Vanderbilt AMDCC

Generating Endothelial-Specific Knockouts of Endothelial Nitric Oxide Synthase and Prostacyclin Synthase

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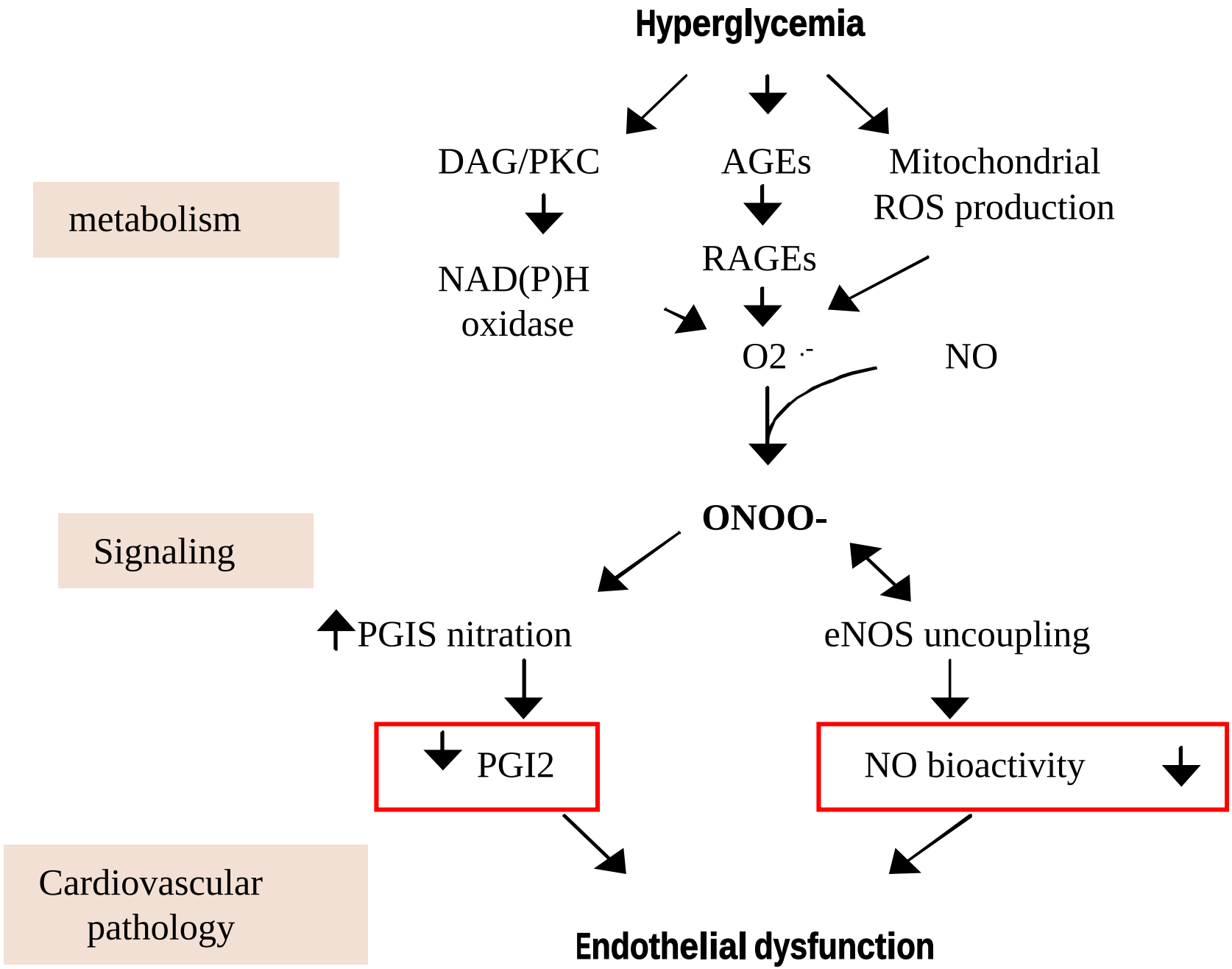
9-18-2007


Hyperglycemia
DAG/PKC
AGEs
Mitochondrial ROS production
metabolism
NAD(P)H oxidase
RAGEs
O2 ·-
NO
ONOO-
Signaling
PGIS nitration
eNOS uncoupling
PGI2
NO bioactivity
Cardiovascular pathology
Endothelial dysfunction

eNOS

In microvasculature, eNOS-derived NO controls basal tone and vascular permeability and inhibits platelet aggregation and neutrophil adhesion.Hyperglycemia *per se* produces reductions in eNOS expression, eNOS activity and NO availability in cultured cells

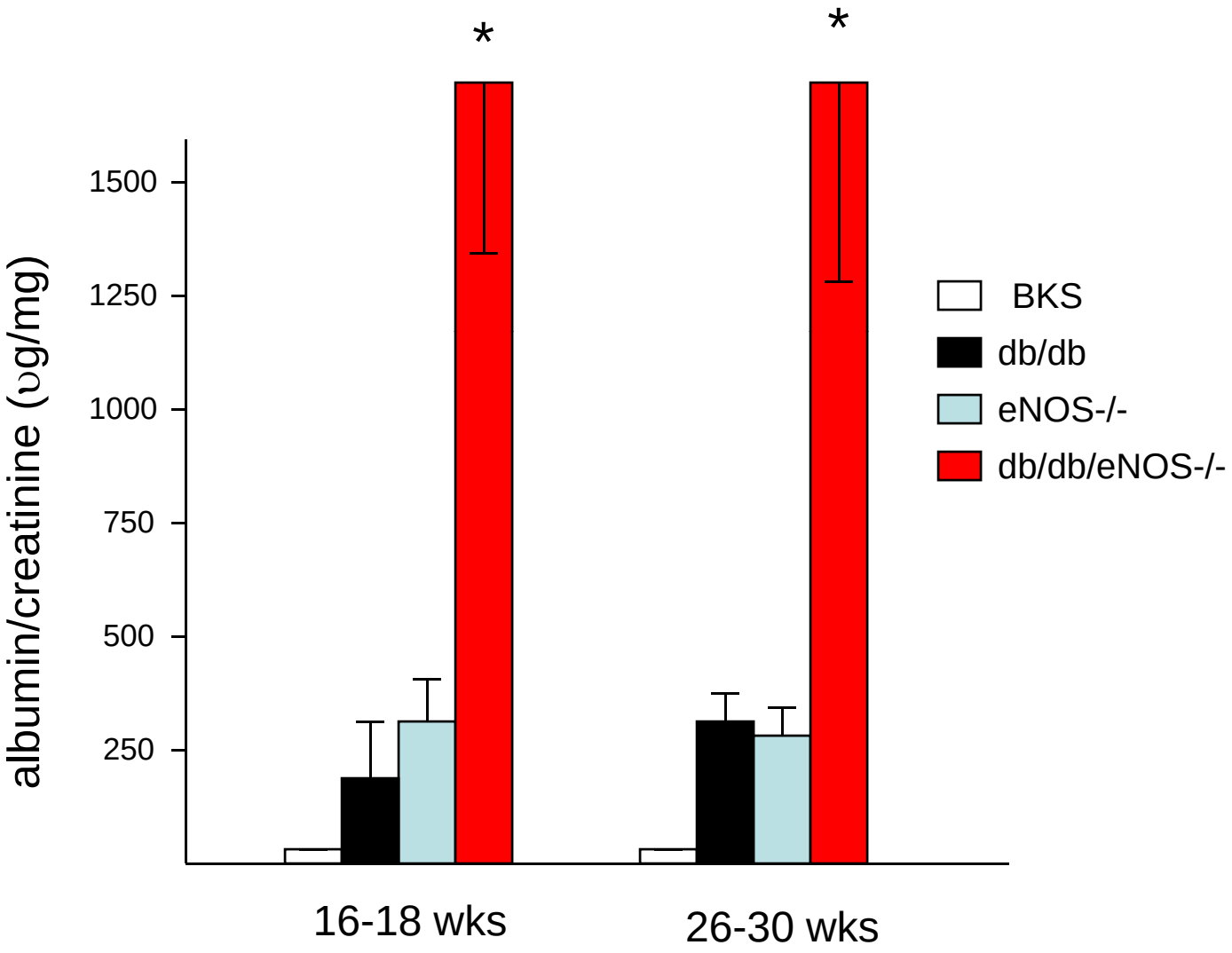
In early diabetes eNOS mRNA and protein expression increases in the vasculature in early diabetes but activity markedly decreases.

In established diabetes of longer duration, vascular eNOS expression decreases.

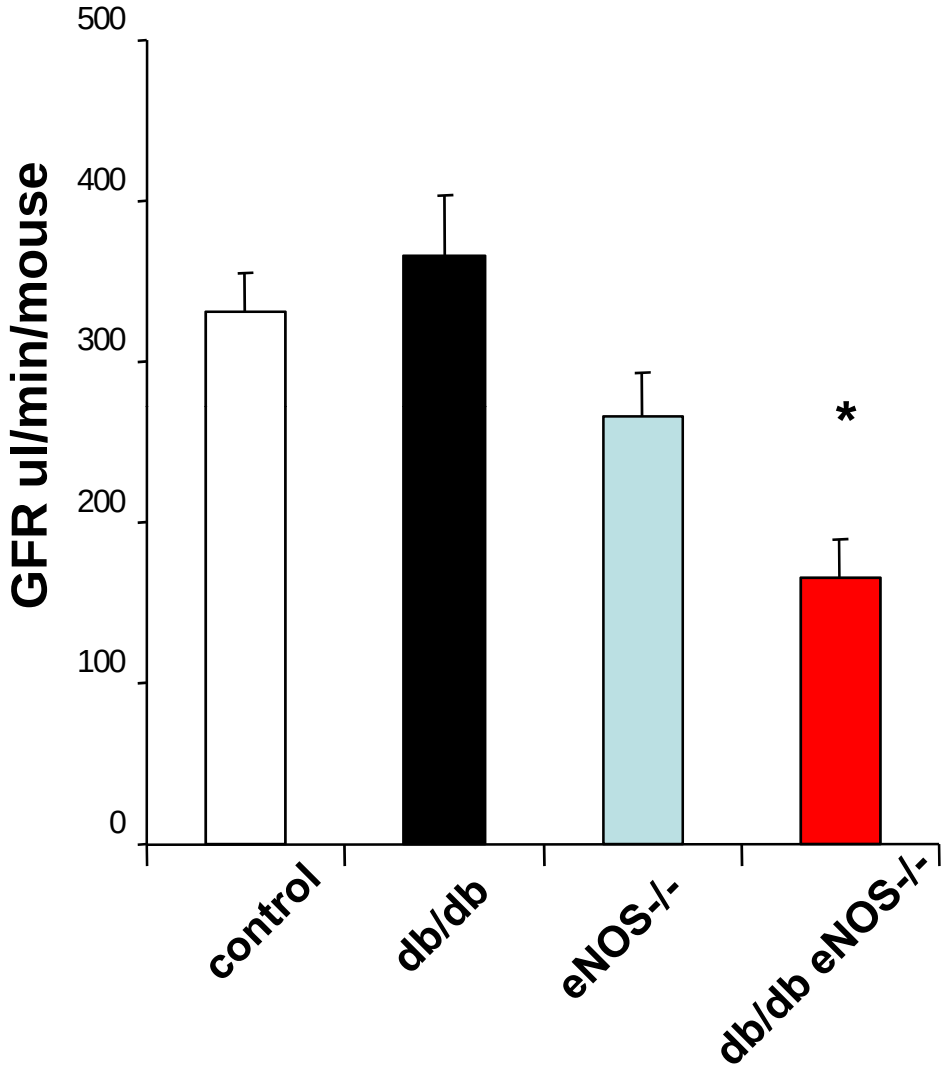
In humans, the eNOS gene is found on chromosome 7q. Genome wide scans have indicated regions on 7q, 18q and 22q that may influence proteinuria and/or development of diabetic nephropathy in type 2 diabetes. Of note, evidence for a region on 7q overlapped all studies.

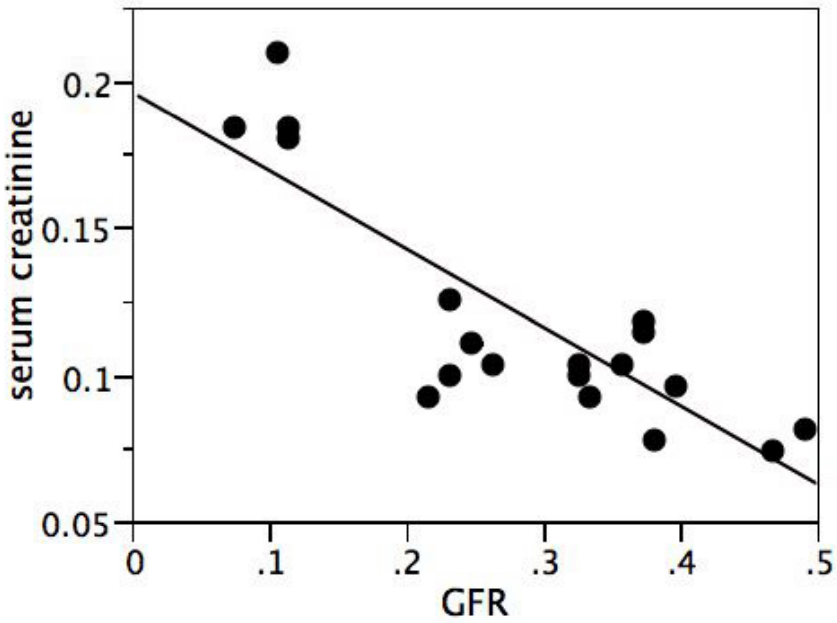
In some studies, SNPs associated with decreased eNOS activity have been associated with development of advanced nephropathy in type I and type II diabetic patients, as well as other causes of ESRD.

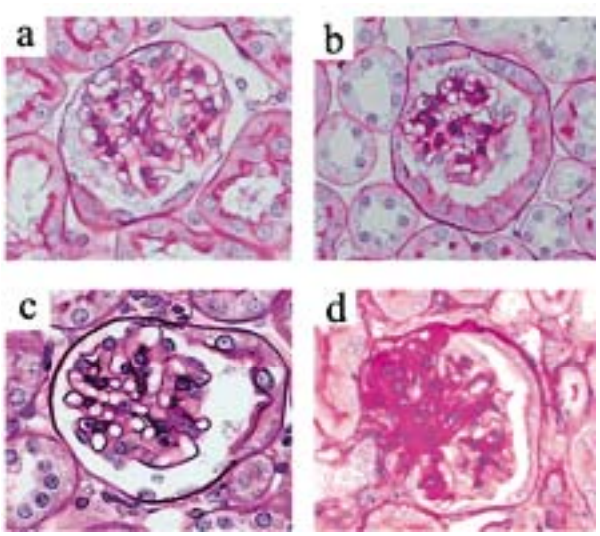
Albuminuria

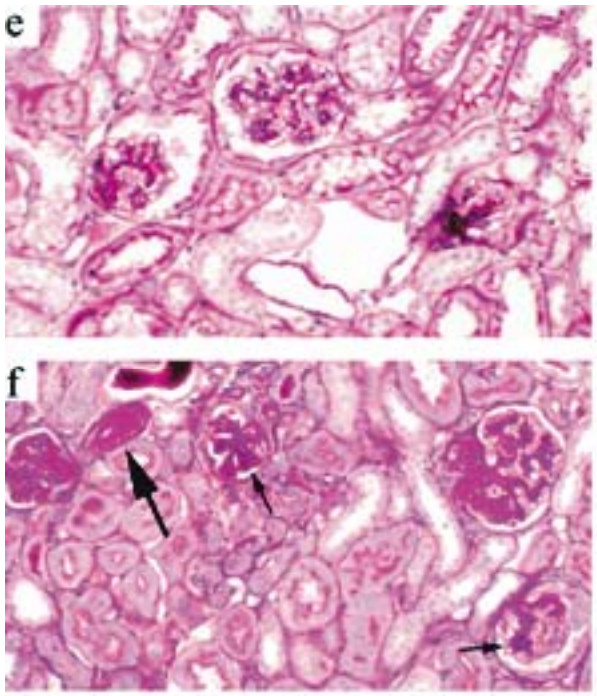


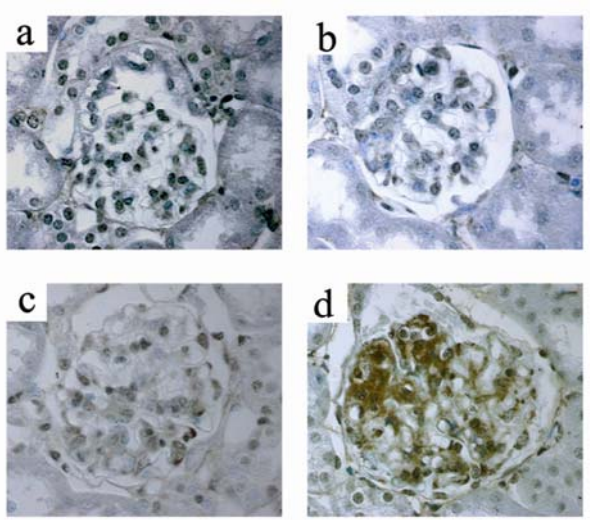
GFR

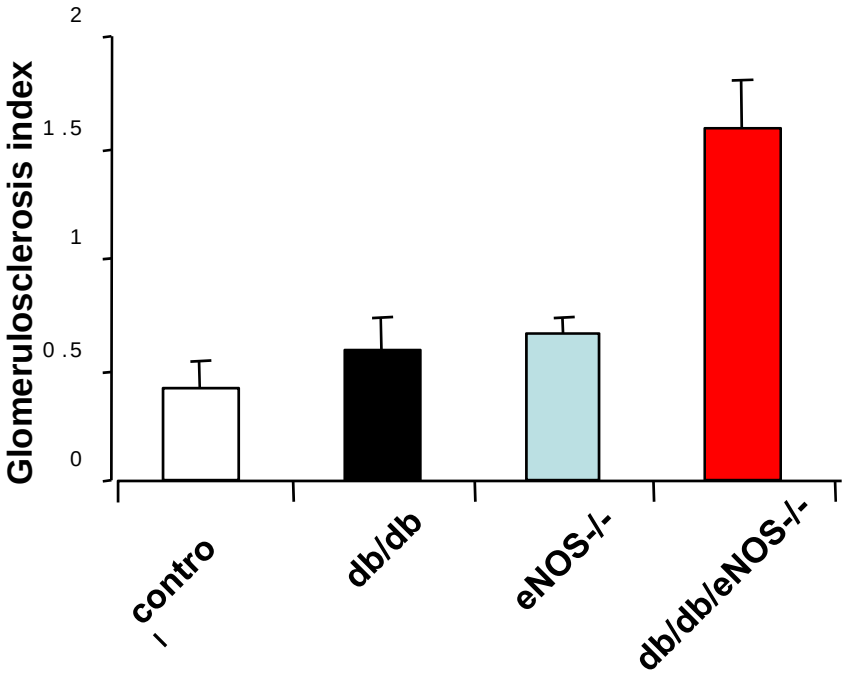

GFR ul/min/mouse
500
400
300
200
100
0
*
control
db/db
eNOS-/-
db/db eNOS-/-

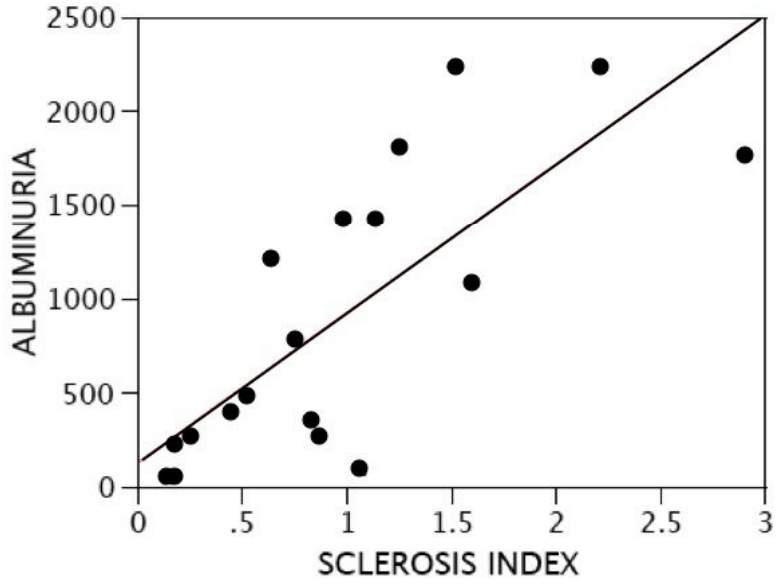

serum creatinine
0.2
0.15
0.1
0.05
0
.1
.2
.3
.4
.5
GFR


a
b
c
d

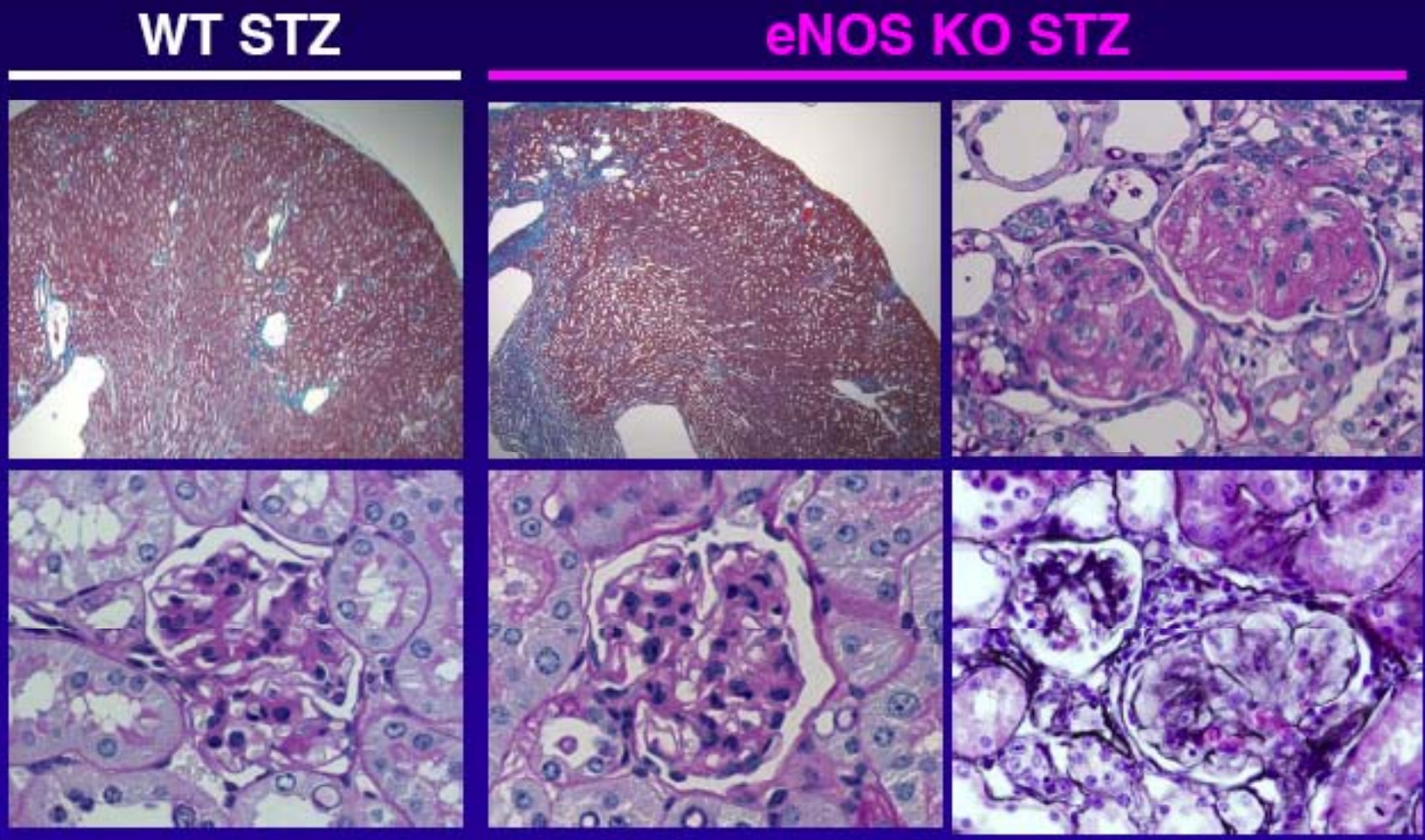

e
f


a
b
c
d


Glomerulosclerosis index
2
1.5
1
0.5
0
contro
l
db/db
eNOS-/-
db/db/eNOS-/-

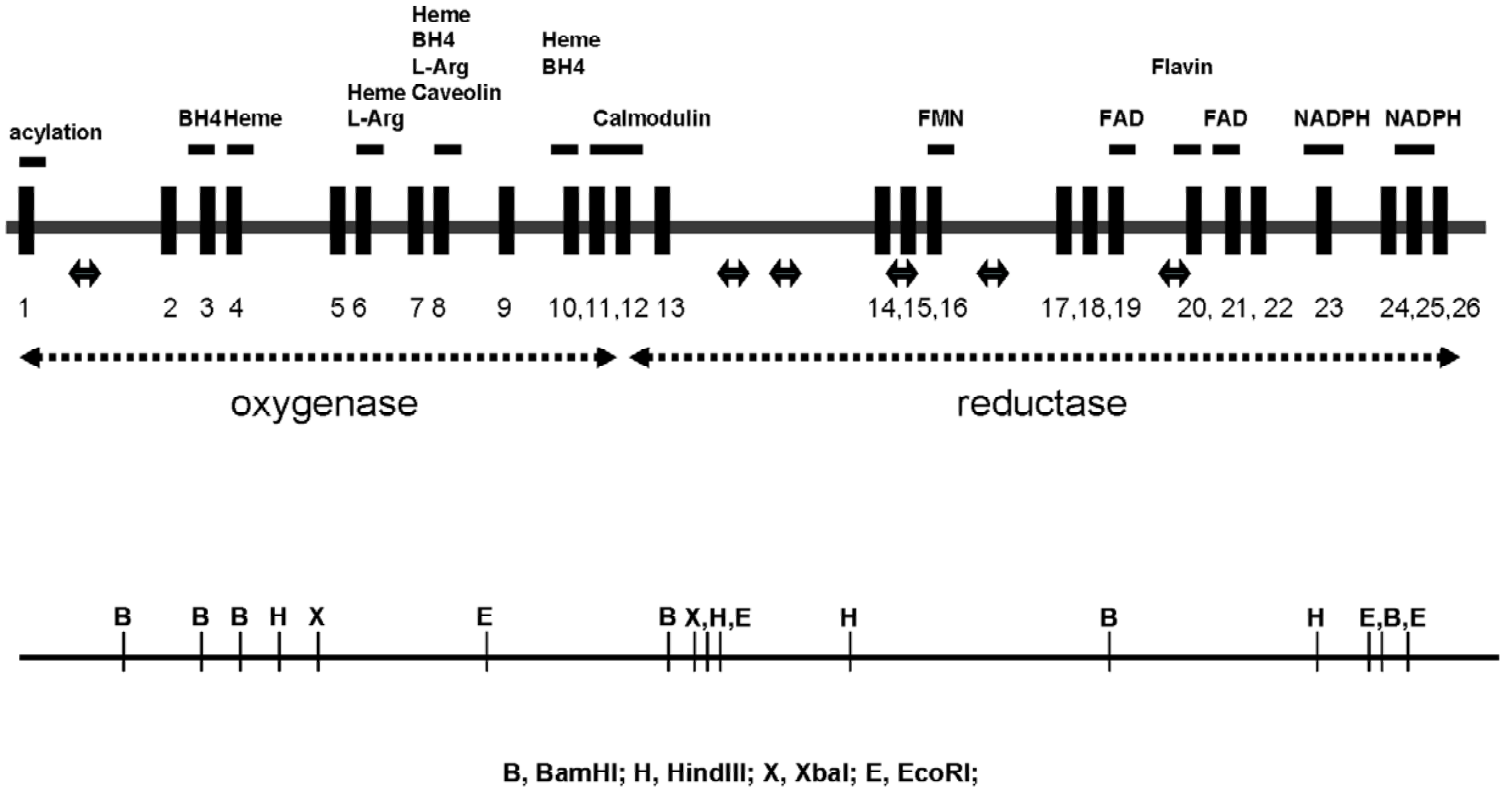

ALBUMINURIA
2500
2000
1500
1000
500
0
0
.5
1
1.5
2
2.5
3
SCLEROSIS INDEX

Histological findings (STZ 20w)



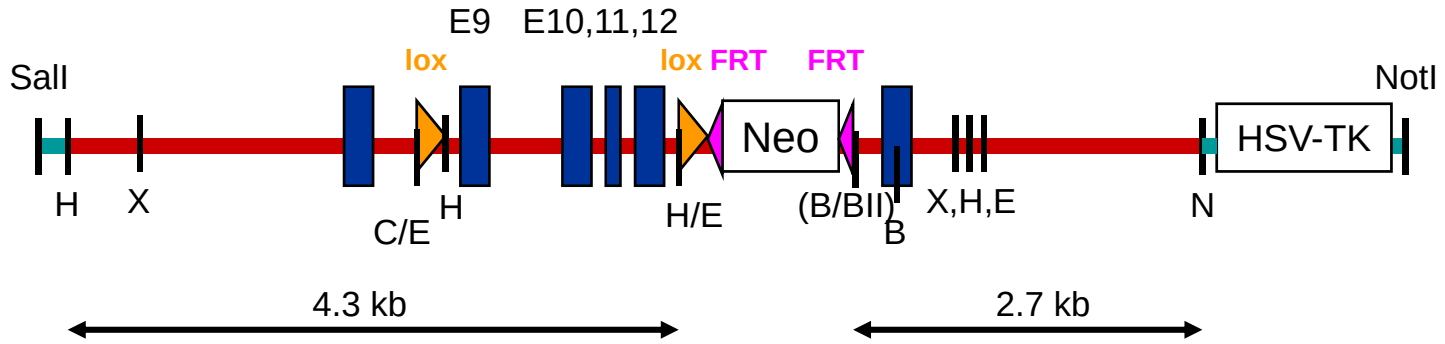
In addition to vasculature, eNOS is also expressed in:

Cardiac myocytes
Thick ascending limb
Pulmonary epithelial cells
Adipocytes
Platelets
Hippocampus
Dorsal root ganglia

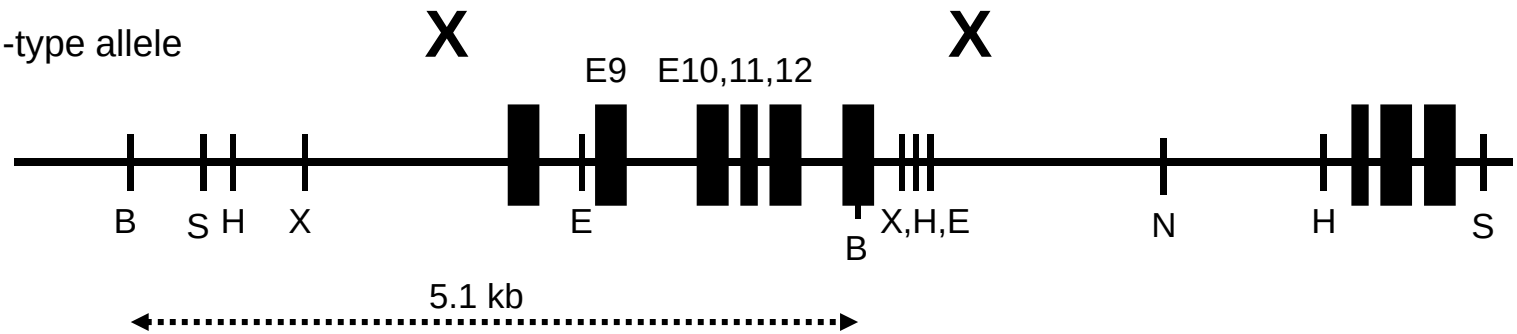


Genomic organization of mouse eNOS gene. Schematic diagram of the mouse eNOS gene locus (19.6kb, 26 exons, GenBank NC_ 000071) is shown. Exons are indicated by vertical black rectangles. The binding sites of BH4, Heme, L-Arg, Caveolin, Calmodulin, FMN, FAD, Flavin, and NADPH are also displayed. Arrows indicate potential regulatory regions whose sequences are highly conserved between human and mouse eNOS genomes.

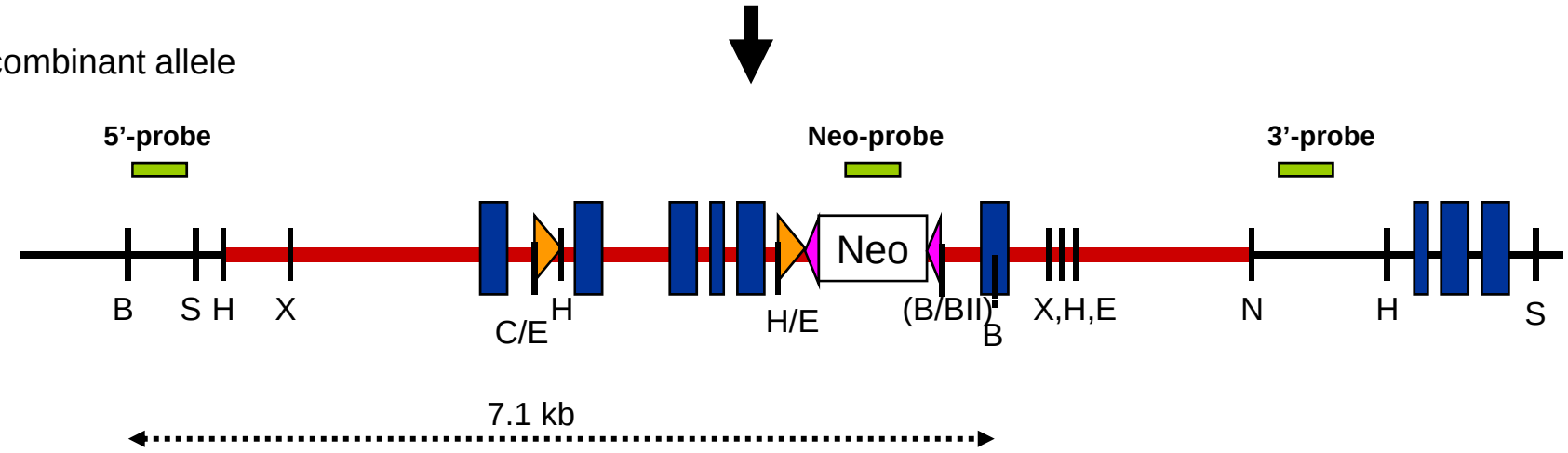
Targeting Vector (Lox FRT-eNOS)



Wild-type allele



Recombinant allele



Enzyme digestion

Enzyme digestion

Linearization of the targeting Vector: **Sal I**

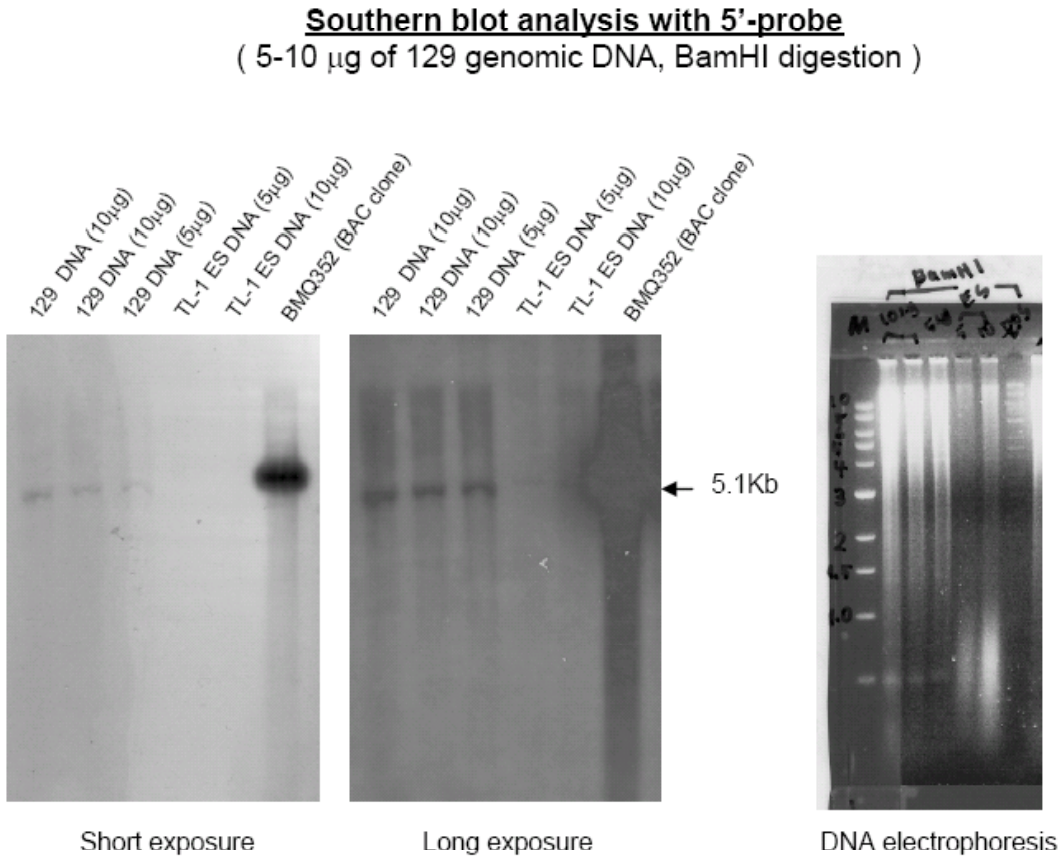
Screening with 5'-probe: **BamH I**

	Wild-type allele	Targeted allele
BamH I	5.1 kb	7.1 kb

Screening with 3'-probe: **Sac I (or KpnI or ScaI or SpeI)**

	Wild-type allele	Targeted allele
Sac I	7.6 kb	9.6 kb

Southern blot of genomic DNA



Vector

Parent targeting vector

Plasmid Map

Plasmid Name: FRT.loxP Date: 4/June/96

Description: A targeting vector containing 2-loxP sites, 2-FRT sites, and the TK and Neo cassettes from pNTK(A).

Vector: BS+ (KS) Size: 2961 bp

Inserts: 2kb Neo; 2kb TK; Size: ~4300 bp
2x54 bp FRT oligo couple; Total: ~7261 bp
2x100 bp loxP oligo couple

Resistances: Amp

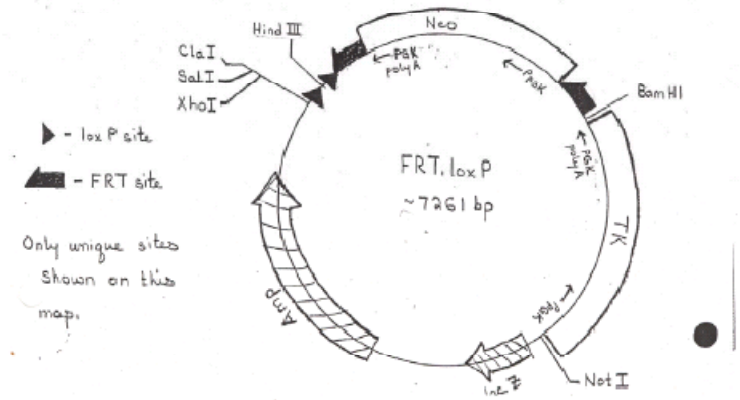
Verification Method: RE digestion and sequence

Bacterial Strain Transformed: DH5α

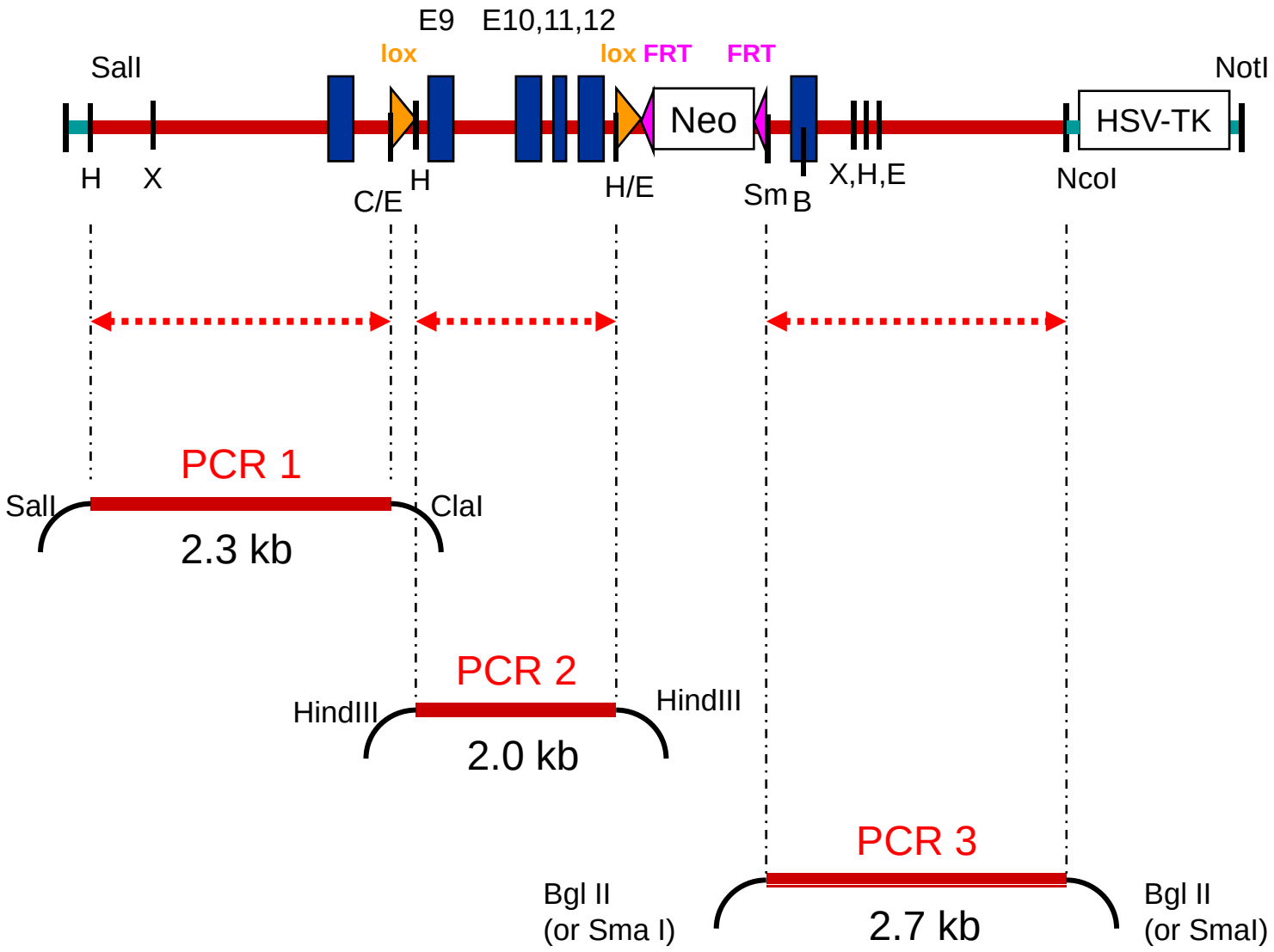
Stock Location: 3D-2-5 (bacteria) + 8-3-3 (p-dna)

Constructed by: Kathy Shelton

Other Comments:



Targeting Vector (Lox FRT-eNOS)

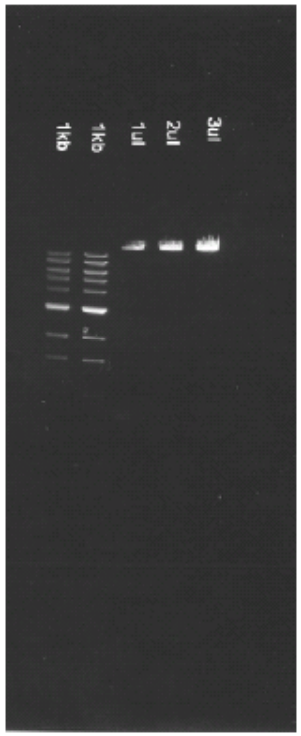


Sal1 linealization

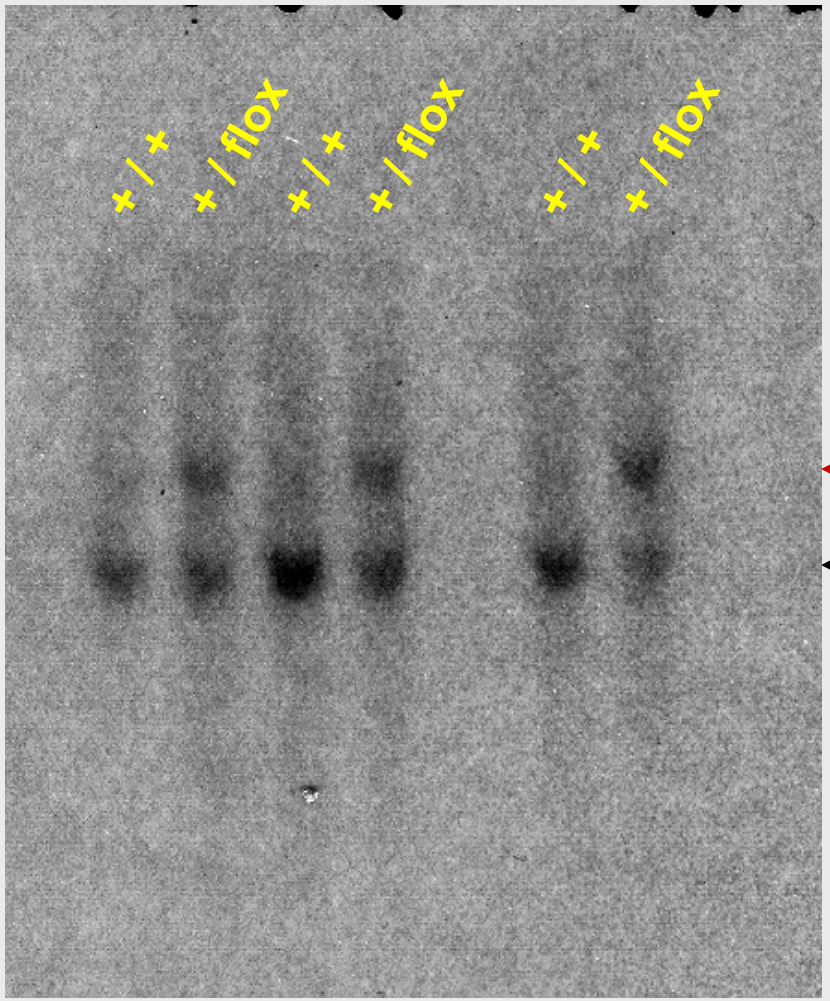
Sall linealization of the targeting vector

The size of the targeting vector: ~14.3 kb

1kb marker (from top); 10, 8, 6, 5, 4,
3=brightest, 2, 1.5, 1.0 kb



Southern blot analysis of ES clones



Targeted allele
(7.1 kb)

wild-type
(5.1 kb)

5’-probe (BamHI digestion)

Targeting Prostacyclin Synthase

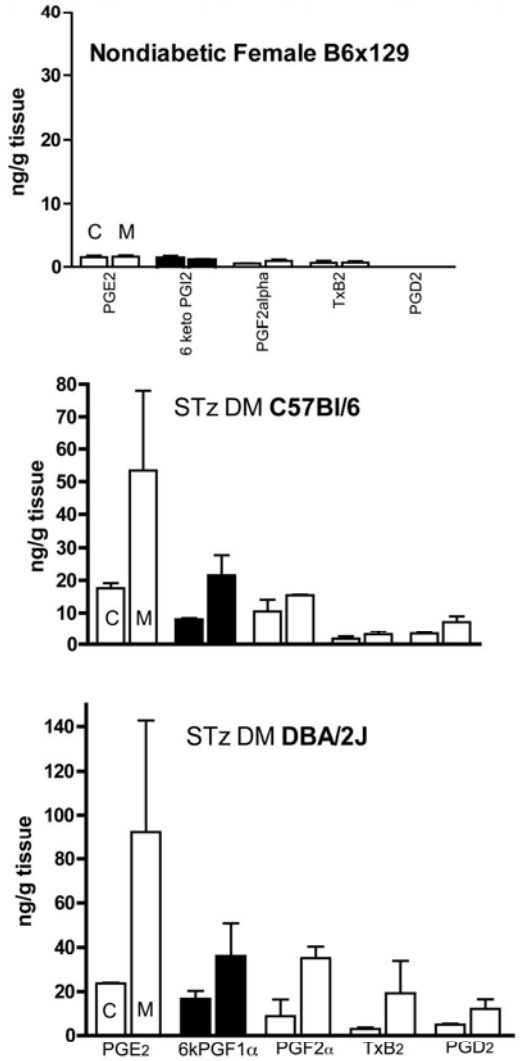
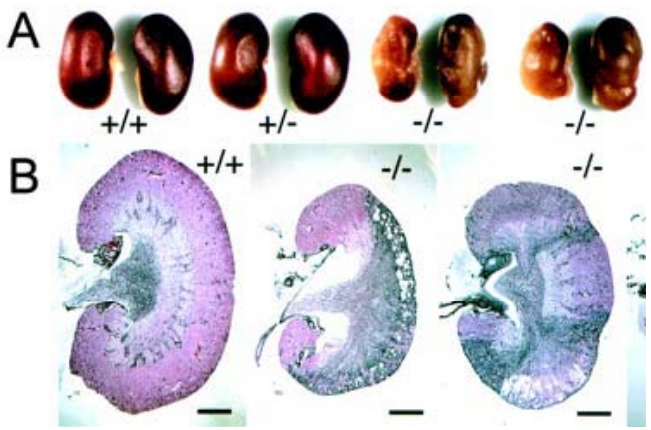
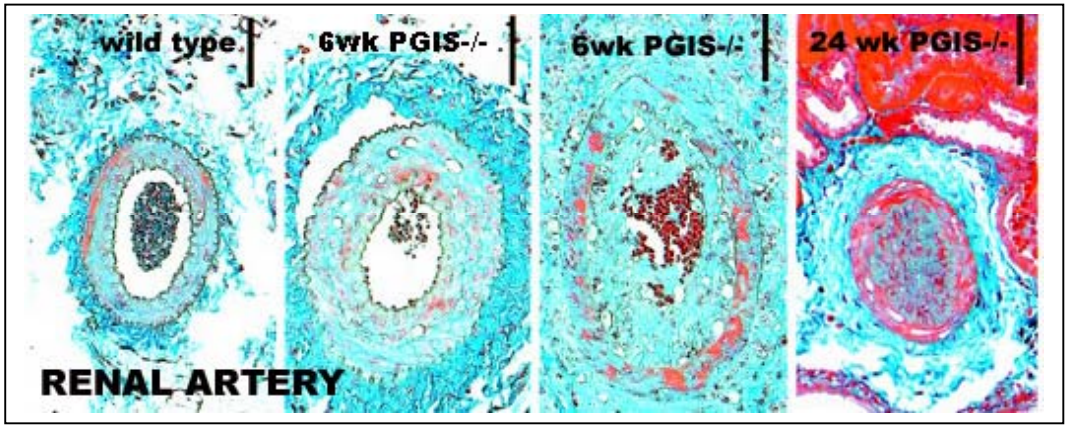
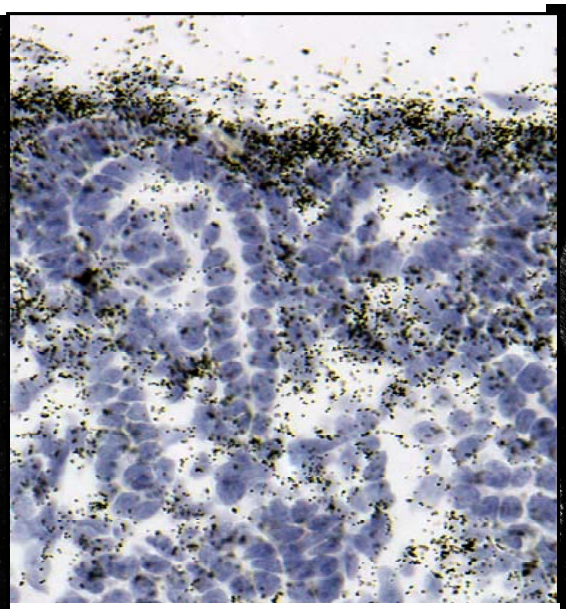


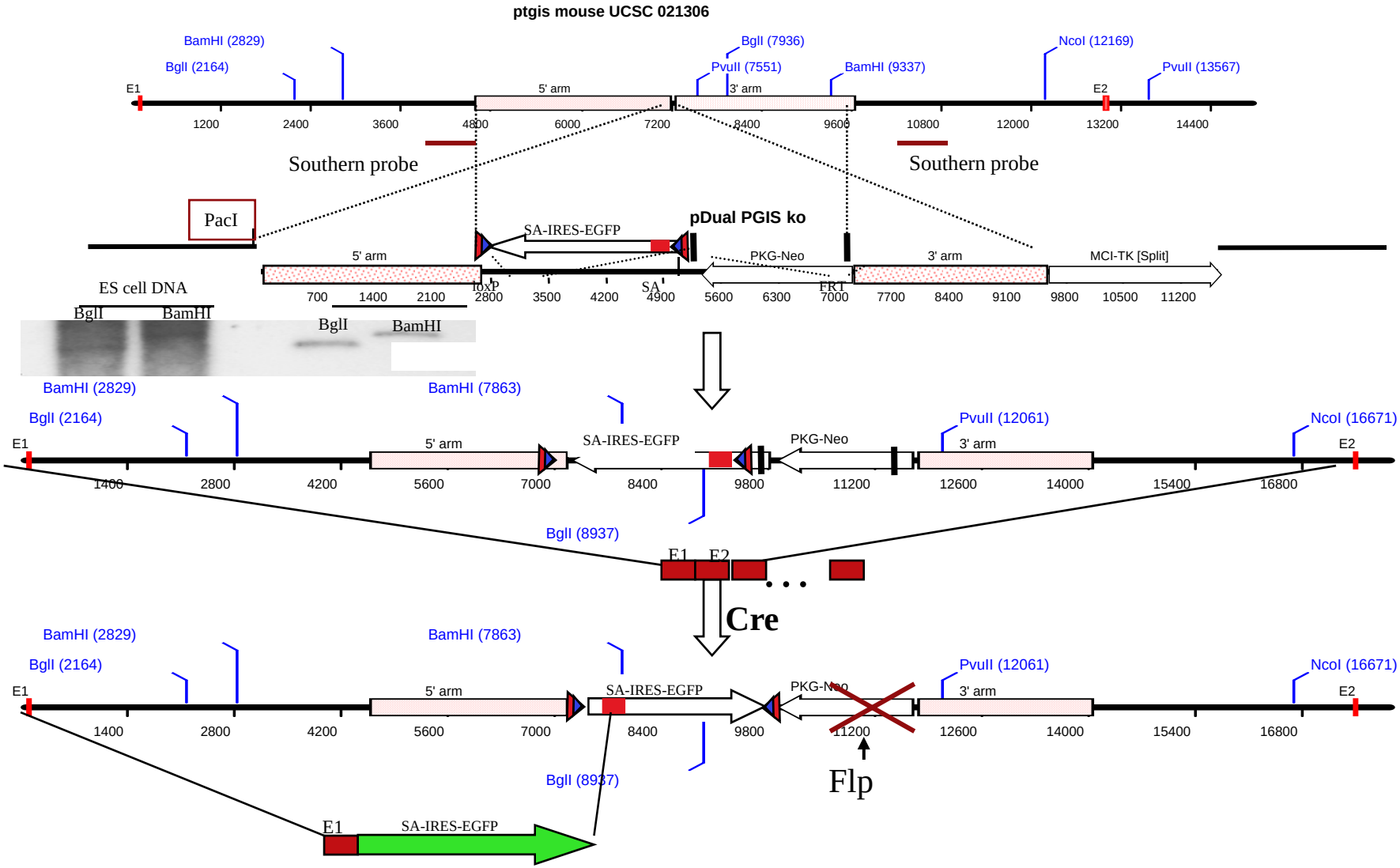
Figure 4: Prostaglandin profile in r enal cortex (C) and medulla (M). Top panel: non-diabetic mice. Middle panel: diabetic C57BL/6J; Bottom panel Diabetic DBA2/J mice.





PGI Synthase Expression During Renal Development


postnatal Day 0


ptgis mouse UCSC 021306
BamHI (2829)
BglI (2164)
BglI (7936)
PvuII (7551)
BamHI (9337)
NcoI (12169)
PvuII (13567)
E1
5' arm
3' arm
E2
1200
2400
3600
4800
6000
7200
8400
9600
10800
12000
13200
14400
Southern probe
Southern probe
PacI
pDual PGIS ko
SA-IRES-EGFP
5' arm
PKG-Neo
3' arm
MCI-TK [Split]
loxP
SA
FRT
700
1400
2100
2800
3500
4200
4900
5600
6300
7000
7700
8400
9100
9800
10500
11200
ES cell DNA
BglI
BamHI
BglI
BamHI
BamHI (2829)
BamHI (7863)
BglI (2164)
PvuII (12061)
NcoI (16671)
E1
5' arm
SA-IRES-EGFP
PKG-Neo
3' arm
E2
1400
2800
4200
5600
7000
8400
9800
11200
12600
14000
15400
16800
BglI (8937)
E1
E2
Cre
BamHI (2829)
BamHI (7863)
BglI (2164)
PvuII (12061)
NcoI (16671)
E1
5' arm
SA-IRES-EGFP
PKG-Neo
3' arm
E2
1400
2800
4200
5600
7000
8400
9800
11200
12600
14000
15400
16800
BglI (8937)
Flp
E1
SA-IRES-EGFP

Breeding strategy for endothelial specific eNOS and PGIS knockouts

Step 1: Obtain eNOS or PGIS$^{flox/+}$ DBA/2J and Tie2Cre mice

Step 2: Cross eNOS or PGIS$^{flox/+}$ DBA/2J or Akita with Tie2Cre DBA/2J to yield diabetic and non-diabetic Tie2Cre mice heterozygous for the floxed allele (i.e. eNOS or PGIS$^{flox/+}$ Akita).

Step 3: Intercross Tie2Cre; eNOS or PGIS$^{flox/+}$ with Akita eNOS or PGIS$^{flox/+}$ to obtain Tie2Cre; eNOS or PGIS$^{flox/flox}$ mice that are both diabetic (Akita+) and non-diabetic (Akita negative). As required, T1D can also be induced in Akita negative mice using low dose STZ.

1) Backcross of Tie-2 Cre to DBA2- underway
2) Backcross of Tal/SCL-CreERT to DBA2- underway
3) DBA2-eNOS-/- mice- available
4) KKHIJ-eNOS-/- mice- available

Publications- 9/2006-9/2007

1) Kanetsuna, Y., Hirano, K., Nagata, M., Gannon, M.A., Takahashi, K. Harris, R.C., Breyer, M.D. and Takahashi, T. Characterization of Diabetic Nephropathy in a Transgenic Model of Nonobese Diabetes Am. J. Physiol Renal 291:F1315-22, 2006.

2) Zhao, H J, Wang, S, Cheng, H, Zhang, M-Z, Takahashi, T, Fogo, AB, Breyer, MD, and Harris, RC. Endothelial nitric oxide sythase deficiency produces accelerated nephropathy in diabetic mice. JASN 17:2664-9, 2006.

3) Tchekneva EE, Rinchik EM, Polosukhina D, Davis LS, Kadkina V, Mohamed Y, Dunn SR, Sharma K, Qi Z, Fogo AB, Breyer MD. A sensitized screen of N-ethyl-N-nitrosourea-mutagenized mice identifies dominant mutants predisposed to diabetic nephropathy. JASN 18:103-12, 2007.

4) Kanetsuna, Y, Takahashi, K, Nagata, M, Gannon, MA, Breyer, MD, Harris, RC and Takahashi, T. eNOS Deficiency Confers Susceptibility to Diabetic Nephropathy in Nephropathy Resistant Inbred Mice. Am. J. Pathology 170:1473-84, 2007

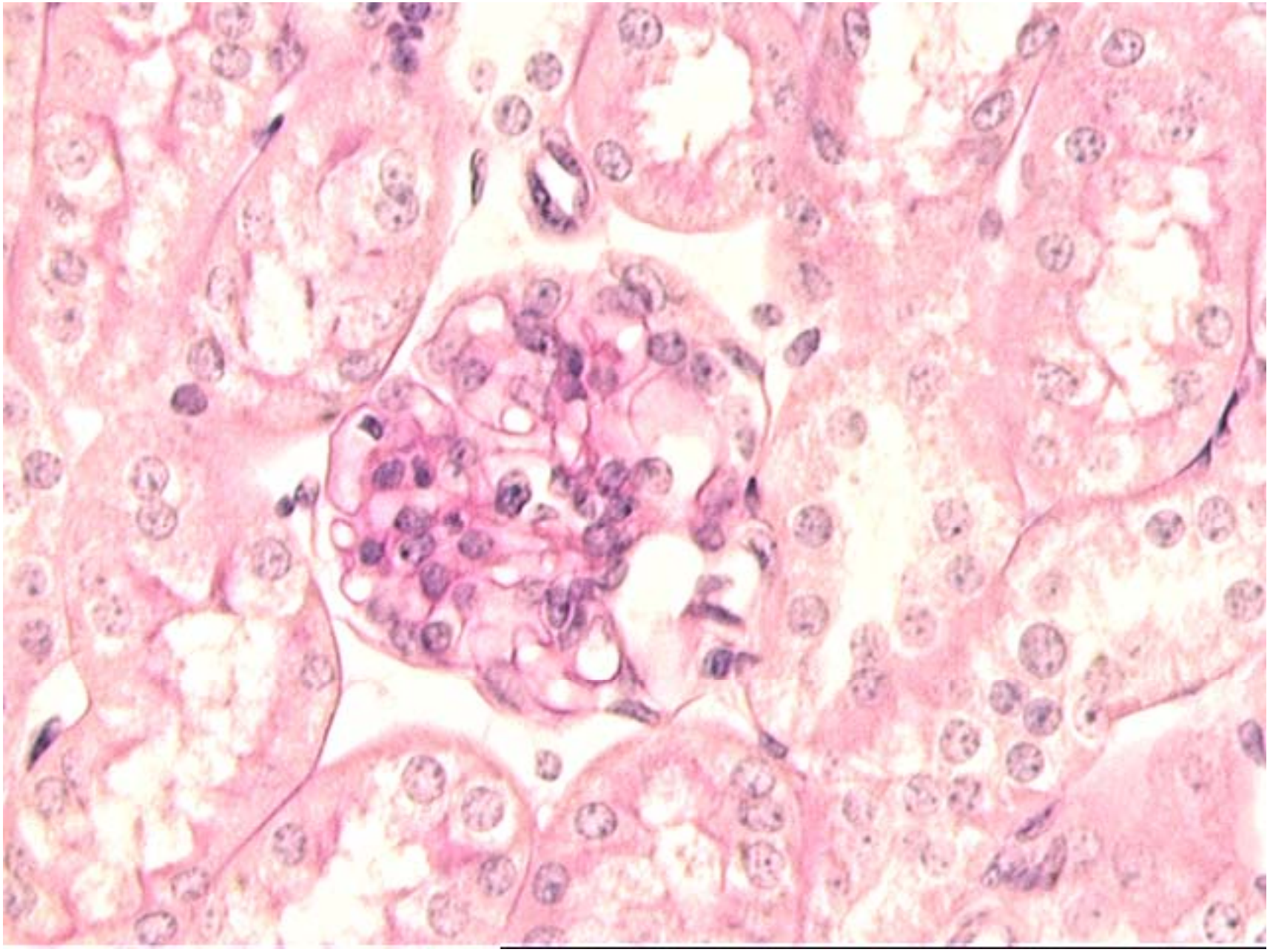
5) Breyer MD, Tchekneva E, Qi Z, Takahashi T, Fogo AB, Zhao HJ and Harris RC. Genetics of diabetic nephropathy: lessons from mice. Semin Nephrol. 27:237-47, 2007.

6) Breyer, MD and Harris, RC Diabetic Nephropathy, in The Molecular and Genetic Basis of Kidney Disease, D Mount and M Pollak, ed. 2007.

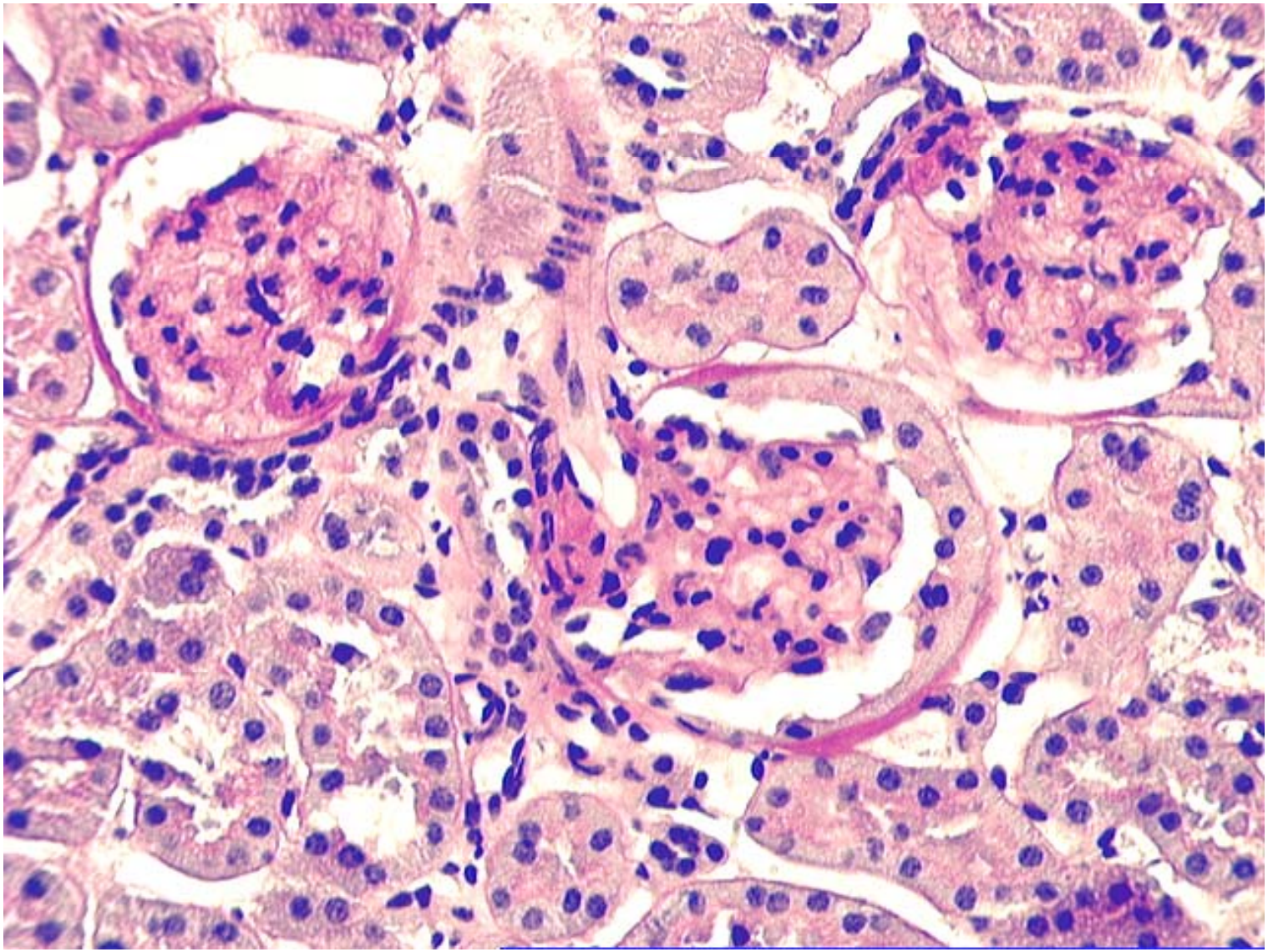
7) Harris, R.C. Diabetic Nephropathy. In Cecil's Textbook of Medicine L.Goldman and D. Ausiello, ed. 2007

8) Breyer, MD, Tchekneva, E, Qi, Z, Takahashi, T, Fogo, AB and Harris, RC. Examining diabetic nephropathy through the lens of mouse genetics. Current Diabetes Reports In Press

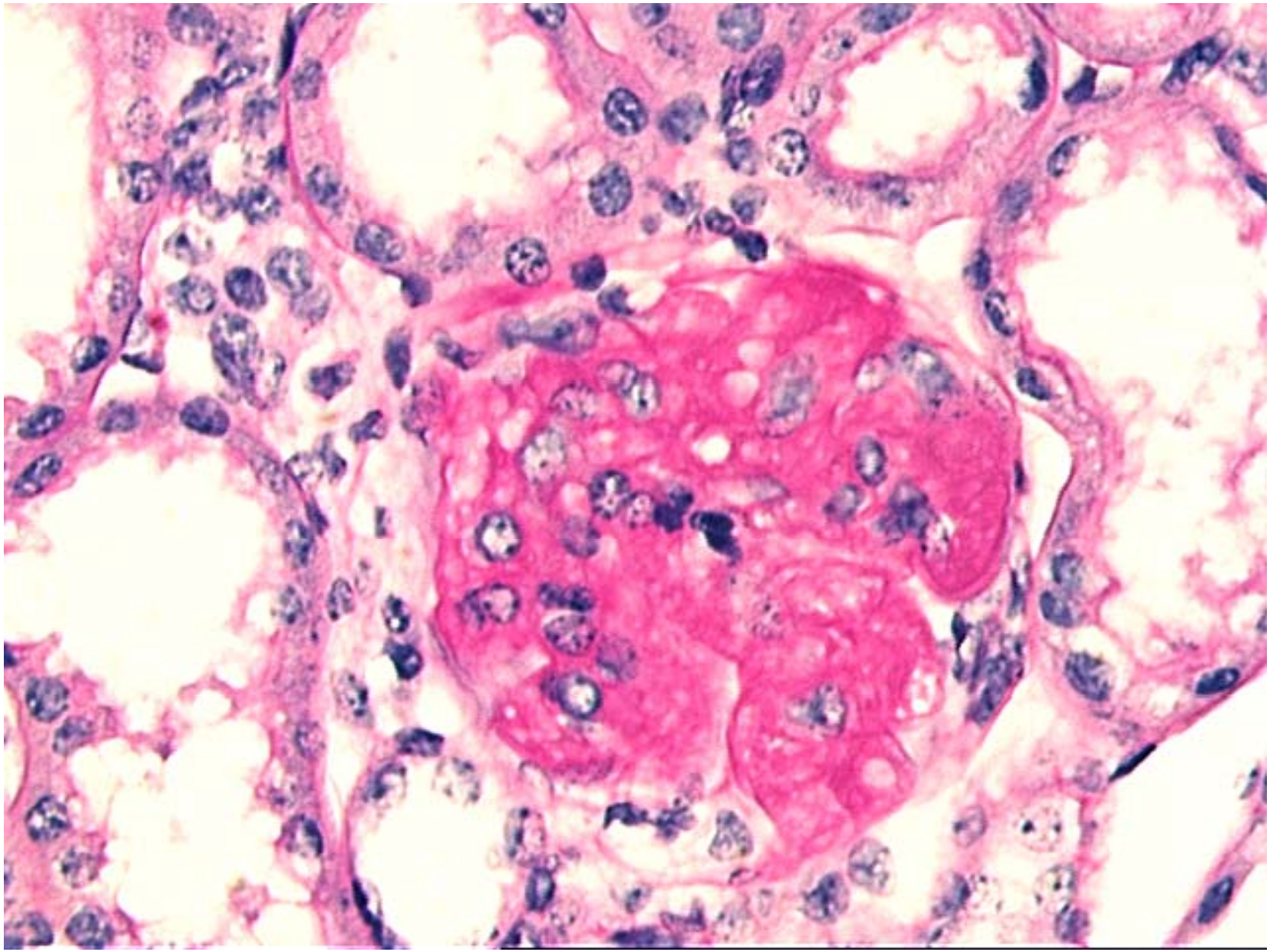
db/db/eNOS-/- 16 wks PAS



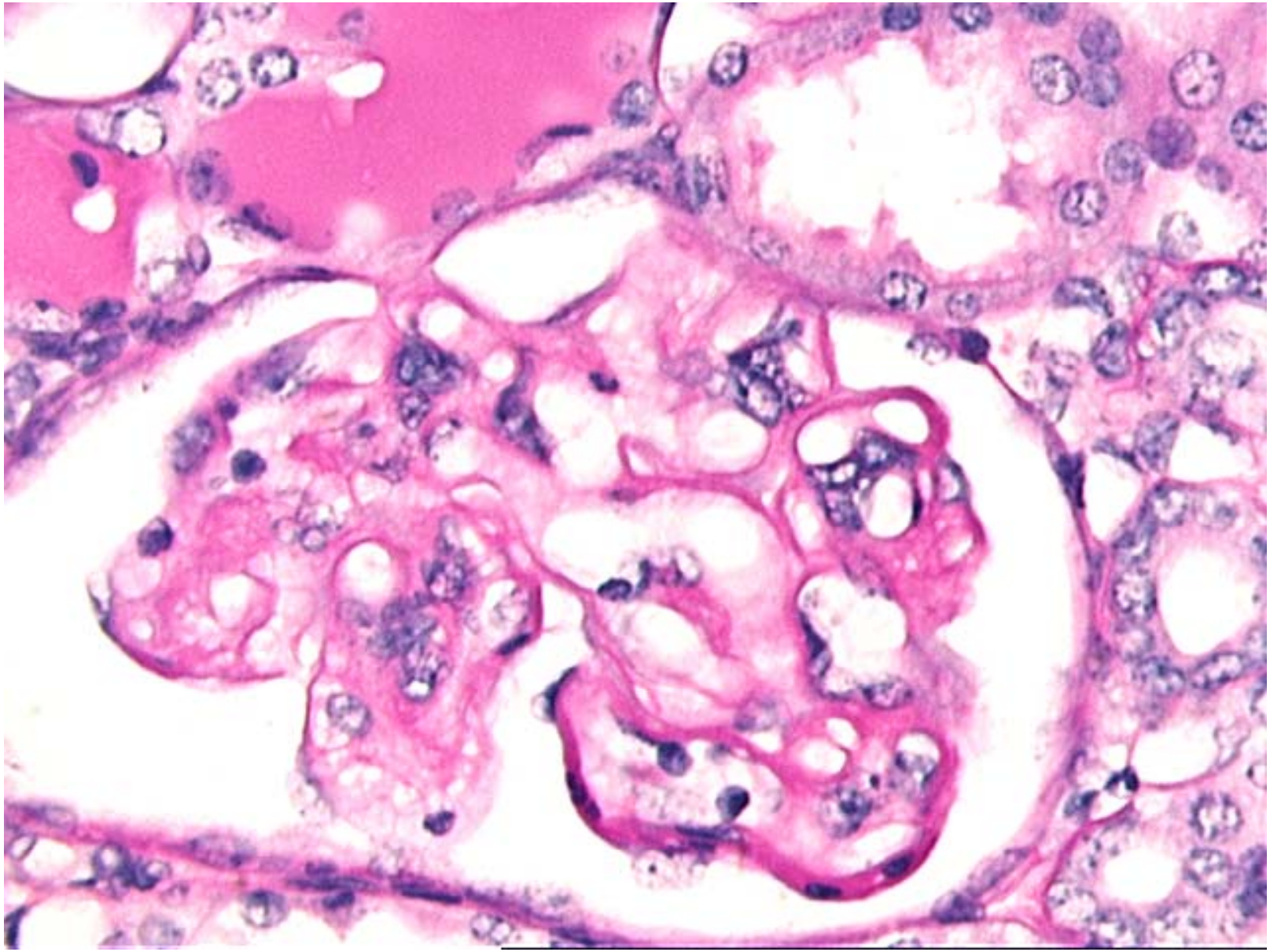
db/db/eNOS-/- 26 wks PAS



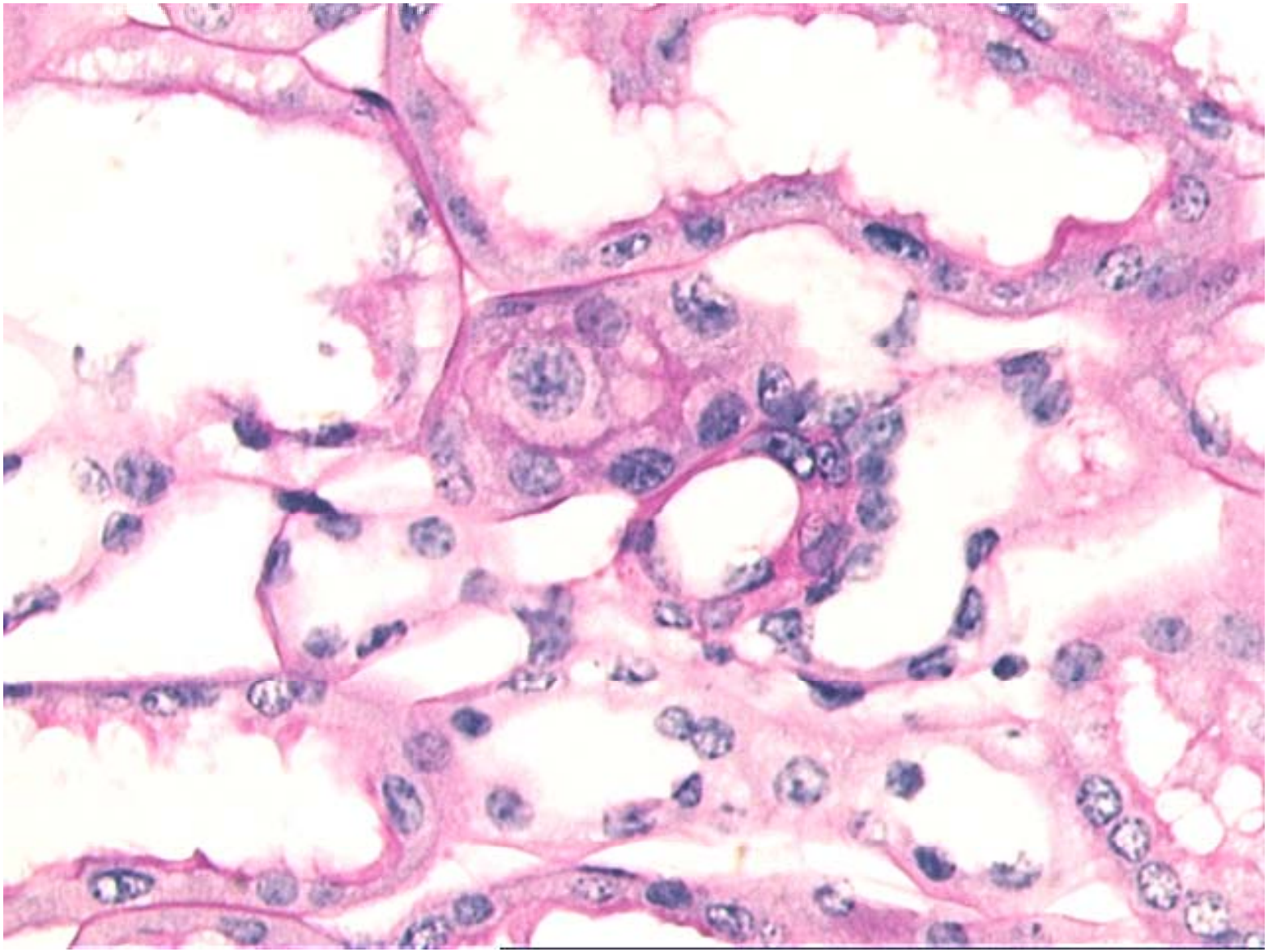
db/db/eNOS-/- 26 wks PAS



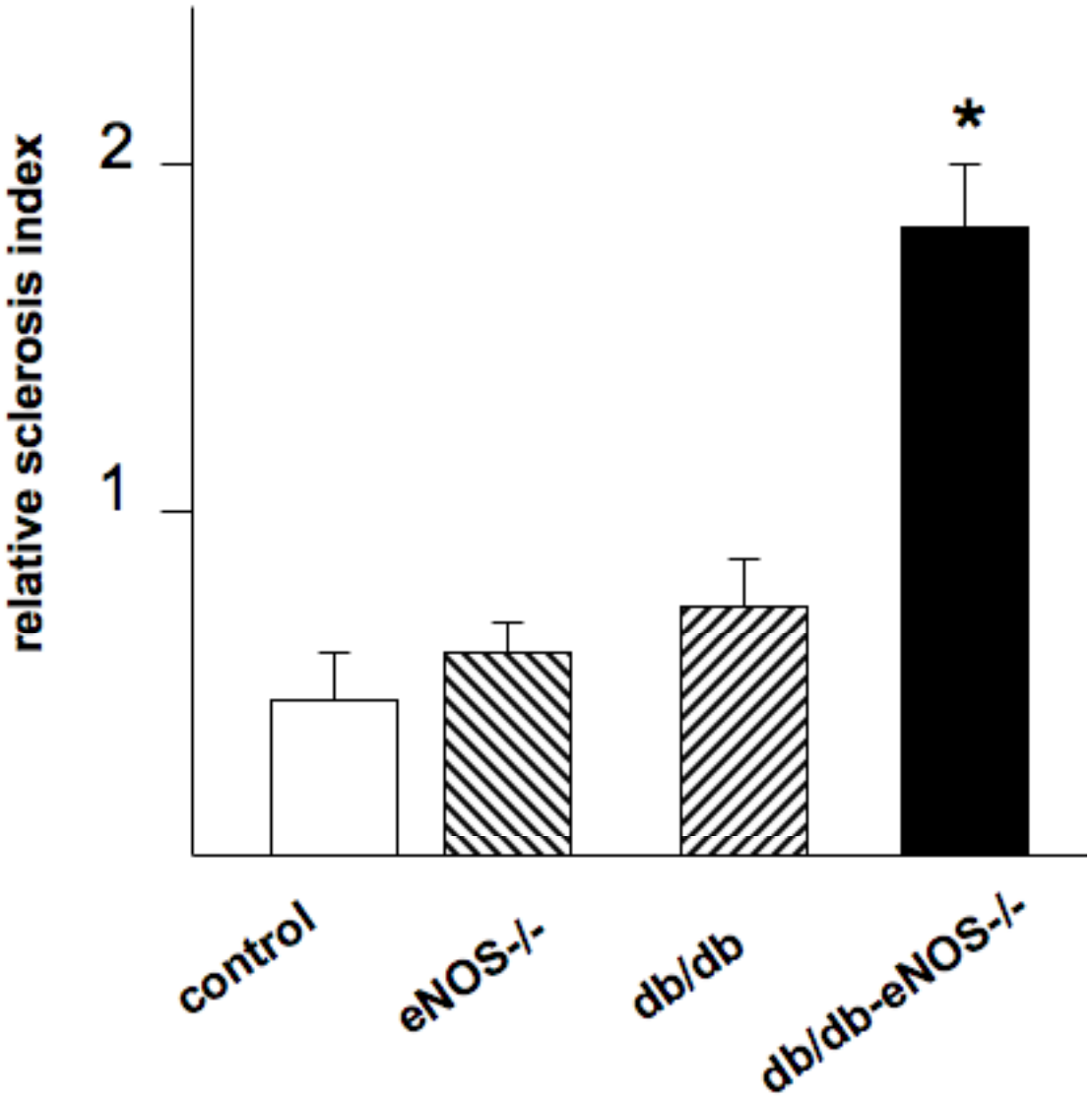
db/db/eNOS-/- 26 wks PAS



db/db/eNOS-/- 26 wks PAS

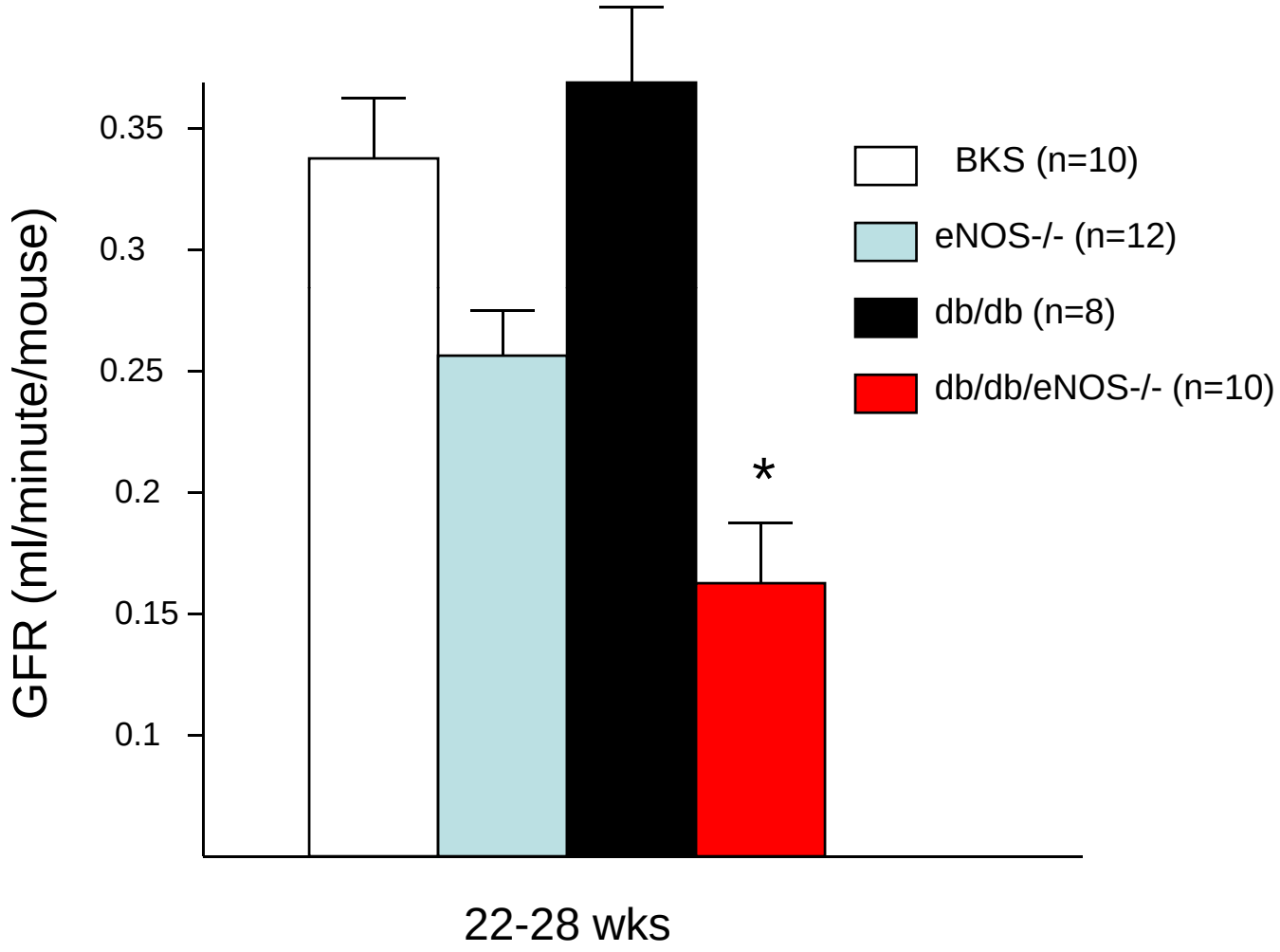


Sclerosis Index- 26 week old mice

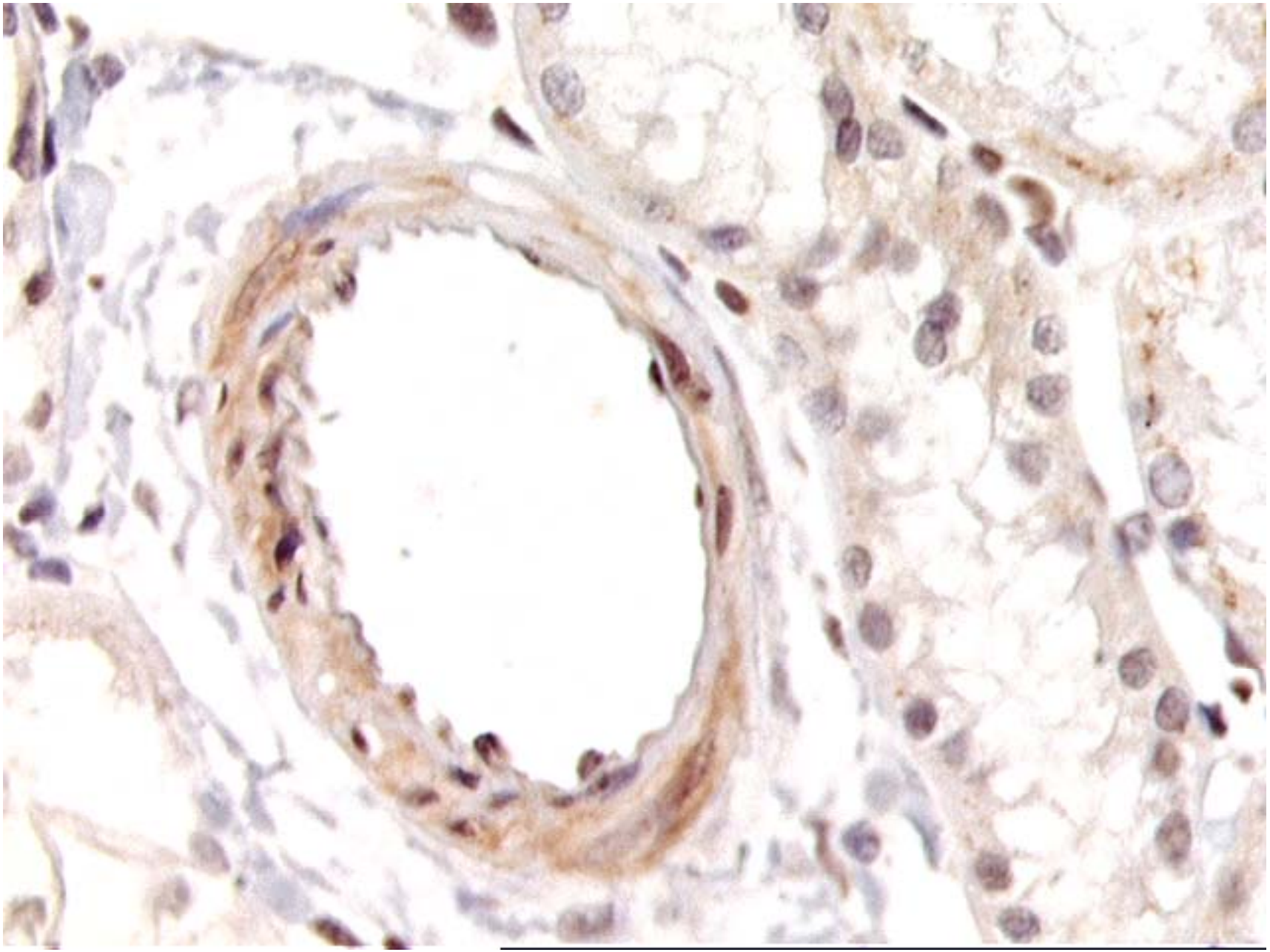


n=6-8

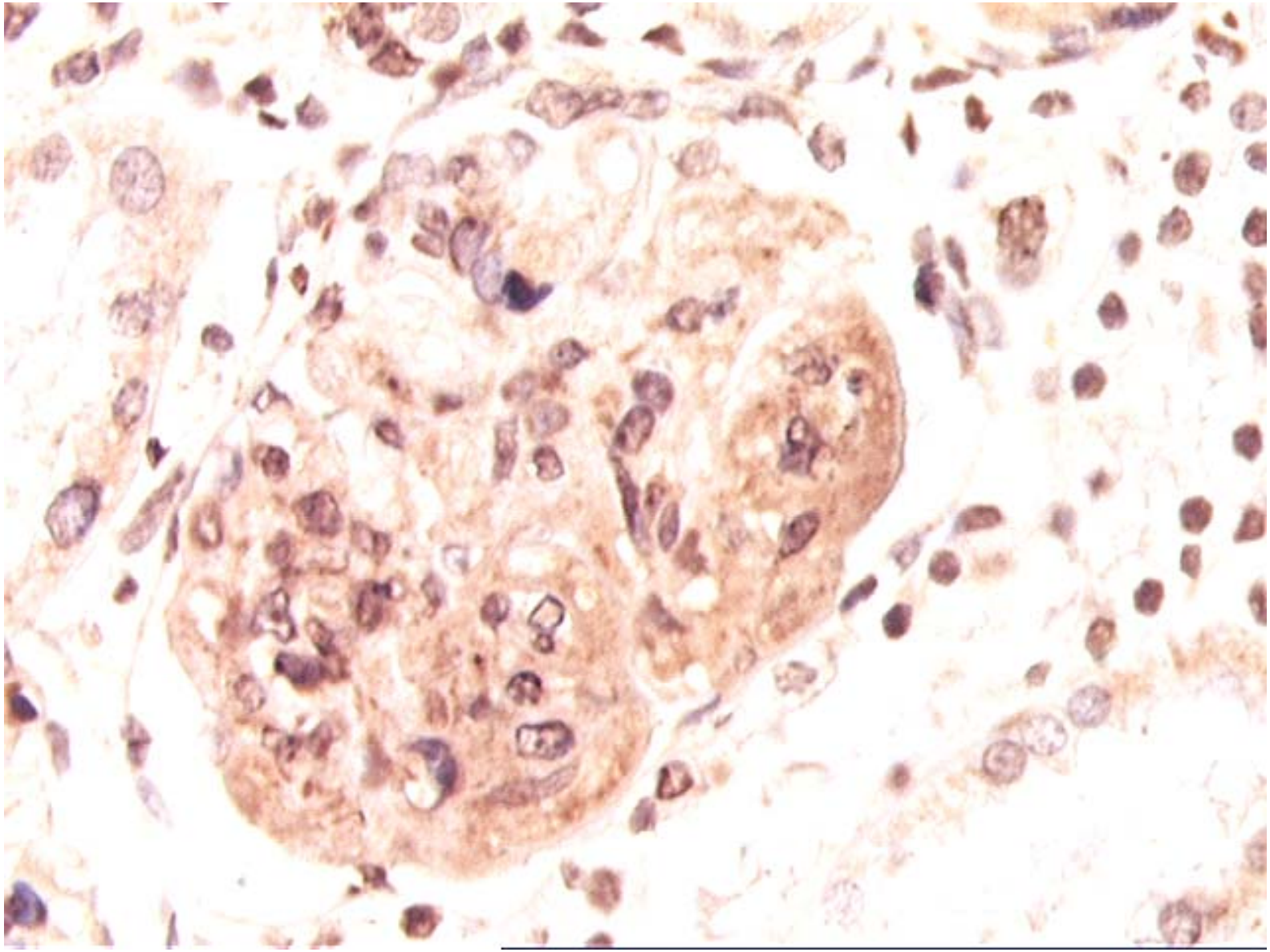
Glomerular Filtration Rate



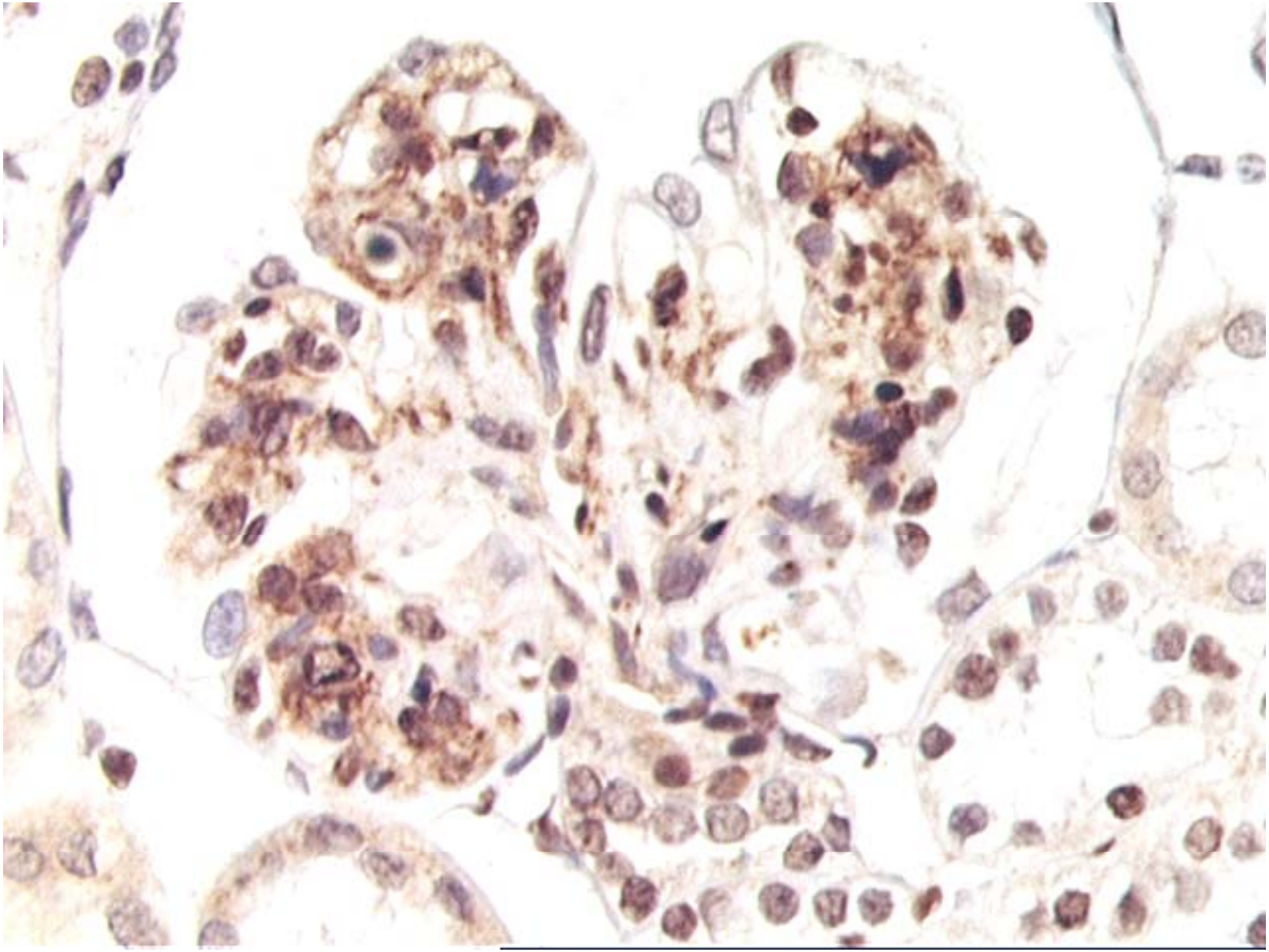
db/db/eNOS-/- 26 wks anti-RAGE antibody



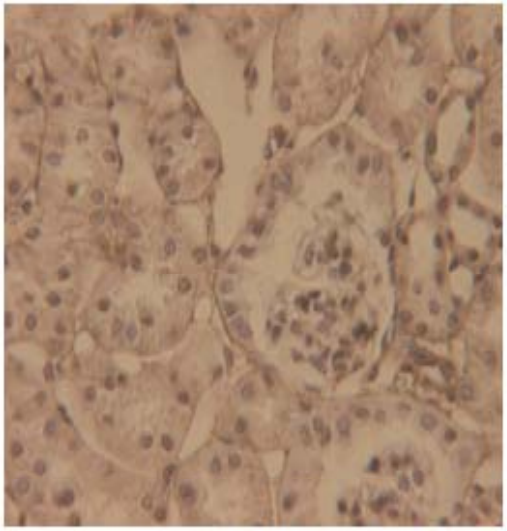
db/db/eNOS-/- 26 wks anti-RAGE antibody



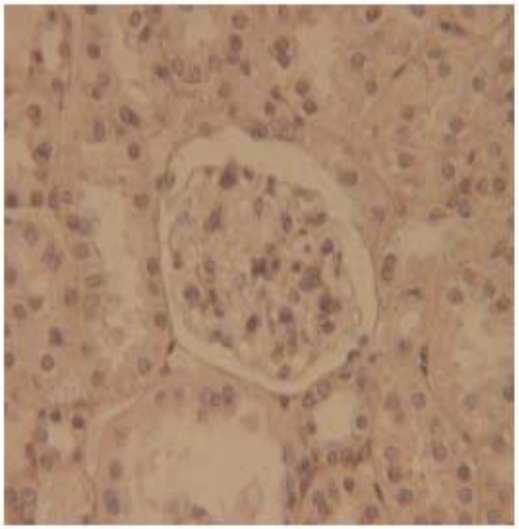
db/db/eNOS-/- 26 wks anti-RAGE antibody



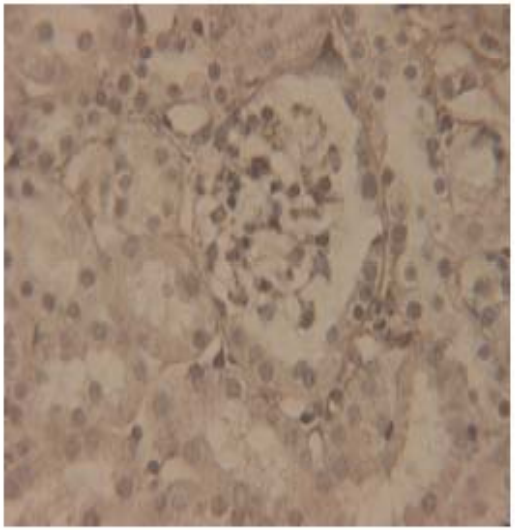
fibronectin



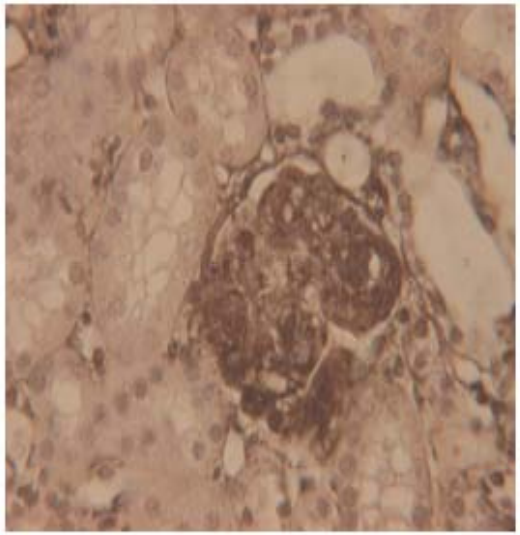
misty/misty



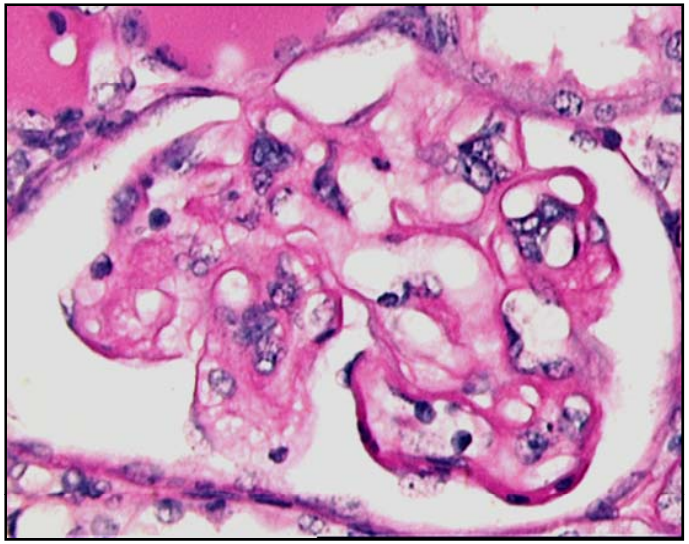
db/db



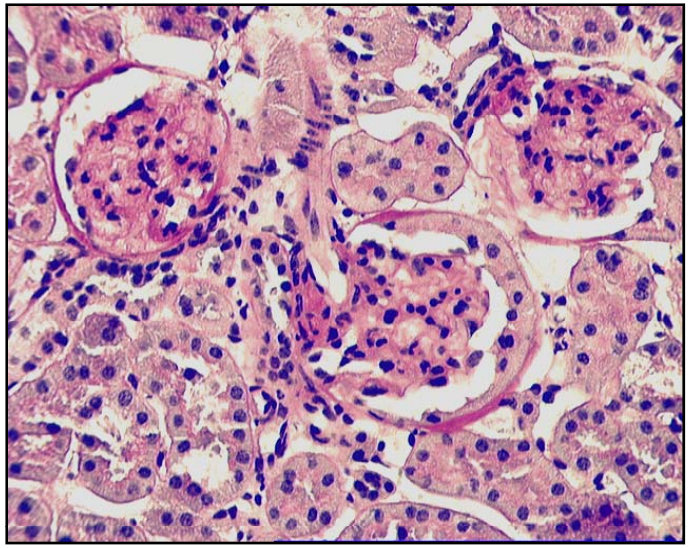
misty/misty / eNOS$^{-/-}$



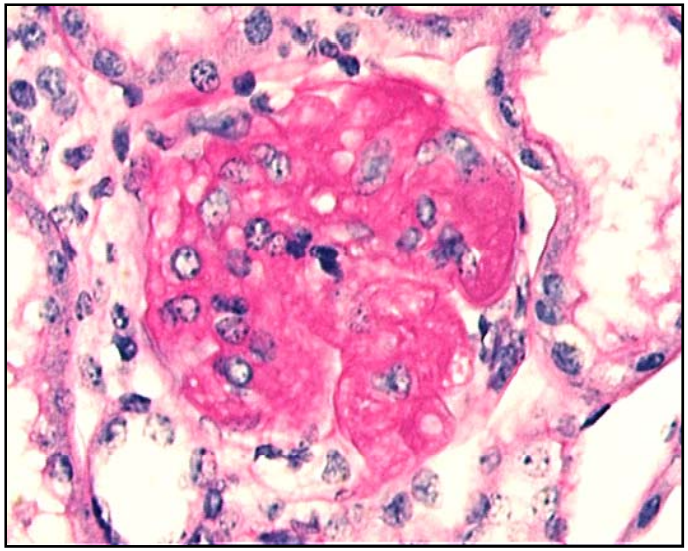
db/db / eNOS$^{-/-}$



db/db eNOS-/- 16 wks



db/db eNOS-/- 26 wks



db/db eNOS-/- 26 wks

Background

- Our previous experiments indicated, nephropathy in eNOS-/- mice was markedly more robust in both type 1 and 2 diabetes.
- On the BLKS background, eNOS-/- mice develop glomerular lesions that closely mimic human diabetic nephropathy, characterized by decreased GFR, massive proteinuria, arteriolar hyalinosis, nodular focal sclerosis and mesangiolysis.
- However, the mechanism by which eNOS inhibition exacerbates development of diabetic nephropathy is still incompletely understood.
- eNOS is expressed in a wide variety of non-endothelial (and non-renal) tissue sites, including cardiac myocytes and adipocytes. eNOS-/- mice develop significant hypertension even in the absence of diabetes and develop progressive arteriolar glomerular sclerosis (the time course and histology appearance are distinct).

- Although our preliminary findings are consistent with our hypothesis that endothelial eNOS dysfunction plays an important role in development of diabetic nephropathy, as well as other diabetic microvascular (and macrovascular) damage, we cannot discount potential contributions by other potential systemic alterations seen in the global eNOS knockout mouse.
- We propose that development of a conditional knockout mouse that will allow lineagespecific eNOS gene disruption would circumvent these problems, offering a unique means to investigate the role of endothelial eNOS-derived NO in diabetic nephropathy.
- Finally, this conditional knockout study may also circumvent the postnatal death in eNOS-/- mice which has made it difficult for us to prepare eNOS-/- diabetic mice.