Identification of SLC41A3 as a novel player in magnesium homeostasis

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Regulation of the body Mg2+ balance takes place in the distal convoluted tubule (DCT), where transcellular reabsorption determines the final urinary Mg2+ excretion. The basolateral Mg2+ extrusion mechanism in the DCT is still unknown, but recent findings suggest that SLC41 proteins contribute to Mg2+ extrusion. The aim of this study was, therefore, to characterize the functional role of SLC41A3 in Mg2+ homeostasis using the Slc41a3 knockout (Slc41a3−/−) mouse. By quantitative PCR analysis it was shown that Slc41a3 is the only SLC41 isoform with enriched expression in the DCT. Interestingly, serum and urine electrolyte determinations demonstrated that Slc41a3−/− mice suffer from hypomagnesemia. The intestinal Mg2+ absorption capacity was measured using the stable 25Mg2+ isotope in mice fed a low Mg2+ diet. 25Mg2+ uptake was similar in wildtype (Slc41a3+/+) and Slc41a3−/− mice, although Slc41a3−/− animals exhibited increased intestinal mRNA expression of Mg2+ transporters Trpm6 and Slc41a1. Remarkably, some of the Slc41a3−/− mice developed severe unilateral hydronephrosis. In conclusion, Slc41a3 was established as a new factor for Mg2+ handling.

The distal convoluted tubule (DCT) is essential for magnesium (Mg2+) homeostasis1. Although only 10% of the filtered Mg2+ is reabsorbed in this segment of the kidney, it determines the final urinary Mg2+ excretion since no reabsorption takes place beyond this segment1. In the DCT, Mg2+ enters the cell via the Transient Receptor Potential Melastatin type 6 (TRPM6) divalent cation channel2, while the basolateral Mg2+ extrusion mechanism remains to be identified. Disturbances in this transcellular transport process result in hypomagnesemia, which is characterized by a variety of clinical symptoms including cardiac arrhythmias, muscle cramps and fatigue1. Additionally, drugs directly affecting the DCT such as epidermal growth factor receptor inhibitors, calcineurin inhibitors, and diuretics reduce blood Mg2+ levels1. Therefore, understanding the molecular mechanism underlying Mg2+ reabsorption in the DCT is of major importance for the treatment of patients with hypomagnesemia.

In a recent transcriptome screening study, Solute carrier family 41 member 3 (Slc41a3) was identified as a highly regulated gene by dietary Mg2+ intake1. In this study mice were fed with Mg2+-enriched or Mg2+-deficient diets after which the DCT segments were isolated. Using a quantitative gene expression microarray, the Slc41a3−/− genes in the DCT were identified. Slc41a3 was among the most significant genes with increased expression in response to a Mg2+-deficient diet.

SLC41A3 was originally described by Quamme and colleagues as part of the solute carrier family 41 of putative Mg2+ transporters5. Although SLC41A3 has never been studied in detail, a few reports have addressed the structure, regulation and function of its close homologue Slc41a1 (52% identity at amino acid level)6–8. There is controversy about the structure of Slc41a1; some groups support a 10 transmembrane domain structure, while others suggest 11 transmembrane domains with an extracellular carboxyl (C)-terminus8,9. At the functional level, initial experiments in Xenopus laevis oocytes demonstrated Mg2+ currents for SLC41A1 and SLC41A3 using physiological Mg2+ concentrations, suggesting a channel-like function5,10. Indeed, SLC41 proteins posses conserved pore regions with the MgtE bacterial Mg2+ channel11,12. In contrast, recent experiments using Mg2+-sensitive fluorescent probes showed Na+-dependent Mg2+ transport, suggesting that SLC41A1 may be the long-sought Na+/Mg2+-exchanger13. Interestingly, an exon-skipping mutation in Slc41a1 resulted in a nephrophthiasis-like phenotype in a patient eventually requiring renal transplantation14. Whether SLC41A3 may have a similar function as SLC41A1 has never been examined. Interestingly, in contrast to Slc41a3, Mg2+-sensitive regulation of Slc41a1 expression was not observed in the DCT transcriptome study14.

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The aim of the present study was, therefore, to characterize the role of SLC41A3 in renal and intestinal Mg$^{2+}$ (re)absorption. For this purpose, Slc41a3 knockout mice (Slc41a3$^{-/-}$) were analysed for electrolyte homeostasis, intestinal function and renal abnormalities. Furthermore, by challenging the mice with Mg$^{2+}$-deficient diets, compensatory mechanisms in intestine, kidney and brain were examined in detail. Intestinal Mg$^{2+}$ absorption studies were performed using the $^{25}$Mg$^{2+}$ isotope to address the functional role of SLC41A3 in the intestine.

**Results**

**Expression profile of Slc41 isoforms in kidney and DCT.** To examine the role of the SLC41 protein family in renal Mg$^{2+}$ transport, a tissue expression screening for Slc41a1, Slc41a2 and Slc41a3 was performed using RT-quantitative PCR (RT-qPCR, Fig. 1). All three isoforms showed a ubiquitous expression pattern. Robust Slc41a1 expression was detected in brain, heart and lung. Slc41a2 was predominantly expressed in the proximal intestine whereas Slc41a3 expression was highest in heart, lung and small intestine. However, in RT-PCR analysis of isolated DCT segments Slc41a3 messenger RNA (mRNA) transcript levels showed a significant 10-fold enrichment compared to total kidney mRNA. Slc41a1 and Slc41a2 expression was not enriched in DCT compared to
other segments, while the latter transporter was even significantly decreased, suggesting that SLC41A3 is the most relevant SLC41 family member for Mg^{2+} reabsorption in the DCT.

**Slc41a3 mouse breeding.** To examine the function of SLC41A3 in Mg^{2+} homeostasis, Slec41a3^{-/-} mice were generated. Breeding of heterozygous (Slc41a3^{+/−}) mice resulted in a normal Mendelian inheritance pattern in the offspring. Of a total of 181 mice, 32% were genotyped Slec41a3^{+/+}, 46% Slec41a3^{−/+} and 22% Slec41a3^{−/−}. Since Shirpa (SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment) screening by the Mouse Genetics Project reported abnormal locomotor coordination of Slec41a3^{−/−} mice fed with a high fat diet (MGI: 1918949), special attention to behavioral observations was given during the breeding procedure. However, no abnormalities in behavior of the mice including ataxia and locomotor behavior were observed. By visual inspection, it was not possible to distinguish between Slec41a3^{+/+}, Slec41a3^{−/+} and Slec41a3^{−/−} mice based on behavior or external phenotype. All offspring was genotyped for the insertion of the knockout cassette and presence of the wild type Slec41a3 allele (Fig. 2B).

**Hypomagnesemia in Slc41a3^{−/−} mice.** To investigate the role of SLC41A3 in Mg^{2+} homeostasis, mice were subjected to normal or low Mg^{2+}-containing diets for 14 days. Blood, 24-hour urine and feces were collected at day 9 and 14 of the experiment using metabolic cages (Fig. 3A). At the end of the experiment, the mice were sacrificed and tissues were collected for further analysis. No significant differences were observed between the body weights of Slec41a3^{+/+} mice and Slec41a3^{−/+} and Slec41a3^{−/−} littermates (Table 1). Food and water intake, urinary volume and fecal excretion were comparable among all mouse genotypes on the same diet (Table 1). On the normal diet, serum Mg^{2+} concentrations of Slec41a3^{−/−} mice were significantly decreased by 29 ± 2% compared to Slec41a3^{+/+} mice (1.30 ± 0.05 mM vs. 1.04 ± 0.01 mM, Fig. 3B). Urinary Mg^{2+} excretion was not different (Fig. 3C). Furthermore, both serum and urinary calcium (Ca^{2+}) levels were not significantly altered in Slec41a3^{−/−} mice compared to Slec41a3^{+/−} mice, underlining the specificity of the hypomagnesemia in these Slec41a3^{−/−} mice (Fig. 3D,E). Slec41a3^{−/−} mice fed a Mg^{2+}-deficient diet for two weeks, displayed hypomagnesemia (serum Mg^{2+} levels of 0.43 ± 0.04 mM, Fig. 3B). Although the serum Mg^{2+} levels of Slec41a3^{−/−} mice on a low Mg^{2+} diet were lower (0.32 ± 0.02 mM) and decreased to a similar extent as the Slec41a3^{−/−} mice on the normal Mg^{2+} diet (26%), they did not reach a significant difference compared to Slec41a3^{+/−} mice (p = 0.14). On the low Mg^{2+} diet, no differences in urinary Mg^{2+} and Ca^{2+} excretion or serum Ca^{2+} concentrations were measured among the groups (Fig. 3C–E).

**Hydronephrosis in subset of Slc41a3^{−/−} mice.** Interestingly, in 10% of the male Slec41a3^{+/−} mice on the low Mg^{2+} diet a severely increased kidney size was observed in the left kidney caused by a hydronephrosis (Fig. 4A). The kidney volume was 6–8x larger than the other kidney of the same animal and caused a serious organ rearrangement in the peritoneal cavity. The unilateral hydronephrotic kidney was observed in 10% of the Slec41a3^{−/−} mice in the low Mg^{2+} group and was never detected in Slec41a3^{−/−} mice fed the normal Mg^{2+} diet or in the Slec41a3^{−/+} mice. To further assess the morphology of the hydronephrosis, the kidney tissue was stained using standard hematoxylin and eosin (H&E) stainings (Fig. 4B, left image). The presence of transitional epithelium around the dilated tissue and the absence of dilated ureters suggested that the origin of the hydronephrosis is located in the renal calyx or renal pelvis. Indeed, umbrella cells were detected to further confirm the urethral origin of the tissue (Fig. 4B, middle image). However, major parts of the tissue lining the fluid-filled cavity were not covered with transitional epithelium, but existed of fibrous connective tissue with flattened epithelium or absence of epithelium. The ureter immediately next to the cyst was slender and filled with cosinophilic material, suggesting an obstruction at the pyelo-ureteral junction and absence of urinary flow (Fig. 4B, right image). However, no clear anatomical cause for obstruction could be identified. To access whether more initial stages of the development of the hydronephrosis could be detected, kidneys from Slec41a3^{−/−} mice fed with the normal Mg^{2+} diet without apparent abnormalities at the exterior of the kidney were processed using a standard H&E staining (Fig. 4C). Although a subset of tubules and blood vessels displayed some dilatation in the cortex and the medulla of Slec41a3^{−/+} and Slec41a3^{−/−} mice, quantification of the surface size of these dilations did not show significant differences between kidneys from Slec41a3^{−/+} and Slec41a3^{−/−} mice (Fig. 4D).
Expression of Mg\(^{2+}\) transporters in kidney and colon of Slc41a3\(^{-/-}\) mice. The kidney and colon are the main sites of Mg\(^{2+}\) (re)absorption. To examine the compensatory mechanisms of the renal Mg\(^{2+}\) wasting in Slc41a3\(^{-/-}\) mice, the expression level of renal Mg\(^{2+}\) transporters was analyzed using RT-qPCR (Fig. 5). The mRNA transcript levels of \textit{Trpm6}, \textit{Slc41a1}, \textit{Cnnm2} and \textit{Parvalbumin} were not significantly different between Slc41a3\(^{+/+}\) and Slc41a3\(^{-/-}\) mice (Fig. 5A–D). Renal \textit{Slc41a1} expression was increased in the low Mg\(^{2+}\) diet group mice compared to mice on the normal Mg\(^{2+}\) diet for all genotypes (Fig. 5B). Furthermore, the renal mRNA levels of \textit{Egf}, \textit{Ncc}, \textit{Cldn16} and \textit{Cldn19} were not altered in Slc41a3\(^{-/-}\) mice in comparison with their Slc41a3\(^{+/+}\) littermates (Supplementary Fig. 1). Subsequently, the expression of Mg\(^{2+}\) transporters in the colon, where Mg\(^{2+}\) is actively absorbed from the diet, was examined by RT-qPCR. Particularly, in the low Mg\(^{2+}\) groups, gene expression levels of \textit{Slc41a1}, \textit{Cnnm4} and \textit{Trpm7} were increased in the colon of Slc41a3\(^{-/-}\) animals compared to colon of their Slc41a3\(^{+/+}\) littermates (Fig. 5E–H). In contrast, no significant difference in colonic \textit{Trpm6} expression was detected between Slc41a3\(^{+/+}\) and Slc41a3\(^{-/-}\) mice. Since Slc41a3 is also expressed in brain (Fig. 1) and SLC41A1 is associated with Parkinson’s disease\(^{16,17}\), the expression levels of \textit{Trpm7} and \textit{Slc41a1} in the brain were determined by RT-qPCR. No significant changes in brain mRNA expression levels of \textit{Trpm7} and \textit{Slc41a1} were observed among the genotypes (Supplementary Fig. 2).

Intestinal Mg\(^{2+}\) absorption is similar in Slc41a3\(^{+/+}\) and Slc41a3\(^{-/-}\) mice. To further examine the role Slc41a3\(^{-/-}\) in the intestine, the Mg\(^{2+}\) absorption capacity was determined using the stable \(^{25}\)Mg\(^{2+}\) isotope\(^{18}\).
The mice were subjected to 10 days of low Mg²⁺ diet prior to the ²⁵Mg²⁺ absorption analysis. At the final day of the experiment, the mice were administered ²⁵Mg²⁺ by oral gavage and subsequently blood was taken from the tail up to 60 minutes after the administration. The natural abundance of ²⁵Mg²⁺ in blood is 10%, which was doubled during 60 minutes of ²⁵Mg²⁺ uptake to more than 20% (Fig. 6A). However, no significant differences in Mg²⁺ absorption were detected between Slocala3−/+ and Slocala3−/− mice (Fig. 6A). Determination of the mRNA expression levels of Trpm6 and Slocala1 in intestinal Mg²⁺ uptake showed that the expression of both genes is magnified in duodenum and colon of Slocala3−/− mice (Fig. 6B–E).

**Discussion**

This study identified Slocala1 as a novel player in Mg²⁺ homeostasis. This conclusion is based on the following results: i) Slocala3 is specifically expressed in the DCT and in the intestine where Mg²⁺ is (re)absorbed; ii) Slocala3−/− mice suffer from hypomagnesemia and normomagnesiuria indicating a possible renal Mg²⁺ leak; iii) intestinal Mg²⁺ transporters including Trpm6 and Slocala1 are upregulated in Slocala3−/− mice. Additionally, this study shows that some Slocala3−/− mice fed a Mg²⁺−deficient diet develop hydronephrosis.

The urinary electrolyte levels in the hypomagnesemic Slocala3−/− mice point to a specific renal Mg²⁺ leak, mimicking the phenotype of patients with renal Mg²⁺ wasting19–21, namely low serum Mg²⁺ levels accompanied by normal urinary Mg²⁺ excretion. Under normal physiological circumstances, the kidney should be able to compensate for reduced blood Mg²⁺ levels by increasing renal Mg²⁺ reabsorption. However, the Slocala3−/− mice failed to counteract their urinary Mg²⁺ wasting. Moreover, the urinary Mg²⁺ excretion was reduced in all mice fed with Mg²⁺−deficient diets, showing that Slocala3−/− mice still have the ability to raise renal Mg²⁺ reabsorption despite inactivation of Slocala1. However, in Slocala3−/− fed a low Mg²⁺ diet for 14 days, serum Mg²⁺ concentrations were 26% lower than in Slocala3+/+ littermates. Although this finding did not reach statistically significant (p = 0.14), this suggests that the Mg²⁺ reabsorption capacity is impaired in Slocala3−/− mice fed the Mg²⁺−deficient diet. However, longer treatment may be necessary to observe a significant reduction of serum Mg²⁺ concentrations. Additionally, the intestinal ²⁵Mg²⁺ absorption was normal in Slocala3−/− mice, potentially because increased Trpm7 and Slocala1 expression compensates for the loss of Slocala1 function.

In kidney, Slocala1 was, in contrast to its close homologue Slocala1, highly enriched in DCT. Likewise, the expression of Slocala3, but not of Slocala1, in DCT is highly dependent on dietary Mg²⁺ intake. These results are comparable to the established findings concerning the Mg²⁺ channels TRPM6 and TRPM7. TRPM6 provides the specific luminal Mg²⁺ uptake mechanism in kidney and intestine22,23. Hence, the expression of TRPM6 is localized to the DCT and the colon where it is highly regulated by dietary Mg²⁺ availability, EGF, insulin, ATP and estrogens23–26. In contrast, TRPM7 is ubiquitously expressed and is involved in basic cellular Mg²⁺ homeostasis27,28. Its expression is insensitive to dietary Mg²⁺ changes22. A comparable concept could apply to Slocala1 proteins, in which Slocala1 would be specific for epithelial Mg²⁺ uptake in the DCT and Slocala1 would serve as ubiquitously expressed general Mg²⁺ transporter.

The molecular function of Slocala1 in the DCT remains elusive. Slocala1 activity has only been examined in Xenopus laevis oocytes by voltage-clamp, showing Mg²⁺ currents with a kₘ within the physiological range for blood Mg²⁺ concentrations25. However, when overexpressed in human embryonic kidney 293 (HEK293) cells Mg²⁺ currents could not be detected (unpublished data from our lab). The function of the close homologue Slocala1 has been examined in more detail. As Slocala1, Slocala1 mediates Mg²⁺ currents in Xenopus laevis oocytes20. Moreover, its Mg²⁺ transport capacity was further established in the TRPM7−deficient DT40 cell line, where Slocala1 restored cell growth29. Conversely, Slocala1 failed to complement TRPM7−deficient DT40 cells, suggesting that Slocala1 transport activity may depend on partner proteins. Importantly, recent data suggest that Slocala1 acts as a Na⁺/Mg²⁺−exchanger being localized at the basolateral plasma membrane30–34. Although Slocala1 and Slocala3 are very homologous and may have a similar mode of action, definitive evidence for the function and plasma membrane localization of Slocala1 is currently lacking. Future cellular and functional studies should confirm the Mg²⁺ transporting function of Slocala1.

Although Slocala3 is expressed in the intestine, Slocala3−/− mice exhibited normal intestinal Mg²⁺ absorption compared to their Slocala3+/+ littermates. However, the expression of Mg²⁺ transporters Slocala1 and Trpm6 was increased in duodenum of Slocala3−/− mice, suggesting that loss of Slocala1 function induces a compensatory

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**Table 1. Metabolic parameters of Slocala3−/− mice.**

| Parameter                      | Normal Mg²⁺ diet | Low Mg²⁺ diet |
|-------------------------------|------------------|---------------|
|                               | Slocala3+/+ | Slocala3−/− | Slocala3+/+ | Slocala3−/− | Slocala3+/+ | Slocala3−/− |
| Weight – day 0 (g)            | 18.0 ± 2.1 | 18.4 ± 1.6 | 17.5 ± 2.2 | 18.4 ± 2.1 | 18.7 ± 2.0 | 17.7 ± 3.2 |
| Weight – day 14 (g)           | 20.5 ± 2.1 | 20.5 ± 1.9 | 20.2 ± 2.5 | 19.8 ± 2.6 | 20.3 ± 2.4 | 19.1 ± 3.3 |
| Food intake (g)               | 3.97 ± 0.50 | 4.26 ± 0.23 | 4.43 ± 0.28 | 3.46 ± 0.52 | 3.56 ± 0.42 | 3.76 ± 0.56 |
| Water intake (mL)             | 4.95 ± 1.23 | 4.98 ± 1.13 | 4.77 ± 0.88 | 4.00 ± 0.82 | 4.65 ± 1.12 | 4.03 ± 0.61 |
| Feces weight (g)              | 1.86 ± 0.38 | 1.94 ± 0.39 | 2.09 ± 0.22 | 0.34 ± 0.12 | 0.38 ± 0.08 | 0.34 ± 0.13 |
| Urine volume (mL)             | 1.25 ± 0.31 | 1.12 ± 0.37 | 1.22 ± 0.29 | 1.53 ± 0.66 | 1.61 ± 0.54 | 1.46 ± 0.48 |
| Serum Na⁺ (mmol/L)            | 152 ± 1    | 150 ± 1    | 153 ± 1    | 152 ± 0    | 152 ± 0    | 153 ± 0    |
| Serum K⁺ (mmol/L)             | 4.5 ± 0.1  | 4.6 ± 0.1  | 4.5 ± 0.1  | 4.2 ± 0.2  | 4.4 ± 0.1  | 4.8 ± 0.1  |
| Urine Na⁺ (μmol/24 hrs)       | 185 ± 13   | 184 ± 20   | 214 ± 11   | 204 ± 13   | 169 ± 17   | 176 ± 26   |
| Urine K⁺ (μmol/24 hrs)        | 539 ± 26   | 538 ± 39   | 562 ± 25   | 559 ± 39   | 460 ± 46   | 502 ± 69   |

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mechanism to facilitate normal Mg\(^{2+}\) absorption. Specifically, the upregulation of Slc41a1 is of interest because the structure of SLC41A1 is largely similar to SLC41A3\(^{-}\). Importantly, the pore region of SLC41A1 and SLC41A3 proteins is conserved from MgtE bacterial Mg\(^{2+}\) channels and essential for their function\(^{8,11,12}\). If the increased expression of Slc41a1 indeed compensates for defects in intestinal Mg\(^{2+}\) absorption in Slc41a3\(^{-}\)/− mice, this would suggest that SLC41A1 and SLC41A3 are functionally redundant. Future studies with Slc41a1 and Slc41a3 double knockout mice should substantiate the hypothesis that SLC41A1 and SLC41A3 function cooperatively to regulate Mg\(^{2+}\) handling.

The development of hydronephrosis in Slc41a3\(^{-}\)/− mice is a striking finding of our study. H&E stainings showed transitional epithelia including umbrella cells lining the hydronephrotic region, excluding a cystic origin of the kidney malformation\(^{30}\). However, important parts of the lining tissue of the hydronephrosis were not covered with transitional epithelium, but with a layer of fibrous connective tissue that normally originates from the renal capsule. A similar phenotype of perinephric pseudocysts, in which fluid accumulates in a fibrous sac surrounding the kidney, has been observed previously in humans, mice and cats\(^{31-33}\). In a previously described mouse study using the C57BL/6J strain, extensive histological analysis showed that unilateral perinephric pseudocyst can be formed from an initial hydronephrosis that at some point ruptures when the integrity of the lining

Figure 4. Slc41a3\(^{-}\)/− mice develop hydronephrosis on low Mg\(^{2+}\) diets. (A) Images of hydronephrotic kidneys found in 2 out of 20 Slc41a3\(^{-}\)/− mice fed low Mg\(^{2+}\) diets. (B) H&E stainings of the hydronephrosis kidney detected in Slc41a3\(^{-}\)/− mice showing transitional epithelium lining the hydronephrosis. The left image gives an overview of the total kidney (bar: 1 mm). The middle image shows the transitional epithelium with a black arrow indicating the umbrella cells (bar: 20 \(\mu\)m). The right image shows a slender proximal ureter filled with eosinophilic material, indicating stasis (bar: 20 \(\mu\)m). (C) Alizarin red staining of the hydronephrosis kidney did not show Ca\(^{2+}\) deposits (bar: \(\mu\)m). (D) H&E stainings of normal kidney tissue from Slc41a3\(^{-}\)/− mice fed with normal Mg\(^{2+}\) demonstrating venous and tubular dilations (left image bar: 100 \(\mu\)m, right image bar: 20 \(\mu\)m). (E) Quantification of dilated surface in Slc41a3\(^{-}\)/+ and Slc41a3\(^{-}\)/− mice. Values are presented as means ± SEM (n = 10).
The wall is compromised\textsuperscript{31}. The urine may then leak into the subcapsular space between the renal capsule and the remnant kidney. In concordance with these findings, histological analysis of the \textit{Slc41a3}\textsuperscript{−/−} mice demonstrated both transitional epithelium and connective tissue lining the cavity, suggesting that an initial hydronephrosis may have ruptured resulting in a perinephric pseudocyst.

Hydronephrosis is normally the consequence of obstruction of the ureter resulting in fluid retention in the renal calyx and pelvis\textsuperscript{34}. Anatomical obstructions were not found in \textit{Slc41a3}\textsuperscript{−/−} mice and, therefore, the cause of

Figure 5. Compensatory mechanisms for the loss of \textit{Slc41a3}. (A–H) The mRNA expression levels of \textit{Trpm6} (A,E), \textit{Slc41a1} (B,F), \textit{Cnnm2} (C), \textit{Parvalbumin} (D), \textit{Cnnm4} (G) and \textit{Trpm7} (H) in kidney (A–D) or colon (E–H) of \textit{Slc41a3}\textsuperscript{+/+} (black bars), \textit{Slc41a3}\textsuperscript{−/+} (striped bars) and \textit{Slc41a3}\textsuperscript{−/−} (white bars) mice fed with a low or a normal Mg\textsuperscript{2+}-containing diet for 14 days were measured by RT-qPCR. Relative gene expression was analyzed using the Livak method (2\textsuperscript{−ΔΔCt}), where results are normalized against \textit{Gapdh} expression (reference gene). Data represent means ± SEM (n = 10) and are expressed as fold difference when compared to the gene expression in normal diet fed \textit{Slc41a3}\textsuperscript{+/+} mice. *P < 0.05 indicates a statistically significance compared to \textit{Slc41a3}\textsuperscript{+/+} mice fed the same diet.
the hydronephrosis could not be identified. Importantly, only a subset of the Slc41a3−/− mice developed hydronephrosis, suggesting that Slc41a3−/− mice are more sensitive to the development of hydronephrosis, but that inactivation of Slc41a3 is not causative of hydronephrosis per se. Speculatively, the dietary Mg2⁺ availability could have contributed to the development of the hydronephrosis. It is widely acknowledged that Mg2⁺ can prevent urolithiasis by reducing the formation of calcium oxalate (CaC₂O₄) and calcium phosphate (Ca(H₂PO₄)₂) stones and deposits.³⁵–³⁷. Urolithiasis can cause obstruction of the ureter and, therefore, result in hydronephrosis.³⁸ However, alizarin red stainings did not show Ca²⁺ deposits in Slc41a3−/− mice on the low Mg²⁺ diet despite their low urinary Mg²⁺ excretion. H&E stainings demonstrated a slender proximal ureter containing amorphic eosinophilic material, possibly due to stasis. Although the slender proximal ureter suggests a stenosis at the ureteropelvic junction, no anatomical cause for obstruction could be identified. It could be hypothesized that a functional defect in peristalsis caused hydronephrosis in Slc41a3−/− mice, but to substantiate this further studies are required.

Figure 6. Increased expression of intestinal Mg²⁺ transporters compensate for Slc41a3 KO. (A) 60 minutes intestinal ⁴⁰Mg²⁺ absorption in Slc41a3+/+ (solid line) and Slc41a3−/− (dashed line) mice after 10 days on low Mg²⁺ diets. B-E. The mRNA expression levels of Trpm6 (B,D) and Slc41a1 (C,E) in duodenum (B,C) or colon (D,E) of Slc41a3+/+ and Slc41a3−/− mice fed with a low Mg²⁺-containing diet for 10 days were measured by RT-qPCR. Relative gene expression was analyzed using the Livak method (2−ΔΔCt), where results are normalized against Gapdh expression (reference gene). Values are presented as means ± SEM (n = 10) and are expressed as fold difference when compared to the gene expression in normal diet fed Slc41a3+/+ mice. *P < 0.05 is considered statistically significant compared to Slc41a3+/+ mice.
Interestingly, a mutation of SLC41A1 was recently shown to be causative for a nephronophthisis-like (NPHP-like) phenotype in an Italian family. The kidneys of the patients showed irregular echogenicity when examined by kidney ultrasonography, suggesting renal cysts or hydronephrosis. However, hydronephrosis could be excluded by subsequent histological analysis, which showed periglomerular fibrosis, tubular ectasia, tubular basement membrane disruption and tubulointerstitial infiltrations. The aforementioned signs of inflammation and fibrosis were absent in the remnant kidney tissue of SLC41A1(-/-) mice. Moreover, in contrast to the NPHP-like phenotype in patients with SLC41A1 mutations, the kidney size was markedly increased in the SLC41A1(-/-) mice that suffered from hydronephrosis. More patients with mutations in SLC41A1 should be identified and examined for hydronephrosis to allow final conclusions on the kidney phenotype of these patients.

In conclusion, our study of the SLC41A3 mice has identified SLC41A3 as a novel player in Mg²⁺ (re)absorption and potentially a new factor in the formation of hydronephrosis. Consequently, SLC41A3 should be included in genetic screenings for hypomagnesemic patients.

Methods

Expression analysis. Three C57BL/6 mice were sacrificed; kidney, duodenum, ileum, jejunum, cecum, colon, brain, lung, liver, spleen, muscle, and heart tissues were collected. For the collection of DCT material, transgenic parvalbumin-eGFP mice were used as described previously (kind gift from Dr. Monyer, University of Heidelberg, Germany). In short, mice were anesthetized by a mixture injection of domitor (0.01 mg/g of body weight) and ketamine (0.1 mg/g of body weight). Subsequently, the mice were perfused with 10 ml of Krebs buffer (in mM: 145 NaCl, 5 KCl, 10 HEPES/NaOH pH 7.4, 1 NaH₂PO₄, 2.5 CaCl₂, 1.8 MgSO₄, 5 glucose) through the heart. The kidneys were removed, minced, and digested in collagenase (1 mg/ml collagenase A (Worthington, Lakewood, NJ, USA), 0.6 mg/ml hyaluronidase) in Krebs buffer. The digested tubules sized between 40 and 100 μm were sorted based on GFP fluorescence by COPAS (Complex Object Parametric Analysis and Sorting, Union Biometrica, Holliston, MA, USA). Per mouse, 4,000 eGFP-positive fluorescent DCT tubules were collected, and an additional 4,000 control tubules were sorted from the same kidney sample without selection for eGFP-positive cells.

RNA Isolation and cDNA synthesis. Total RNA was isolated using TRIzol total RNA isolation agent (Invitrogen, Bleiswijk, the Netherlands) according to the manufacturer’s protocol. Obtained RNA was precipitated in ethanol, washed, and dissolved in nuclease-free ultrapure water. RNA concentrations were measured spectrophotometrically and purity was determined: in all samples, the ratio of optical density at 260 and 280 nm wavelength was >1.8. Next, 1 μg of RNA was subjected to DNase treatment (Promega, Fitchburg, WI, USA) to prevent genomic DNA contamination. Subsequently, RNA was reverse-transcribed by Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Bleiswijk, the Netherlands) according to the manufacturer’s instructions (1 h at 37 °C). Samples were then diluted 1:10 with nuclease-free ultrapure water and stored at −20 °C until further use.

Real time quantitative PCR. Relative mRNA expression was assessed by quantitative real-time polymerase chain reaction (RT-qPCR). Primers used for RT-qPCR were designed using the Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are shown in Supplementary Table 1. Two and a half μL of cDNA template and an optimal concentration (which was determined for each gene during primer validation and was of 400 nM) of forward and reverse primers were added to 6.25 μL 2 × iQ™SYBR® Green supermix (Bio-Rad, Veendendaal, the Netherlands). The total volume was adjusted to 12.5 μL with diethylpyrocarbonate (DEPC)-treated deionized H₂O. RT-qPCR (7 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C) was carried out using a CFX96 detection system (Bio-Rad, Veendendaal, the Netherlands). As a negative control, the cDNA template was substituted for DEPC-treated water. Additionally, to ensure that residual genomic DNA was not being ampliﬁed, control samples, in which reverse transcription was omitted during cDNA synthesis, were included in the plates during measurements. All samples were normalized to the expression level of the standard mouse-specific reference gene Gapdh. Gene expression data were calculated using the Livak method (2−ΔΔCt) and they represent the mean fold difference from the calibrator/control group.

For primer validation, standard curves with serially diluted cDNA were generated and primer concentration was optimized to ensure the efficiencies of RT-qPCR (95–105%). The construction of SYBR Green dissociation curves after completion of 40 PCR cycles revealed the presence of single amplicons for each primer pair. Amplicon size was conﬁrmed by electrophoresis in 1.5% (w/v) agarose gel.

Animals. All experimental protocols and procedures involving animals were approved by the animal ethics board of Radboud University (Nijmegen, The Netherlands) and were in compliance with National and European guidelines. Heterozygous male and female (Slc41a3+/−) mice of the Slc41a3tm1a(KOMP)Wtsi strain were purchased from Knock Out Mouse Project repository (KOMP, Davis, CA, USA MGI: 1918949) and crossedbred to C57Bl/6N wild-type mice. The heterozygous offspring was used to generate Slc41a3−/− mice. Littermates were housed in a temperature- and light-controlled room with standard pellet chow and deionized drinking water available ad libitum.

Diet study. 20 Slc41a3−/−, 20 Slc41a3+/− and 20 Slc41a3+/− mice aged between 8–12 weeks were randomly selected for this experiment (50% male, 50% female). The animals were housed in metabolic cages for 48hrs (24hrs adaptation, 24hrs sampling) prior to collect urine and feces. Subsequently, the mice were randomly divided to a group and fed with normal (0.19% wt/wt Mg²⁺, SSNIFF Spezialitäten GmbH, Soest, Germany) and low Mg²⁺ diets (0.02% wt/wt Mg²⁺, SSNIFF) (n = 10 per group per genotype) for 14 days. Blood samples were taken before and after the diets via submandibular facial vein puncture. The last 48hrs of the experiment the animals were housed in the metabolic cages again to collect urine and feces. Then, animals were sacrificed, blood was collected and kidney, brain and colon tissues were sampled and frozen immediately in liquid nitrogen for further analysis.
**25Mg**²⁺ absorption study. Intestinal absorption of Mg²⁺ was measured by analyzing serum ²⁵Mg⁺⁺ as percentage of total Mg²⁺⁺ in 25 male animals aged 8–10 weeks (10 Scl41a3−/−, 10 Scl41a3−/+) were fasted (food, not water) overnight on wire-mesh raised floors to prevent coprophagia. At time-point 0, mice were administered a solution containing 44 mM **25Mg**²⁺ (MgO, isotopic enrichment of >98%, CorteNet, Voisins-Le-Bretonneux, France), 125 mM NaCl, 17 mM Tris-HCl pH 7.5. 18 g/L fructose. Animals were administered a volume of 15 μL/g bodyweight via oral gavage. Subsequently, blood was taken at serial time-points via a small tail-cut and collected in Microvette serum tubes (Sarstedt, Ettten-Leur, The Netherlands). After serum collection from the coagulated blood samples, sera were digested in nitric acid (65% concentrated, Sigma, Menzel-Glaser, Braunschweig, Germany) and stored at −20°C. Paraffin sections were deparaffinated and rehydrated and stained with a Hematoxylin and eosin (H&E) staining. Slides were put in Hematoxylin and differentiating agent in tapwater. Thereafter the slides were counterstained with Eosin Y, dehydrated and mounted. Pictures were made with a Zeiss (Oberkochen, Germany) microscope.

**25Mg**²⁺ and Ca²⁺ measurements. Serum and urine total Mg²⁺ and Ca²⁺ concentrations were determined using a colorimetric assay kit according to the manufacturer’s protocol (Roche Diagnostics, Woerden, the Netherlands). Urine volume was measured to calculate 24 hours excretion.

**Statistical analysis.** Data are expressed as mean ± SEM. Statistical comparisons were analyzed by two-way ANOVA with a Tukey's multiple comparison test. When only two experimental groups were affected by only one factor of variance, an unpaired Student’s t-test was used (Figs 1 and 6). P < 0.05 was considered statistically significant.

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