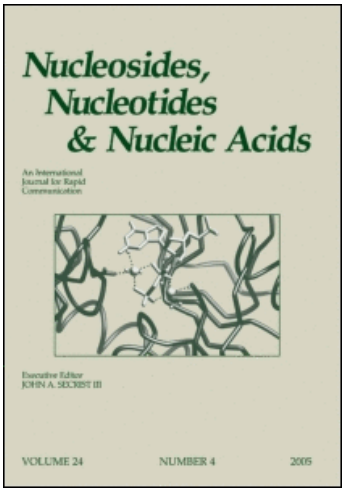

Nucleosides, Nucleotides and Nucleic Acids

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Establishment and Analysis of *SLC22A12* (URAT1) Knockout Mouse

M. Hosoyamada[a]; Y. Takiue[a]; H. Morisaki[b]; J. Cheng[b]; M. Ikawa[c]; M. Okabe[c]; T. Morisaki[b]; K. Ichida[d]; T. Hosoya[e]; T. Shibasaki[a]

[a] Pharmacotherapeutics, Faculty of Pharmacy, Keio University, Tokyo, Japan [b] Bioscience, National Cardiovascular Center Research Institute, Osaka, Japan [c] Genome Information Research Center, Osaka University, Osaka, Japan [d] Pathophysiology, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan [e] Internal Medicine (Kidney and Hypertension), Jikei University School of Medicine, Tokyo, Japan

Online publication date: 11 June 2010

To cite this Article Hosoyamada, M. , Takiue, Y. , Morisaki, H. , Cheng, J. , Ikawa, M. , Okabe, M. , Morisaki, T. , Ichida, K. , Hosoya, T. and Shibasaki, T.(2010) 'Establishment and Analysis of *SLC22A12* (URAT1) Knockout Mouse', Nucleosides, Nucleotides and Nucleic Acids, 29: 4, 314 — 320

To link to this Article: DOI: 10.1080/15257771003738634

URL: http://dx.doi.org/10.1080/15257771003738634

Nucleosides, Nucleotides and Nucleic Acids, 29:314–320, 2010

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257771003738634

Taylor & Francis
Taylor & Francis Group

ESTABLISHMENT AND ANALYSIS OF *SLC22A12* (URAT1) KNOCKOUT MOUSE

M. Hosoyamada,[1] Y. Takiue,[1] H. Morisaki,[2] J. Cheng,[2] M. Ikawa,[3] M. Okabe,[3] T. Morisaki,[2] K. Ichida,[4] T. Hosoya,[5] and T. Shibasaki[1]

[1]*Pharmacotherapeutics, Faculty of Pharmacy, Keio University, Tokyo, Japan*
[2]*Bioscience, National Cardiovascular Center Research Institute, Osaka, Japan*
[3]*Genome Information Research Center, Osaka University, Osaka, Japan*
[4]*Pathophysiology, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan*
[5]*Internal Medicine (Kidney and Hypertension), Jikei University School of Medicine, Tokyo, Japan*

□ *In order to elucidate the mechanisms of post-exercise acute renal failure, one of the complications of hereditary renal hypouricemia, we have targeted the mouse* Slc22a12 *gene by the exchange of exons 1–4 with pMC1neo-polyA. The knockout mice revealed no gross anomalies. The concentration ratio of urinary urate/creatinine of the knockout mice was significantly higher than that of wildtype mice, indicating an attenuated renal reabsorption of urate. The plasma levels of urate were around 11 μM and were similar among the genotypes. Although the fractional excretion of urate of knockout mice was tend to higher than that of wildtype mice, the urate reabsorption ability remained in the kidney of knockout mice, indicating a urate reabsorptive transporter other than Urat1.*

Keywords Urat1; knockout mice; urate; allantoin

INTRODUCTION

Post-exercise acute renal failure is a serious complication of hereditary renal hypouricemia 1 (OMIM: #220150), which is caused by mutation in *SLC22A12*, the gene encoding the renal urate transporter Urat.[1] In order to elucidate the pathological mechanisms and possible prevention of post-exercise acute renal failure, we have established *Slc22a12* (Urat1) knockout mice as an animal model of hereditary renal hypouricemia.

Address correspondence to M. Hosoyamada, Pharmacotherapeutics, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105 8512, Japan. E-mail: hosoyamada-mk@pha.keio.ac.jp

MATERIALS AND METHODS

Generation of Urat1 Knockout Mice

A 13.6 kb DNA clone containing *Slc22a12* gene was isolated from a mouse 129/sv genomic DNA library (MoBioTec, Germany) using a digoxigenin-labeled probe prepared by PCR with sense primer (5′-ccctctctctcctgggttagctcacagtac-3′) and antisense primer (5′-tgtgatgagcctgctttccccttggctctg-3′). A replacement targeting vector was constructed by incorporating a 1.2 kb segment of inverted directed pMC1neo-polyA and a 1.9 kb segment of pSK-DTA (Figure 1A) was linearized by BlpI digestion and electroporated into embryonic stem (ES) cells obtained from mouse strain 129/sv. A homologous recombinant ES clone was selected by genomic Southern analysis using probe 1 prepared with sense primer (5′-gactgtctagaggcggccttaactag-3′) and antisense primer (5′-ctgctgagccgcaggagcccacagacgccgctgg-3′). Successfully transfected ES cells were injected into developing blastocysts from C57BL/6J mice to obtain

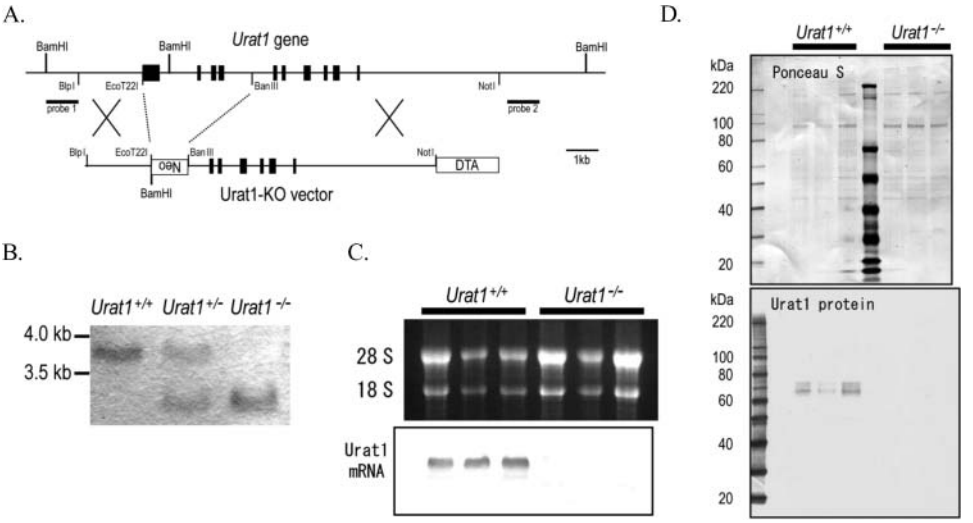

FIGURE 1 Establishment of *Slc22a12* (Urat1) knockout mice. A) Schema of targeted disruption of *Slc22a12*. Homologous recombination was performed to replace a 3.4 kb segment including exons 1–4 of *Slc22a12* with Neo cassette resulting in formation of a null allele. The positions of probe 1 for genomic Southern analysis are indicated. B) Genomic Southern analysis for genotyping of a typical litter from a cross between mice heterozygous for the Urat1 null allele. BamHI-digested genomic DNA was analyzed with probe 1 as indicated in panel A. C) Northern analysis. Total RNA from the kidneys of 3 WT (WT 1–3) and 3 Urat1 KO (KO 1–3) mice was electrophoresed through a denaturing gel (upper panel) and then transferred to a nylon membrane and hybridized to a digoxigenin-labeled mouse Urat1 cDNA (lower panel). D) Western analysis. Membrane fractions of kidneys from 3 WT (WT 1–3) and 3 Urat1 KO (KO 1–3) mice were separated by SDS-PAGE and then transferred to a nitrocellulose membrane (upper panel, stained with Ponceau-S). Mouse Urat1 protein was detected with the anti-Urat1 antibody.

chimeric mice, which were bred with C57BL/6J mice to obtain mice heterozygous for Urat1 deficiency. Gene targeting of Urat1-/- offspring obtained by mating of heterozygous mice was confirmed by genomic Southern analysis using probe 1.

Northern Analysis

Total RNAs (2 μg each) prepared by ISOGEN kit (Nippongene, Japan) from the kidneys of three Urat1+/+ and three Urat1−/− mice were mixed with ethidium bromide and separated on a formaldehyde denaturing agarose gel for obtaining a picture. The separated total RNA was transferred on a nylon membrane (Hybond-N^+, GE, UK) by capillary blotting with 20 × SSC, hybridized at 55°C with a digoxigenin-labeled full length mouse Urat1 cDNA probe, and washed in 0.1 × SSC at 65°C for the detection of mouse Urat1 mRNA.[2]

Western Analysis

Membrane proteins (2 μg) of the mouse kidney prepared as described before[2] were separated by SDS-PAGE under non-reducing conditions with NuPAGE Bis-Tris 4–12% gel (Invitrogen, Tokyo, Japan) and transferred to a nitrocellulose membrane using iBlot module (Invitrogen). Mouse Urat1 protein was detected using the ECL kit (GE) with a novel affinity-purified anti-mouse Urat1 antibody that was obtained from the serum of a rabbit immunized by KLH-conjugated mouse Urat1 C-terminal peptide, CHDT-PDGSILMSTRL (Sigma Genosys, Japan). The nitrocellulose membrane was stained with Ponceau S to check for blotting failure.

Measurement of Allantoin, Urate, and Creatinine by HPLC

The Urat1 mutation was backcrossed onto the C57BL/6J strain for four generations to produce Urat1−/− offspring with a similar genetic background. The 24-hour urine samples of seven 13-week old wildtype mice (WT) and eight 13-week old knockout mice (KO) were collected using metabolic cages (Tecniplast, Italy). The blood of them were collected with heparin from the abdominal vein of the mice anesthetized with ether and 50 mg/kg pentobarbital i.p. 40 μl of acetonitrile (ACN) and 10 μl of plasma sample or 100 times diluted urine sample were delivered into Ultrafree-MC 0.22 μm PVDF membrane filter unit (Millipore, USA) to remove proteins by centrifugation. The ACN and any aqueous material were evaporated to dryness in a centrifuge evaporator (Ikemoto-Rika, Japan). The residue containing the analyte was resuspended in 10 μl of HPLC mobile phase (20 mM ammonium formate). Separation was achieved at a flow rate of 0.200 mL/min on a 250

mm × 2 mm, 3 μm particle size ODS column, Unison UK-C18, (Imtakt, Japan) at 25°C (Hitachi LaChrom Elite PDA system).

RESULTS

Confirmation of Targeting of *Slc22a12* (Urat1)

Homologous recombination was used to replace a segment of exons 1–4 of *Slc22a12* with pMC1neo-polyA, effectively generating a null allele (Figure 1A). Mice heterozygous for this allele were bred to yield knockout type offspring, which were typed by genomic Southern analysis of genomic DNA using probe 1 (Figure 1B). The 3.7 kb of wild allele and 3.0 kb of targeted allele were detected and coincided with the predicted fragment sizes of 3,738 bp of wildtype allele and 2,912 bp of targeted allele from the gene database (Genbank #AC124394). Thus, the *Slc22a12* gene was targeted successfully. The Urat1−/− mice revealed no gross anomalies and grew and bred normally.

The absence of Urat1 mRNA was demonstrated in the total RNA of knockout mice by Northern analysis (Figure 1C, lower panel). The possibility of degradation of total RNA in the knockout mouse sample was eliminated by demonstrating that the ribosomal RNAs were intact (Figure 1C, upper panel), therefore, the loss of expression of the Urat1 gene in the kidney of knockout mice was confirmed. Moreover, the absence of Urat1 protein was demonstrated in the membrane fraction of the kidney of knockout mice by Western blotting using a novel anti-mouse Urat1 antibody (Figure 1D, lower panel). The possibility of incomplete transfer of knockout mouse samples was also eliminated by the staining of the intact protein on the membrane (Figure 1D, upper panel), therefore, the novel anti-mouse Urat1 antibody recognizes mouse Urat1 molecule specifically.

Urate Excretion in *Slc22a12* (Urat1) Knockout Mice

For the detection of allantoin, urate and creatinine, a novel HPLC method was developed for the simultaneous detection of the three analytes and reduction of organic solvent use. Detection of allantoin, urate, and creatinine peaks were achieved at 235 nm at 3.40 ± 0.02, 5.40 ± 0.02, and 6.10 ± 0.02 minutes, respectively. The purity of the peaks was confirmed by a PDA spectrum (data not shown). The coefficient values (CV) of this method were 0.4∼4.2% for urate (0.05∼10 mg/dL), 0.4∼6.4% for creatinine (0.01∼10 mg/dL), and 0.1∼3.7% for allantoin (0.5∼50 mg/dL). The linearity of standard curves were also confirmed ($r^2 = 1.00$) at the same range of concentrations (Figure 2A).

There was a significant increase in the amount of urinary urate excretion (255.7 ± 4.3 mmol/mol Cr in WT and 355.6 ± 35.8 mmol/mol Cr in

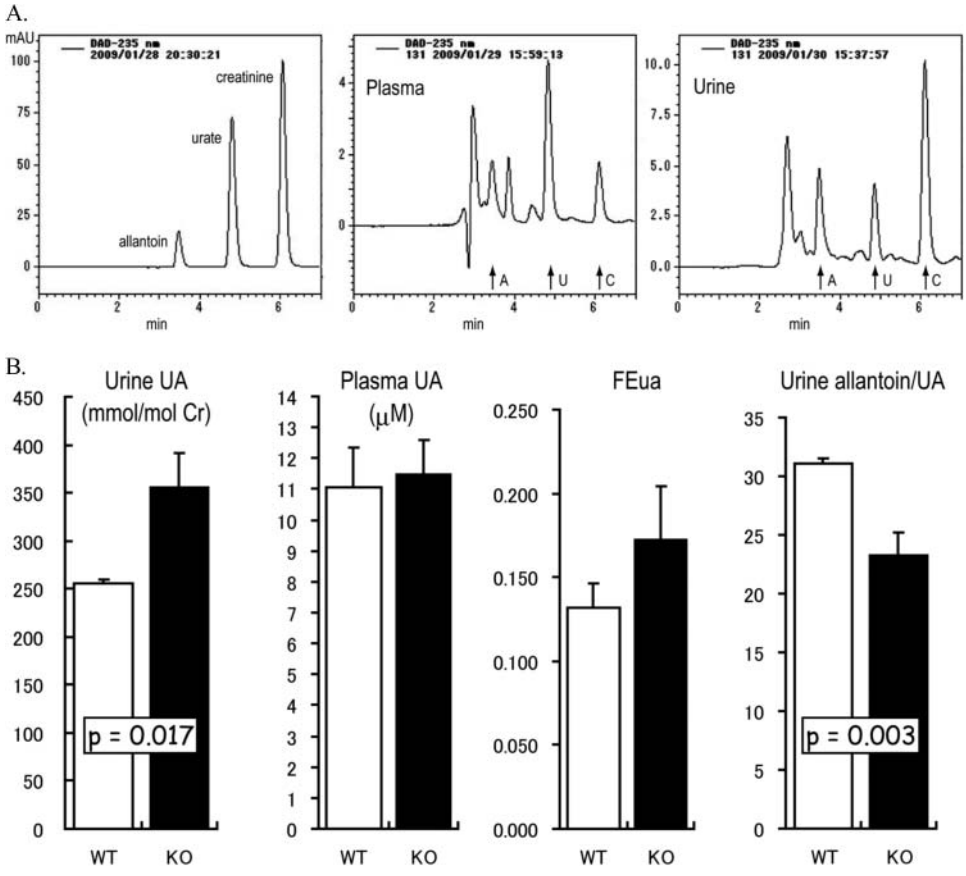

FIGURE 2 Renal urate handling of *Slc22a12* (Urat1) knockout mice. A) Typical chromatograms of standard (left), plasma (middle), and diluted urine (right) samples. The peaks of allantoin (A), urate (U), and creatinine (C) are indicated in the chromatograms. B) Renal urate handling of WT (open column, n = 7) and KO mice (closed column, n = 8). Urinary concentrations of urate are presented normalized to that of creatinine. Plasma urate concentrations were measured from blood samples collected with heparin under anesthesia. The fractional excretion of urate (FEua) was calculated as the ratio of urine urate normalized to urine creatinine to plasma urate normalized to plasma creatinine. The urinary allantoin-to-urate ratios were calculated. Data represent mean ± SE.

KO, p = 0.017 by Student's *t* test) and a significant decrease in the urinary allantoin/urate ratio in knockout mice (31.0 ± 0.5 in WT and 23.2 ± 2.0 in KO, p = 0.003 by Student's *t* test), indicating an attenuated renal reabsorption of urate and a decreased allantoin production from reabsorbed urate. However, plasma levels of urate were around 11 μM and were similar among the genotypes (11.1 ± 1.3 μM in WT and 11.5 ± 1.1 μM in KO, p = 0.815 by Student's *t* test). It was also failed to demonstrate the difference of FEua among the genotypes (0.13 ± 0.01 in WT and 0.17 ± 0.03 in KO, p = 0.273 by Student's *t* test; Figure 2B).

DISCUSSION

Eraly et. al. already reported on another RST (Urat1) knockout mouse, in which the third exon of the RST gene (*Slc22a12*) was replaced by a β-galactosidase transgene flanked by upstream splice acceptor sites and downstream transcriptional as well as translational stop signals.[3] Both our knockout mice and Eraly's mice were demonstrated by Northern analysis to be defective in the transcription of *Slc22a12*, and were successfully produced as *Slc22a12* knockout mice. Both our knockout mice and Eraly's RST-null mice appeared grossly normal.

Our novel anti-Urat1 antibody was verified to recognize the Urat1 molecule in the kidney specifically. Although we have tried to produce anti-Urat1 antibody with three antigen peptides (two N-terminal peptides and one C-terminal peptide) using two rabbits for each antigen, only one antibody recognized the Urat1 molecule specifically. Thus, knockout mice seem to be useful for verification of peptide antibodies.

Although the defect of URAT1 causes renal hypouricemia in humans, both Eraly's and our study revealed that there was no difference in plasma urate levels between WT and KO mice. Urate oxidase in mouse liver degrades reabsorbed urate from the kidney and this action likely extinguishes the difference in plasma urate levels between the WT and KO mice. The significant elevation of the daily urate excretion in KO mice was not accompanied by a change in the excretion of purine metabolites; urinary excretions of urate and allantoin were 1.745 ± 0.155 μmol/g/day in WT and 1.661 ± 0.156 μmol/g/day in KO. Therefore, the significant elevation of the daily urate excretion in KO mice was not due to an increase of purine metabolism but to a decrease of urate reabsorption in the kidney. Although a decrease of urate degradation in the liver also induces the elevation of urinary urate excretion, renal specific transporter Urat1 seems to have no effect on liver function.

The plasma urate level of wildtype mice in Eraly's paper was about 0.11 μM, two orders of magnitude lower than the urate level of our wildtype mice, 11.1 μM, which was almost the same as that reported by Dan et. al.[4] If the plasma urate level in Eraly's paper is a misprint of 0.11 mM (110 μM), it may be the same mistake that urate levels in mouse blood have been reported incorrectly one order of magnitude higher than our and Dan's data. Dan also described that xanthine oxidase activity in the rodent serum caused the urate level to increase up to 10 times of the real value in vitro after collection of blood sample.[4]

Both Eraly's and our study revealed that FEua of Urat1 (RST) knockout mice was below 1.0; therefore, an urate reabsorptive transporter (or transporters) other than Urat1 must be expressed in the kidney. (This is coincident with the FEua of the renal hypouricemia patients with a homozygous URAT1 defect, which was below 1.0.[5]) The urate reabsorptive transporter Glut 9[6,7] appears a likely candidate for such a role.

In conclusion, we succeeded in targeting the Urat1 gene in mice. After back-crossing, we will start to study the mechanism and prevention of post-exercise acute renal failure.

REFERENCES

1. Enomoto, A.; Kimura, H.; Chairoungdua, A.; Shigeta, Y.; Jutabha, P.; Cha, S.H.; Hosoyamada, M.; Takeda, M.; Sekine, T.; Igarashi, T.; Matsuo, H.; Kikuchi, Y.; Oda, T.; Ichida, K.; Hosoya, T.; Shimokata, K.; Niwa, T.; Kanai, Y.; Endou, H. Molecular identification of a renal urate anion exchanger that regulates blood urate levels. *Nature* **2002**, 417, 447–452.
2. Hosoyamada, M.; Ichida, K.; Enomoto, A.; Hosoya, T.; Endou, H. Function and localization of urate transporter 1 in mouse kidney. *J. Am. Soc. Nephrol.* **2004**, 15, 261–268.
3. Eraly, S.A.; Vallon, V.; Rieg, T.; Gangoiti, J.A.; Wikoff, W.R.; Siuzdak, G.; Barshop, B.A.; Nigam, S.K. Multiple organic anion transporters contribute to net renal excretion of uric acid. *Physiol. Genomics* **2008**, 33, 180–192.
4. Dan, T.; Tanaka, H.; Koga, H. Mechanism of uricosuric action of AA-193 in DBA/2N mice. *J. Pharmacol. Exp. Ther.* **1990**, 253, 437–443.
5. Ichida, K.; Hosoyamada, M.; Hisatome, I.; Enomoto, A.; Hikita, M.; Endou, H.; Hosoya, T. Clinical and molecular analysis of patients with renal hypouricemia in Japan-influence of URAT1 gene on urinary urate excretion. *J. Am. Soc. Nephrol.* **2004**, 15, 164–173.
6. Anzai, N.; Ichida, K.; Jutabha, P.; Kimura, T.; Babu, E.; Jin, C.J.; Srivastava, S.; Kitamura, K.; Hisatome, I.; Endou, H.; Sakurai, H. Plasma urate level is directly regulated by a voltage-driven urate efflux transporter URATv1 (SLC2A9) in humans. *J. Biol. Chem.* **2008**, 283, 26834–26838.
7. Matsuo, H.; Chiba, T.; Nagamori, S.; Nakayama, A.; Domoto, H.; Phetdee, K.; Wiriyasermkul, P.; Kikuchi, Y.; Oda, T.; Nishiyama, J.; Nakamura, T.; Morimoto, Y.; Kamakura, K.; Sakurai, Y.; Nonoyama, S.; Kanai, Y.; Shinomiya, N. Mutations in glucose transporter 9 gene SLC2A9 cause renal hypouricemia. *Am. J. Hum. Genet.* **2008**, 83, 744–751.