Podocytes play an indispensable role as a filtration barrier for macromolecules in the glomerulus. Damage of podocytes is a key step triggering the progression of glomerulosclerosis. A large volume of evidence indicates that angiotensin (Ang) II acting on the Ang II type 1 receptor (AT₁) plays important roles in this process. Blockade of Ang II synthesis with Ang I–converting enzyme (ACE) inhibitors or of Ang II action with AT₁ receptor blocker (ARB) is a clinically established therapeutic measure for slowing the progression of chronic kidney diseases. ACE inhibitors and ARBs have been shown to attenuate podocyte damage, proteinuria, and development of glomerulosclerosis in a variety of animal models, including, among others,1–7 the subtotal nephrectomy model8 and diabetic nephropathy models.9,10

Continuous infusion of Ang II in normal rats increased desmin expression11 and suppressed nephrin and podocin mRNA.6 Studies conducted thus far collectively indicate that Ang II is involved in triggering, enhancing, and expanding podocyte injury and in the progression of glomerular injury toward sclerosis through mechanisms beyond its effect on systemic blood pressure.

Because podocyte injury along with proteinuria ubiquitously precedes progressive development of glomerulosclerosis, and because Ang II inhibition attenuates podocyte damage and progressive glomerulosclerosis, it appears reasonable to speculate that inhibition of AT₁ on podocytes is the key to the protective effect of pharmacological Ang II blockade. In fact, several lines of evidence indicate that Ang II has direct cellular effects on podocytes. Cultured mouse podocytes express mRNA and protein for AT₁,5,12 Podocytes in isolated glomeruli express functional AT₁, and Ang II depolarizes and increases intracellular Ca²⁺.13,14 In cultured podocytes, Ang II, via AT₁, increases α3(IV) collagen and...
vascular endothelial growth factor, decreases nephrin, heparan sulfate proteoglycans, and α-actinin 4; augments reactive oxygen species production; and induces redistribution of zona occludens 1 and reorganization of F-actin cytoskeleton. More directly, transgenic rats overexpressing the AT1 receptor selectively in podocytes develop glomerulosclerosis. These data are consistent with the notion that the beneficial effect of Ang II blockade on glomerulosclerosis is attributed to its direct inhibitory effects on Ang II action on podocytes.

Earlier, we established a transgenic mouse line (NEP25) that expresses human (h) CD25 (ie, interleukin 2 receptor) selectively on podocytes. Because hCD25 does not react with mouse interleukin 2 ligand, it is highly unlikely that expression of hCD25, per se, affects podocyte function, including Ang II signaling. By injecting an hCD25-targeted recombinant immunotoxin, anti-Tac(Fv)-PE38 (LMB2), podocyte-selective injury can be induced in NEP25 mice. LMB2 is a recombinant chimeric protein composed of PE38 (a mutant form of pseudomonas exotoxin A) and the Fv domain of monoclonal antiCD25 antibody. LMB2 (mass: 63 kDa) can cross the glomerular basement membrane, and its half-life in the circulation is 35 minutes in mice. After a single injection of LMB2 (at 0.625 ng/g of body weight [BW]), NEP25 mice develop moderate proteinuria, which peaks 1 to 2 weeks after the injection and gradually decreases. Within 2 weeks, NEP25 mice have minor podocyte injury. After 3 weeks, they show progressive damage of podocytes and other glomerular cells and develop focal segmental glomerulosclerosis.

To clarify the role of Ang II on podocytes during the progression of glomerulosclerosis, we generated podocyte-specific AT1−/− deficient mice. Unlike humans, mice have 2 AT1 receptor genes, Agtr1a and Agtr1b, each encoding AT1A and AT1B receptor subtype, respectively. In the mouse kidney, >99% of AT1 mRNA is derived from Agtr1a, and Agtr1a inactivation does not lead to activation of Agtr1b. We, therefore, used podocyte-specific Agtr1a null-mutant mice.

### Methods

The Tokai University Animal Experimentation Committee approved the protocol, in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Generation of Podocyte-Specific Agtr1a Null-Mutant Mice

Detailed methods for generation of Agtr1aloxp mice (C57BL/6 background) are described in the online Data Supplement (please see http://hyper.ahajournals.org.) Nephrin-Cre mice were reported previously. In the present study, line 10 of Nephrin-Cre mice was used. They were backcrossed with the C57BL/6 strain >3 times and used for mating with Agtr1aloxp mice. To induce podocyte-specific injury, mice carrying Agtr1aloxp and Nephrin-Cre were further mated with NEP25 mice on the C57BL/6 genetic background.

### Determination of Genotype of Podocytes

From mice carrying the Agtr1aloxp/+;Crt(+) /TRE-SV40T/podocin-rTA/ROSA26loxP, or Agtr1aloxp/+;Crt(+) /TRE-SV40T/podocin-rTA/ROSA26loxP, genotype, glomeruli were isolated by perfusing with Dynabeads (Dynal ASA). Glomeruli were cultured on laminin-coated dishes in the presence of doxycycline (1 μg/mL) for 5 days. Sprouting cells were sparsely replated and cultured until they formed colonies. Cells were then fixed in 2% glutaraldehyde/PBS for 10 minutes and stained for lacZ in a staining solution (2 mmol/L of K3Fe(CN)6, 5 mmol/L of K4[CN]6, and 1 mg/mL of 5-bromo-4-chloro-3-indolyl β-D-galactoside in PBS [pH 7.4]) at 37°C for 4 hours. After washing with PBS, isolated colonies were surrounded by O-rings, and cells were lysed in a solution (10 mmol/L of Tris-HCl, 1 mmol/L of EDTA, 1% Tween 20, and 0.4 mg/mL of proteinase K) at 55°C for 12 hours. The lysate containing genomic DNA was harvested, heated to inactivate proteinase K, and used as a template of PCR.

The following 3 primers were used: AT5, ATCCCTGAATGCTATTTTGT (Figure 1). Embryonic stem (ES) cell DNA carrying Agtr1aloxp/+;Crt(+) /TRE-SV40T/podocin-rTA/ROSA26loxP. Both show the Agtr1aloxp genotype. Six colonies (not shown) similarly showed the Agtr1aloxp+/- genotype. Lanes 8 to 12, LacZ-tagged podocyte clones from mice carrying Agtr1aloxp/+;Crt(+) /TRE-SV40T/podocin-rTA/ROSA26loxP. All show the Agtr1aloxp+/- genotype. Thirteen (87%) of 15 clones examined showed the Agtr1aloxp+/- genotype.

### Determination of Relative Amount of AT1A and AT1B mRNA

Glomeruli were harvested from Agtr1aloxp/+;Crt(−) and Agtr1aloxp/+;Crt(+) mice without LMB2 or Agtr1aloxp/+;Crt(−)/Nep25 and Agtr1aloxp/+;Crt(+) /Nep25 mice 7 days after the addition of 0.625 ng/g of BW of LMB2 (each n = 4). Total RNA was extracted, and cDNA was synthesized. As reported previously,
PCR was carried out using primers GCATCATTTTGGTGTTGGG and GAAGAAAGGCACAATGCC, which are common to both AT1A and AT1B sequences. Only the PCR product derived from AT1A mRNA has an EcoRI site. Therefore, EcoRI-cleaved and uncleaved bands represent AT1A and AT1B mRNA, respectively. AT1A and AT1B mRNAs were also quantified by real-time RT-PCR. TaqMan Primer Probe sets for Agtr1a, Agtr1b, and 18s RNA were used with Applied Biosystems 7300 Real-Time PCR systems. The amplification efficiency for AT1A and AT1B was normalized by a standard template containing an equal molar of AT1A and AT1B cDNAs.

Real-time RT-PCR was also performed in RNA extracted from primary cultured podocytes after the first and second passages. Primary cultured podocytes were obtained from wild-type mice (n=3), as described previously.27

Experimental Protocol of Immunotoxin-Induced Nephropathy

To study the effect of podocyte-specific AT1 inactivation, 5 female and 8 male mice carrying Agtr1aloxP/loxP/Cre−/yNEP25 and 10 female and 8 male mice carrying Agtr1aloxP/loxP/Cre+/yNEP25 (3 to 7 months of age) were used. Twenty-four–hour urine was collected before and 7, 14, 21, and 28 days after LMB2 injection. They were euthanized 28 days after LMB2 injection.

To study the effect of losartan, an AT1 receptor blocker, 8 female NEP25 mice with a C57BL/6 genetic background (5 months of age) were treated with losartan (0.5 g/L, in drinking water, ~25 μg/g of BW) from 5 days before LMB2 injection until the end of the experiment. Nine age- and sex-matched NEP25 mice were used as controls. In both groups of mice, 0.625 ng/g of BW of LMB2 were intravenously injected under diethyl ether anesthesia. Conscious controls. In both groups of mice, 0.625 ng/g of BW of LMB2 were intravenously injected under diethyl ether anesthesia. Conscious controls.

Morphological Analysis

Glomerulosclerosis was evaluated in PAS-stained paraffin sections (2-μm thick). Each glomerulus was graded on a 0 to 4 scale, which represents the sclerotic area involving 0%, 1% to 25%, 26% to 50%, 51% to 75%, or >75% of the glomerulus. Scores for all of the glomeruli on a section were averaged and defined as the sclerosis index for each mouse.

For evaluating podocyte injury, paraffin sections were stained for nephrin using guinea pig polyclonal antibody (GP-N2, Progen). For semiquantification of nephrin staining, each quadrant of each glomerulus was scored as 0 (no staining), 1 (diminished), or 2 (normal), with total glomerular score range calculated from 0 (complete loss) to 8 (normal). Scores for all of the glomeruli on a section for each mouse (>80) were averaged and defined as the nephrin index.

Statistical Analysis

Results are expressed as mean±SE. Student t test was used to analyze the difference between 2 groups in blood pressure. One-way ANOVA was used to compare blood pressure among 3 groups. Albumin/creatinine ratio was measured repeatedly, and global test assessing the between-group effect was performed through multivariate ANOVA to prevent inflation of type I error through multiple comparisons. When overall effect was detected with multivariate ANOVA, Student t test was performed at individual time points. Comparison of sclerosis index and nephrin index was performed by the Mann-Whitney U test. Values were regarded as significant at 2-sided P<0.05.

Results

Generation of Agtr1aloxP/loxP Mice

To investigate the direct effect of Ang II on podocytes, we generated podocyte-specific Agtr1a-null mutant mice. Using homologous recombination in ES cells, we established mutant mice carrying Agtr1aflpft, in which 2 loxP sites were inserted at the upstream and the lower stream to the coding exon of Agtr1a (Figure S1, please see the online Data Supplement at http://hyper.ahajournals.org).

Homozygous (Agtr1aloxPloxP) and heterozygous (Agtr1aloxP+loxP) mice showed no apparent abnormal phenotype. Northern blotting analysis revealed that Agtr1aloxPloxP and Agtr1aloxP+ mice similarly expressed AT1A mRNA in the kidney compared with wild-type mice (data not shown). Systolic blood pressures were 97±13 and 105±8 mm Hg in Agtr1aloxPloxP and Agtr1aloxP+ mice, respectively; similar to that in wild-type littermates (100±12 mm Hg). Agtr1aloxPloxP showed normal renal morphology. These data confirm that the insertion of the loxP sequences did not disturb the expression and the function of AT1A mRNA.

Confirmation of Agtr1a Disruption in Podocytes of Agtr1aloxPloxP/Cre(+) Mice

The Agtr1aloxPloxP line was mated with a Nephrin-Cre line, which expresses Cre recombinase selectively in podocytes. Previously, we tested the efficiency of Cre-mediated recombination in podocytes by mating Nephrin-Cre mice with ROSA26loxP+, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP+, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP+, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP+, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP+, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP+, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP+, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP+, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP+, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP+, a tester strain.

Next we tested whether recombination occurs in similar efficiency in podocytes of Agtr1aloxPloxP/Nephrin-Cre (Agtr1aloxPloxP/Cre[+]) mice. Reliable antinouse AT1 antibodies suitable for immunohistochemical study were not available to us, and we, therefore, cultured podocytes and then cloned and determined the Agtr1a genotype by PCR. For this purpose, Agtr1aloxPloxP/Cre(+), mice were mated with TRE-SV40/Todocin-rtTA mice, which express SV40 T antigen in podocytes in the presence of doxycycline. To mark the podocyte-lineage with lacZ, the mice were further mated with the ROSA26loxP, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP+, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP+, a tester strain. One-hundred percent of podocytes were lacZ positive in Nephrin-Cre/ROSA26loxP+, a tester strain.
Basal Phenotype of Agtr1a Mice showed that all of the lacZ-positive clones had the Agtr1aloxP/loxP genotype. These confirmed that Cre-mediated recombination of Agtr1aloxP occurs efficiently in podocytes, and most podocytes in Agtr1aloxP mice showed similar degrees of glomerulosclerosis (B and C) and a similar degree of downregulation of nephrin (E and F, arrows). A through C, periodic acid Schiff, ×200; D through F, antinephrin, ×400.

Effect of Podocyte-Specific Inactivation of AT1 on Progression of Glomerulosclerosis

To examine whether podocyte-specific deletion of AT1 receptor can slow the progression of glomerulosclerosis, we mated Agtr1aloxPloxP/Cre(−)/NEP25 and Agtr1aloxPloxP/Cre(+) mice with the NEP25 line, generating Agtr1aloxPloxP/Cre(−)/NEP25 and Agtr1aloxPloxP/Cre(+) mice. Without LMB2, Agtr1aloxPloxP/Cre(−)/NEP25 mice showed no proteinuria (Figure 2A, before LMB2), and renal morphology and nephrin staining were normal (Figure 3A and D) with a sclerosis index of 0 and a nephrin score of 8. After injection of 0.625 ng/g of BW, both types of mice showed moderate proteinuria, which peaked 2 weeks after the injection, with no statistical difference at any time point (Figure 2A).

The degree of focal segmental glomerulosclerosis was similar in both mouse groups at 4 weeks. Agtr1aloxPloxP/
Cre(+) /NEP25 mice had a sclerosis index of 0.65±0.16, which was not statistically different from that of Agtr1aloxP/loxP /Cre(−) /NEP25 mice, at 0.82±0.27 (Figures 2B and 3). Both types of mice had glomeruli with similarly diminished nephrin staining. The nephrin index in Agtr1aloxP/loxP /Cre(+) /NEP25 mice was, on average, 5.78±0.45, which was not statistically different from that of Agtr1aloxP/loxP /Cre(−) /NEP25 mice, at 5.63±0.58 (Figures 2C and 3). Thus, podocyte-specific inactivation of AT1 showed no impact on the progression of glomerulosclerosis triggered by podocyte injury.

**ARB Attenuates Progression of Glomerulosclerosis in NEP25 Model**

We next examined whether ARB can protect against the glomerular injury in NEP25 transgenic mice, which progressively develops after the injection of LMB2 (0.625 ng/g of BW). The ARB-treated mice showed significantly lower systolic blood pressure than the control mice without treatment (66±6 versus 101±5 mm Hg). NEP25 mice without ARB showed moderate proteinuria with a marked decrease in ARB NEP25 (urinary protein:creatinine ratio: 66:6±20.7 versus 22.8±10.3 mg/mg) 7 days after LMB2 injection (Figure 4A). Twenty-one days after injection, NEP25 mice showed focal segmental sclerosis with a sclerosis index averaging 0.83±0.36, demonstrating marked protection in ARB NEP25 mice (0.01±0.01; Figure 4B and 5).

Podocyte injury, assessed by nephrin staining, was significantly attenuated in NEP25 versus ARB NEP25 (nephrin staining index: 5.61±0.76 versus 7.96±0.01, 0 to 8 scale; Figures 4C and 5). Similar protection was observed in Agtr1aloxP/loxP /Cre(+) /NEP25 mice treated with ARB (Figure 6). Agtr1aloxP/loxP /Cre(−) /NEP25 mice treated with ARB starting after LMB2 injection (Figure 6 vs. 101

**Effect of Podocyte-Specific Inactivation of AT1 on Ang II–Induced Microalbuminuria**

We next examined the effect of podocyte-specific inactivation of AT1 on microalbuminuria induced by Ang II infusion. A pressor dose of Ang II (1000 ng/kg per minute) was continuously infused for 14 days using miniosmotic pumps in Agtr1aloxP/loxP /Cre(+) and Agtr1aloxP/loxP /Cre(−) mice. Systolic blood pressure was similarly elevated in both groups (149.3±11.2 versus 143.5±14.3, respectively). As shown in Figure 7, both strains of mice showed a similar degree of microalbuminuria at all of the time points examined. Under...
this experimental condition, no mouse in these groups showed glomerular sclerosis or downregulation of nephrin staining.

Discussion

In the present study, blockage of Ang II, by either an ARB or ACE inhibitor, attenuated proteinuria, podocyte injury, and glomerulosclerosis in the NEP25 model in a fashion similar to those of other models for podocyte injury. Treatment with hydralazine showed no renal protective effect, although it effectively decreased blood pressure, indicating that the protective effect of Ang II blockage is independent of its systemic blood pressure–lowering effect. Because an ACE inhibitor was also effective, the beneficial effect of ARB is ascribed to inhibition of Ang II, not to an activation of non-Ang II receptor(s).

As discussed earlier, podocytes in vitro, as well as in vivo, have been shown to express functional AT1 receptors, attenuated proteinuria, podocyte injury, and glomerulosclerosis in the NEP25 model in a fashion similar to those of other models for podocyte injury. Treatment with hydralazine showed no renal protective effect, although it effectively decreased blood pressure, indicating that the protective effect of Ang II blockage is independent of its systemic blood pressure–lowering effect. Because an ACE inhibitor was also effective, the beneficial effect of ARB is ascribed to inhibition of Ang II, not to an activation of non-Ang II receptor(s).

Figure 7. Microalbuminuria induced by Ang II infusion. Agtr1dloxP/loxP/Cre(-) and Agtr1dloxP/loxP/Cre(+) mice were continuously infused with Ang II (1000 ng/kg per minute) for 14 days. There was no significant difference in urinary albumin:creatinine ratio (ACR) at any time point between the 2 types of mice.

In the present study, blockage of Ang II, by either an ARB or ACE inhibitor, attenuated proteinuria, podocyte injury, and glomerulosclerosis in the NEP25 model in a fashion similar to those of other models for podocyte injury. Treatment with hydralazine showed no renal protective effect, although it effectively decreased blood pressure, indicating that the protective effect of Ang II blockage is independent of its systemic blood pressure–lowering effect. Because an ACE inhibitor was also effective, the beneficial effect of ARB is ascribed to inhibition of Ang II, not to an activation of non-Ang II receptor(s).

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References
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**ARB Protection Against Podocyte-Induced Sclerosis is Podocyte AT1-Independent.**

Short title: Podocyte AT1 and Glomerulosclerosis

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Methods

Generation of Agtr1a\textsuperscript{loxP} mice

A targeting vector was constructed for conditional targeting of the Agtr1a gene (Figure S1). The vector contains 13 kb of intron 2, exon 3 (the coding exon) and 2.8 kb of 3' flanking region. At the Bgl II site of intron 2, upstream of the coding exon, one loxP site was inserted. At the Pst I site, downstream of the coding exon, a neomycin resistant gene cassette (pgk-neo) flanked by two loxP sites was inserted. At the 3' end of the vector, an expression cassette for herpes simplex virus thymidine kinase (pgk-tk) was connected. The resultant targeting vector was introduced into E14.1 cells by electroporation. 768 G418 resistant ES colonies were picked up and analyzed by Southern blot analysis. Fourteen clones (18%) had undergone homologous recombination. We selected one clone, expanded it, and used for further experiments. To delete pgk-neo, a Cre expression vector, pCre-Pac plasmid (Kurabo, Osaka, Japan), was transiently introduced into the ES clone by electroporation. 196 puromycin-resistant colonies were analyzed by Southern blot analysis. Twelve colonies had desired recombination, i.e., pgk-neo was deleted while Agtr1a coding exon was preserved. The loxP insertion was confirmed by PCR amplification and sequencing. This allele is designated as Agtr1a\textsuperscript{loxP}. One of the targeted ES cells was injected into C57BL/6 blastocysts. One of the 10 chimeric mice obtained showed germline transmission of Agtr1a\textsuperscript{loxP} when mated with C57BL/6 females. Genotyping for Agtr1a was performed by PCR using primers, TTCAGCCAGATCGAGGAGCGGAGG and CTAACCGTTGAAAATAGCTGTCC, which generate 217 base pair (bp) band in wild-type Agtr1a and 251 bp band in Agtr1a\textsuperscript{loxP}. Heterozygous (Agtr1a\textsuperscript{loxP/+}) mice were backcrossed with C57BL/6 strain more than 10 times before mating with Nephrin-Cre mice.

Results

Generation of podocyte-specific Agtr1a knockout mice

To generate podocyte-specific Agtr1a-null mutant mice, we established mutant mice carrying Agtr1a\textsuperscript{loxP}, in which two loxP sites were inserted before and after the coding exon of Agtr1a (Figure S1). Northern blotting analysis
revealed that $Agtr1a^{loxp/loxp}$ and $Agtr1a^{loxp/+}$ mice similarly expressed AT1A mRNA in the kidney compared to wild-type mice (data not shown). Systolic blood pressure was 97±13 and 103±8 mmHg in $Agtr1a^{loxp/loxp}$ and $Agtr1a^{loxp/+}$ mice, respectively, which was not different statistically from that in wild-type littermates (100±12 mmHg). $Agtr1a^{loxp/loxp}$ and $Agtr1a^{loxp/+}$ mice showed normal renal morphology. These data confirm that the insertion of the loxP sequences did not disturb the expression and the function of AT1A mRNA.

Then, the $Agtr1a^{loxp/loxp}$ line was mated with a Nephrin-Cre line, which expresses Cre recombinase selectively in podocytes. Previously, we tested efficiency of Cre-mediated recombination in podocytes by mating Nephrin-Cre mice with ROSA26$^{loxP}$, a tester strain. 100% of podocytes were lacZ positive in Nephrin-Cre/ROSA26$^{loxP}$. We next tested whether recombination occurs in similar efficiency in podocytes of $Agtr1a^{loxp/loxp}$/Nephrin-Cre ($Agtr1a^{loxp/loxp}$/Cre(+)) mice. Reliable anti-mouse AT1 antibodies are not available to us and we therefore cultured podocytes, and then cloned and determined the $Agtr1a$ genotype by PCR. For this purpose, $Agtr1a^{loxp/loxp}$/Cre(+) mice were mated with TRE-SV40T/podocin-rtTA mice, which express SV40 T antigen in podocytes in the presence of doxycycline. To mark the podocyte-lineage with lacZ, the mice were further mated with ROSA26$^{loxP}$ line.

Glomeruli obtained from $Agtr1a^{loxp/loxp}$/Cre(+)/TRE-SV40T/podocin-rtTA/ROSA26$^{loxP}$ mice were cultured in the presence of doxycycline. Colonies, each stem from a single cell, were stained for lacZ. PCR analysis revealed that 13 out of 15 (87%) lacZ-positive clones examined showed only deleted allele ($Agtr1a$)(Figure S2). Two lacZ positive colonies showed both $Agtr1a^{loxp}$ and $Agtr1a^a$, indicating that they are heterozygote ($Agtr1a^{loxp^+}$). LacZ-negative cobblestone-like cells often grew even without doxycycline. PCR analysis revealed that all three such colonies examined had $Agtr1a^{loxp/loxp}$ genotype. Similar analysis in eight lacZ positive colonies from $Agtr1a^{loxp^+}$/Cre(+)/TRE-SV40T/podocin-rtTA/ROSA26$^{loxP}$ showed that all lacZ-positive clones had $Agtr1a^{+/+}$ genotype. These confirmed that Cre-mediated recombination of $Agtr1a^{loxp}$ occurs efficiently in podocytes and most podocytes in $Agtr1a^{loxp/loxp}$/Cre(+) mice were actually null-mutated for $Agtr1a$. 
Effect of losartan treatment starting after the LMB2 injection on NEP25 mice

Female Agtr1a<sup>loxP/loxP/Cre(-)/NEP25</sup> mice (2-8 months of age) were injected with LMB2 (0.625 ng/g BW). 12 mice were treated with losartan (0.5 g/L in drinking water) 12 hours after the injection of LMB2 until the end of the experiment. They were compared with the 12 age-sex-matched mice injected with LMB2 without losartan treatment. Age did not affect the severity of renal injury induced by LMB2. Mice were sacrificed 28 days after the injection.

Losartan treatment attenuated glomerular injury. Thus, 28 days after the LMB2 injection, urinary albumin/creatinine ratio was, on average, 0.63±0.17, in the losartan group, which was significantly lower than that in the control group, 31.25±9.99 (Figure S2). Glomerulosclerosis and downregulation of nephrin were attenuated in the losartan group. Sclerosis index in the losartan group was, on average, 0.64±0.17, which was significantly lower than that in the control group, 1.80±0.40 (Figure S2). Nephrin index in the losartan group was, on average, 6.09±0.46, which was significantly higher than that in the control group, 4.00±0.76 (Figure S2).

Effect of captopril treatment starting after the LMB2 injection on NEP25 mice

Six female NEP25 mice with C57BL/6 genetic background (4 months of age) were treated with an ACE inhibitor, captopril (0.5 g/l, in drinking water, approximately 25 ng/g BW) 12 hours after LMB2 injection until the end of the experiment. Six age-sex-matched NEP25 mice with LMB2 without captopril were used as controls. Mice were sacrificed 28 days after the injection.

Captopril treatment remarkably well protected glomeruli, with sclerosis index, 0.01±0.01 (vs. 0.35±0.09 in control) and nephrin index, 7.98±0.01 (vs. 7.14±0.22 in control) (Figure S3)

Effect of hydralazine treatment on NEP25 mice

Six female NEP25 mice with C57BL/6 genetic background (3 months of age) were treated with hydralazine (250mg/l, in drinking water,
approximately 12 ng/g BW) from 5 days before LMB2 injection until the end of the experiment. Six age-sex-matched NEP25 mice injected with LMB2 without hydralazine were used as controls. Mice were sacrificed 21 days after the injection.

Systolic blood pressure was measured by tail cuff method two days before the LMB2 injection. The hydralazine-treated mice showed significantly lower systolic blood pressure than the control mice without treatment (114.3±3.7 vs. 85.5±5.2 mmHg).

Both groups showed similar degree of proteinuria, with no significant difference in urinary protein/creatinine ratio at any time point (Figure S4a). With hydralazine treatment, glomerulosclerosis was not attenuated, with sclerosis index, on average 0.11±0.06, which was not statistically different from that in the control group, 0.37±0.11 (Figure S4b). Similarly, there was no significant difference between the two groups in nephrin index (7.75±0.13 vs. 7.03±0.20)(Figure S4c).
Figure S1
Generation of conditional Agtr1a targeted mice.
(a) Wild-type Agtr1a. The entire coding region is included within exon 3.
(b) Construction of the targeting vector. The targeting vector contains 13 kb of intron 2, exon 3, 2.8 kb of 3' flanking region. At the Bgl II site in intron 2, a loxP sequence (shown by a triangle) was inserted. At the Pst I site in 3' flanking region, a neomycin resistant gene cassette (pgk-neo) flanked by two loxP sites was inserted. At the 3' end of the targeting vector, an expression cassette for herpes simplex virus thymidine kinase (pgk-tk) was attached for negative selection.
(c) Structure of mutant Agtr1a (Agtr1aneo) obtained by homologous recombination with (b).
(d) Structure of mutant Agtr1a (Agtr1aloXP) in the ES cell clone used for microinjection. The bulky pgk-neo of Agtr1aneo was removed by transient transfection with pCre-Pac plasmid.
(e) Structure of null Agtr1a allele (Agtr1a-) generated by Cre-mediated recombination. In Agtr1aloXP/loxP/Nephrin-Cre mice, only podocytes carry this allele, while other types of cells have Agtr1aloXP.
(f) Southern analysis for ES cell screening. Genomic DNA digested with BamH I was hybridized with the probe shown in (a). 8 kb bands represent wild-type Agtr1a allele and 5 kb bands (*) represent Agtr1aneo allele.
Figure S2

Effect of ARB treatment starting after LMB2 injection on NEP25 mice.

NEP25 mice were injected with LMB2 (0.625 ng/g BW). Mice with ARB treatment starting 12 hours after LMB2 injection (closed columns) showed significantly attenuated urinary albumin/creatinine ratio (a), glomerulosclerosis (b) and downregulation of nephrin (c) when examined 4 weeks after the injection, compared to those in control mice without ARB (open columns). * p<0.05.

In this experiment, Agtr1a<sup>loxP/loxP/Cre(-)/NEP25</sup> mice were used. These and the mice used in the experiment shown in Figure 2 were from the same colony. Without LMB2, both strains of mice showed no sclerosis or podocyte damage with sclerosis index 0, and nephrin index 8.

* p<0.05.
NEP25 mice were injected with LMB2 (0.625 ng/g BW). Mice with captopril treatment starting 12 hours after LMB2 injection (closed columns) showed significantly attenuated urinary albumin/creatinine ratio 1-4 weeks after the injection (a), glomerulosclerosis (b) and downregulation of nephrin (c) when compared to those in control mice without captopril (open columns). Without LMB2, both strains of mice showed no sclerosis or podocyte damage with sclerosis index 0, and nephrin index 8.

* p<0.05.
Figure S4

*Effect of hydralazine on NEP25 mice.*

Hydralazine treatment starting 5 days before LMB2 injection (0.625 ng/g BW) exerted no impact on urinary total protein/creatinine ratio (a), glomerulosclerosis (b) or downregulation of nephrin (c).