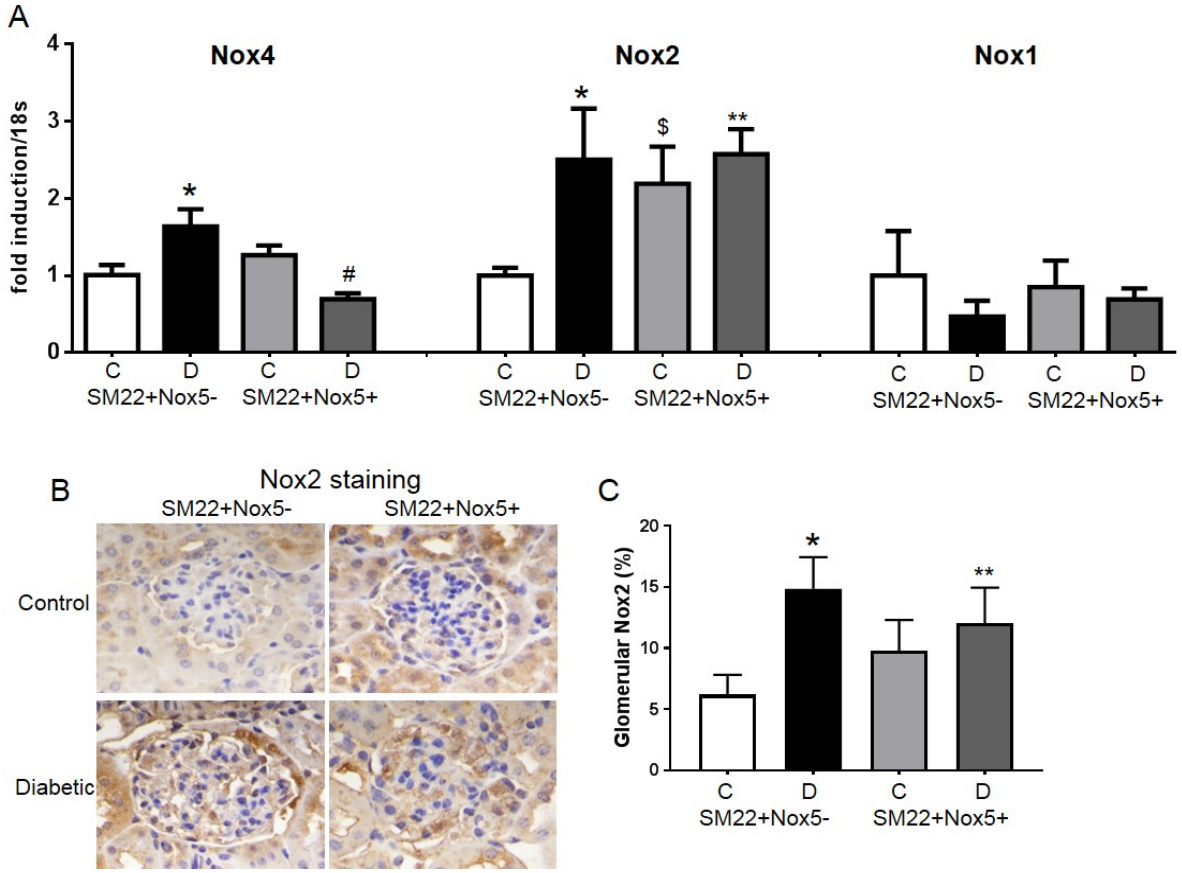
SUPPLEMENTARY DATA

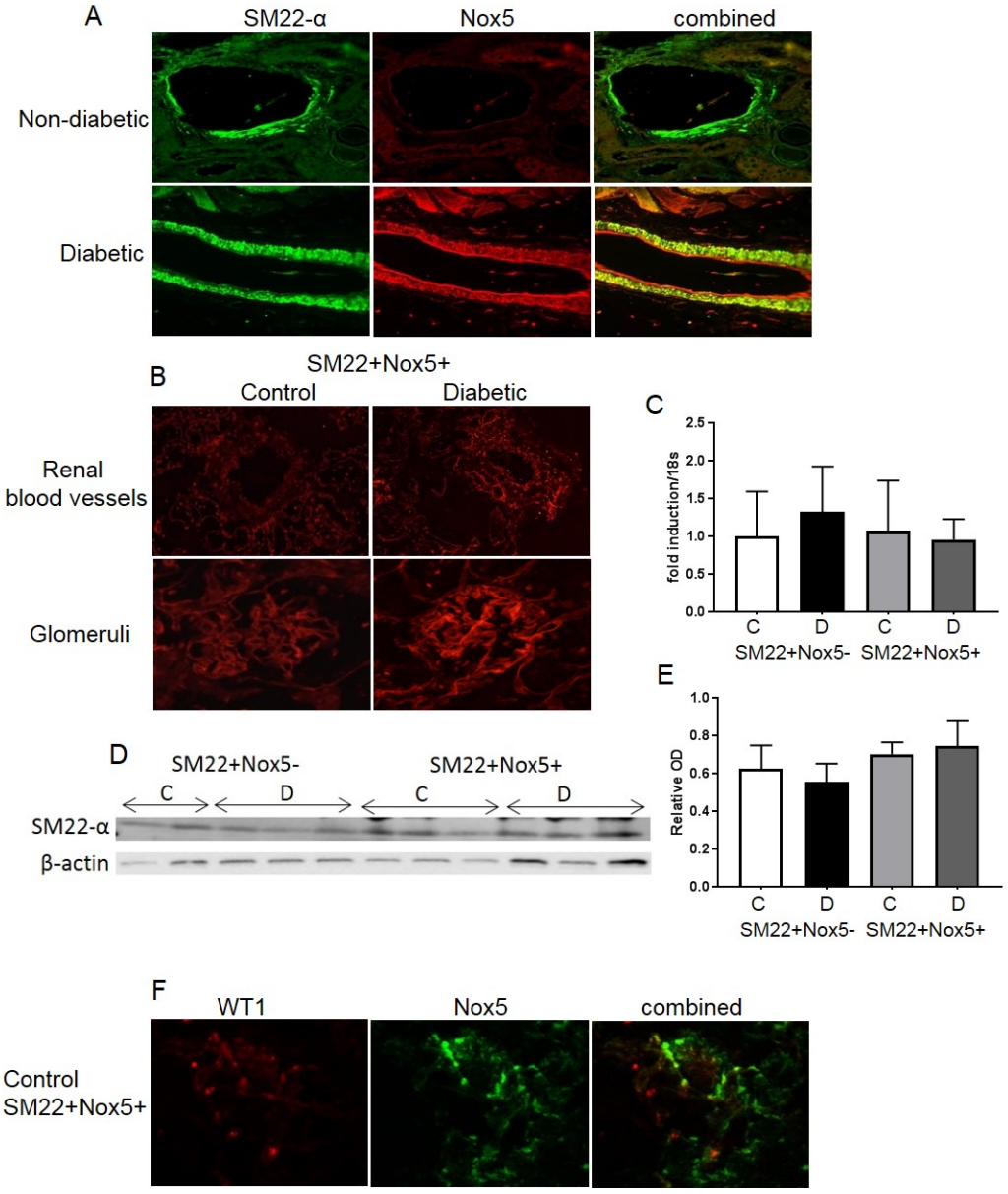
NADPH oxidase-Nox5 accelerates renal injuries in diabetic nephropathy by Jha et al.

Supplementary Figure 1. Gene expression analysis of Nox1, Nox2 and Nox4 by RT-PCR (**A**) and immunostaining for Nox2 (**B**) and its quantitation (**C**) in the glomerular fraction of control (C) and diabetic (D) SM22+Nox5- and SM22+Nox5+ mice after 10 weeks of diabetes (n= 6/group for RT-PCR and n=3 for immunostaining). Data are shown as mean ± SEM. *p<0.05 vs. control SM22+Nox5- mice; **p<0.05 vs. control SM22+Nox5- mice; $^{\$}$p<0.05 vs. control SM22+Nox5- mice and $^{\#}$p<0.05 vs. diabetic SM22+Nox5- mice.



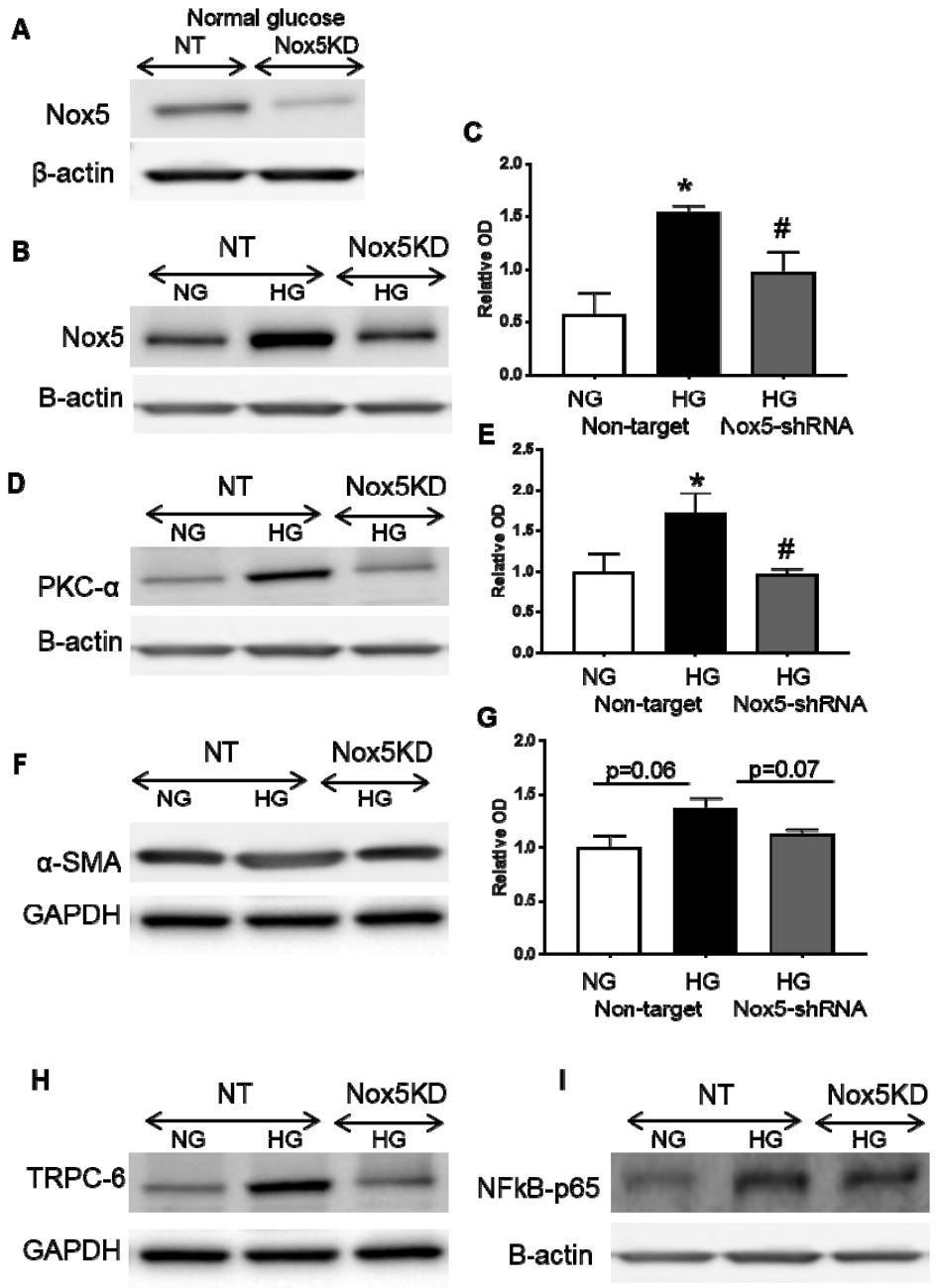
SUPPLEMENTARY DATA

Supplementary Figure 2. Figure 2A: Co-localization of Nox5 (red staining) and transgelin (SM22-α; green staining) in renal blood vessels of kidney biopsies from non-diabetic and diabetic individuals (magnification x 20); **Figure 2B:** immunofluorescence for Nox5 in renal blood vessels and glomeruli of control and diabetic SM22+Nox5+ mice; **Figure 2C-E:** gene (**C)** and protein **(D and E)** expression of SM22- α (23KDa) in in the glomerular fraction of control (C) and diabetic (D) SM22+Nox5- and SM22+Nox5+ mice; **Figure 2F:** Co-localization of Nox5 and WT1 (a podocyte specific marker) in glomeruli which shows Nox5 expression is not present in podocytes in SM22+Nox5+ transgenic mice (magnification x 20).



SUPPLEMENTARY DATA

Supplementary Figure 3. Protein expression of Nox5 (Fig.3A-C); PKC-α (Fig.3D-E); α-SMA (Fig.3F-G); TRPC-6 (Fig.H) and NFkB-p65 (Fig.I) by western blot in non-target (NT) and Nox5 knockdown (Nox5KD) human mesangial cells in response to normal glucose (NG, 5mM) and high glucose (HG, 25mM) after 2 days. Results were expressed relative to non-target plus NG. Data are mean ± SEM (n= 3/group). $^{*}p< 0.05$ vs non-target plus NG and $^{\#}p<0.05$ vs non-target plus HG.



SUPPLEMENTARY DATA

Supplementary table 1. Mouse probe and primer sequences for qRT-PCR

Genes	**Probe Sequence** 5'FAM-3"TAMRA	**Forward Primer 5'-3'**	**Reverse Primer 5'-3'**
Nox1	CTAGAATAGCTACTGCCCACC	GACCAATGTGGGACAATGAGTTT	CCCCCACCGCAGACTTG
Nox2	CAACTGGACAGGAACCT	AGTGCGTGTTGCTCGACAAG	CCAAGCTACCATCTTATGGAAAGT
Nox4	CATTTTGCTATTTCATCAAA	AAAAATATCACACACTGAATTCGAGACT	TGGGTCCACAGCAGAAAACTC
MCP1	AATGGGTCCAGACATAC	GTCTGTGCTGACCCCAAGAAG	TGGTTCCGATCCAGGTTTTTA
Collagen I	ATCGACCCTAACCAAG	GACTGGAAGAGCGGAGAGTACTG	CCTTGATGGCGTCCAGGTT
Collagen IV	CAGTGCCCTAACGGT	GGCGGTACACAGTCAGACCAT	GGAATAGCCGATCCACAGTGA
Fibronectin	CCCCGTCAGGCTTA	ACATGGCTTTAGGCGGACAA	ACATTCGGCAGGTATGGTCTTG
α-SMA	TGCCAGATCTTTTCC	GACGCTGAAGTATCCGATAGAACA	GGCCACACGAAGCTCGTTAT
P21	AGAGCCACAGGCACC	TCCACAGCGATATCCAGACATT	CGGACATCACCAGGATTGG
PKC-α	CGATCCCAGTCCCAG	AGACAAAGACCGGCGACTGT	TTAGCTCTGAGACACCAAAGGAAA
SM22	SYBER	TGTCCTAGTCCTCTTACCCTAGTGTCT	GAGTGCCTTGTTTAGCAGCAAGT

FAM, 5'-Fluorescein; α-SMA, smooth muscle actin; MCP1, Monocyte chemoattractant protein 1; p21, Cyclin-dependent kinase inhibitor 1A, and PKC-α, protein kinase C – α and PKC-β, protein kinase C- β.

SUPPLEMENTARY DATA

Supplementary table 2. Human probe and primer sequences for qRT-PCR

Genes	**Probe Sequence** 5'FAM-3"TAMRA	**Forward Primer 5'-3'**	**Reverse Primer 5'-3'**
Nox5	CCTGCTGACTAAACTG	CGGTCTTTCGAGTGGTTTGTG	CCTCGGCCTGGTCCATCT
MCP-1	CAGGAAACCAATATCCA	CAAAGCAGGGCTCGAGTTG	CCTGGGACTAGACTTGATGTCTCA
TRPC6	TTTGCAAGGGCCAAAC	GACGCTGATGTGGAGTGGAA	TCTGCCCTCCTCAAAGTAGGAA
PKC-α	CGATCCCAGTCCCAG	TCAGACAAAGACCGACGACTGT	TCCGAAACTCCAAAGGAAAGG
PKC-β	AGCCACTGCACCGAC	ACGAGGTGAAGAACCACAAATTC	CCCGAAGCCCCAGATGA
Collagen IV	ATTTGCGTAACTAACACACC	CAATATGAAAACCGTAAAGTGCCTTATA	CAGCAAGTAGAGGTCAATGAAGCA
Fibronectin	TGCCATTTGCTCCTGC	AGAACAGTGGCAGAAGGAATATCTC	CCCGCTGGCCTCCAA
CTGF	ACTGCCTGGTCCAGAC	GCGGCTTACCGACTGGAA	GGAACAGGCGCTCCACTCT
α-SMA	TGCCAGATCTTTTCC	ACCCTGAAGTACCCGATAGAACAT	CAACACGAAGCTCATTGTAGAAAGA

FAM, 5'-Fluorescein; α-SMA, smooth muscle actin; CTGF, connective tissue transforming growth factor; MCP1, Monocyte chemoattractant protein 1; TRPC6, transient receptor potential cation channel subfamily C member 6; PKC-α, protein kinase C – α and PKC-β, protein kinase C- β.

SUPPLEMENTARY DATA

DETAILED MATERIAL

Histological assessment

Kidney sections (3μm) were stained with Periodic Acid–Schiff (PAS) for measurement of mesangial expansion and glomerulosclerotic injury (GSI). Mesangial area was analyzed (percentage of glomerular area) from digital pictures of glomeruli (20 glomeruli per kidney per animal) using Image-Pro plus 7.0 software (Media Cybernetics, Bethesda, MD), as described previously (8; 14). GSI was graded based on the severity of glomerular damage, including mesangial matrix expansion, hyalinosis with focal adhesion, capillary dilation, glomerular tuft occlusion, and sclerosis, as previously described (16). Twenty glomeruli per kidney were assessed in a masked fashion.

Immunofluorescence

Human kidney biopsies and mice renal frozen sections (8 μm) were fixed in 4% paraformaldehyde for 15 minutes, permeabilized using 0.1% tritonX and incubated in a blocking buffer (1% BSA, 0.25% TritonX in PBS, pH 7.4). Double staining for Nox5 and SM22- α in human kidney biopsies and mouse kidney tissue as well as for Nox5 and WT1 in mouse kidney tissue was performed by incubation with primary antibody to Nox5 (1:100, rabbit polyclonal; Abcam, Cambridge, MA) and SM22-α (1:100, goat polyclonal; Abcam, Cambridge, MA) as well as Nox5 (1:100, goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) and WT-1 (1:100, rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibody incubation was followed by incubation with secondary antibody Alexa Fluor 568 (1:400, donkey anti-rabbit, Invitrogen, Eugene, Oregon) and Alexa Fluor 488 (1:400, donkey anti-goat, Invitrogen, Eugene, Oregon). The sections were washed with PBS and were coverslipped using Prolog Fold antifade reagent with DAPI (Invitrogen, Carlsbad, CA).

Dihydroethidium (DHE) staining: DHE staining was used to detect superoxide levels in the frozen kidney sections and in human mesangial cells. DHE freely permeates cell membranes and reacts with superoxide anions to form ethidium which intercalates with DNA and shows red fluorescence. Human mesangial cells grown on coverslips and frozen kidney sections were washed twice with PBS. DHE (10 μM, Invitrogen) in Krebs buffer was added to cells and tissues and incubated at 37 °C for 40 minutes, washed with distilled water, dried and mounted with ProLong Gold anti-fade reagent (Invitrogen) as mounting medium. All stained sections were examined using an Olympus (Tokyo) BX61 fluorescence microscope, and images were captured on Zeiss 510 Meta laser scanning confocal microscope (Zeiss) using LSM 510 Software (version 3.2 SP2; Zeiss) (20x magnification).

Immunohistochemistry

Immunohistochemical staining was performed as described previously (8; 14). Briefly, 4μm kidney paraffin sections were stained for collagen IV (1:300, goat anti- type IV collagen, Southern Biotech, Birmingham, AL), fibronectin (1:1000, rabbit anti-fibronectin, Dako Cytomation, Glostrup), nitrotyrosine (1:100, rabbit anti-nitrotyrosine, Millipore, Billercia, MA), F4/80 (1:50, rat monoclonal anti- F4/80, Abcam, Cambridge, MA), PKC-α (1:100, rabbit anti- PKC-α, Santa Cruz Biotechnology, Cambridge) and Nox2 (1:100, goat anti-Nox2, Santa Cruz Biotechnology, Cambridge). Sections for collagen IV, fibronectin and nitrotyrosine were digested with 0.4% pepsin (Sigma Chemical Co) in 0.01M HCl at 37°C. This was followed by incubation with 0.5% milk diluted in TBS to block nonspecific binding. Similarly, sections for F4/80 were incubated with protein blocking agent prior to incubation with the primary anti-F4/80 antibody overnight at 4°C and staining further amplified by the Dako Catalysed Signal Amplification Kit, according to instructions (Dako Cytomation, Glostrup). Subsequently, sections were incubated with the primary antibody overnight at 4°C followed by avidin/biotin blocking. Thereafter, biotinylated anti-goat Ig (1:500) for collagen IV, biotinylated anti-rabbit Ig (1:500) for fibronectin, nitrotyrosine and PKC-α and biotinylated anti-rat Ig (1:200) for F4/80 (Vector Laboratories, Burlingame, CA) were applied as the secondary antibody, followed by horseradish peroxidase-conjugated streptavidin (VECTASTAIN Elite ABC Staining Kit; Vector Laboratories). Peroxidase conjugates were subsequently visualized using 3,3'-diaminobenzidine (DAB)

tetrahydrochloride (Sigma-Aldrich, St Louis, MO, USA) in 0.08% H_2O_2/TBS. Finally, sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted. All sections were examined under light microscopy (Olympus BX-50; Olympus Optical, Tokyo) and digitized with a high-resolution camera. For the quantification of the proportional area of staining, 20 glomeruli (×400) were analysed using Image-Pro plus 7.0 (Media Cybernetics; Bethesda, MD) (8; 14). All assessments were performed in a blinded manner. Six to eight kidneys were investigated in each group.

In vitro western blot

Twenty μg protein samples from non-target and Nox5 knockdown human mesangial cells exposed to normal glucose and high glucose was electrophoresed on 10% acrylamide gels under non-reducing conditions. Western blot analysis was then performed after incubation with a primary antibody to Nox5 (86 kDa, rabbit polyclonal; Abcam, Cambridge, MA); PKC-α (83 kDa, rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA); α-SMA (42 kDa, rabbit polyclonal; Abcam, Cambridge, MA), TRPC-6 (106 kDa, rabbit polyclonal; Abcam, Cambridge, MA) and NFkB-p65 (65 KDa, rabbit polyclonal, Invitrogen, Carlsbad, CA) and assessed with a goat anti-rabbit (Dako Corp., Carpinteria, CA) secondary antibody. Membranes were subsequently probed for β-actin (42 kDa, Sigma-Aldrich) and GAPDH (37 KDa, Abcam) for determination of equal loading of samples. Blots were detected using the ECL detection kit (Sigma-Aldrich, St Louise, MO).