**Online supplement**

**Supplementary methods and results**

*Obedience of guideline for animal experiments*

The Animal Experimentation Committee of Tokai University and the Institutional Animal Care and Use Committee of Vanderbilt University Medical School approved the protocol, which is in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Laboratory Animals.

*Generation of* Agt-*loxP mice*

A targeting vector was constructed for conditional targeting of the *Agt* gene (Figure S1). The vector contains 8.8 kb *Agt* homologous fragment containing exons 2 and 3 and a portion of exon 4. One loxP site was inserted at the *BglII* site, ~0.2 kb upstream to exon 2, which encodes the sequence of AII. At the *XhoI* site in intron 2, a neomycin resistant gene cassette (*pgk-neo*) flanked by two loxP sites was inserted. For negative selection, an expression cassette for herpes simplex virus thymidine kinase (*pgk-tk*) was attached at the 3' end of the vector. The resultant targeting vector was introduced into E14.1 cells by electroporation. 544 G418 resistant ES colonies were picked up and analyzed by Southern blot analysis. 30 clones (2.4 %) were revealed to have undergone homologous recombination. We selected one clone, and expanded it for experimental use. To delete *pgk-neo*, a Cre expression vector, *pCre-Pac* plasmid (Kurabo, Osaka, Japan, carrying puromycin-resistant gene) was transiently introduced into the ES clone by electroporation. 222 puromycin-resistant colonies were analyzed by Southern blot analysis, of which 73 colonies had desired recombination, i.e., *pgk-neo* was deleted while *Agt* exon 2 was preserved. The loxP insertion was confirmed by PCR amplification and sequencing. This allele is hereafter designated as $Agt^{loxP}$. One of the targeted ES cell clones was used for injection into C57BL/6 blastocysts. Four of the 6 chimeric male mice obtained showed germline transmission of $Agt^{loxP}$ when mated with C57BL/6 females. Genotyping for *Agt* was performed by PCR using primers, CATGGTGAGTTCAAGACCAGCTGG and TCCGGGTGGAAAGCACACTCATCC, which generate 200 base pair (bp) band for wild-type *Agt* and 240 bp band for $Agt^{loxP}$. Heterozygous ($Agt^{loxP/+}$) mice were backcrossed with C57BL/6 strain for more than 10 times before mating with *KAP-Cre* or *Alb-Cre* mice.

Homozygous ($Agt^{loxP/loxP}$) mice without Cre gene showed normal phenotypes. Systolic blood pressure, *Agt* mRNA in the kidney and the liver, and renal histology were not different from those of wild-type mice (Figure S4), indicating that insertion of the loxP sites does not affect *Agt* gene expression.

*Generation of tissue-specific* Agt *KO mice*

A 4 kb promoter fragment spanning from *XbaI* site to 20 bp upstream to the initiation codon was subcloned from a plasmid clone containing mouse kidney androgen regulated protein (KAP) genome (generous gift by Dr. Uchida). This fragment was combined with a rabbit β globin intron fragment with splice donor and acceptor sites and the Cre gene. The resultant transgene fragment was microinjected into fertilized eggs obtained from mating between C57BL/6N X DBA2 F1 and C57BL/6N mice.

Five *KAP-Cre* transgenic mice were obtained. Heminephrectomy and immunostaining for Cre were performed for each mouse. One transgenic line with highest Cre expression in the proximal tubule was chosen and used for subsequent studies. This line was backcrossed with C57BL/6N line 10 times before mating with mice carrying *Agt-loxP*.

Efficiency of Cre-mediated recombination by *KAP-Cre* was tested by mating with *CAT-Z*, a tester strain. In adult male *KAP-Cre/CAT-Z* double transgenic mice, ~80 % of proximal tubular cells of the S3 segment were stained for the positive marker β galactosidase (Figure S2).

*KAP-Cre* mice were mated with $Agt^{loxP/+}$ mice to generate *KAP-Cre*/$Agt^{loxP/loxP}$. Since KAP promoter is androgen dependent, only male mice were used in the present study. To enhance renal *Agt* deletion, all mice were subcutaneously injected with testosterone (50 μg/g BW, 5 days) more than two weeks before experiments and used as kidney *Agt* KO mice. Mice with other genotypes, when compared with mice carrying *KAP-Cre*, were also treated with testosterone in the same manner.

PCR was performed to detect the Cre transgenes, using the following primers. For *Alb-Cre*, AGGACATGGACAAGGTCGAG and TGGAGTGGCAACTTCCAAG; for *KAP-Cre*, GTCCATGGTGATACAAGGGACATC and CATAAAGGTCCTTCCCAAACCCCT.

*RT-PCR*

Total RNA was extracted from the kidney, and then, cDNA was synthesized. Using TaqMan primer probe sets and Applied Biosystems 7300 Real Time PCR Systems, relative amount was determined for *Agt* mRNA and 18S rRNA, using a single standard RNA extracted from a wild-type kidney. Data are shown as percentage of the median of each control sample.

*Western analysis*

Kidney homogenates were separated by SDS-PAGE and blotted to PVDF membrane. Rabbit polyclonal anti- mouse/rat Agt antibody (IBL, Japan) and rabbit anti-mouse βactin antibody (Sigma) were used at X800 and X5000 dilution, respectively.

*Measurement of Agt protein*

Agt protein concentration was determined in 24 hour-urine or EDTA-plasma, using a specific ELISA

kit (IBL). Specificity of this assay was verified by observing undetectable level of plasma Agt from whole body *Agt* KO mice.

*Physiological analysis*

Conscious systolic blood pressure was measured by tail cuff method using MK-2000 (Muromachi Kikai, Tokyo, Japan). Twenty-four-hour urine specimens were collected using metabolic cages. Concentrations of creatinine and albumin in the urine were determined by enzymatic method and immunonephelometry method, respectively, in an outside laboratory (SRL, Tokyo, Japan).

*Histological analysis*

Kidney samples were fixed in 4 % buffered paraformaldehyde and embedded in paraffin. 2-μm sections were used for PAS staining and immunostaining. Rabbit polyclonal anti mouse/rat Agt antibody (IBL, Japan), rabbit polyclonal anti megalin antibody, and rabbit anti mouse renin antibody (generous gift from Dr. Inagami of Vanderbilt University, U. S. A.) were used in the present study. For Agt, slides were heated at 100°C for 15 minutes.

**Supplemental Figure Legends**

**Figure S1**

Generation of ES cells carrying $Agt^{loxP}$

(A) Wild-type *Agt* gene. AII peptide is encoded in exon 2. (B) Targeting vector. One loxP site (shown by triangle) was inserted at the *BglII* site (BII), about 0.2kb upstream to exon 2. At the *XhoI* site in intron 2, a neomycin resistant gene cassette ($Neo^r$) flanked by two loxP sites was inserted. (C) $Agt^{loxP\text{-}Neo}$ allele, which is obtained by homologous recombination. (D) $Agt^{loxP}$ allele, which is obtained by Cre-loxP mediated deletion of the $Neo^r$ cassette. (E) Southern bot analysis for ES cell screening. With *XbaI* and *SalI* digestion, probe 1 detects 3.3 kb band in $Agt^{loxP\text{-}Neo}$ and 5.2 kb band in wild-type *Agt*. Clone 77 (left lane) was found to carry $Agt^{loxP\text{-}Neo}$. (F) With *EcoRV* digestion, probe 2 detects 7.7 kb band in $Agt^{loxP\text{-}Neo}$ and 12 kb band in wild-type *Agt*. These two clones carry $Agt^{loxP\text{-}Neo}$. (G) ES cells carrying $Agt^{loxP\text{-}Neo}$ were transfected with pCre-Pac plasmid to delete $Neo^r$. With *EcoRV* digestion, probe 2 detects 12 kb band in $Agt^{loxP}$, which is identical to the band of wild-type *Agt*. The clone of the middle lane was found to carry $Agt^{loxP}$. In the left lane, $Neo^r$ was not deleted, and in the right lane, both exon 2 and $Neo^r$ were deleted. (H) With *BglII* digestion, probe 3 detects 2.8 kb band in $Agt^{loxP}$ and 2.1 kb band in wild-type *Agt*. Clone 7 (right lane) was found to carry $Agt^{loxP}$.

*Xb*, *XbaI*, *EV*, *EcoRV*, *BII, BglII*. Only relevant restriction enzyme sites are shown.

**Figure S2**

PTC-specific Cre recombination activity in KAP-Cre mice

KAP-Cre transgenic mouse line was mated with a tester strain, CAT-Z, and kidneys of adult male double transgenic mice were counterstained with either nuclear fast red (A) or PAS (B). (A) Cre recombinase activity shown by blue staining was observed predominantly in the outer stripe of outer medulla and the medullary ray. (B) High magnification picture of the boundary of the inner and outer stripes of the outer medulla. Blue lacZ staining was observed in the S3 segment, which was characterized by its brush borders. Non-PTC cells in the kidney and non-renal cells were not stained. Approximately 80% of the S3 segment, where *Agt* mRNA is predominantly synthesized, was positive for lacZ. When generating kidney *Agt* KO mice, adult male mice were treated with testosterone to increase the efficiency of *Agt* gene disruption.

**Figure S3**

Representative amplification curves of real-time RT PCR analyses for *Agt* mRNA

Real time RT-PCR was performed for *Agt* mRNA on the RNA extracted from the whole kidney of

KAP-Cre/*Agt*$^{loxP/loxP}$ (kidney *Agt* KO) and control (*Agt*$^{loxP/loxP}$) mice. Data from 5 control mice and 10 kidney *Agt* KO mice, which showed similar 18S RNA amplification, are presented. Data of *Agt*/18S RNA ratio from all samples (9 control and 15 kidney Agt KO samples) are shown in Figure 1A.

**Figure S4**

Real-time RT PCR analyses for *Agt* mRNA

Control mice carrying *Agt*$^{loxP/loxP}$, but not Cre gene (Cont), have *Agt* mRNA/18S rRNA level similar to that of wild-type mice (WT) in both the kidney and the liver. In the liver of liver *Agt* KO mice (Liv KO), *Agt* mRNA/18S rRNA is decreased to 0.2 % of that in control mice.

Horizontal bars represent median values.

**Figure S5**

Renin immunostaining.

In control and kidney *Agt* KO mice, only a few juxtaglomerular cells were stained for renin. In liver *Agt* KO mice, renin staining was markedly extended proximally in the afferent arteriole. Scale bar, 50 μm.

**Figure S6**

Histology of NEP25 mice

NEP25 mice were injected with either 1.25 ng/g BW of LMB2 or saline. Seven days later, although mice injected with LMB2 showed massive proteinuria, light microscopic findings were unremarkable.

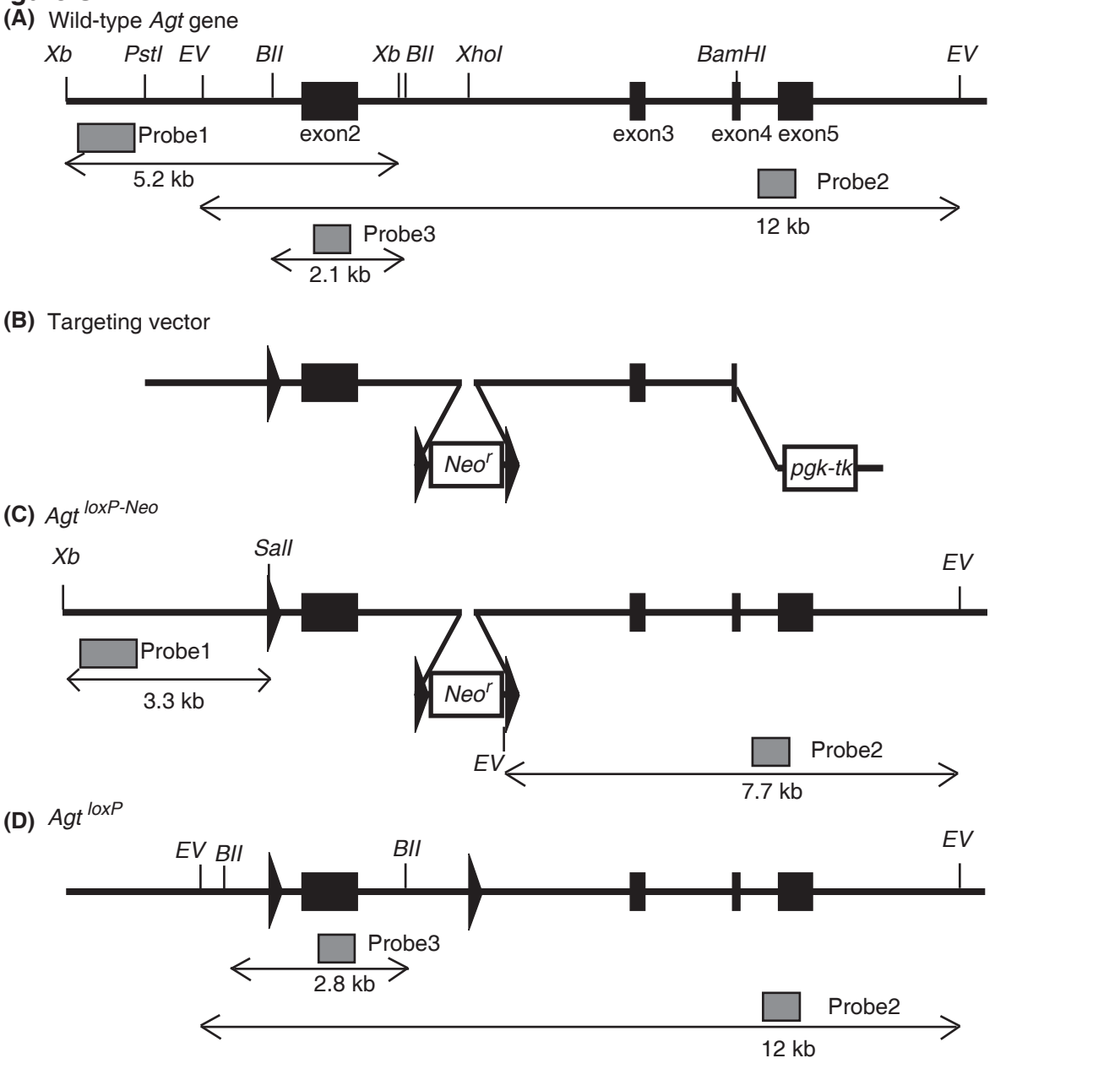
Scale bar, 50 μm.

**Figure S7**

Compensatory increase in Agt reabsorption by megalin-intact tubules of mosaic megalin KO mice

Kidneys from mosaic megalin KO and control mice were embedded in the same paraffin block. Serial sections were stained for Agt and megalin. Representative pictures of deep cortex are shown. In control mice, all proximal tubule cells uniformly express megalin (B). Although faint, Agt staining is seen in the adjacent sections (A). In mosaic *megalin* KO mice, approximately half of proximal tubule cells express megalin (D). These megalin-intact cells incorporate more Agt than those in control mice (C vs A). Scale bar, 50 μm.

**Figure S1**


(A) Wild-type Agt gene
Xb
PstI
EV
BII
Xb
BII
XhoI
BamHI
EV
Probe1
exon2
exon3
exon4
exon5
5.2 kb
Probe2
12 kb
Probe3
2.1 kb
(B) Targeting vector
Neo^r
pgk-tk
(C) Agt loxP-Neo
Xb
SalI
EV
Probe1
3.3 kb
Neo^r
EV
Probe2
7.7 kb
(D) Agt loxP
EV
BII
BII
EV
Probe3
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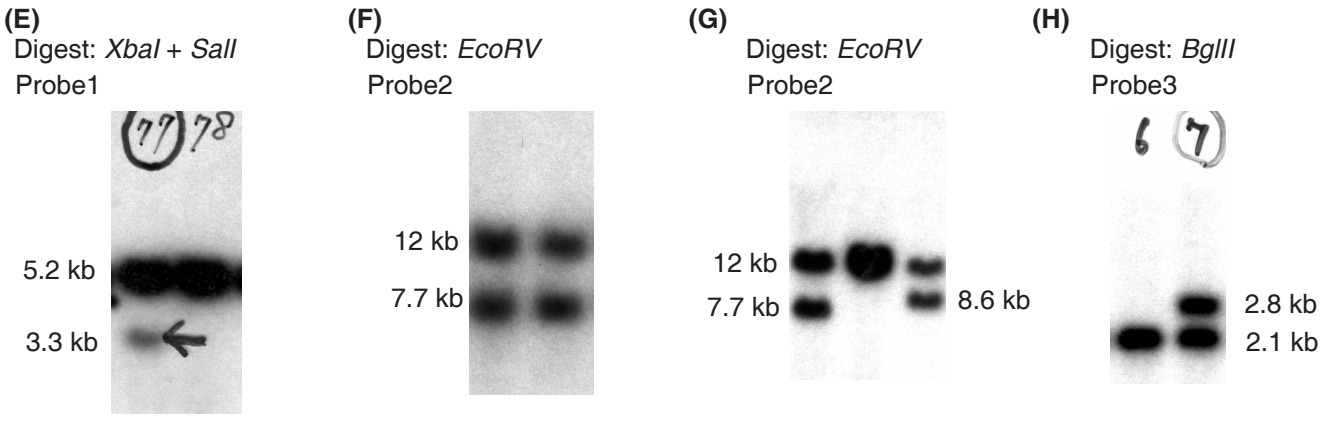
Probe2
12 kb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(F)
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Probe2
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(G)
Digest: EcoRV
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2.1 kb

# Figure S2

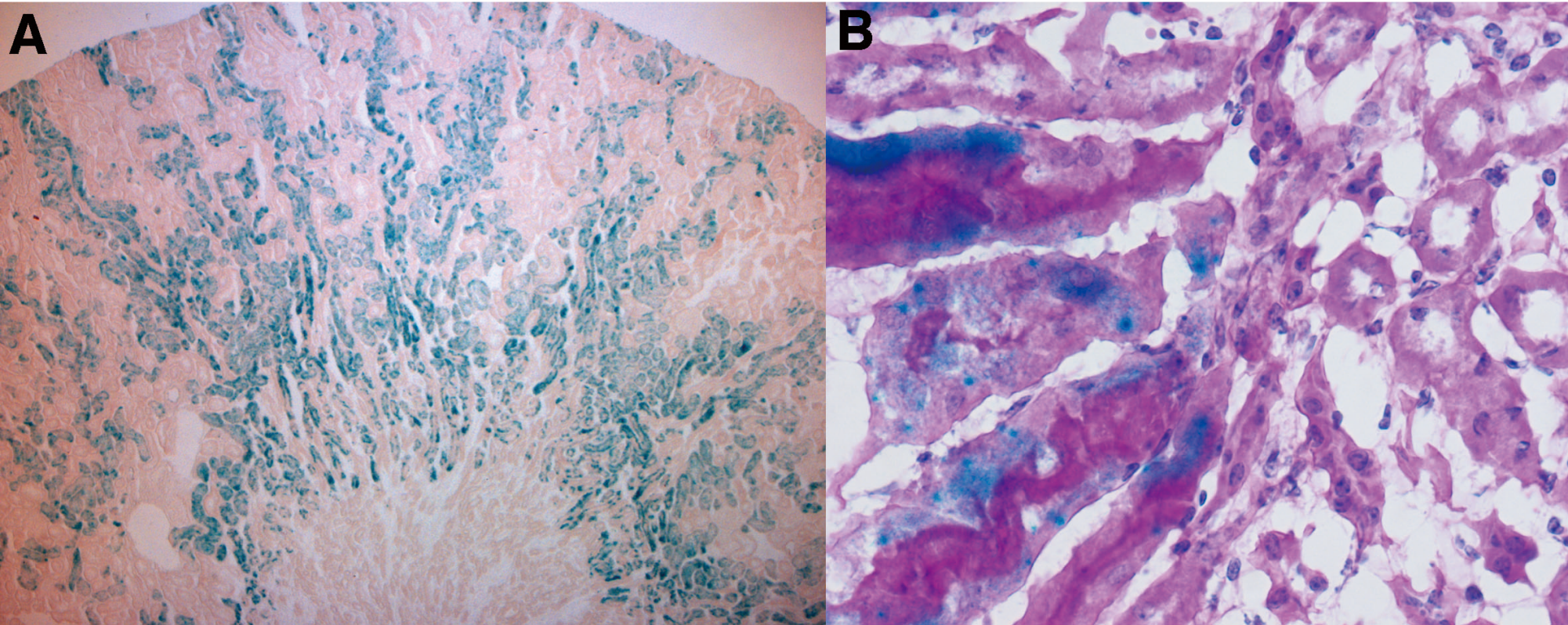
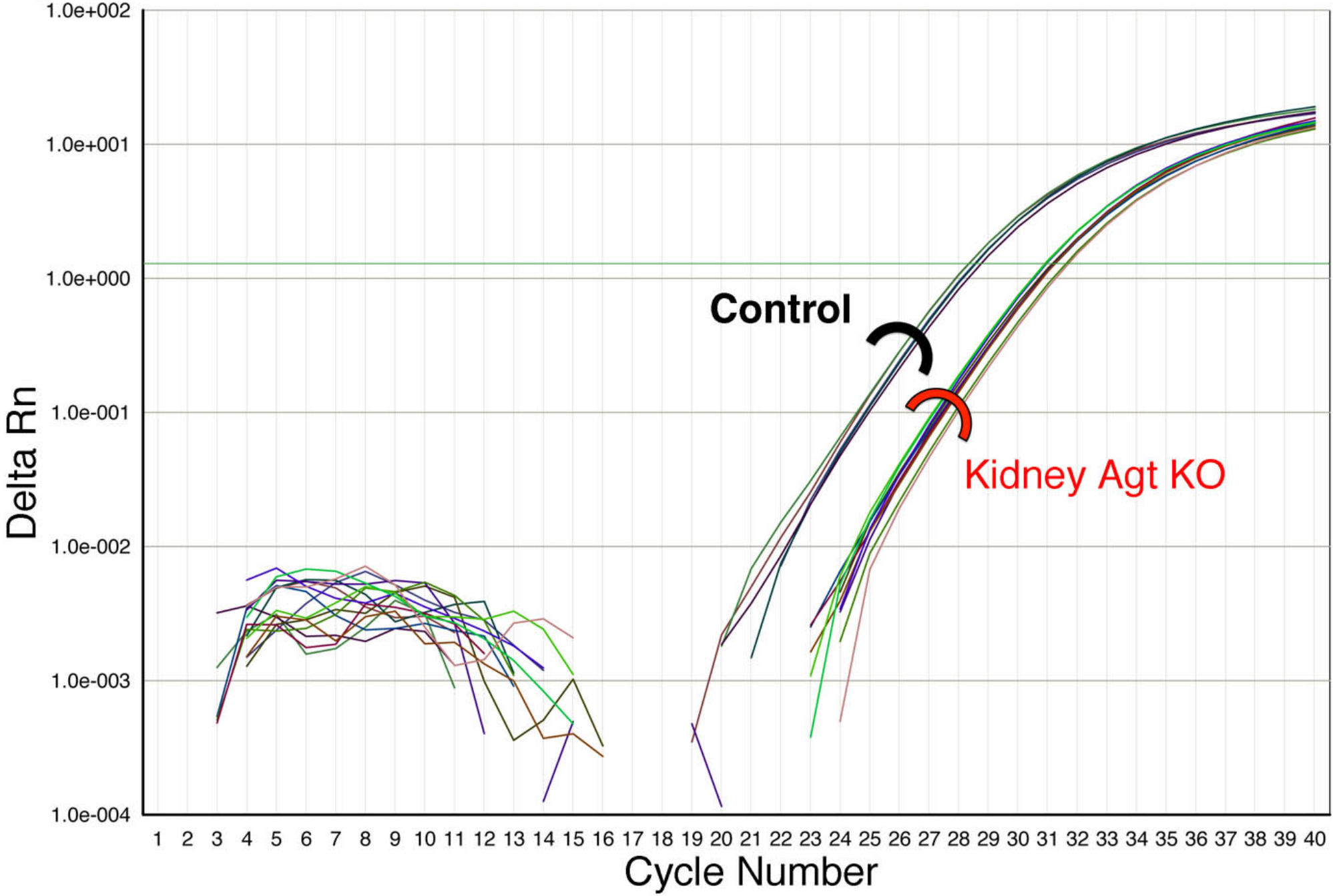


**Figure S3**


1.0e+002
1.0e+001
1.0e+000
1.0e-001
1.0e-002
1.0e-003
1.0e-004
Delta Rn
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Cycle Number
Control
Kidney Agt KO

# Figure S4

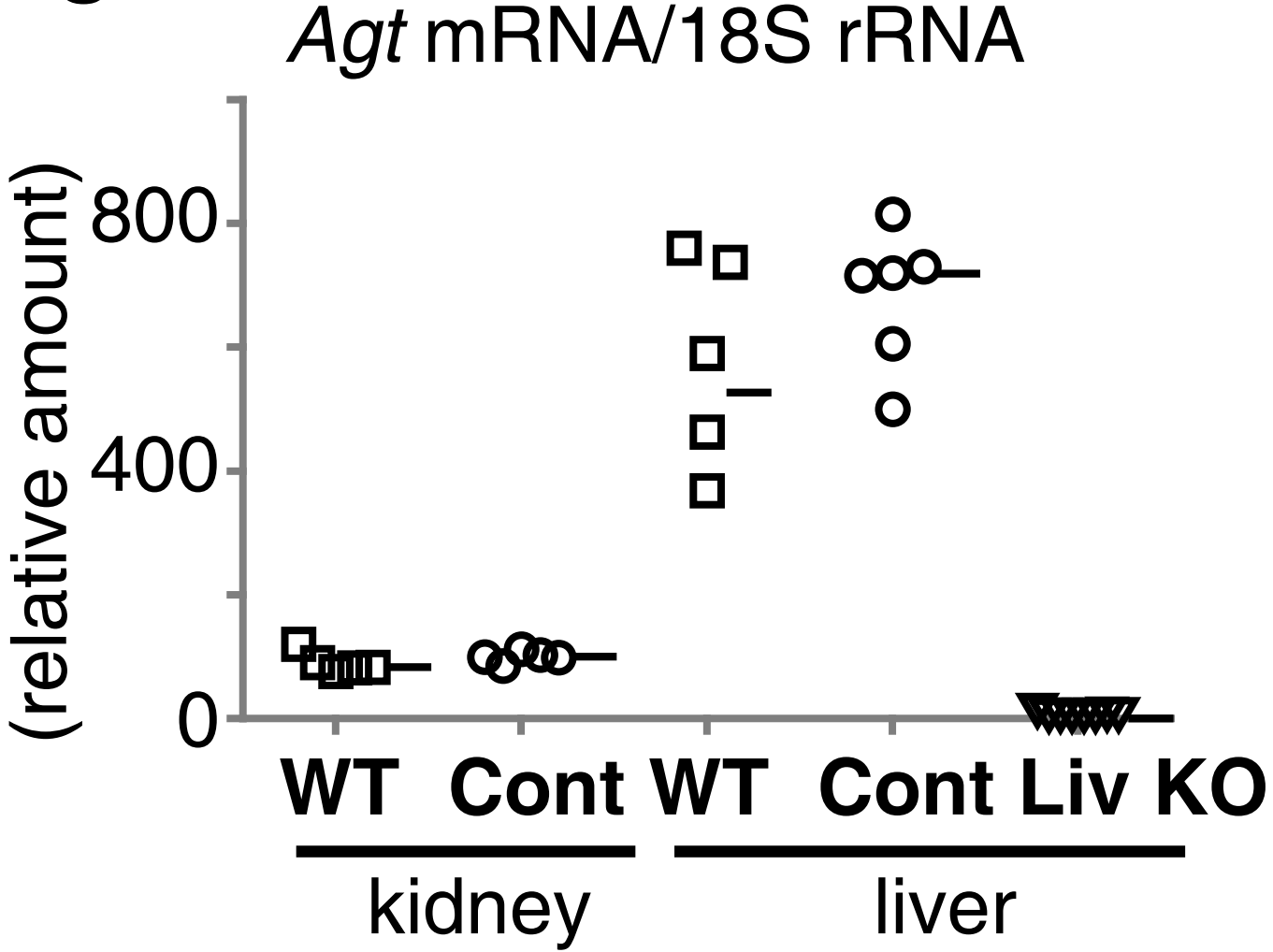
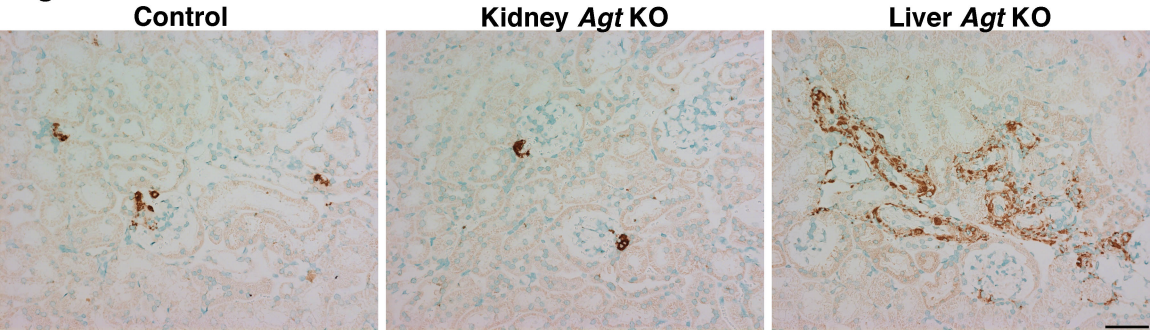
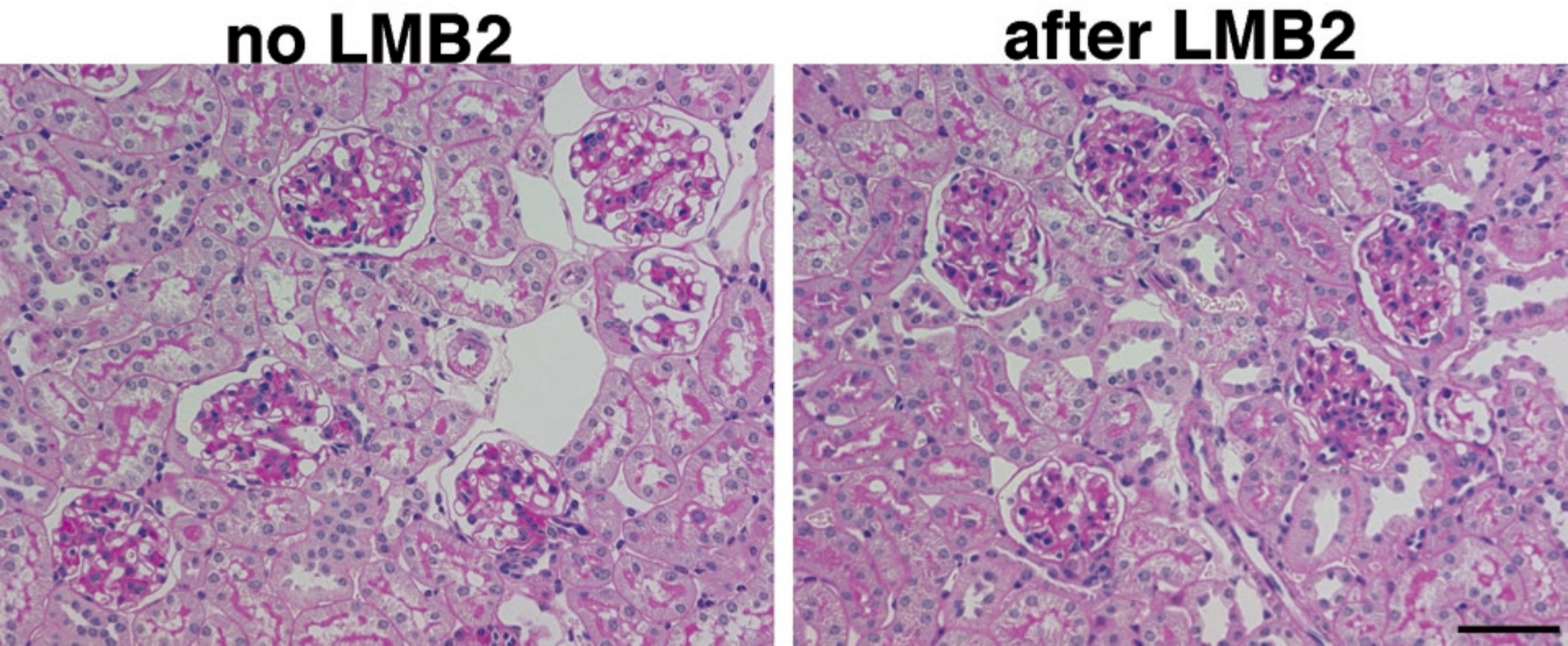

*Agt* mRNA/18S rRNA
(relative amount)
800
400
0
WT
Cont
WT
Cont
Liv KO
kidney
liver

**Figure S5**



# Figure S6



# Figure S7