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

Short title: Podocyte AT1 and Glomerulosclerosis

Taiji Matsusaka¹, 4, 5, Takako Asano⁹, Fumio Niimura⁶, Masaru Kinomura⁵, Akihiko Shimizu¹⁰, Ayumi Shintani¹¹, Ira Pastan⁸, Agnes B. Fogo¹, ², ³, Iekuni Ichikawa¹, ³, ⁷

Departments of ¹Pediatrics, ²Pathology, ³Medicine, and ¹¹Biostatistics, Vanderbilt University Medical Center, Nashville, TN, USA
⁴Institute of Medical Science, ⁵Departments of Internal Medicine, ⁶Pediatrics and ⁷Bioethics, Tokai University School of Medicine, Isehara, Kanagawa, Japan
⁸Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland, USA
⁹Department of Pediatrics, National Defense Medical College, Tokorozawa, Saitama, Japan
¹⁰Division of Kidney and Hypertension, Department of Internal Medicine, Jikei University School of Medicine, Tokyo, Japan

Corresponding Author:
Taiji Matsusaka
Department of Internal Medicine
Tokai University School of Medicine
143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan
Email: taijim@is.icc.u-tokai.ac.jp
FAX: 81-463-90-1611
Phone: 81-463-93-1121
Methods

Generation of Agtr1a<sup>loxP</sup> 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<sup>loxP</sup>. One of the targeted ES cells was injected into C57BL/6 blastocysts. One of the 10 chimeric mice obtained showed germline transmission of Agtr1a<sup>loxP</sup> when mated with C57BL/6 females. Genotyping for Agtr1a was performed by PCR using primers, TTCAGCCAGATCGAGGAGCGGAGG and CTAACCGTTGAAATAGCTGTCC, which generate 217 base pair (bp) band in wild-type Agtr1a and 251 bp band in Agtr1a<sup>loxP</sup>. Heterozygous (Agtr1a<sup>loxP</sup>+/−) 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<sup>loxP</sup>, 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 $97 \pm 13$ and $103 \pm 8$ mmHg in Agtr1a^{loxP/loxP} and Agtr1a^{loxP/+} mice, respectively, which was not different statistically from that in wild-type littermates ($100 \pm 12$ 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, 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 $Agtr1a^{loxP/loxP}/Cre(-)/NEP25$ 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-thk*) 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/loxF/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 *BamHI* 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^{loxP/loxP}/Cre(-)/NEP25 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.
Effect of captopril treatment starting after LMB2 injection on NEP25 mice.

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).