

---

---

# Renal Uptake of $^{99m}\text{Tc}$ -Dimercaptosuccinic Acid Is Dependent on Normal Proximal Tubule Receptor–Mediated Endocytosis

Kathrin Weyer<sup>1</sup>, Rikke Nielsen<sup>1</sup>, Steen V. Petersen<sup>1</sup>, Erik I. Christensen<sup>1</sup>, Michael Rehling<sup>2</sup>, and Henrik Birn<sup>1,3</sup>

<sup>1</sup>Department of Biomedicine, Aarhus University, Aarhus, Denmark; <sup>2</sup>Department of Clinical Physiology and Nuclear Medicine, Aarhus University Hospital, Aarhus, Denmark; and <sup>3</sup>Department of Nephrology, Aarhus University Hospital, Aarhus, Denmark

$^{99m}\text{Tc}$ -labeled dimercaptosuccinic acid ( $^{99m}\text{Tc}$ -DMSA) accumulates in the kidney cortex and is widely used for imaging of the renal parenchyma. Despite its extensive clinical use, the mechanism for renal targeting of the tracer is unresolved. Megalin and cubilin are cooperating receptors essential to the proximal tubule endocytic uptake of proteins from the glomerular ultrafiltrate. We have used megalin/cubilin-deficient mice produced by gene knockout to determine whether receptor-mediated endocytosis is responsible for the renal uptake of  $^{99m}\text{Tc}$ -DMSA. **Methods:** Control or megalin/cubilin-deficient mice were injected intravenously with 0.5 MBq of  $^{99m}\text{Tc}$ -DMSA or  $^{99m}\text{Tc}$ -mercaptoacetyltryglycine (MAG3). Whole-body scintigrams and the activity in plasma, urine, and the kidneys were examined 6 h after injection. The size and identity of  $^{99m}\text{Tc}$ -DMSA-bound proteins in urine were analyzed by fractionation by centrifugation and separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by autoradiography and mass spectrometry. **Results:** No renal accumulation of  $^{99m}\text{Tc}$ -DMSA was identified in scintigrams of megalin/cubilin-deficient mice. The renal accumulated activity of the tracer was reduced to 11.4% ( $\pm 2.5\%$ ,  $n = 7$ ) of the normal uptake in control mice, correlating with a reduction in renal megalin/cubilin expression in knockout mice to about 10% of normal. The reduced renal uptake in megalin/cubilin-deficient mice was accompanied by an increase in the urinary excretion of  $^{99m}\text{Tc}$ -DMSA. Size separation of the urine by ultracentrifugation and sodium dodecyl sulfate polyacrylamide gel electrophoresis demonstrated that in megalin/cubilin-deficient mice an increased amount of  $^{99m}\text{Tc}$ -DMSA was excreted in an approximately 27-kDa form, which by mass spectrometry was identified as the plasma protein  $\alpha 1$ -microglobulin, an established megalin/cubilin ligand. **Conclusion:**  $^{99m}\text{Tc}$ -DMSA is filtered bound to  $\alpha 1$ -microglobulin and accumulates in the kidneys by megalin/cubilin-mediated endocytosis of the  $^{99m}\text{Tc}$ -DMSA protein complex. Renal accumulation of  $^{99m}\text{Tc}$ -DMSA is thus critically dependent on megalin/cubilin receptor function and therefore is a marker of proximal tubule endocytic activity.

**Key Words:** renogram; megalin; cubilin; kidney

**J Nucl Med 2013; 54:159–165**

DOI: 10.2967/jnumed.112.110528

**D**imercaptosuccinic acid (DMSA) labeled with  $^{99m}\text{Tc}$  is a major renal cortical imaging agent used in the diagnosis of renal parenchymal disorders (1).  $^{99m}\text{Tc}$ -DMSA is highly accumulated in the kidney cortex (2), but the mechanism for the renal targeting of the tracer has not been established. Two main routes of  $^{99m}\text{Tc}$ -DMSA tubular uptake have been proposed: by basolateral uptake from plasma by peritubular extraction and by tubular reabsorption from the glomerular ultrafiltrate. However, the significance of both routes is debated (3–12). The sodium-dependent dicarboxylate transporter NaDC-3 has been implicated in the basolateral uptake of  $^{99m}\text{Tc}$ -DMSA from peritubular capillaries into proximal tubule cells (13). The mechanism by which  $^{99m}\text{Tc}$ -DMSA may be reabsorbed from the glomerular ultrafiltrate is unknown; however, studies have indicated that this route may contribute substantially to the renal uptake of the tracer (4,5,14). The potential importance of this pathway is further stressed by recent observations of a decreased renal uptake of  $^{99m}\text{Tc}$ -DMSA in patients with proximal tubular endocytic dysfunctions, such as Dent's disease, Lowes syndrome, and Fanconi syndrome (10,15,16).

The cooperating receptors megalin and cubilin are responsible for the endocytic uptake of proteins from the glomerular ultrafiltrate in the proximal tubule (17,18). Megalin, a member of the low-density lipoprotein receptor family, is a 600-kDa transmembrane glycoprotein with more than 50 known ligands, including a variety of plasma proteins. Megalin functions in a complex with the 460-kDa extracellular protein cubilin. Cubilin also binds several plasma proteins, including albumin, but depends on megalin for the endocytosis of cubilin–ligand complexes (19,20). Accordingly, megalin/cubilin-deficient mice excrete several low-molecular-weight proteins in the urine, including  $\beta 2$ -microglobulin,  $\alpha 1$ -microglobulin, and retinol-binding protein, characteristic of tubular proteinuria caused by proximal tubule endocytic dysfunction (20–22). Megalin/cubilin dysfunction has been implicated in the pathogenesis

Received Jun. 29, 2012; revision accepted Aug. 15, 2012.

For correspondence or reprints contact: Kathrin Weyer, Department of Biomedicine, Aarhus University, Wilhelm Meyers Allé 3, Building 1234, DK-8000, Aarhus C, Denmark.

E-mail: kwey@ana.au.dk

Published online Dec. 11, 2012.

COPYRIGHT © 2013 by the Society of Nuclear Medicine and Molecular Imaging, Inc.

of various renal diseases, such as the early stages of diabetic and hypertensive nephropathy, and other conditions of proteinuria (23). However, there are currently no assays for direct quantitation and imaging of proximal tubule receptor-mediated endocytic function.

Our aim was to investigate if the megalin/cubilin receptor complex is important for renal  $^{99m}\text{Tc}$ -DMSA accumulation. Using conditional megalin/cubilin-deficient mice and examining the renal uptake and urinary excretion of tracer, we identified a crucial role of the megalin/cubilin receptors for the accumulation of  $^{99m}\text{Tc}$ -DMSA and suggest a mechanism of uptake involving glomerular filtration and proximal tubule endocytosis of protein-bound tracer.

## MATERIALS AND METHODS

### Animals

Animal breeding and experiments were performed in a certified animal facility according to provisions approved by the Danish Animal Experiments Inspectorate. The conditional megalin/cubilin-deficient mice used were  $\text{Meg}^{\text{lox/lox}}$ ,  $\text{Cubn}^{\text{lox/lox}}$ ,  $\text{Wnt4-Cre}^+$  mice, where the Cre-recombinase gene is driven by the Wnt4 promoter (22). Genotyping was made on tail DNA by polymerase chain reaction as described before (20). Cre-negative littermates were used as controls. The mice were on a mixed C57BL/6-129/Svj background and 8–12 wk old at the time of use. The body weights of megalin/cubilin-deficient and control mice were  $27.4 \pm 4.8$  g and  $26.9 \pm 1.8$  g ( $P = 0.39$ ), respectively. Megalin/cubilin-deficient mice displayed left and right kidney weights of  $91.8 \pm 31.5$  and  $112.7 \pm 42.8$  mg, respectively, which was not significantly different from the kidney weights of controls of  $79.3 \pm 20.0$  and  $102.2 \pm 20.4$  mg ( $P = 0.20$  and  $P = 0.28$ ), respectively.

### Radiotracers

DMSA was prepared using a commercially available kit, Renosis DMSA (RH-Isotopagenturet). Mercaptoacetyltriglycine (MAG3) was prepared using a commercially available kit, MAG3 Technescan (DRN 4334; RH-Isotopagenturet). The tracers were labeled according to the manufacturer's instructions by adding 1,000 MBq of freshly eluted  $^{99m}\text{TcO}_4^-$  (pertechnetate) to a final concentration of 200 MBq per mL. Radiochemical purity was above 95%.

### $^{99m}\text{Tc}$ -DMSA and $^{99m}\text{Tc}$ -MAG3 Uptake Studies in Mice

Megalin/cubilin-deficient or control mice were anesthetized with isoflurane for a few minutes for the intravenous injection in the femoral vein of 0.5 MBq of  $^{99m}\text{Tc}$ -DMSA or  $^{99m}\text{Tc}$ -MAG3 in a total volume of 0.1 mL of saline. The mice were placed in metabolic cages for 6 h for urine collection. At the end of the 6-h urine collection, the mice injected with  $^{99m}\text{Tc}$ -DMSA ( $n = 7$  in each group) were anesthetized, and the remaining urine in the bladder and the kidneys was collected. A blood sample was collected into a syringe containing heparin. Blood and urine samples were centrifuged for 10 min at 1,600g. Specimens (50  $\mu\text{L}$ ) of plasma and urine, and the whole kidneys, were counted using a  $\gamma$ -counter (Packard Biosciences). Renal imaging was performed by placing megalin/cubilin-deficient mice injected with  $^{99m}\text{Tc}$ -DMSA or control mice ( $n = 3$  in each group) directly on the detector of a  $\gamma$ -camera (BrightView, Philips Healthcare) with a low-energy high-resolution collimator. Planar images were acquired over 10 min in a  $256 \times 256$  matrix.

### Size Fractionation of Urine

Urine from megalin/cubilin-deficient mice injected with  $^{99m}\text{Tc}$ -DMSA or control mice ( $n = 3$  in each group) was pooled, and 800  $\mu\text{L}$  were applied on centrifuge filters (Amicon; Millipore) with a molecular weight cutoff at 100, 30, 10, 3, and less than 3 kDa, and separated by centrifugation at 4,000g. Samples were counted using a  $\gamma$ -counter before and after the fractionation.

### Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Protein Visualization

Urine samples (15  $\mu\text{L}$ ) collected from megalin/cubilin-deficient mice injected with  $^{99m}\text{Tc}$ -DMSA or control mice ( $n = 3$  in each group) were separated under nonreducing conditions by SDS-PAGE (16%). For protein identification, gels were silver-stained using Proteosilver Plus according to the manufacturer's instructions (Sigma-Aldrich) and dried, and the position of the  $^{99m}\text{Tc}$ -labeled protein was determined by autoradiography. For immunodetection, urine samples collected from megalin/cubilin-deficient mice or controls ( $n = 3$  in each group) were separated by unreduced SDS-PAGE (16%) and immunoblotted as described previously (24), using rabbit-antirat  $\alpha 1$ -microglobulin (Agrisera).

### Mass Spectrometry (MS)

The in-gel digestion procedure of proteins separated by SDS-PAGE was performed essentially as described before (25). The samples were digested by sequence-grade porcine trypsin, peptides were recovered by reverse-phase absorption ( $\text{C}_{18}$  StageTip; Proxeon Biosystems), and the sample was eluted directly onto the matrix-assisted laser desorption/ionization (MALDI) target using 1  $\mu\text{L}$  of  $\alpha$ -cyano-4-hydroxycinnamic acid in 70% acetonitrile and 0.1% trifluoroacetic acid. Peptides were subsequently analyzed using an Autoflex Smartbeam III instrument (Bruker Daltonics GmbH) operated in both MS and MS/MS mode (LIFT). Before analyses, the instrument was calibrated by external calibration using a peptide mix containing 7 calibrants (Bruker Daltonics). On the basis of the generated mass spectra, proteins were identified using the Mascot search engine and the Swiss-Prot database as reference.

### Kidney Tissue Preparation and Analysis

After being counted on the  $\gamma$ -counter, kidney extracts were prepared from the collected kidneys of the megalin/cubilin-deficient mice injected with  $^{99m}\text{Tc}$ -DMSA or from control mice. In brief, kidney cortices were homogenized in ice-cold 0.3 M sucrose, 25 mM imidazole (pH 7.2), 8.5 M leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM ethylenediaminetetraacetic acid for 30 s with an Ultra-Turrax T8 homogenizer (IKA-Werke) and centrifuged at 4,000g for 15 min at 4°C. Cleared supernatants (10  $\mu\text{g}$  of protein) were analyzed by SDS-PAGE (16%) and visualized by immunoblotting (24), using sheep-antirat megalin (26), rabbit-antirat cubilin (27), and rabbit antiactin (A5060; Sigma-Aldrich). Band intensities were quantified using the Odyssey software (version 1.2; LI-COR Biosciences). The megalin and cubilin protein levels in the kidneys were expressed relative to actin protein levels.

### In Vitro Binding Assay

$^{99m}\text{Tc}$ -DMSA (0.005 MBq) was incubated for 1 h at 37°C with buffer or purified human  $\alpha 1$ -microglobulin (AbD Serotec) at a final concentration of 0.05 mg/mL, in a total volume of 250  $\mu\text{L}$  diluted in phosphate-buffered saline (pH 7.4). Samples (50  $\mu\text{L}$ ) were counted with a  $\gamma$ -counter before and after fractionation

on a filter (molecular weight cutoff, 30 kDa; Amicon) by centrifugation at 4,000g. The experiment was performed in triplicate.

### Immunohistochemistry

Kidneys from megalin/cubilin-deficient or control mice ( $n = 3$  in each group) were fixed by retrograde perfusion with 2% paraformaldehyde, and sections were processed as reported before (20), using sheep-antirat megalin and rabbit-antirat  $\alpha$ 1-microglobulin. Images were obtained using a confocal laser-scanning microscope (LSM 510-META; Carl Zeiss) and processed using Zeiss Zen software (2009, Light Edition).

### Statistics

Data represent mean  $\pm$  SD. Groups were compared using a Student unpaired  $t$  test, assuming equal variance. A  $P$  value of less than 0.05 was considered significant.

## RESULTS

### Renal Uptake and Urinary Excretion of $^{99m}\text{Tc}$ -DMSA

Accumulation of  $^{99m}\text{Tc}$ -DMSA uptake in the kidneys of normal control mice was evident by whole-body  $\gamma$ -camera scintigraphy 6 h after intravenous injection of  $^{99m}\text{Tc}$ -DMSA (Fig. 1A). In contrast, no accumulation was seen in the kidneys of megalin/cubilin-deficient mice (Fig. 1B). When quantified by  $\gamma$ -counting, the uptake of  $^{99m}\text{Tc}$ -DMSA in isolated kidneys from megalin/cubilin-deficient mice was reduced to  $1.8\% \pm 0.4\%$  of the injected dose per 100 mg of kidney tissue (Fig. 2A), corresponding to 11.4% of the normal uptake in controls ( $n = 7$  in each group). This uptake was associated with an approximate 90% increase in the urinary excretion of  $^{99m}\text{Tc}$ -DMSA (Fig. 2B). Thus, whereas the total renal activity of megalin/cubilin-deficient mice was  $8,882 \pm 3,264$  cpm, compared with  $66,656 \pm 9,336$  cpm ( $P < 0.0001$ ) in controls, the total activity of  $^{99m}\text{Tc}$ -DMSA extracted by the kidneys (kidney plus urine activity) was not significantly different between megalin/cubilin-deficient mice and control mice ( $7.9 \cdot 10^3 \pm 1.6 \cdot 10^3$  vs.  $6.5 \cdot 10^3 \pm 1.2 \cdot 10^3$  cpm/g mouse,  $P = 0.07$ ).

The approximate 90% reduction in kidney uptake of the tracer correlates with a megalin and cubilin protein knockdown efficiency of  $90.8\% \pm 8.7\%$  and  $89.8\% \pm 6.1\%$ , respectively, as evaluated by immunoblotting of kidney cortex homogenates from megalin/cubilin-deficient

mice, compared with controls, which is similar to previous reports in these mice (22). The plasma level of  $^{99m}\text{Tc}$ -DMSA in megalin/cubilin-deficient mice and controls was not significantly different (Fig. 2C).

### Urinary Excretion of $^{99m}\text{Tc}$ -MAG3

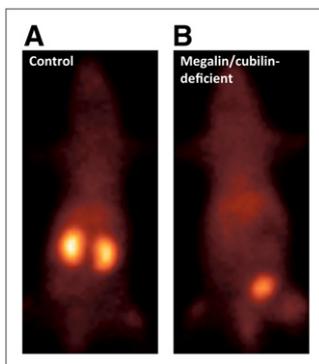
No significant difference ( $P = 0.11$ ) in the urinary excretion of  $^{99m}\text{Tc}$ -MAG3 was observed between megalin/cubilin-deficient ( $n = 7$ ) and control mice ( $n = 7$ , Fig. 3) after 6 h. Although we cannot rule out a potential difference in the kinetic parameters of  $^{99m}\text{Tc}$ -MAG3 handling, the total excretion of  $^{99m}\text{Tc}$ -MAG3 appears to be unaffected by the megalin/cubilin deficiency.

### Urinary Excretion of Protein-Bound $^{99m}\text{Tc}$ -DMSA

The molecular weight of the  $^{99m}\text{Tc}$  activity in urine was determined through size fractionation by successive application of urine to centrifuge filters, thereby producing fractions corresponding to molecular weights of more than 100, 30–100, 10–30, 3–10, and less than 3 kDa. In urine from megalin/cubilin-deficient mice, 60% of the injected dose of  $^{99m}\text{Tc}$ -DMSA was associated with fractions corresponding to a molecular weight of more than 10 kDa, compared with only 17% in urine from control mice (Table 1). In both controls and megalin/cubilin-deficient mice, 2%–3% was bound to small peptides (3–10 kDa), and 19%–20% was excreted as small molecules, including amino acid-bound and free  $^{99m}\text{Tc}$ -DMSA. Thus, the increased  $^{99m}\text{Tc}$ -DMSA activity in the urine of megalin/cubilin-deficient mice reflects a 3.5-fold increase of protein-bound  $^{99m}\text{Tc}$ -DMSA excreted in the urine.

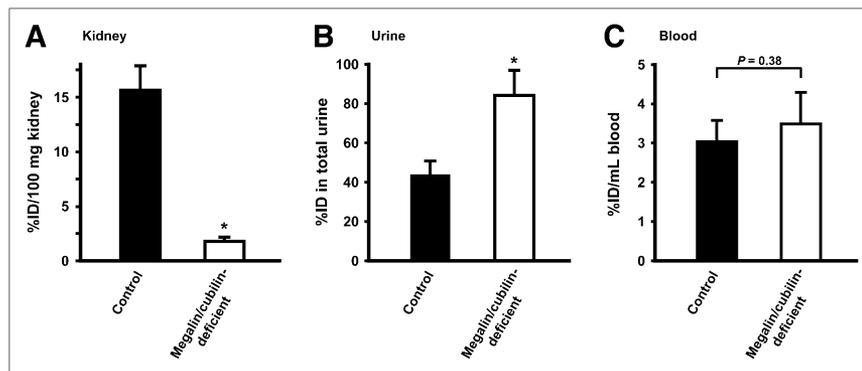
### Identification of $^{99m}\text{Tc}$ -DMSA Bound to $\alpha$ 1-Microglobulin

The analysis of urine samples collected from megalin/cubilin-deficient mice by SDS-PAGE and autoradiography showed that the  $^{99m}\text{Tc}$  radioactivity was associated with a single band of approximately 27 kDa (Fig. 4A). To identify the protein associated with the tracer, the collected urine samples were subjected to SDS-PAGE, and proteins were visualized by silver staining. The sample representing megalin/cubilin-deficient mice presents unique staining at the position corresponding to 27 kDa (Fig. 4B). This band was excised, subjected to in-gel digestion using trypsin, and subsequently identified as the plasma protein  $\alpha$ 1-microglobulin by MALDI MS (Table 2). This finding was further corroborated by immunoblotting (Fig. 4C). The interaction between  $^{99m}\text{Tc}$ -DMSA and  $\alpha$ 1-microglobulin was further evaluated in vitro using purified  $\alpha$ 1-microglobulin, whereby approximately 80% of the  $^{99m}\text{Tc}$ -DMSA activity was found bound to  $\alpha$ 1-microglobulin after 1 h of incubation. Finally, immunohistochemical staining of kidney sections from control and megalin/cubilin-deficient mice demonstrated proximal tubule uptake of  $\alpha$ 1-microglobulin in megalin-expressing cells only, confirming that megalin is essential for uptake of  $\alpha$ 1-microglobulin (Fig. 5).



**FIGURE 1.** Megalin/cubilin-dependent renal accumulation of  $^{99m}\text{Tc}$ -DMSA. Whole-body scintigram of mice 6 h after injection of  $^{99m}\text{Tc}$ -DMSA shows accumulation in kidneys of control mice (A) but no accumulation in kidneys of megalin/cubilin-deficient mice (B). In megalin/cubilin-deficient mice, evidence of accumulation of tracer in bladder was seen. Images shown are representative of similar analyses ( $n = 3$  in each group).

**FIGURE 2.** Renal uptake and urinary excretion of  $^{99m}\text{Tc}$ -DMSA by megalin/cubilin-deficient and control mice. Renal uptake (A), urinary excretion (B), and plasma levels (C) of  $^{99m}\text{Tc}$ -DMSA in megalin/cubilin-deficient mice are compared with controls. Columns represent mean of each group ( $n = 7$ )  $\pm$  SD. \*Significantly different from control mice ( $P < 0.0001$ ).



## DISCUSSION

We show that in megalin/cubilin-deficient mice, the renal accumulation of  $^{99m}\text{Tc}$ -DMSA is almost abolished and associated with a corresponding increase in urinary excretion of the tracer. The increased  $^{99m}\text{Tc}$ -DMSA activity in the urine is found to be associated with high-molecular-weight fractions, suggesting protein binding. MS and *in vitro* binding studies identified  $\alpha 1$ -microglobulin as the  $^{99m}\text{Tc}$ -DMSA binding protein present in urine.  $\alpha 1$ -microglobulin is a 27-kDa plasma protein filtered in the glomeruli and an established ligand for both megalin and cubilin (21,24). This is confirmed by immunohistochemistry showing uptake of  $\alpha 1$ -microglobulin in megalin-expressing tubule cells only. On the basis of these observations, we proposed a new model for renal accumulation of  $^{99m}\text{Tc}$ -DMSA (Fig. 6). In this model,  $^{99m}\text{Tc}$ -DMSA binds to  $\alpha 1$ -microglobulin, which is filtered as a complex in the normal renal glomeruli followed by megalin/cubilin-mediated endocytic uptake and accumulation of the tracer in the kidney proximal tubules.

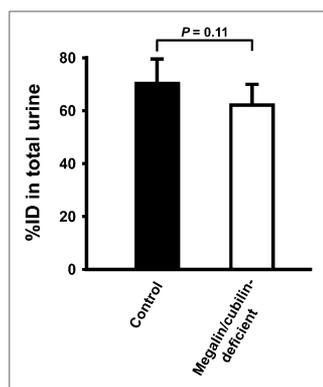
An approximate 10% residual renal uptake of  $^{99m}\text{Tc}$ -DMSA was observed in the kidneys from megalin/cubilin-deficient mice. Although an additional and minor pathway for renal uptake of  $^{99m}\text{Tc}$ -DMSA cannot be excluded, this corresponds to the possibility that the approximately 10% remaining megalin expression in these mice is a likely explanation for the residual uptake. This argument is also consistent with a previous study using kidney-specific megalin-

deficient mice, in which a renal uptake of 35%–55% of  $^{99m}\text{Tc}$ -DMSA was reported in megalin-deficient mice, compared with normal controls, although the data were not shown in the study (12). Previous analyses, however, showed that these megalin-deficient mice have a partial and variable (35%–50%) megalin expression in the kidneys (28), explaining this variation in  $^{99m}\text{Tc}$ -DMSA uptake.

The renal  $^{99m}\text{Tc}$ -DMSA uptake in megalin/cubilin-deficient mice was reduced to  $1.8\% \pm 0.4\%$  of the total injected dose per 100 mg of kidney tissue, whereas the uptake in control mice was  $15.6\% \pm 2.3\%$ , similar to what has been reported in rats (12). Importantly, the total activity of  $^{99m}\text{Tc}$ -DMSA ( $7.9 \cdot 10^3 \pm 1.6 \cdot 10^3$  cpm/g) extracted by the kidneys (kidney plus urine activity) of megalin/cubilin-deficient mice was not significantly different from that of control mice ( $6.5 \cdot 10^3 \pm 1.2 \cdot 10^3$  cpm/g) ( $P = 0.07$ ), indicating similar renal extraction and filtration of  $^{99m}\text{Tc}$ -DMSA.

$\alpha 1$ -microglobulin, a member of the lipocalin protein superfamily, is synthesized in the liver, freely filtered by glomeruli, and reabsorbed by renal proximal tubule cells, where it is catabolized (29). Under normal conditions, little filtered  $\alpha 1$ -microglobulin appears in the final excreted urine; however, urinary excretion of  $\alpha 1$ -microglobulin has been demonstrated previously in megalin-deficient mice (21) and  $\alpha 1$ -microglobulin was recently shown to be excreted in the urine of a patient with cubilin dysfunction, where additional binding experiments confirmed that both megalin and cubilin bind  $\alpha 1$ -microglobulin (24). An in-

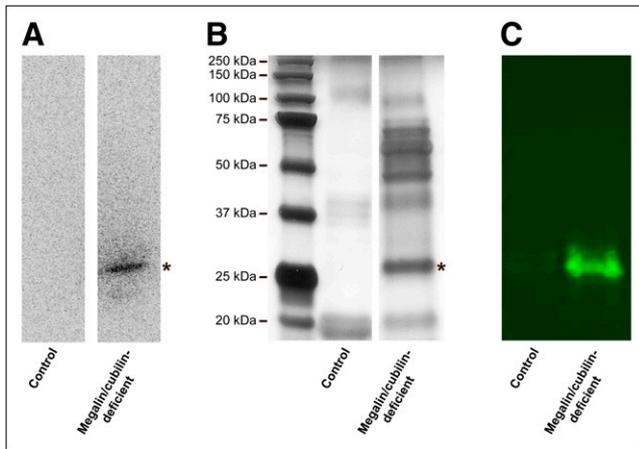
**FIGURE 3.** Urinary excretion of  $^{99m}\text{Tc}$ -MAG3 by megalin/cubilin-deficient and control mice. Columns represent mean of each group ( $n = 7$ )  $\pm$  SD.



**TABLE 1**  
Ultrafiltration of Urine from Mice Injected with  $^{99m}\text{Tc}$ -DMSA

Molecular weight (kDa)	Control	Megalyn/cubilin-deficient
>100	4	8
30–100	9	43
10–30	4	9
3–10	3	2
<3	20	19

Values are from mixed urines ( $n = 3$ ) and expressed as percentage of injected dose.



**FIGURE 4.** Urinary excretion of protein-bound  $^{99m}\text{Tc}$ -DMSA by megalin/cubilin-deficient mice. (A) SDS-PAGE separation and autoradiography of urine from megalin/cubilin-deficient mice and controls injected with  $^{99m}\text{Tc}$ -DMSA. A single, labeled band of approximately 27 kDa is observed in urine collected from megalin/cubilin-deficient mice only. (B) Urine profile analysis by SDS-PAGE and silver staining of urine from megalin/cubilin-deficient mice and controls. A distinct protein band of 27 kDa was identified in urine from megalin/cubilin-deficient mice (\*). This band was excised and protein identified by MALDI MS as  $\alpha$ 1-microglobulin (Table 2). (C) Immunoblotting identifying  $\alpha$ 1-microglobulin as an approximate 27-kDa band in urine from megalin/cubilin-deficient mice but not from controls. Data shown are representative of 2 (autoradiography and MALDI MS) or 3 (SDS-PAGE, silver staining, and immunoblotting) independent experiments.

crease in the urinary concentration of  $\alpha$ 1-microglobulin therefore indicates proximal tubule cell injury or impaired proximal tubular endocytosis and is considered a valuable marker of renal proximal tubular dysfunction (30–32). Interestingly, similar to our findings in urine from megalin/cubilin-deficient mice, the molecular size of excreted  $^{99m}\text{Tc}$ -DMSA in the urine of patients with idiopathic tubular proteinuria is also in the range of 24–28 kDa (33), supporting binding to a urinary protein of this size. Our study showed no uptake of  $\alpha$ 1-microglobulin in megalin-negative tubular cells independent of cubilin expression. Thus, although  $\alpha$ 1-microglobulin may bind to both cubilin and megalin, megalin is essential for the internalization of the cubilin– $\alpha$ 1-microglobulin complex, as also shown for other proteins binding to both receptors, such as albumin

(20). It has previously been shown that most (65%–90%)  $^{99m}\text{Tc}$ -DMSA in plasma is protein-bound (6,31,34), and it has been proposed that the sulfhydryls in DMSA bind to free cysteine residues.  $\alpha$ 1-microglobulin has a free cysteine side-chain located in a flexible loop, giving the protein reductase and dehydrogenase properties with a broad biologic substrate specificity (35). It is therefore likely that the binding of  $^{99m}\text{Tc}$ -DMSA to  $\alpha$ 1-microglobulin occurs via its free cysteine residue.

On the basis of our findings, the renal uptake of  $^{99m}\text{Tc}$ -DMSA depends on the binding to  $\alpha$ 1-microglobulin, whereby it becomes a ligand for the megalin/cubilin receptors; the glomerular filtration of  $\alpha$ 1-microglobulin; and the activity of proximal tubule megalin/cubilin-mediated endocytosis. Under conditions for which the first 2 factors may be assumed to be constant, a  $^{99m}\text{Tc}$ -DMSA scan will reflect the receptor-mediated endocytic activity in the proximal tubule, as is supported by several studies showing poor  $^{99m}\text{Tc}$ -DMSA renal uptake in patients with disturbed tubular endocytosis and subsequent low-molecular-weight proteinuria, including Dent's disease, Lowes syndrome, and Fanconi syndrome (10,15,16). Reduced  $^{99m}\text{Tc}$ -DMSA uptake in these patients is associated with increased bladder activity, indicating an increased urinary excretion of  $^{99m}\text{Tc}$ -DMSA, and correlates with the degree of tubular proteinuria (10). However, quantitative estimates of megalin/cubilin function will require further studies, including evaluation of the dynamics of  $^{99m}\text{Tc}$ -DMSA uptake and relating it to the glomerular filtration rate in conditions with varying degree of tubulopathy. In the case of heavy proteinuria, an altered tracer distribution could be due to the increased glomerular filtration of proteins, leading to increased urinary loss and reduced  $^{99m}\text{Tc}$ -DMSA uptake, as reported (36). Conversely, diminished filtration would lead to low  $^{99m}\text{Tc}$ -DMSA kidney uptake, even while tubular function is retained, as it is observed under renal artery stenosis and captopril experiments (5,37). Thus,  $^{99m}\text{Tc}$ -DMSA renal uptake should also be cautiously interpreted in the case of alterations in glomerular filtration.

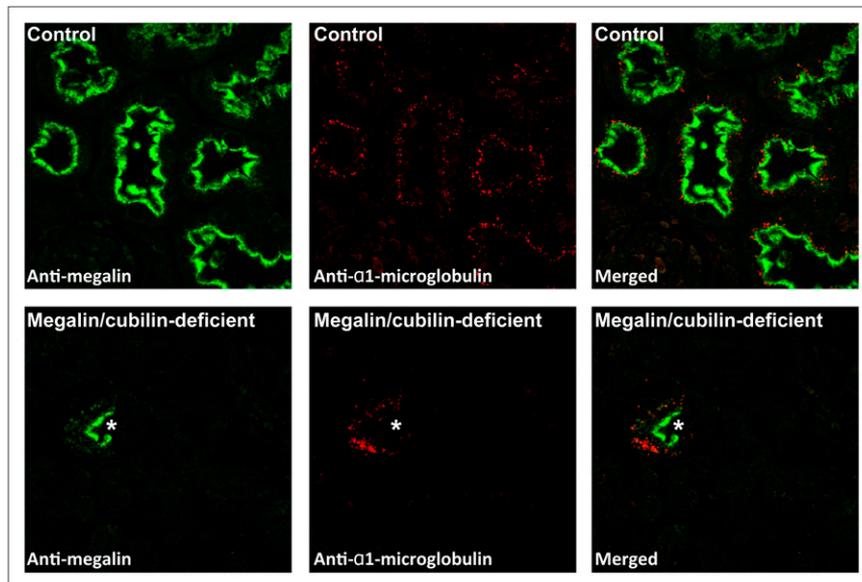
Our findings may also explain previous observations made in a mouse model of Dent's disease caused by knock-out of the renal, endosome-associated chloride channel

**TABLE 2**  
Mass Spectrometry (MS/MS) Analysis of Trypsin-Cleaved 27-kDa Band from Urine Separated by SDS-PAGE

Protein	Measured peptide mass (Da)	Peptide sequence identified by MS/MS
$\alpha$ 1-microglobulin*	1,131.6	K.LYGREPQLR.D
	1,354.6	R.GVCEEITGAYQK.T
	1,510.7	R.RGVCEEITGAYQK.T
	1,809.8	K.WYNLAVGSTCPWLSR.I
	2,090.0	K.DVALNVGISENSIIFMPDR.G

\*Mascot score, 393.

**FIGURE 5.** Megalin/cubilin-dependent renal uptake of  $\alpha$ 1-microglobulin. Immunohistochemical staining is shown for megalin (green) and  $\alpha$ 1-microglobulin (red) in kidney cortex of control and megalin/cubilin-deficient mice. In megalin/cubilin-deficient mice, a few profiles of megalin-expressing proximal tubule cells can be identified (\*) because of mosaic expression. Merged images showed that  $\alpha$ 1-microglobulin cannot be identified in proximal tubular cells lacking megalin.



CIC-5. In *Clcn5* knockout mice, impaired uptake of  $^{99m}\text{Tc}$ -DMSA was observed (11). The *Clcn5* knockout mice are known to display disturbed megalin/cubilin-mediated endocytic activity due to impaired trafficking of the receptors (38), accompanied by an increased urinary excretion of  $\alpha$ 1-microglobulin (39). Thus, although it was proposed that CLC-5 affects trafficking of NaDC transporters (40), it seems likely that dysfunction of megalin/cubilin-mediated endocytosis is the actual cause of impaired

$^{99m}\text{Tc}$ -DMSA uptake in these mice. Interestingly, similar to what our data indicate in megalin/cubilin-deficient mice, the renal uptake of  $^{99m}\text{Tc}$ -MAG3 was normal in *Clcn5* knockout mice (11), suggesting that the basolateral uptake mediated by an organic anion transporter (41), and its excretion by proximal tubular cells, is not disturbed by defective endocytosis.

## CONCLUSION

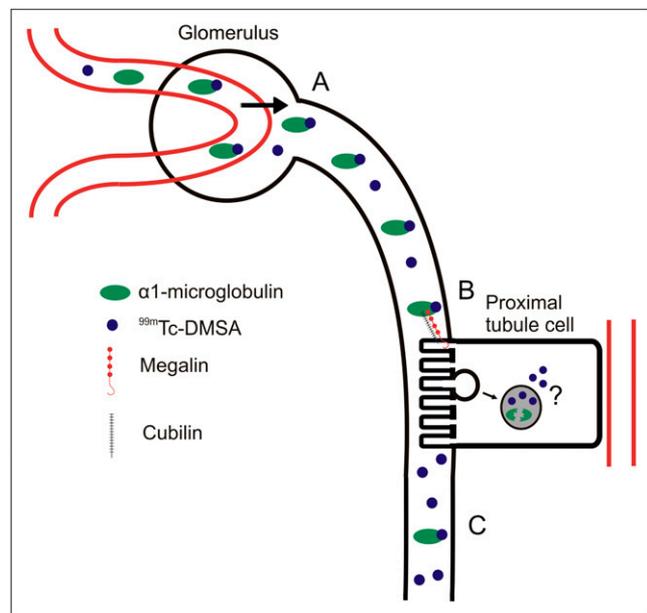
We have shown that megalin/cubilin receptor-mediated endocytosis is essential for normal renal accumulation of the  $^{99m}\text{Tc}$ -DMSA tracer. Renal uptake depends on the binding of  $^{99m}\text{Tc}$ -DMSA to the plasma protein  $\alpha$ 1-microglobulin, followed by glomerular filtration and megalin/cubilin-mediated endocytosis by proximal tubular cells. Thus, the  $^{99m}\text{Tc}$ -DMSA renography is a potential method for the evaluation of proximal tubule endocytic function in patients.

## DISCLOSURE

The costs of publication of this article were defrayed in part by the payment of page charges. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

## ACKNOWLEDGMENTS

The work was supported by the Lundbeck Foundation, the Danish Medical Research Council, Novo Nordisk Foundation, the European Commission EUNEFON (FP7, GA#201590), The Danish Kidney Association, and The Aase and Ejnar Danielsen's Foundation. No other potential conflict of interest relevant to this article was reported. Hanne Sidelmann, Inger Blenker Kristoffersen, Pia Kamuk Nielsen, Helle Salling Gittins, and Lene E. Nielsen are gratefully acknowledged for excellent technical assistance.



**FIGURE 6.** Mechanism for renal uptake of  $^{99m}\text{Tc}$ -DMSA. (A)  $\alpha$ 1-microglobulin-bound  $^{99m}\text{Tc}$ -DMSA is freely filtered by glomeruli and accumulates in renal proximal tubules by endocytosis mediated by multiligand-binding megalin/cubilin receptors (B). (C) Free  $^{99m}\text{Tc}$ -DMSA and trace amounts of  $\alpha$ 1-microglobulin-bound  $^{99m}\text{Tc}$ -DMSA are excreted in urine. Consequently, megalin/cubilin dysfunction leads to abolishment of renal uptake and increased urinary excretion of  $\alpha$ 1-microglobulin-bound  $^{99m}\text{Tc}$ -DMSA.

## REFERENCES

- Durand E, Prigent A. The basics of renal imaging and function studies. *Q J Nucl Med.* 2002;46:249–267.
- Willis KW, Martinez DA, Hedley-Whyte ET, Davis MA, Judy PF, Treves S. Renal localization of <sup>99m</sup>Tc-stannous glucoptonate and <sup>99m</sup>Tc-stannous dimer-captosuccinate in the rat by frozen section autoradiography: the efficiency and resolution of technetium-99m. *Radiat Res.* 1977;69:475–488.
- van Luijk WH, Ensing GJ, Meijer S, Donker AJ, Piers DA. Is the relative <sup>99m</sup>Tc-DMSA clearance a useful marker of proximal tubular dysfunction? *Eur J Nucl Med.* 1984;9:439–442.
- Provoost AP, Van Aken M. Renal handling of technetium-99m DMSA in rats with proximal tubular dysfunction. *J Nucl Med.* 1985;26:1063–1067.
- Peters AM, Jones DH, Evans K, Gordon I. Two routes for <sup>99m</sup>Tc-DMSA uptake into the renal cortical tubular cell. *Eur J Nucl Med.* 1988;14:555–561.
- de Lange MJ, Piers DA, Kosterink JG, et al. Renal handling of technetium-99m DMSA: evidence for glomerular filtration and peritubular uptake. *J Nucl Med.* 1989;30:1219–1223.
- de Lange MJ, Kosterink JG, Piers DA, van Luijk WH, Meijer S. Comments on: “Two routes for renal <sup>99m</sup>Tc-DMSA uptake into the renal cortical tubular cell.” *Eur J Nucl Med.* 1989;15:633.
- Kamper AL, Thomsen HS, Nielsen SL, Strandgaard S. Renal uptake of dimer-captosuccinic acid and glomerular filtration rate in chronic nephropathy at angiotensin converting enzyme inhibition. *Eur J Nucl Med.* 1990;16:85–88.
- Müller-Suur R, Gutsche HU. Tubular reabsorption of technetium-99m-DMSA. *J Nucl Med.* 1995;36:1654–1658.
- Lee BH, Lee SH, Choi HJ, et al. Decreased renal uptake of (99m)Tc-DMSA in patients with tubular proteinuria. *Pediatr Nephrol.* 2009;24:2211–2216.
- Jouret F, Walrand S, Parreira KS, et al. Single photon emission-computed tomography (SPECT) for functional investigation of the proximal tubule in conscious mice. *Am J Physiol Renal Physiol.* 2010;298:F454–F460.
- Melis M, de Swart J, de Visser M, et al. Dynamic and static small-animal SPECT in rats for monitoring renal function after <sup>177</sup>Lu-labeled Tyr3-octreotate radionuclide therapy. *J Nucl Med.* 2010;51:1962–1968.
- Burckhardt BC, Drinkuth B, Menzel C, et al. The renal Na(+)-dependent dicarboxylate transporter, NaDC-3, translocates dimethyl- and disulfhydryl-compounds and contributes to renal heavy metal detoxification. *J Am Soc Nephrol.* 2002;13:2628–2638.
- van Luijk WH, Ensing GJ, Piers DA. Low renal uptake of <sup>99m</sup>Tc-DMSA in patients with proximal tubular dysfunction. *Eur J Nucl Med.* 1983;8:404–405.
- Kim SE, Cho JT, Lee DS, et al. Poor renal uptake of technetium-99m-DMSA and technetium-99m-MDP in a patient with Fanconi syndrome and near normal glomerular filtration rate. *J Korean Med Sci.* 1994;9:29–34.
- Timmis A, Jones C, Paize F. An unusual DMSA scan: answer. *Pediatr Nephrol.* 2012;27:225–227.
- Christensen EI, Birn H. Megalin and cubilin: synergistic endocytic receptors in renal proximal tubule. *Am J Physiol Renal Physiol.* 2001;280:F562–F573.
- Christensen EI, Birn H. Megalin and cubilin: multifunctional endocytic receptors. *Nat Rev Mol Cell Biol.* 2002;3:256–266.
- Birn H, Fyfe JC, Jacobsen C, et al. Cubilin is an albumin binding protein important for renal tubular albumin reabsorption. *J Clin Invest.* 2000;105:1353–1361.
- Amsellem S, Gburek J, Hamard G, et al. Cubilin is essential for albumin reabsorption in the renal proximal tubule. *J Am Soc Nephrol.* 2010;21:1859–1867.
- Leheste JR, Rolinski B, Vorum H, et al. Megalin knockout mice as an animal model of low molecular weight proteinuria. *Am J Pathol.* 1999;155:1361–1370.
- Weyer K, Storm T, Shan J, et al. Mouse model of proximal tubule endocytic dysfunction. *Nephrol Dial Transplant.* 2011;26:3446–3451.
- Saito A, Sato H, Iino N, Takeda T. Molecular mechanisms of receptor-mediated endocytosis in the renal proximal tubular epithelium. *J Biomed Biotechnol.* 2010;2010:403272.
- Storm T, Emma F, Verroust PJ, Hertz JM, Nielsen R, Christensen EI. A patient with cubilin deficiency. *N Engl J Med.* 2011;364:89–91.
- Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc.* 2006;1:2856–2860.
- Moestrup SK, Nielsen S, Andreasen P, et al. Epithelial glycoprotein-330 mediates endocytosis of plasminogen activator-plasminogen activator inhibitor type-1 complexes. *J Biol Chem.* 1993;268:16564–16570.
- Le Panse S, Galceran M, Pontillon F, et al. Immunofunctional properties of a yolk sac epithelial cell line expressing two proteins gp280 and gp330 of the intermicrovillar area of proximal tubule cells: inhibition of endocytosis by the specific antibodies. *Eur J Cell Biol.* 1995;67:120–129.
- Vegt E, Melis M, Eek A, et al. Renal uptake of different radiolabelled peptides is mediated by megalin: SPECT and biodistribution studies in megalin-deficient mice. *Eur J Nucl Med Mol Imaging.* 2011;38:623–632.
- Strober W, Waldmann TA. The role of the kidney in the metabolism of plasma proteins. *Nephron.* 1974;13:35–66.
- Weber MH, Verwiebe R. Alpha 1-microglobulin (protein HC): features of a promising indicator of proximal tubular dysfunction. *Eur J Clin Chem Clin Biochem.* 1992;30:683–691.
- Penders J, Delanghe JR. Alpha 1-microglobulin: clinical laboratory aspects and applications. *Clin Chim Acta.* 2004;346:107–118.
- Guder WG, Hofmann W. Clinical role of urinary low molecular weight proteins: their diagnostic and prognostic implications. *Scand J Clin Lab Invest Suppl.* 2008;241:95–98.
- Suzuki S, Suzuki J, Kume K, et al. Poor renal accumulation of <sup>99m</sup>Tc-DMSA in idiopathic tubular proteinuria. *Nephron.* 1999;81:49–54.
- Freitas RS, Gutfilen B, da Fonseca LM, Bernardo-Filho M. Evaluation of <sup>99m</sup>technetium-radiopharmaceutical binding to blood elements using different trichloroacetic acid concentrations. *Yale J Biol Med.* 1996;69:483–488.
- Akerström B, Logdberg L, Berggard T, Osmark P, Lindqvist A. alpha(1)-Microglobulin: a yellow-brown lipocalin. *Biochim Biophys Acta.* 2000;1482:172–184.
- Evans K, Lythgoe MF, Anderson PJ, Smith T, Gordon I. Biokinetic behavior of technetium-99m-DMSA in children. *J Nucl Med.* 1996;37:1331–1335.
- Hovinga TK, Beukhof JR, van Luyk WH, Piers DA, Donker AJ. Reversible diminished renal <sup>99m</sup>Tc-DMSA uptake during converting-enzyme inhibition in a patient with renal artery stenosis. *Eur J Nucl Med.* 1984;9:144–146.
- Christensen EI, Devuyt O, Dom G, et al. Loss of chloride channel CIC-5 impairs endocytosis by defective trafficking of megalin and cubilin in kidney proximal tubules. *Proc Natl Acad Sci USA.* 2003;100:8472–8477.
- Norden AG, Scheinman SJ, Deschodt-Lanckman MM, et al. Tubular proteinuria defined by a study of Dent’s (CLCN5 mutation) and other tubular diseases. *Kidney Int.* 2000;57:240–249.
- Mo L, Wills NK. CIC-5 chloride channel alters expression of the epithelial sodium channel (ENaC). *J Membr Biol.* 2004;202:21–37.
- Shikano N, Kanai Y, Kawai K, Ishikawa N, Endou H. Transport of <sup>99m</sup>Tc-MAG3 via rat renal organic anion transporter 1. *J Nucl Med.* 2004;45:80–85.