# Kidney-specific upregulation of vitamin $D_3$ target genes in ClC-5 KO mice

T Maritzen[1,2], G Rickheit[1,2], A Schmitt[1] and TJ Jentsch[1]

[1]*Zentrum für Molekulare Neurobiologie Hamburg, ZMNH, Universität Hamburg, Hamburg, Germany*

**Mutations in ClC-5 cause Dent's disease, a disorder associated with low molecular weight proteinuria, hyperphosphaturia, and kidney stones. ClC-5 is a $Cl^-/H^+$-exchanger predominantly expressed in the kidney, where it facilitates the acidification of proximal tubular endosomes. The reduction in proximal tubular endocytosis resulting from a lack of ClC-5 raises the luminal concentration of filtered proteins and peptides like parathyroid hormone (PTH). The increase in PTH may explain the hyperphosphaturia observed in Dent's disease. Expression profiling, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), and hormone measurements were used to investigate whether the disruption of ClC-5 affects other signalling pathways. Although the upregulation of 25(OH)$_2$-vitamin $D_3$ 1$\alpha$-hydroxylase and downregulation of vitamin $D_3$ 24-hydroxylase suggested an increased formation of 1,25(OH)$_2$-vitamin $D_3$, the concentration of this active metabolite was reduced in the serum of ClC-5 knockout (KO) mice. However, target genes of 1,25(OH)$_2$-vitamin $D_3$ were upregulated in KO kidneys. Expression analysis of intestine and bone revealed that the upregulation of 1,25(OH)$_2$-vitamin $D_3$ target genes was kidney intrinsic and not systemic. In spite of reduced serum levels of 1,25(OH)$_2$-vitamin $D_3$ in ClC-5 KO mice, 1,25(OH)$_2$-vitamin $D_3$ is increased in later nephron segments as a consequence of impaired proximal tubular endocytosis. This leads to a kidney-specific stimulation of 1,25(OH)$_2$-vitamin $D_3$ target genes that may contribute to the pathogenesis of Dent's disease. The activation of genes in distal nephron segments by hormones that are normally endocytosed in the proximal tubule may extend to other pathways like those activated by retinoic acid.**

*Kidney International* (2006) **70,** 79–87. doi:10.1038/sj.ki.5000445; published online 3 May 2006

KEYWORDS: chloride channel; channelopathy; nephrolithiasis; nephrocalcinosis; CYP27B1; CYP24A1

**Correspondence:** *TJ Jentsch, Zentrum für Molekulare Neurobiologie Hamburg, ZMNH, Universität Hamburg, Falkenried 94, Hamburg D-20252, Germany. E-mail: Jentsch@zmnh.uni-hamburg.de*

[2]These authors contributed equally to this work

**Received 23 June 2005; revised 17 January 2006; accepted 10 February 2006; published online 3 May 2006**

ClC-5 belongs to the intracellular branch of the CLC gene family of chloride channels and transporters. It is most prominently expressed in the kidney, where it is present in vesicles of proximal tubules (PTs) and of intercalated cells of the collecting duct.[1] Like other intracellular CLC proteins, ClC-5 resides predominantly in the endocytic pathway.[1] Recent data indicate that ClC-5 is an electrogenic $Cl^-/H^+$-exchanger[2,3] rather than a $Cl^-$-channel, as thought previously. Loss-of-function mutations in ClC-5 underlie Dent's disease,[4] an X-linked renal disorder characterized by low molecular weight proteinuria, hyperphosphaturia, hypercalciuria, and more variable symptoms like kidney stones, rickets, nephrocalcinosis, and progressive renal failure.[5]

The mechanisms underlying the pathology of Dent's disease were investigated using knockout (KO) mouse models.[6,7] Consistent with the proposed role of ClC-5 in neutralizing currents of vesicular proton pumps, proximal tubular endosomes of KO animals acidified more slowly and to a lesser extent than wild-type (WT) vesicles.[8] The disruption of ClC-5 led to a severe impairment of both fluid-phase and receptor-mediated endocytosis in the PT.[6] A defect in intracellular trafficking is the likely cause of the observed decreased abundance of megalin,[6] an apical scavenger receptor for a large variety of proteins and other substances. This decrease in megalin expression further reduces receptor-mediated endocytosis.

Whereas the loss of low molecular weight proteins *per se* has no negative impact on the organism, the loss of hormones and vitamins that are normally endocytosed in the PT has important consequences. On the one hand, a significant urinary loss of a vitamin may result in pathologically low serum concentrations. On the other hand, the decrease in proximal tubular endocytosis may raise the concentration of the hormone or vitamin in the lumen of later nephron segments. In the presence of apical hormone receptors, this may cause an abnormally high stimulation of signalling pathways. Indeed, the analysis of ClC-5 KO mice suggested that the decreased endocytosis of parathyroid hormone (PTH) entails an increased stimulation of proximal tubular apical PTH receptors.[6] This, in turn, enhances the endocytosis and degradation of the Na-phosphate co-transporter NaPi-2a that mediates the bulk of phosphate reabsorption in the PT.[6,9] Hence, the hyperphosphaturia observed in Dent's disease may be an indirect consequence of impaired endocytosis.

Additionally, the increased luminal PTH levels should stimulate the transcription of 25(OH)-vitamin $D_3$-1α-hydroxylase (also known as CYP27B1),[8] which is mainly active in the PT. This enzyme converts the vitamin $D_3$ precursor 25(OH)-vitamin $D_3$ into the active compound 1,25$(OH)_2$-vitamin $D_3$. On the other hand, most of the precursor 25(OH)-vitamin $D_3$ is supplied to this mitochondrial enzyme through the apical endocytosis of a 25(OH)-vitamin $D_3$/binding protein complex.[10] As this process depends on ClC-5 and megalin, the delivery of the precursor to the 1α-hydroxylase is severely reduced in the absence of ClC-5. These alterations (increased amount of the enzyme, scarcity of the precursor, and loss of active metabolite into the urine) have opposing effects on 1,25$(OH)_2$-vitamin $D_3$ serum levels. The outcome probably depends on nutritional and genetic factors. In Dent's disease patients, serum 1,25$(OH)_2$-vitamin $D_3$ levels were mostly increased,[5,11] whereas in our ClC-5 KO mouse model, serum levels of the precursor and of the active metabolite were decreased.[8]

1,25$(OH)_2$-vitamin $D_3$ is a key regulator of calcium homeostasis. It influences renal $Ca^{2+}$ excretion, intestinal $Ca^{2+}$ absorption as well as bone $Ca^{2+}$ uptake and release.[12] The increased 1,25$(OH)_2$-vitamin $D_3$ levels in Dent's disease patients may indirectly cause hypercalciuria and kidney stones by stimulating intestinal $Ca^{2+}$ absorption. Our mouse model displayed decreased 1,25$(OH)_2$-vitamin $D_3$ levels in the absence of hypercalciuria,[6,8] whereas a different ClC-5 KO mouse had increased 1,25$(OH)_2$-vitamin $D_3$ levels and hypercalciuria.[7,13] These findings reflect the phenotypical variability found in Dent's disease.

We now used gene expression profiling to identify other possible signalling pathways that might be changed in ClC-5 KO kidneys. We identified 1,25$(OH)_2$-vitamin $D_3$-dependent genes as upregulated in kidney, but not in bone or intestine, suggesting that a luminal increase in 1,25$(OH)_2$-vitamin $D_3$ concentration selectively activates renal 1,25$(OH)_2$-vitamin $D_3$ target genes in nephron segments that are distal to the PT. Renal retinoic acid-dependent genes may be affected by a similar mechanism.

## RESULTS AND DISCUSSION

To further elucidate the pathways leading from the disruption of ClC-5 to the complex pathology of Dent's disease, we performed gene expression profiling of ClC-5 KO kidneys. Of the ~36 000 full-length genes and expressed sequence tag clusters analyzed on the Affymetrix murine genome array (U74v2) (http://www.affymetrix.com/products/arrays/specific/mgu74.affx), the transcript levels of 58 genes significantly differed from WT kidneys (according to the criteria described in Materials and Methods). The 25 genes that were downregulated, and the 33 genes that were upregulated in ClC-5 KO kidneys are given in the Supplementary Tables S2A and S2B, respectively. A selection of genes that were further validated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and that are discussed in this work are listed in Table 1. Additional genes that were evaluated by qRT-PCR are found in the Supplementary Table S1.

### Changes in transcript levels of 1,25$(OH)_2$-vitamin $D_3$-associated genes in ClC-5 KO kidneys

The most striking result of the expression profiling was the changed transcript levels of genes that are targets of 1,25$(OH)_2$-vitamin $D_3$ or are involved in its metabolism (Table 1, Figure 1a–c). The largest increase in transcripts (~350% of WT level) was observed for 25(OH)-vitamin $D_3$-1α-hydroxylase, which generates the active hormone 1,25$(OH)_2$-vitamin $D_3$ by hydroxylating its inactive precursor.[12] This finding confirms previous Northern blot and RT-PCR data.[8] In parallel to the upregulation of the 1α-hydroxylase, transcripts of vitamin $D_3$ 24-hydroxylase (also known as CYP24A1) were decreased to ~10% of WT levels. This enzyme converts the active hormone 1,25$(OH)_2$-vitamin $D_3$ and its precursor 25(OH)-vitamin $D_3$ to the largely inactive metabolites 1,24,25$(OH)_3$-vitamin $D_3$ and 24,25$(OH)_2$-vitamin $D_3$, respectively.[14] The increase of the activating enzyme and the decrease of the inactivating enzyme is expected to generate larger amounts of the active hormone 1,25$(OH)_2$-vitamin $D_3$.

**Table 1 | Genes differentially expressed in kidneys of ClC-5 KO mice[a]**

| Chip (%) | RT (%) | s.e.m. (%) | GeneBank | Gene description |
|---|---|---|---|---|
| *Vitamin D/calcium* | | | | |
| 349 | 498 | 187 | AB006034 | 25-Hydroxyvitamin D 1α-hydroxylase |
| 235 | 212 | 27 | U49430 | Ceruloplasmin |
| 152 | 160 | 13 | AI839138 | Thioredoxin-interacting protein (txnip) |
| 139 | 142 | 7 | AF028071 | Calbindin D-9k |
| — | 146 | 8 | XM_112633 | TrpV5 (ECac1) |
| — | 136 | 31 | NM_022413 | TrpV6 (ECac2) |
| — | 127 | 11 | D26352 | Calbindin D-28k |
| 13 | 4 | 5 | D89669 | Vitamin D 24-hydroxylase (Cyp24a1) |
| *Renin–angiotensin system* | | | | |
| 153 | 135 | 5 | AA690434 | Angiotensin I-converting enzyme II (ACEII) |
| — | 123 | 27 | J00621 | Renin |
| — | 116 | 14 | BC054387 | Angiotensinogen |
| *Miscellaneous* | | | | |
| 59 | 29 | 44 | X66976 | Collagen α1 type VIII (Col8A1) |
| 54 | 64 | 13 | M34094 | Midkine (Mdk) |

Abbreviations: KO, knockout mice; RT, room temperature; s.e.m., standard error of the mean; TrpV, transient receptor potential vanilloid.

[a]The ratio of transcript levels in the ClC-5 KO is given in % of WT levels. The first column gives results from the Affymetrix chip analysis, and the second column from quantitative RT-PCR. The third column gives the s.e.m. for the qRT-PCR data, which were performed with at least three pairs of WT and ClC-5 KO animals. As several interesting genes were not represented on the used Affymetrix chips, selected genes were investigated by qRT-PCR only. The third and fourth columns give the GeneBank Accession number for the genes, and a description of their gene product, respectively. Genes are grouped into functional categories. Complete lists of genes significantly deviating from WT are given in the Supplementary data, and the complete Affymetrix data set has been submitted to the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under Accession number E-MEXP-495.