected by glass pipette, placed in 96-well plate (10 glomeruli per well), incubated for 30 min at 37^0C in an isooncotic solution, and images were obtained for all glomeruli. Three wells (30 glomeruli) were used for each treatment. PBS (300 μl) was added to each well, and glomeruli were incubated at 37^0C for another 30 min in hypooncotic medium; then images were obtained. Images were used for area measurement for each glomeruli by CellSens software (Olympus) and glomerular volume was calculated. The glomerular volume change was shown as a percent of non-treated glomeruli volume.

Immunofluorescent staining and Western Blots

Rabbit MT-SP1 (Anti-Matriptase/MT-SP1, Cat# IM1014, Calbiochem) antibody was used for immunostaining and Western blot (WB) of THP1 cells. HGEC were treated with 1 μM of human recombinant MSP1 (R&D Systems, Cat# 352-MS) with or without RON inhibitor (200 nM) for 15, 30, 180 minutes and 24 hrs. WB analysis was performed with rabbit anti-p44/p42 MAPK (Erk1/2) (cat#4695), rabbit anti-phospho-p44/p42 Erk1/2 (Cat# 4377), rabbit anti-pan-Akt (Cat# 4685) and rabbit anti-phospho-Akt (Cat# 4660) antibodies (all from Cell Signaling Technology). Mouse anti-β-actin antibodies were from Sigma-Aldrich (Cat# A-1978). Phalloidin-FITC conjugate was from Sigma-Aldrich (Cat# P5282).

MTT assay for cell viability and growth

Cells were treated with 1 μM of MSP1 for 24 hrs. MTT solution (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, 250 μg/ml) was added for 2 h at 37^0C followed by DMSO, and optical density was read at 630 nm. Five 5 wells were analyzed for treatment and control in three independent experiments.

Meta analysis

Analysis of gene expression profiles was performed using Geo database NCBI Data Set GDS3203 and Data Set GDS2036.

Supplemental References:

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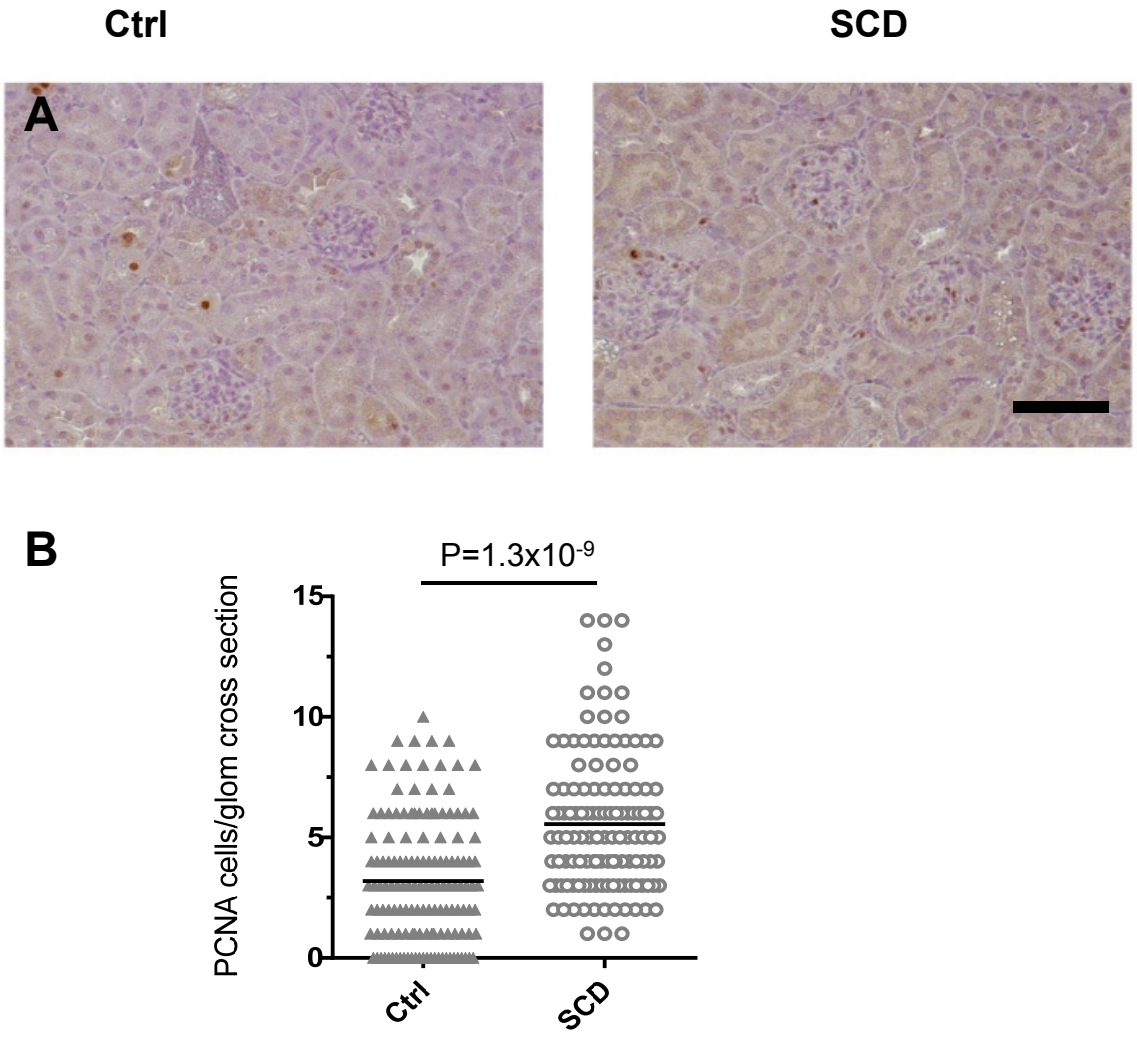
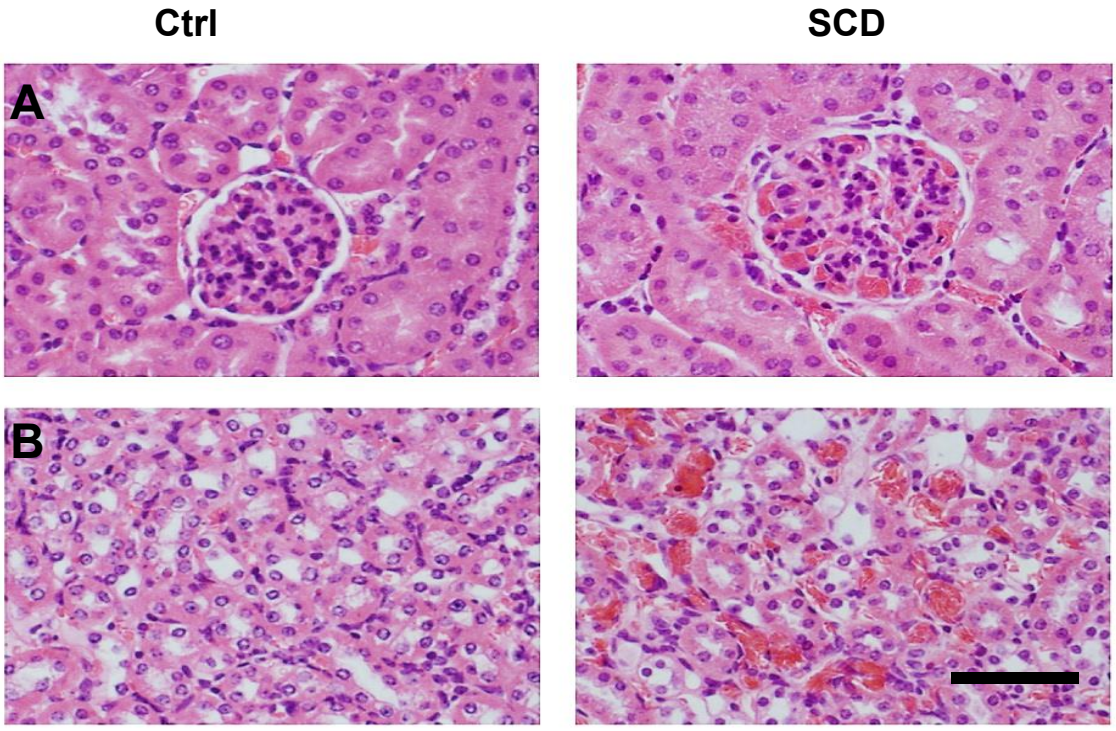
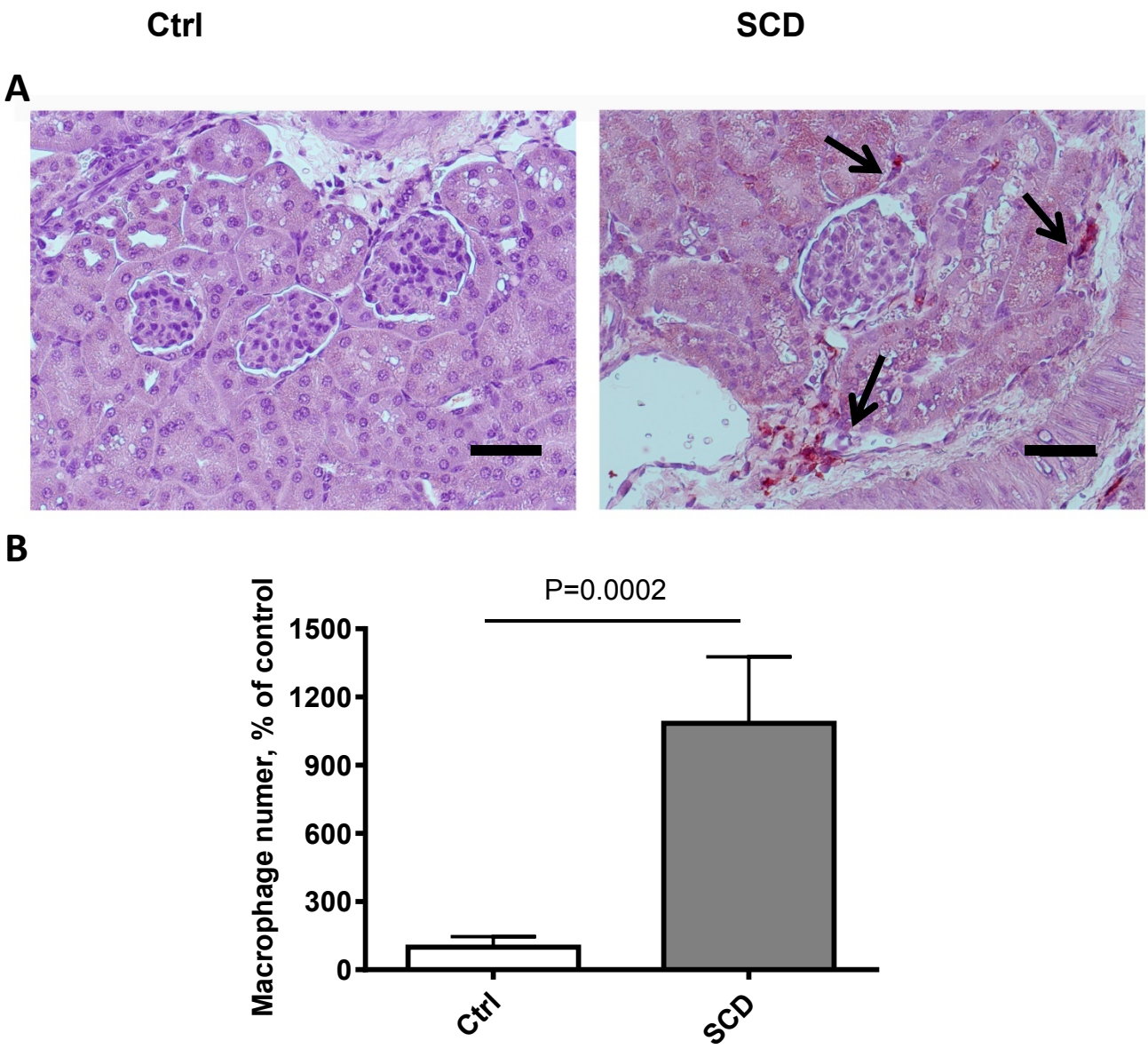


Supplemental Figure 1. *Proliferation of glomerular cells is increased in SCD mice.*

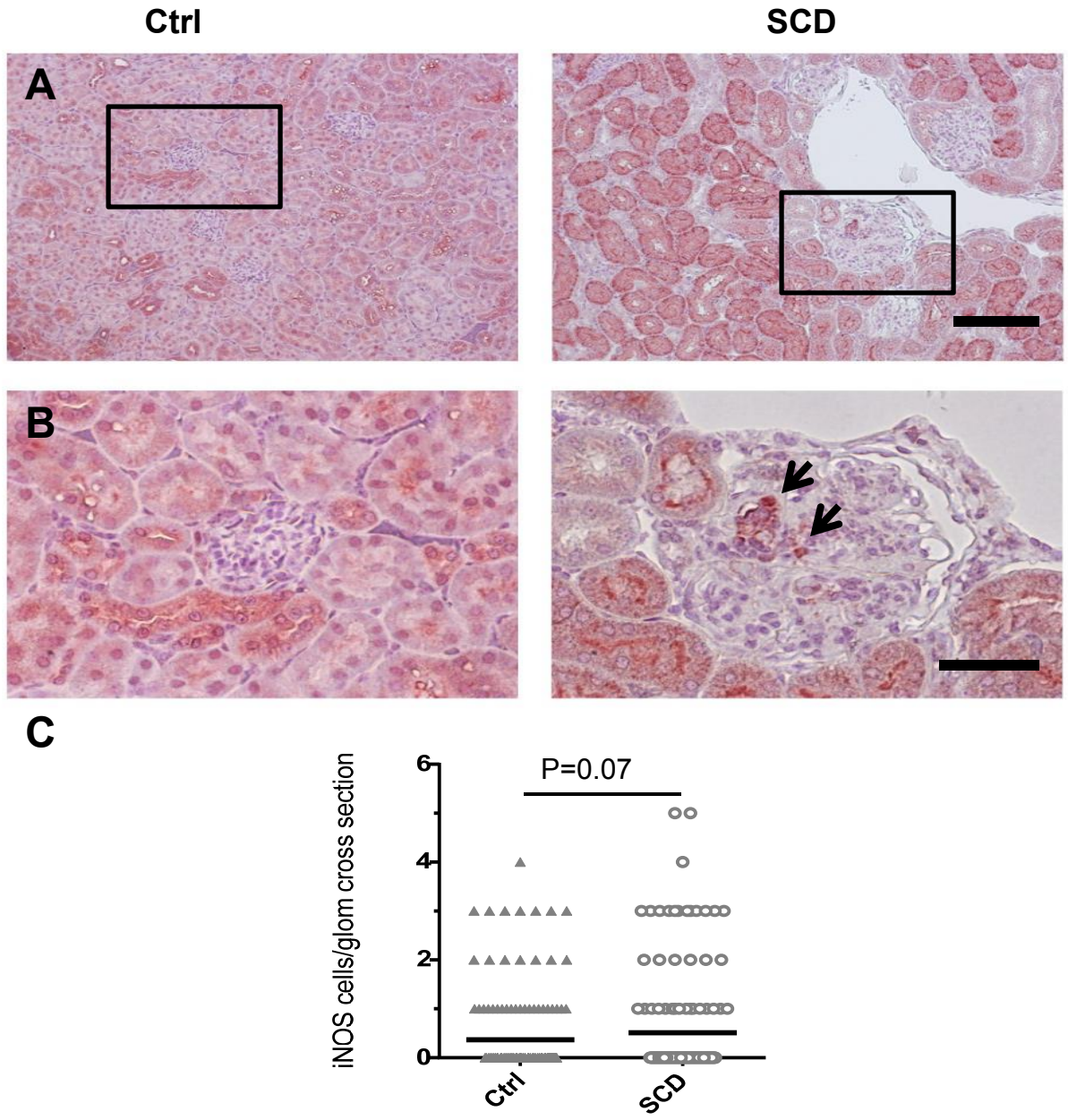
(A) Representative picture of PCNA staining of renal sections (brown nuclear staining). PCNA kit was obtained from Invitrogen (Cat#93-1143). (B) Quantification of PCNA expression in glomeruli is performed in 5 mice per group. Means are shown. Each dot represents a value obtained from one glomerulus cross-section. Bar size on the microphotographs is 80 µm..



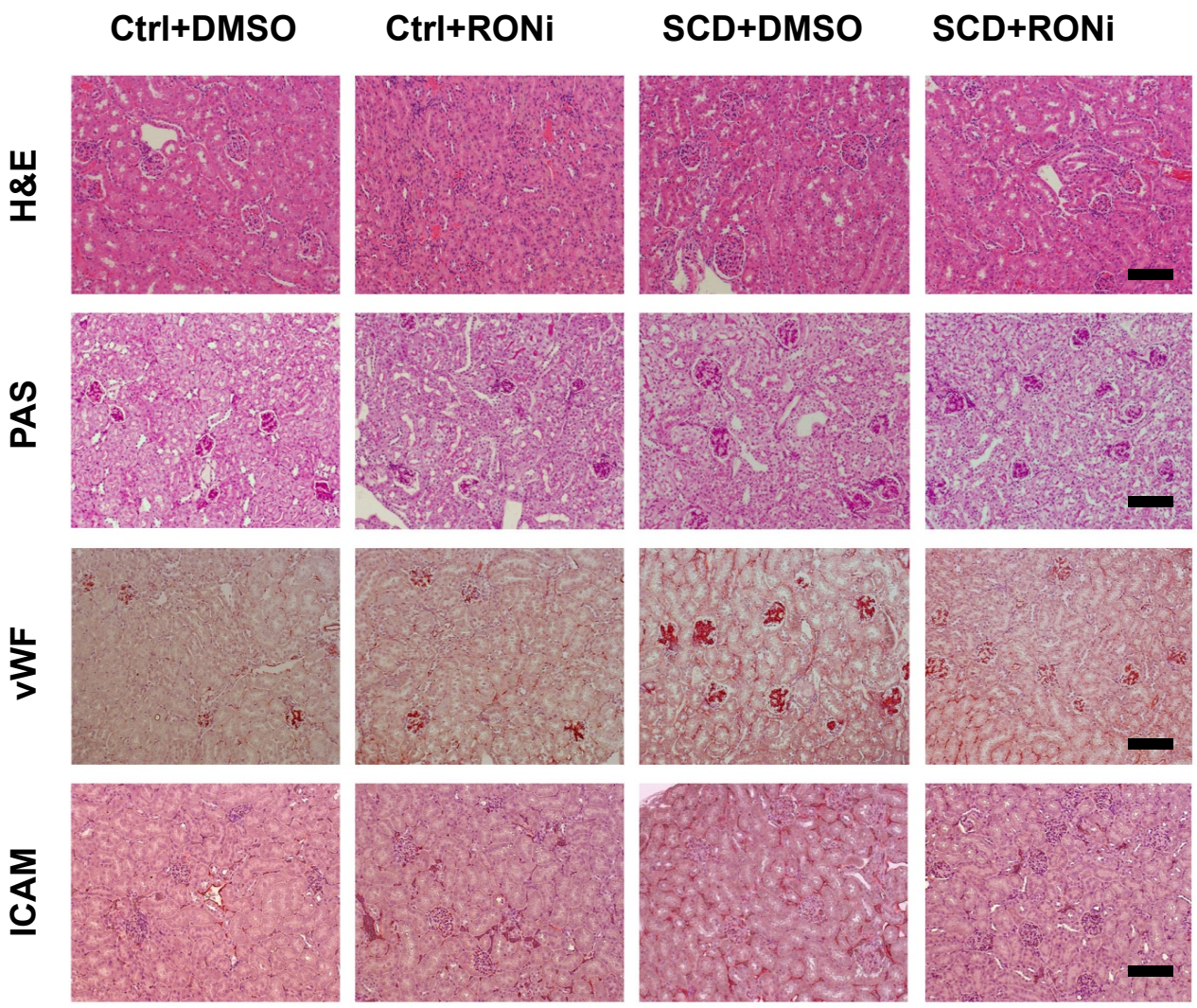
Supplemental Figure 2. *Renal capillary is markedly congested in SCD mice.*
(A-B) Representative pictures of glomerular, cortical peritubular capillaries (A) and papilla capillaries (B) H&E staining. Bar size is 40 μm.



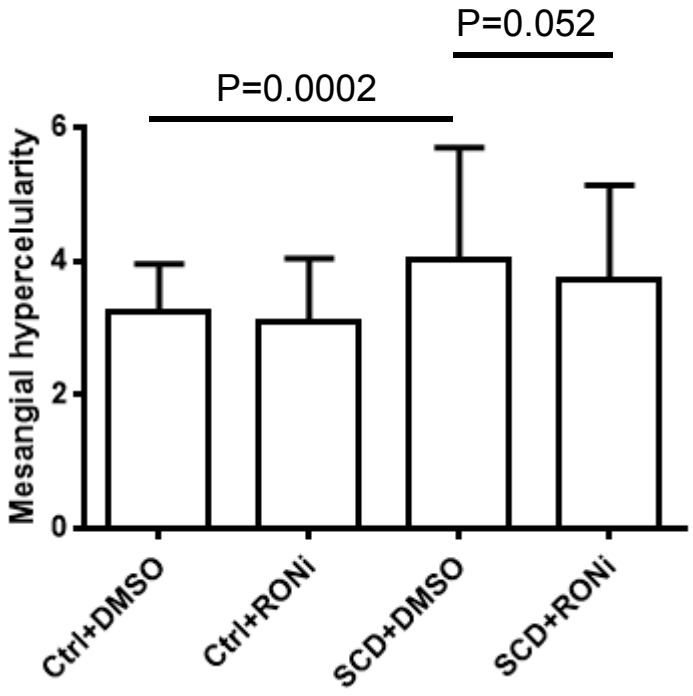
Supplemental Figure 3. *Renal interstitial macrophage infiltration is increased in SCD mice.*
(A) Representative picture of renal interstitial macrophages (F4/80) immunostaining (red color, arrows). Bar size is 120 µm. (B) Quantification of macrophages infiltration (5 mice per group). Means and standard deviations are shown.



Supplemental Figure 4. *Low levels of iNOS expression in the infiltrating renal macrophage in SCD mice.*
(A) Representative picture of macrophage immunostaining (red color, arrow). Squares show enlarged area (B). (C) Quantification of iNOS positive macrophages per glomeruli cross section is shown. Five mice per group were used for each staining. Means are shown. Each dot represents a value obtained from one glomerulus cross-section. Bar sizes on the microphotographs are 120 μm (A)) and 40 μm (B).



Supplemental Figure 5. *Treatment of SCD mice with RON inhibitor ameliorates endothelial injury.*
Representative picture of renal sections of control and SCD mice treated with either RONi or vehicle. (A) H&E staining. (B) PAS staining.(C) von Willebrand factor (vWF) immunostaining (red color). (C) Intercellular Adhesion Molecule (ICAM) immunostaining (red color). Bar sizes are 120 µm.



Supplemental Figure 6. *Treatment of SCD mice with RON inhibitor does not reduce mesangial hyper-cellularity.*
Quantification of mesangial cell number per glomerular capillary loop cross-section (PAS staining). Five mice per group were used for quantification. Means and SD are shown.