Title:

Urinary Protein Analysis in Mice Lacking Major Urinary Proteins

Running head:

URINARY PROTEINS IN MUP-KNOCKOUT MICE

Authors:

Osamu Suzuki, Minako Koura, Kozue Uchio-Yamada, and Mitsuho Sasaki

Authors’ Address:

Laboratory of Animal Models for Human Diseases, National Institutes of Biomedical Innovation, Health and Nutrition, Saito-Asagi 7-6-8, Ibaraki City, Osaka 568-0085, JAPAN

Correspondence should be addressed to:

Osamu Suzuki (D.V.M., Ph.D.), Laboratory of Animal Models for Human Diseases, National Institutes of Biomedical Innovation, Health and Nutrition, Saito-Asagi 7-6-8, Ibaraki City, Osaka 567-0085, JAPAN, Tel: +81-72-641-9852, Fax: +81-72-641-9857; E-mail: osuzuki@nibiohn.go.jp
**ABSTRACT**

Mouse urine contains major urinary proteins (MUPs) that are not found in human urine. Therefore, even healthy mice exhibit proteinuria, unlike healthy humans, making it challenging to use mice as models for human diseases. It was also unknown whether dipsticks for urinalysis could measure protein concentrations precisely in urine containing MUPs. To resolve these problems, we produced MUP-knockout (Mup-KO) mice by removing the Mup gene cluster using Cas9 proteins and two guide RNAs and characterized the urinary proteins in these mice. We measured the urinary protein concentrations in Mup-KO and wild-type mice using a protein quantitation kit and dipsticks. We also examined the urinary protein composition using SDS-PAGE and two-dimensional electrophoresis (2DE).

The urinary protein concentration was significantly lower ($p < 0.001$) in Mup-KO mice (17.9 ± 1.8 mg/dl, mean ± SD, n = 3) than in wild-type mice (73.7 ± 8.2 mg/dl, n = 3). This difference was not reflected in the dipstick values, perhaps due to the low sensitivity to MUPs. This suggests that dipsticks have limited ability to measure changes in MUPs with precision. SDS-PAGE and 2DE confirmed that Mup-KO mice, like humans, had no MUPs in their urine, whereas wild-type mice had abundant MUPs in their urine. The absence of the masking effect of MUPs in 2DE would enable clear comparisons of urinary proteins, especially low-molecular-weight proteins. Thus, Mup-KO mice may provide a useful model for human urinalysis.

Key words: genome editing, knockout model, major urinary proteins, urinalysis
Introduction

Urinary protein profiles are valuable for diagnosing urological diseases. While normal human urine contains little protein (<15 mg/dl), proteinuria in humans occurs for various reasons [1-3]. Urinary proteins can be classified into four categories [2]: (1) glomerular proteins such as albumin (~66.5 kDa), which appear in urine due to increased filtration of macromolecules through glomeruli; (2) proteins such as β2-microglobulin (11.8 kDa) and α1-microglobulin (~30 kDa), tubular proteins, which appear due to increased excretion of normally filtered low-molecular-weight proteins because of impaired reabsorption by the proximal tubules (the patterns of low-molecular-weight urinary proteins ranging from 10 to 23 kDa have clinical and prognostic significance [4]); (3) proteins such as Tamm–Horsfall proteins (92 kDa), secretory proteins, which are oversecreted in the tubules [5]; and (4) overflow proteins such as Bence–Jones proteins (22–24 kDa), which appear when the plasma concentration of low-molecular-weight proteins exceeds the capacity of the tubules to reabsorb the filtered proteins [6]. Low-molecular-weight proteins are important diagnostically. Although simple screening tests such as dipstick tests, are available to detect albumin, the analysis of low-molecular-weight proteins needs more sophisticated assays, such as electrophoresis, isoelectric focusing, and chromatography [3].

As research models for human urological diseases, mice have the shortcoming that even healthy mice exhibit proteinuria, which is a species-specific characteristic and not pathological [7]. The main proteins responsible for the physiological proteinuria of mice are major urinary proteins (MUPs), which are members of the lipocalin superfamily [8, 9] that regulate chemical communication and nutrient metabolism [10]. MUPs bind to small molecules, such as pheromones, and also act as pheromones themselves. Seventeen mouse urinary volatile organic compounds are bound to MUPs [11]. Humans do not express MUPs, as they have only one MUP pseudogene [12]. In mice, all 22 Mup genes and 29 of the 30
Mup-ps pseudogenes are encoded as a gene cluster on chromosome 4 [8]. MUPs are ~20 kDa proteins that are synthesized primarily in the liver in response to various hormones [13, 14], transported by the bloodstream, and excreted into urine. The high level of MUPs in urine may mask the presence or absence of urinary proteins of similar sizes in analyses of urinary proteins. MUPs are present in both urine and blood, and the small molecule-binding activity of MUPs may cause mice to differ from humans in drug efficacy tests. Moreover, the level of MUP production depends on the strain [15] and nutritional status [16]. MUPs may modulate drug efficacy, which could also reduce drug efficacy.

To overcome this disadvantage of the mouse model in urology, we produced MUP-knockout (Mup-KO) mice by genome editing with the CRISPR/Cas9 system. Our strategy was similar to that of Yang et al. [17] and included a comparison of the urinary protein profiles of KO and wild-type mice. We also examined the effect of MUPs on protein measurements using dipsticks for urinalysis, because the sensitivity of dipsticks depends on the proteins [18].

**Materials and Methods**

**Animals**

We used C57BL/6NCr mice to produce KO and ICR mice as uterine foster mothers. All animal experiments were conducted in accordance with the guidelines for animal experiments of the National Institutes of Biomedical Innovation, Health, and Nutrition, Osaka, Japan (authorization number: DS30-34).

**Production of Mup-KO mice by genome editing**

We deleted an ~2-Mb cluster of Mup genes on chromosome 4 (Fig. 1) in C57BL/6NCr mice by the TAKE method [19] using Cas9 proteins (Integrated DNA
Technologies, Coralville, IA, USA) and two guide RNAs (gRNAs, Integrated DNA Technologies) targeting both ends of the cluster (see Fig. 1B for details). We verified the removal of the gene cluster in genetically modified mice using PCR (see Fig. 1B, 1C, and 1D for details). The primer sequences used were Mup4-F (CAGGATTGCACCTTCTCTGTGAGACA), Mup4-R (TTTCTTCCCTGTGTTCTGGCAAATA), and Mup21-F (AAAAAGCCCACTGAAACCAGAGAGT). The Mup-KO mice are available from our mouse repository (Laboratory Animal Resource Bank, https://animal.nibiohn.go.jp/e_mup-ko.html).

Urine collection

We used 0.5-ml Eppendorf tubes to collect urine from 8- to 9-week-old mice following spontaneous urination of the mice when handled [20]. The urine was stored in a −25°C freezer until use. Human urine (a mixture of urine collected from five healthy males and five healthy females, with informed consent) was purchased from BioIVT (Royston, UK).

Quantification of urinary proteins

Urinary protein concentrations were measured using a fluorescence-based protein assay for total proteins (EZQ™ Protein Quantitation Kit, Thermo Fisher Scientific, Waltham, MA, USA) and dipsticks for urinalysis (Uropaper III, Eiken, Tokyo, Japan).

SDS-PAGE of urine samples

Proteins in urine (3.25 μl urine/lane) from Mup-KO and wild-type mice were separated by SDS-PAGE using a NuPAGE Bis-Tris gel (4–12%) and NuPAGE MES SDS
running buffer (Thermo Fisher Scientific). Precision Plus protein™ standard (unstained; Bio-Rad, Hercules, CA, USA) was used as a molecular weight marker. The proteins in the gels were stained with SYPRO Ruby (Thermo Fisher Scientific), and the stained gels were photographed using an image scanner (FX-Pro, Bio-Rad).

Two-dimensional electrophoresis (2DE) of urine samples

Urinary proteins from Mup-KO and wild-type mice and humans were purified using a 2D clean-up kit (Bio-Rad). Then, they were reduced and alkylated using a reduction–alkylation kit (Bio-Rad). The urinary proteins (~18 μg) were separated by 2DE (first dimension, isoelectric focusing using IPG strips (pH 3–10); second dimension, NuPAGE 4–12% Bis-Tris gels + NuPAGE MES SDS running buffer, Thermo Fisher Scientific). Precision Plus protein™ standard (unstained; Bio-Rad) was used as a molecular weight marker. The proteins in the gels were stained with SYPRO Ruby, and gel images were captured using an image scanner (FX-Pro).

Statistical analysis

The urinary protein concentrations measured by the EZQ quantitation kit were compared between Mup-KO and wild-type mice using Student’s t-test after normality and equality of variance were confirmed by Shapiro-Wilk and Brown-Forsythe tests, respectively. The urinary protein concentrations measured with dipsticks were compared using the chi-square test. Differences were considered significant if $p < 0.05$. 
Results

Production of Mup-KO mice by genome editing

We introduced Cas9/gRNA complexes into 92 pronuclear embryos by electroporation. Transfer of 87 two-cell embryos into uterine foster mothers resulted in the birth of 19 pups. Genotyping PCR for KO alleles revealed that eight of 17 weaned pups had the desired mutations (Fig. 1C). Genotyping PCR for wild-type alleles indicated that one (#2) of the eight pups with KO alleles was homozygous for the KO alleles (Fig. 1D). The other seven pups were heterozygous. The genetically modified mice were produced with the same efficiency as reported by Yang et al. [17]. We intercrossed founder mice (F0) and used F1 generation mice homozygous for the Mup-KO alleles as Mup-KO mice. The Mup-KO mice appeared healthy and reproduced normally.

Quantification of urinary proteins

The urinary protein concentrations assayed by the EZQ protein quantitation kit were significantly lower in male Mup-KO mice than in male wild-type mice ($p < 0.001$, Student’s t-test; normality and equality of variance confirmed by the Shapiro-Wilk and Brown-Forsythe tests, $p = 0.686$ and $p = 0.127$, respectively; Fig. 2A). Protein measurement using urine dipsticks showed the same tendency (Mup-KO < wild-type), but the difference between the two groups was not significant ($p = 0.368$, chi-square test; Fig. 2B).

SDS-PAGE of urine samples

Protein analysis using SDS-PAGE gels loaded with equal volumes of urine, not protein, per lane indicated no MUP proteins in the urine of Mup-KO mice, whereas there were ~20-kDa MUP proteins in the urine of wild-type mice (Fig. 2C). No differences in
proteins other than MUPs were detected, and no gender differences in urinary MUP proteins were detected in wild-type mice.

2DE of urine samples

Figure 3 shows 2DE images for urinary proteins from male Mup-KO and wild-type mice and humans. The 2DE image for Mup-KO mouse proteins was similar to that for human proteins in terms of the absence of the prominent spot at ~20 kDa, representing MUPs, seen in the 2DE image for wild-type mouse proteins. The enlarged panels in Fig. 3 show that the spot pattern for Mup-KO urinary proteins (i.e., the spots in circled regions) was more similar to that for human urinary proteins than that for wild-type urinary proteins.

Discussion

In this study, we confirmed that the urinary protein profile of mice was comparable to that of humans after knocking out the *Mup* gene cluster. Therefore, Mup-KO mice might be a useful mouse model of human kidney diseases and a tool for evaluating the roles of MUPs in mice. Dipsticks for urinalysis had low sensitivity to MUPs. This indicates that dipsticks can measure the concentration of urinary proteins other than MUPs in wild-type mice but are not suitable for detecting changes in MUPs in urine.

KO of the *Mup* gene cluster significantly reduced the protein concentration in mouse urine to a level equivalent to that in healthy humans (< 15 mg/dl, Fig. 2A). The contribution of MUPs to the urinary protein concentration was 56 mg/dl or 76% of total mouse urinary proteins. Although this contribution is lower than that reported in the literature (99% or greater) [7], MUPs are still considered major mouse urinary proteins. In addition, the removal of MUPs did not seem to change the concentrations of other proteins determined by SDS-PAGE (Fig. 2C). Both male and female wild-type mice had large quantities of urinary MUPs.
proteins, whereas both male and female KO mice had lost urinary MUP proteins. Thus, no noticeable difference was found between males and females. An ~25-kDa band was detected only in wild-type females, as shown in Fig. 2C, but proteins of this band seem unlikely to be MUPs because the 25kDa band is not always detected (data not shown), as shown in the study of Liu et al. [21]; moreover, bands have also been detected in some MUP-KO mice (data not shown). Although we did not identify the ~25-kDa proteins in the present study, lipocalin 2 [22] is one possible candidate.

Protein quantification by dipstick is not sensitive to MUPs. The contribution of MUPs to the protein concentration measured by dipsticks was minor (Fig. 2B), despite the large reduction in protein concentration following MUP removal, which was confirmed by the EZQ assay (Fig. 2A) and SDS-PAGE (Fig. 2C). The effect of MUPs on dipstick measurements was one of the main concerns in this study, since urinary protein concentrations of mice in experiments are often measured with dipsticks [23], which use protein-error methods [24] based on bromophenol blue or tetrabromophenol blue. However, the sensitivity of these methods depends on the proteins [18], and in human urine, the protein concentration measured reflects mainly the albumin level. Our results indicate that dipstick measurements in mice mainly depends on the albumin level as in humans, and that the presence of MUPs may be negligible in analyses of urinary albumin. On the other hand, the “true” total protein concentration, including MUPs, in mouse urine is difficult to measure using dipsticks. In addition to low molecular weights, acidic isoelectric points (pI) might be involved in the low response of dipsticks to MUPs (pI: ~4, estimated from 2DE in Fig. 3) because uromodulin (Tamm-Horsfall protein, pI: ~3.5 [25]) is one of the low responders [18] despite its high molecular weight (~85kDa).

Our comparisons of urinary protein profiles examined by SDS-PAGE (Fig. 2C) and 2DE (Fig. 3) demonstrate the advantage of Mup-KO mice; that is, we can compare protein
status in more detail among mice and between mice and humans using Mup-KO mice compared with wild-type mice because it is no longer necessary to consider the masking effect of abundant MUPs. Specifically, we can compare ~20 kDa proteins, including many proteins with clinical and prognostic significance [4]. The similarity of some spots in the 2DE profiles of Mup-KO mice and humans (Fig. 3) also suggests an advantage of Mup-KO mice as a model for human diseases.

Mup-KO mice might be a useful mouse model of human kidney diseases because of the absence of MUPs, which are also absent in humans, and because there is no masking effect due to the presence of these abundant MUPs when comparing low-molecular-weight urinary proteins. These mice are also useful for research on the roles of MUPs in mice, but care should be taken when choosing a protein quantification method that detects MUPs, since dipsticks for urinalysis are not sensitive enough to detect MUPs.

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FIGURES

Fig. 1

Modification of the Mup gene cluster. A: The Mup gene cluster is an approximately 2-Mb region, including pseudogenes. All Mup genes are encoded from the reverse strand.

B: Genome-editing strategy using CRISPR/Cas9 and primer positions for genotyping PCR. An ~2-Mb-long cluster of Mup genes on chromosome 4 was removed from in-vitro-fertilized C57BL/6 embryos by electroporation (TAKE method [19]) of Cas9 proteins and two gRNAs targeting both ends of the cluster (TCTTCCGATCGATACAGCATTGG for the 5′-UTR of Mup21 and CTTTCAGGACCACGTCTTTCAGG for the 3′-UTR of Mup4). PAM sequences are shown in red. We confirmed the removal of the gene cluster by PCR using two primers (Mup21-F and Mup4-R). The presence of wild-type alleles was tested by PCR using two
primers (Mup4-F and Mup4-R). C: Genotyping PCR for knockout (KO) alleles in weaned pups. Removal of the *Mup* gene cluster (Mup-KO) was confirmed by PCR according to the presence of amplified bands at ~150 bp. Eight (circled) of 17 pups had the desired mutations. D. Genotyping PCR for wild-type alleles in eight pups harboring KO alleles (circled in C). The presence of an intact *Mup4* gene was confirmed by PCR, which revealed the presence of amplified bands at ~227 bp. One (#2, circled) of the eight pups was homozygous for KO alleles. Subsequent urinary protein analysis (data not shown) revealed that pup #14 was heterozygous despite the presence of a weak wild-type band. The amplicons in C and D were separated by 2% agarose electrophoresis (E-gel EX, Thermo Fisher Scientific). M: 100 bp ladder (New England Biolabs, Ipswich, MA, USA).

Fig. 2

Analyses of urinary proteins. A: The urinary protein concentration assayed by the EZQ protein quantitation kit was significantly lower (*p* < 0.001) in male Mup-KO mice (17.9 ± 1.8 mg/dl, mean ± SD, *n* = 3) than in male wild-type mice (73.7 ± 8.2 mg/dl, *n* = 3). B: Protein concentrations measured using dipsticks were ± (15 mg/dl) to + (30 mg/dl) in Mup-KO mice and + (30 mg/dl) to 2+ (100 mg/dl) in wild-type mice. The dipsticks were not sensitive enough to detect MUPs, although they did show the same trend (Mup-KO < wild-type) as the EZQ assay (*p* = 0.368). C: SDS-PAGE images show that both male and female Mup-KO mice lost urinary MUP proteins (~20 kDa) compared with male and female wild-type mice. Note that the ~25-kDa proteins in female wild-type mice were not MUPs (not identified in the present study). M: molecular weight markers.
Fig. 3

Comparison of urinary proteins in 2DE gels (~18 μg/gel). A prominent MUP spot at ~20 kDa was clearly seen in the 2DE image for wild-type mice but not in that for Mup-KO mice or humans. Because of the absence of MUPs, more urinary proteins other than MUPs are seen in the gel for Mup-KO mice compared with wild-type mice. In addition, enlarged panels indicate that the spot pattern for Mup-KO urinary proteins (e.g., spots in circled region) was more similar to that for human urinary proteins than that for wild-type urinary proteins.
Figure 1.
Figure 2.

A

Urinary protein concentration (mg/dL, MEAN±SD)

Mup-KO
n=3

Wild-type
n=3

p < 0.001

B

Mup-KO (n=3)  Wild-type (n=3)

C

Males

Females

Mup-KO  Wild-type

Mup-KO  Wild-type

25 KDa

25 KDa
Figure 3.