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# Regulation of gene expression by dietary $Ca^{2+}$ in kidneys of 25-hydroxyvitamin $D_3$-1α-hydroxylase knockout mice

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**Regulation of gene expression by dietary $Ca^{2+}$ in kidneys of 25-hydroxyvitamin $d_3$-1α-hydroxylase knockout mice.**

*Background.* Pseudovitamin D deficiency rickets (PDDR) is an autosomal disease, characterized by undetectable levels of 1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$), rickets and secondary hyperparathyroidism. Mice in which the 25-hydroxyvitamin $D_3$-1α-hydroxylase (1α-OHase) gene was inactivated, presented the same clinical phenotype as patients with PDDR.

*Methods.* cDNA Microarray technology was used on kidneys of 1α-OHase knockout mice to study the expression profile of renal genes in this $Ca^{2+}$-related disorder. Genome wide molecular events that occur during the rescue of these mice by high dietary $Ca^{2+}$ intake were studied by the use of 15K cDNA microarray chips.

*Results.* 1α-OHase knockout mice fed a normal $Ca^{2+}$ diet developed severe hypocalcemia, rickets and died with an average life span of 12 ± 2 weeks. Intriguingly, 1α-$OHase^{-/-}$ mice supplemented with an enriched $Ca^{2+}$ diet were normocalcemic and not significantly different from wild-type mice. Inactivation of the 1α-OHase gene resulted in a significant regulation of ± 1000 genes, whereas dietary $Ca^{2+}$ supplementation of the 1α-$OHase^{-/-}$ mice revealed ± 2000 controlled genes. Interestingly, 557 transcripts were regulated in both situations implicating the involvement in the dietary $Ca^{2+}$-mediated rescue mechanism of the 1α-$OHase^{-/-}$ mice. Conspicuous regulated genes encoded for signaling molecules like the PDZ-domain containing protein channel interacting protein, FK binding protein type 4, kinases, and importantly $Ca^{2+}$ transporting proteins including the $Na^+$-$Ca^{2+}$ exchanger, calbindin-$D_{28K}$ and the $Ca^{2+}$ sensor calmodulin.

*Conclusion.* Dietary $Ca^{2+}$ intake normalized disturbances in the $Ca^{2+}$ homeostasis due to vitamin D deficiency that were accompanied by the regulation of a subset of renal genes, including well-known renal $Ca^{2+}$ transport protein genes, but also genes not previously identified as playing a role in renal $Ca^{2+}$ handling.

**Key words:** TRPV5, ECaC1, vitamin D, calcium reabsorption, PDDR.

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Vitamin D is a major regulator of $Ca^{2+}$ and phosphate homeostasis and is essential for proper development and maintenance of bone. The active form of vitamin D, 1α,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$) is synthesized from its precursor 25-hydroxyvitamin $D_3$ by the renal cytochrome P450 enzyme 25-hydroxyvitamin $D_3$-1α-hydroxylase (1α-OHase). The importance of this enzyme is reflected by severe disorders resulting from mutations identified in the gene encoding 1α-Ohase, including pseudovitamin D-deficiency rickets (PDDR) also known as vitamin D-dependent rickets type I (VDDR-I). Recently, the genetic association of 1α-OHase with PDDR has been confirmed by inactivation of the 1α-OHase gene in mice [1, 2]. Two laboratories independently generated 1α-OHase knockout mice strains that represent valuable animal models for PDDR since they display undetectable $1,25(OH)_2D_3$ levels, hypocalcemia, secondary hyperparathyroidism, and failure to thrive [1, 2]. The 1α-OHase knockout mice (1α-$OHase^{-/-}$) developed distinct histologic evidence of rickets and osteomalacia.

The absence of vitamin D is expected to cause widespread changes in gene expression in the kidney. On one hand, vitamin D receptor (VDR) activation will be decreased resulting in altered gene expression. On the other hand, $Ca^{2+}$ sensing by the $Ca^{2+}$ sensing receptor (CaSR) will be changed due to the hypocalcemia in 1α-OHase mice. The importance of VDR activation versus CaSR activation has recently been challenged by the observation that many of the phenotypic effects of VDR knockout animals can be prevented by a "rescue diet" with lactose, $Ca^{2+}$, and phosphate [3–5]. This observation is supportive for the view that vitamin D becomes relevant at the point where an organism is denied an abundant $Ca^{2+}$ supply.

In a previous study, we have found indications for these $Ca^{2+}$-related actions in the 1α-$OHase^{-/-}$ knockout mice [6]. This study showed that high dietary $Ca^{2+}$ intake normalizes the expression levels in 1α-$OHase^{-/-}$ of the $Ca^{2+}$

transport proteins, including the epithelial $Ca^{2+}$ channel (TRPV5), calbindin-$D_{28K}$, $Na^{+}$-$Ca^{2+}$ exchanger (NCX1), and plasma membrane adenosine triphosphatase (ATPase) (PMCA1b), and contributes in this way to the normalization of blood $Ca^{2+}$ levels [6].

The aim of the present study was to identify new genes important for the maintenance of the $Ca^{2+}$ balance. The hypothesis is that vitamin D deficiency results in widespread gene expression alterations in the kidney, which are in part due to diminished CaSR activation by decreased plasma $Ca^{2+}$ concentrations. Furthermore, we hypothesized that genes, differentially expressed in 1α-OHase$^{-/-}$ mice, could be rescued by dietary $Ca^{2+}$ via a vitamin D–independent mechanism. To address these questions, DNA microarray analysis of gene expression was compared between 1α-OHase$^{-/-}$ and 1α-OHase$^{+/-}$ mice supplemented with dietary $Ca^{2+}$.

## METHODS

### Animal protocol

25-hydroxyvitamin $D_3$-1α-hydroxylase knockout mice were recently generated by targeted ablation of exon 8 encoding the HEME binding domain of the enzyme [1]. 1α-OHase knockout mice were genotyped by Southern blot analysis at an age of 3 weeks directly after the weaning period as described previously [1]. Initial characterization of the 1α-OHase knockout mice demonstrated that there are no significant differences between wild-type (1α-OHase$^{+/+}$) and heterozygous 1α-OHase knockout mice (1α-OHase$^{+/-}$) [1]. The heterozygous were, therefore, used as control animals. 1α-OHase$^{+/-}$ and homozygous 1α-OHase (1α-OHase$^{-/-}$) knockout mice were fed from weeks 3 to 8 either a normal diet [1.1% (wt/wt) $Ca^{2+}$, 0.8% (wt/wt) phosphorus, and 0% (wt/wt) lactose], a $Ca^{2+}$-enriched diet [2% (wt/wt) $Ca^{2+}$, 1.25% (wt/wt) phosphorus, and 20% (wt/wt) lactose] (Harlan Tekled, Wisconsin, MA, USA). Animals ($N = 6$ in each group) were sacrificed at an age of 8 weeks and blood and kidney samples were taken. The Animal Ethics Board of the University of Nijmegen (Nijmegen) and Shriners Hospital for Children (Montreal) approved all animal experimental procedures.

### RNA isolation

RNA was extracted from whole kidney from four subjects per group using the TRIzol reagent using the procedure recommended by the supplier (Invitrogen Life Sciences, Carlsbad, CA, USA). The quality and quantity of the RNA samples was determined with the Bioanalyzer (Agilent, Palo Alto, CA, USA) using the Eukaryote Total RNA Nano Assay.

### Reverse transcription/labeling

Samples were pooled per group in equal amounts of total RNA per subject. For each reaction, 5 μg of total RNA was annealed to 1 μg of Oligo-$dT_{12–18}$ primer (Invitrogen Life Sciences) at 70°C for 15 minutes. Then, reverse transcription (RT) was performed in a 30 μL reaction containing 200 U SuperScript II reverse transcriptase (Invitrogen Life Sciences), 2 nmol deoxyguanosine triphosphate (dGTP), deoxyadenosine triphosphate (dATP), and deoxycytidimine triphosphate (dCTP), 0.5 nmol deoxydithiothreitol (dTTP) (Amersham Biosciences Corp., Biscataway, NJ, USA), 1.5 nmol of aminoallyl-deoxyuridine triphosphate (dUTP) (Amersham Biosciences Corp.) at 42°C for 2 hours. After 2 minutes at 95°C, a 20 μL mixture of 1 mol/L NaOH (Riedel-de Haën, Seelze, Germany) and 0.5 mol/L of ethylenediaminetetraacetic acid (EDTA), pH 8.0 (Sigma Chemical Co., St. Louis, MO, USA) were added and incubated at 65°C for 30 minutes to hydrolyze RNA. The reaction was neutralized by adding 25 μL of 1 mol/L of Hepes, pH 7.5 (Sigma Chemical Co.). Samples were purified on Microcon-30 columns (Millipore, Billerica, MA, USA) and reduced to a volume of 8 μL. Cy3 and Cy5 monofunctional reactive dyes (Amersham Biosciences UK Unlimited, Buckinghamshire, UK) dissolved in dimethyl sulfoxide (DMSO) (Riedel-de Haën) and 1 μL of 0.5 mol/L sodium bicarbonate (pH 9.0; Sigma, St Louis, MO) were added to the cDNA samples and incubated at room temperature for 1 hour. Dyes were quenched by incubation with 4.5 μL of 4 mol/L Hydroxylamine (Aldrich Chemicals, Co., Milwaukee, MI, USA) at room temperature for 15 minutes. Labeled samples were purified on Chromaspin-30 columns (BD Biosciences Clontech, Palo Alto, CA, USA). Samples from 1α-OHase$^{-/-}$ mice and 1α-OHase$^{+/-}$ mice with $Ca^{2+}$ supplementation were labeled with Cy3 and samples from 1α-OHase$^{+/-}$ mice and 1α-OHase$^{-/-}$ mice with $Ca^{2+}$ supplementation were labeled with Cy5.

### Hybridization of cDNA chips

Mouse NIH 15K cDNA chips (Ontario Microarray Centre, Toronto University, Canada) were prehybridized for at least 45 minutes in a filtered (0.22 micron Minisartplus filter, Sartorius AG, Göttingen, Germany) and preheated buffer (37°C) containing 1 g bovine serum albumin (BSA) (Sigma Chemical Co.), 25 mL 20× standard sodium citrate (SSC), 25 mL pure formamide (Merck, Darmstadt, Germany), 1 mL 10% (wt/vol) sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories, Hercules, CA, USA), in a total volume of 100 mL. After washing the slides with distilled water and isopropanol (Mallinkrodt Baker BV, Deventer, The Netherlands), slides were spun dry. Slides were then loaded with 100 to 150 ng labeled cDNA in a filtered (0.22 micron) and preheated buffer

containing 25% (wt/vol) formamide (Bio-Rad Laboratories), 5× SSC [sodium chloride) (Riedel-de Haën)]; sodium citrate dihydrate (Merck, Dramstadt, Germany); 0.1% (wt/vol) SDS; 200 μg/mL of Herring Sperm DNA (Invitrogen Life Sciences); and tRNA (Roche Molecular Diagnostics GmbH, Mannheim, Germany) that had been denatured at 95°C for 5 minutes and incubated at 42°C overnight in acclimatized hybridization chambers (Corning Incorporated Life Sciences, Acton, MA, USA). Slides were then washed first, in 100 mL 1× SSC with 0.2% (wt/vol) SDS; second, in 0.1× SSC with 0.2% (wt/vol) SDS; and finally, in 0.1× SSC.

### Scanning/quantification of cDNA chips

Slides were scanned on the ScanArray 4000XL (BioDiscovery, Marina del Rey, CA, USA) at laser intensities of 85 to 90 and photomultiplier tube sensitivity of 60 to 65 at a resolution of 10 μm. Images were quantified using Imagine software (BioDiscovery). Raw mean intensity data were averaged per duplicate spot and normalized on median ratios on the chip by correcting the Cy5 values. Spots with both Cy3 and Cy5 intensities below background plus two times the standard deviation of the background signal and spots with intensities exceeding 45000 were excluded from further analysis. Genes were considered significantly regulated when $\log_2$ transformed ratios were $\geq 0.7$ or $\leq -0.7$.

### Clustering analysis and gene ontology annotations

Hierarchical clustering of microarray data was performed using the expression profiler tool EPCLUST (European Bioinformatics Institute; Jaak Vilo, Cambridge, UK). Average linkage (average distance) clustering based on correlation measure-based distance was performed on data for which ratios were $>0.7$ or $<-0.7$ in at least one comparison. Ratios of the genes were coupled to the biologic processes classification of the Gene Ontology Consortium (GO Consortium) [7] using proprietary software and Access 2000 (Microsoft Corporation, Redmond, WA, USA). For each biologic process category, the total number of annotated genes and the percentage of significantly changed genes were counted, and the average ratio was calculated. Interesting categories were defined as groups containing at least five genes and that had either a mean overall ratio of $>0.3$ or $<0.3$ or had at least 20% of the genes of that group changed significantly.

### Serum $Ca^{2+}$ measurements

Serum $Ca^{2+}$ concentrations were measured using a colorimetric assay kit as described previously [8].

### Statistical analysis

The serum $Ca^{2+}$ data were expressed as the mean ± SEM. Overall statistical significance was determined by analysis of variance (ANOVA). In the case of significance ($P < 0.05$), individual groups were compared by contrast analysis according to Scheffé.

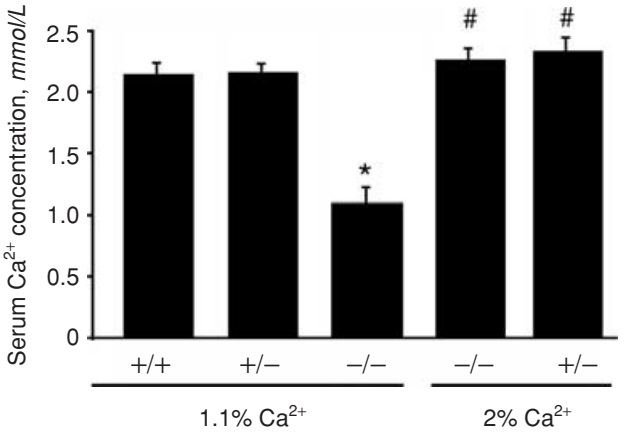


**Fig. 1. The effect high dietary $Ca^{2+}$ intake on the serum $Ca^{2+}$ concentration in 1α-hydroxylase (1α-OHase$^{-/-}$) mice.** Values are presented as means ± SEM ($N = 6$). $^{*}P < 0.05$, significant different from 1α-OHase$^{+/+}$ and 1α-OHase$^{+/-}$ mice fed a normal $Ca^{2+}$ diet containing 1.1% (wt/vol) $Ca^{2+}$; $^{\#}P < 0.05$, significant different from 1α-OHase$^{-/-}$ mice on normal $Ca^{2+}$ diet containing 1.1% (wt/wt) $Ca^{2+}$.

## RESULTS

### Phenotypical characterization of the 1α-OHase$^{-/-}$ mice

Inactivation of the 1α-OHase gene in mice resulted in severe hypocalcemia with serum $Ca^{2+}$ concentrations around 1.2 mmol/L, whereas 1α-OHase$^{+/-}$ mice, which are not distinguishable from 1α-OHase$^{+/+}$ mice, displayed normal plasma $Ca^{2+}$ concentrations (Fig. 1). 1α-OHase$^{-/-}$ mice fed a regular $Ca^{2+}$ diet containing 1.1% (wt/wt) $Ca^{2+}$ developed rickets and died with an average life span of 12 ± 2 weeks (data not shown). Interestingly, supplementation of these mice with an enriched $Ca^{2+}$ diet containing 2% (wt/wt) $Ca^{2+}$ normalized plasma $Ca^{2+}$ concentrations that were not significantly different from 1α-OHase control mice (Fig. 1). This latter diet resulted in a normal development and life span of these supplemented mice.

### Gene expression profiles of 1α-OHase$^{-/-}$ mice compared with 1α-OHase$^{+/-}$ mice

Around 1000 genes were differently regulated in 1α-OHase$^{-/-}$ mice versus the 1α-OHase$^{+/-}$ mice, whereas roughly 2000 genes were significantly regulated by dietary $Ca^{2+}$ in the vitamin D–deficient mice. Interestingly, 557 transcripts, that account for 4% of the total number of 15,000 scanned genes, were regulated under both conditions. This is demonstrated in the Venn diagrams in Figure 2A. Moreover, only minor changes in expression were induced by dietary $Ca^{2+}$ in 1α-OHase$^{+/-}$ mice. Figure 2B and C shows the numbers of up- and down-regulated genes in all the experimental conditions

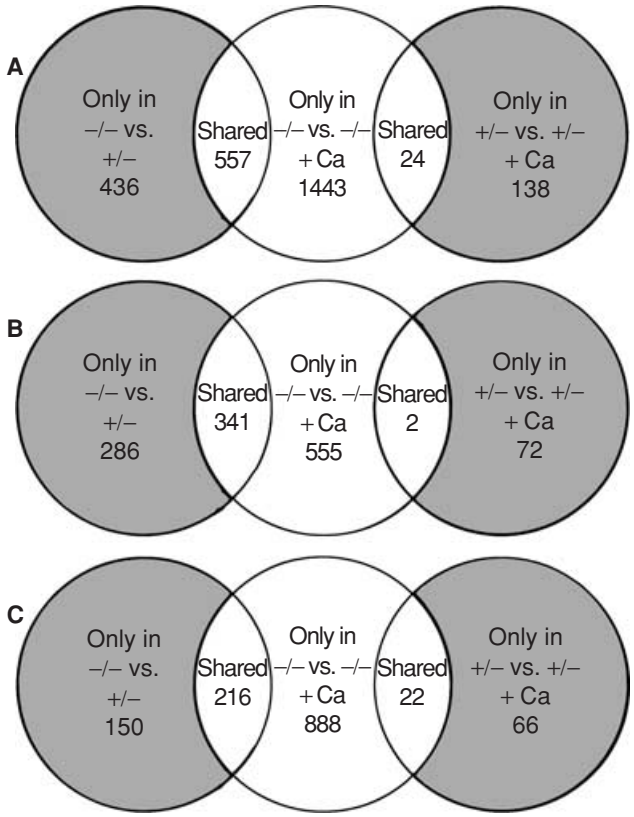


**Fig. 2. Number of genes regulated when comparing the three conditions 1α-hydroxylase (1α-OHase$^{-/-}$) mice versus 1α-OHase$^{+/-}$ mice (left), 1α-OHase$^{-/-}$ mice versus 1α-OHase$^{-/-}$ mice supplemented with dietary $Ca^{2+}$ (middle) and 1α-OHase$^{+/-}$ mice versus $Ca^{2+}$ supplemented 1α-OHase$^{+/-}$ mice (right).** The number of total-regulated (*A*), up-regulated (*B*), or down-regulated (*C*) transcripts on the DNA chip are depicted. Genes are shown in the overlapping regions that are regulated in two conditions.

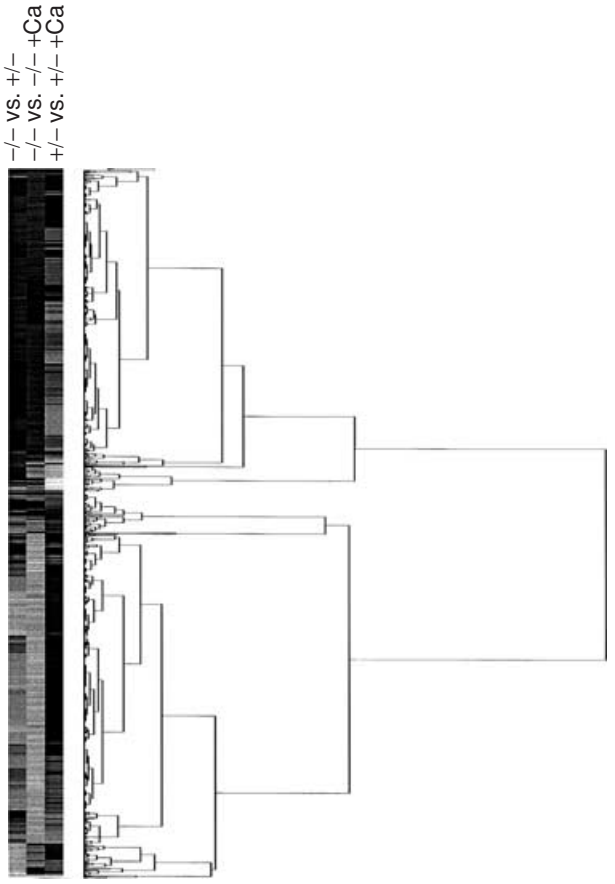


**Fig. 3. Hierarchical clustering of all three comparisons [1α-hydroxylase (1α-OHase$^{-/-}$) mice versus 1α-OHase$^{+/-}$ mice, 1α-OHase$^{-/-}$ mice versus 1α-OHase$^{-/-}$ mice treated with $Ca^{2+}$ and 1α-OHase$^{+/-}$ mice compared to 1α-OHase$^{+/-}$ mice fed the enriched $Ca^{2+}$ diet].** Genes that are significantly up- (dark) or down-regulated (light) are shown.

and includes an overview of the gene expression profile of each condition separately and in combination.

Next, we performed a hierarchical clustering of the three comparisons in the present study: 1α-OHase$^{-/-}$ versus 1α-OHase$^{+/-}$, 1α-OHase$^{-/-}$ versus 1α-OHase$^{-/-}$ supplemented with $Ca^{2+}$ and 1α-OHase$^{+/-}$ compared to 1α-OHase$^{+/-}$ treated with $Ca^{2+}$ (Fig. 3). A large portion of the expression profiles of knockout versus 1α-OHase$^{+/-}$ and 1α-OHase$^{-/-}$ versus 1α-OHase$^{-/-}$ treated with $Ca^{2+}$ were similarly regulated, but there were also some genes differentially regulated. The comparison of 1α-OHase$^{-/-}$ mice versus 1α-OHase$^{-/-}$ mice treated with $Ca^{2+}$ and 1α-OHase$^{+/-}$ mice compared to 1α-OHase$^{+/-}$ mice supplemented with $Ca^{2+}$ yielded far less similarity, but a distinct group of genes was coregulated.

Figure 4 shows representative differential expression of 1α-OHase$^{-/-}$ versus 1α-OHase$^{+/-}$ mice in an intensity-dependent fashion with Cy3/Cy5 scatter plots. Up-regulated and down-regulated genes formed two distinct populations that clearly differentiate from the non-regulated genes. Of interest is the group of genes (depicted $\log_2$ above 0.7 and below –0.7) that were inversely regulated as a consequence of 1α-OHase gene inactivation (Fig. 4A) and dietary $Ca^{2+}$ supplementation in the vitamin D–deficient state (Fig. 4B). Genes depicted in yellow were regulated in a similar direction in the described comparisons.

## Gene expression changes specifically related to $Ca^{2+}$ supplementation

Further analysis of the genes that were inversely regulated by dietary $Ca^{2+}$ supplementation and $1,25(OH)_2D_3$ deficiency provides information about interesting candidates involved in the $1,25(OH)_2D_3$-independent rescue mechanism by dietary $Ca^{2+}$ (Table 1) (the complete data set of the regulated genes is accessible via *http://www.genomics.med.uu.nl/pub/bb/kidney/*). Two important cDNA transcripts, known to encode for $Ca^{2+}$ transporting proteins in the distal part of the nephron, calbindin-$D_{28K}$ and the $Na^+$-$Ca^{2+}$ exchanger (NCX1),

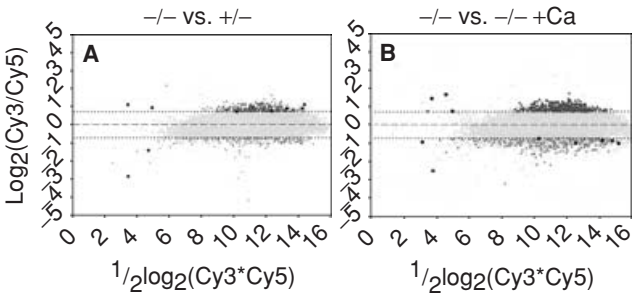


**Fig. 4. Differential expression patterns depicted in an intensity-dependent fashion using Cy3/Cy5 scatter plots.** Regulated genes by inactivation of the 1α-hydroxylase (1α-Ohase) gene (*A*) and supplementation of dietary $Ca^{2+}$ in the vitamin D–deficient state (*B*) form two distinct populations of genes. Genes that are regulated in two conditions (when 1α-OHase$^{+/-}$ mice are compared with 1α-OHase$^{-/-}$ mice and when 1α-OHase$^{-/-}$ supplemented with dietary $Ca^{2+}$) are shown above the 0.7 $\log_2$ line (up-regulated in both comparisons) and below the −0.7 $\log_2$ line (down-regulated in both comparisons). Genes that are regulated in opposite directions in both comparisons are shown as large black dots.

were down-regulated in the 1α-OHase$^{-/-}$ mice and up-regulated by dietary $Ca^{2+}$ in the 1,25$(OH)_2D_3$-deficient animal. Previously, these genes were analyzed by quantitative RT-polymerase chain reaction (PCR) in the same animal model and were found to be regulated in a similar manner [6]. Analysis of other genes that were significantly regulated revealed several genes previously unknown to be involved in the $Ca^{2+}$ homeostasis. New candidates include the $Ca^{2+}$-related genes like FK506 binding protein 4, calmodulin, and several ion transporters (i.e., the $K^+$ channel protein Kcnq1, the $Na^+$-$H^+$ exchanger, and the voltage-dependent $Ca^{2+}$ channel α1 subunit). Several kinases and kinase substrates controlling distinct signalling pathways were regulated by dietary $Ca^{2+}$ as well as a channel-interacting PDZ domain protein (Table 1).

Additional analysis was performed to study genes that followed the $Ca^{2+}$ availability in 1α-OHase$^{-/-}$ and 1α-OHase$^{+/-}$ mice. Although 1α-OHase$^{+/-}$ mice did not show elevated levels of serum $Ca^{2+}$ (Fig. 1), several genes were found to be down-regulated in 1α-OHase$^{-/-}$ mice without dietary supplementation and up-regulated when 1α-OHase$^{+/-}$ mice were supplemented with $Ca^{2+}$ (Table 2). These genes are thought to be interesting candidates that are controlled by $Ca^{2+}$ irrespective of the vitamin D state of the animal. Among them were genes involved in mitogen-activated protein kinase (MAPK)/tyrosine kinase signaling and genes that are responsive to intracellular $Ca^{2+}$ concentrations (Table 2). Subsequently, genes regulated by the 1α-OHase gene inactivation, but not sensitive to dietary $Ca^{2+}$ supplementation, were identified and depicted in Table 3. Typical genes regulated by vitamin D only were identified in several function groups, including $Ca^{2+}$ transport proteins and ion transporters.

### Analysis using GO Consortium functional classification of genes

Differentially regulated genes were grouped into functional profiles using GO Consortium annotation. In both the comparison of the 1α-OHase$^{-/-}$ with the 1α-OHase$^{+/-}$ mice and the comparison of the 1α-OHase$^{-/-}$ mice with the 1α-OHase$^{-/-}$ mice receiving the high $Ca^{2+}$ diet, alterations in some functional categories could be identified. Of all 307 categories of biologic processes, 109 had five or more genes that were represented on the cDNA chip. Of these, 14 processes were considered to be changed in the 1α-OHase$^{-/-}$ mice versus 1α-OHase$^{+/-}$ mice and 32 processes in 1α-OHase$^{-/-}$ mice versus 1α-OHase$^{-/-}$ mice supplemented with $Ca^{2+}$ comparison. Eleven categories were overlapping in both comparisons (Fig. 5). Inactivation of the 1α-OHase gene and supplementation of $Ca^{2+}$ in 1α-OHase$^{-/-}$ mice resulted in a regulation of genes involved in protein biosynthesis. Remarkably, a subset of the biologic processes were regulated in a similar fashion in both comparisons. Biologic processes such as glycolysis, peroxidase reaction, hydrogen transport, tricarbolic acid cycle, iron homeostasis, and lymph gland development were decreased in 1α-OHase$^{-/-}$ mice and reactivated through dietary $Ca^{2+}$ supplementation. Genes involved in muscle development and induction of apoptosis were slightly up-regulated due to the absence of 1,25$(OH)_2D_3$, which were not affected by $Ca^{2+}$ supplementation of the 1α-OHase$^{-/-}$ mice.

## DISCUSSION

In the present study, we demonstrated that dietary $Ca^{2+}$ intake is an important regulator of the $Ca^{2+}$ homeostasis in vitamin D–deficient 1α-OHase$^{-/-}$ mice, ultimately normalizing blood $Ca^{2+}$ levels. Microarray analysis revealed that high dietary $Ca^{2+}$ intake restores the disturbed genetic profile observed in 1α-OHase$^{-/-}$ mice, which is accompanied by a functional rescue of these 1,25$(OH)_2D_3$-deficient animals. Gene expression analysis showed simultaneous regulation of genes with potential importance for $Ca^{2+}$ handling that were not previously identified as such and a subset of genes that were regulated by dietary $Ca^{2+}$ independently of vitamin $D_3$.

Recently, Dardenne et al [1] generated an 1α-OHase knockout strain that represents a valuable animal model for PDDR since these mice display all the clinical symptoms observed in patients, including hypocalcemia and rickets. These pathologic symptoms in the 1α-OHase$^{-/-}$ mice resulted in an early death with an average life span of 12 weeks. Normalization of the plasma $Ca^{2+}$ concentrations by dietary $Ca^{2+}$ supplementation was associated with a functional rescue of the 1α-OHase$^{-/-}$ mice that made the recovered mice phenotypically undistinguishable from wild-type mice. A previous study indicated that the $Ca^{2+}$ transport proteins, including TRPV5

**Table 1.** Summary of candidate genes identified using cDNA microarrays

| Function group | Gene name | Regulation ($Log_2$ ratios) | | GenBank accession number |
|---|---|---|---|---|
| | | −/− vs. +/− | −/−$Ca^{2+}$ vs. −/− | |
| Calcium-related | Calbindin-$D_{28K}$ | −0.86 | 0.83 | D26352 |
| | Calmodulin-I | 0.81 | −0.85 | U16850 |
| Immunology | FK506 binding protein 4 | −0.93 | 1.26 | NM010219 |
| Matrix/structural proteins | Putative membrane protein GENX-3745 gene | 0.82 | −0.74 | AJ270952 |
| | Putative membrane protein 1190006A08Rik | −0.77 | 1.28 | XM129531 |
| Protein synthesis/translation control | Poly(A) binding protein II (Pabpn1) | 0.99 | −0.88 | U93050 |
| Receptor/channel/transporter | Kcnq1 gene for potassium channel protein (Tssc8) | 0.74 | −1.36 | NM008434 |
| | Voltage-dependent calcium channel alpha1 subunit (Cacn4) | −0.84 | 1.03 | D43746 |
| | Voltage-dependent anion channel 1 (Vdac1) | −0.77 | 0.99 | NM011694 |
| | Sodium/calcium exchanger Slc8a1 (NCX1) | −1.18 | 1.57 | NM011406 |
| Signaling/communication | Sodium/hydrogen exchanger, isoform 3 regulator 1 (NHERF-1) Slc9a3r1 | −0.91 | 1.23 | NM012030 |
| | Serine/threonine kinase (Stk18) | 0.90 | −1.03 | L29479 |
| | Guanylate kinase 1 (Guk1) | 0.88 | −0.84 | NM008193 |
| | Channel-interacting PDZ domain protein (Cipp) | 0.84 | −0.72 | NM172696 |
| | v-crk-associated tyrosine kinase substrate (Crkas) | 0.79 | −0.71 | NM009954 |
| | Protein kinase C substrate 80K-H (Prkcsh) | −0.84 | 0.92 | NM008925 |
| | G protein-coupled receptor kinase 5 (GRK5) | 0.83 | −0.96 | AF040756 |

Depicted genes are reversely regulated by inactivation of the 1α-hydroxylase (1α-OHase) gene and supplementation by dietary $Ca^{2+}$ in the vitamin D–deficient state. The $log_2$ ratios represent the change in expression when 1α-OHase$^{-/-}$ mice are compared to 1α-OHase$^{+/-}$ mice and when 1α-OHase$^{-/-}$ mice are supplemented with $Ca^{2+}$. The complete data set of the regulated genes is accessible via *http://www.genomics.med.uu.nl/pub/bb/kidney/*.

(previously named ECaC1), calbindin-$D_{28K}$, and NCX1, were down-regulated in the 1α-OHase mice [6]. Interestingly, the expression of these proteins was normalized by dietary $Ca^{2+}$ supplementation.

The present analysis demonstrated that known $Ca^{2+}$ transport proteins represented on the cDNA microarray, including calbindin-$D_{28K}$ and NCX1, were regulated by dietary $Ca^{2+}$. Dietary $Ca^{2+}$ supplementation in the 1α-OHase$^{-/-}$ mice had a maximum effect on NCX1 expression suggesting that this basolateral extrusion protein is an important mechanism in the process of transcellular $Ca^{2+}$ reabsorption. In line with these findings are functional studies in cell model systems demonstrating that basolateral extrusion of $Ca^{2+}$ is mainly achieved by NCX1 [9, 10]. Interestingly, calbindin-$D_{9K}$ was not regulated by dietary $Ca^{2+}$ supplementation. The expression of this $Ca^{2+}$ binding protein was solely controlled by vitamin D in line with previous studies [6].

Dardenne et al [11] investigated recently bone histology and histomorphometry in 1α-OHase$^{-/-}$ mice supplemented with $Ca^{2+}$ confirming that the rickets and osteomalacia were prevented. The $Ca^{2+}$ rescue diet also restored the biomechanical properties of the bone tissue within normal parameters. Blood biochemical analysis revealed that the $Ca^{2+}$ diet corrected the secondary hyperparathyroidism. This points at an important role for the decreased prevailing calcium levels per se, independent of parathyroid hormone (PTH) and vitamin D. This significant effect of hypocalcemia could indicate a decrease in CaSR activation. The CaSR is an important gateway by which extracellular $Ca^{2+}$ can affect a variety of second messenger systems, such as the phopsholipase $A_2$ ($PLA_2$) and phopsholipase C (PLC), adenylate cyclase, and MAPKs. The downstream actions of CaSR on gene expression involve a large number of cellular processes, most notably secretory pathways of hormones, channels, and transporters (e.g., the thiazide-sensitive NaCl cotransporter, $K^+$ channels and aquaporin-2) and apoptosis-related genes. In various clinical conditions associated with a disturbed $Ca^{2+}$ homeostasis, vitamin D analogues are administered. The treatment of choice for PDDR and for patients with chronic renal failure is long-term replacement therapy with $1,25(OH)_2D_3$. Notably, the currently applied strategy of vitamin D and $Ca^{2+}$ supplementation to patients with chronic renal failure has been associated with undesirable effects, such as vascular calcification and calciphylaxis. It would be interesting to compare the normalization of plasma $Ca^{2+}$ levels by $Ca^{2+}$ supplementation with the treatment with vitamin D analogues in these patient groups.

Hierarchical cluster analysis of the 15K cDNAs demonstrated a remarkable overlap in the expression profiles between inactivation of the 1α-OHase gene compared to 1α-OHase$^{-/-}$ mice and supplementation of dietary $Ca^{2+}$

**Table 2.** List of genes that are regulated by the $Ca^{2+}$ availability in 1α-hydroxylase (1α-OHase$^{-/-}$) and 1α-OHase$^{+/-}$ mice with their corresponding ratios

| Symbol | Gene name | Process | Regulation ($Log_2$ ratios) −/− vs. −/− $Ca^{2+}$ | Regulation ($Log_2$ ratios) +/− $Ca^{2+}$ vs. +/− | GenBank Accession No |
|---|---|---|---|---|---|
| p70 (s6k)/p85 (s6k) | P70/p85 s6 kinase | MAPK signaling/cell proliferation/growth | −1.20 | 0.77 | AJ000654 |
| Pbef | Pre-B-cell colony-enhancing factor | Activation in lymphocytes | −0.84 | 0.77 | BC018358 |
| ECH1 | Enoyl coenzyme A hydratase 1 | Fatty acid beta-oxidation | −1.12 | 0.50 | NM_016772 |
| Fu | FUSED serine/threonine kinase | | −0.72 | 0.58 | O35625 |
| Gsta4 | Glutathione S-transferase, alpha 4 | Antioxidative reponse | −1.56 | 0.98 | NM_010357 |
| BMP-1 | Bone morphogenetic protein | Development | −0.95 | 0.51 | AK004995 |
| P4HA1 | Prolyl 4-hydroxylase alpha (I)-subunit | Matrix/bone formation | −1.23 | 0.59 | BC009654 |
| Snta1 | Syntrophin, acidic 1 | Neuronal differentiation | −0.77 | 0.56 | NM_009228 |
| GC-globulin | Vitamin D-binding protein (GC) | | −0.88 | 1.14 | BC010762 |
| UGT1 | UDP glucuronosyltransferase | Detoxification | −1.34 | 0.96 | BC012716 |
| APC4 | Anaphase-promoting complex subunit 4 | Cell cycle | −1.29 | 0.55 | Q9UJX5 |
| RARRES2/TIG2 | Retinoic acid receptor responder (tazarotene induced) 2 | | −1.04 | 1.11 | NM_002889 |
| BNIP3/NIP3 | BCL2/adenovirus E1B 19 kDa interacting protein 1 | Calcium storage | −1.19 | 0.77 | X82564 |
| JAG1 | Jagged 1 (Alagille syndrome) | Differentiation | 0.69 | −0.77 | NM_000214 |
| HSD17B4 | Hydroxysteroid 17-beta dehydrogenase 4 | Testosterone biosynthesis | −0.76 | 0.54 | NM_008292 |

The $log_2$ ratios represent the change in expression when supplemented with $Ca^{2+}$ in 1α-OHase$^{-/-}$ mice and in 1α-OHase$^{+/-}$ mice.

**Table 3.** List of genes that are significantly regulated by inactivation of the 1α-hydroxylase (1α-Ohase) gene and not by dietary $Ca^{2+}$ supplementation with their corresponding ratios

| Symbol | Gene name | Process | Regulation ($log_2$ ratios) −/− vs. +/− | Regulation ($log_2$ ratios) −/− vs. −/−Ca | GenBank accession number |
|---|---|---|---|---|---|
| ATP6g1 | ATPase $H^+$-transporting lysosomal proton pump | Proton transport | −0.72 | −0.049 | NM_004888 |
| Abcc3 | ATP binding cassette sub-family C (CFTR/MRP) | Ion transport | 0.79 | 0.46 | NM_029600 |
| Calb3 | Calcium binding protein $D_{9K}$ | $Ca^{2+}$ transport | −1.37 | −0.003 | NM_009789 |
| Cdk4 | Cyclin-dependent kinase 4 | Signaling | 0.76 | 0.54 | NM_009870 |
| ECM1 | Extracellular matrix protein 1 | Adhesion | 0.70 | 0.41 | NM_007899 |
| SLC7A | Cation amino acid transporter | Amino acid transport | 1.21 | 0.26 | NM_003982 |
| COL3A | COL3A1 gene for collagen | Bone | −0.84 | 0.49 | XM_129745 |
| KCNN4 | $K^+$ small conductance $Ca^{2+}$ activated channel | $K^+$ channel | 0.89 | −0.67 | NM_008433 |
| Hsp86 - 1 | Heat shock protein Hsp86 - 1 | Chaperone | −0.98 | −0.56 | XM_109307 |
| P2RX4 | P2b4c receptor unit | ATP-gated ion channel | 1.00 | 0.64 | AJ251461 |
| D7RP2e | Kidney testosteron-regulated RP2 | Signaling | −0.77 | −0.23 | NM_033080 |

The $log_2$ ratios represent the change in expression when 1α-OHase$^{-/-}$ mice are compared with 1α-OHase$^{+/-}$ and 1α-OHase$^{-/-}$ mice versus 1α-OHase$^{-/-}$ mice supplemented with $Ca^{2+}$.

in 1α-OHase$^{-/-}$, whereas only a minor overlap was observed between 1α-OHase$^{-/-}$ with 1α-OHase$^{+/-}$ mice. Interestingly, several transcripts, unknown to be involved in $Ca^{2+}$ homeostasis, were significantly regulated. Previous studies from our group have shown that TRPV5, not present on the microarray chip, facilitates the rate-limiting step in the process of transcellular $Ca^{2+}$ transport [12]. With respect to the gatekeeper function of this apical $Ca^{2+}$ influx channel, it will be interesting to discuss three regulated cDNAs encoding a channel-interacting PDZ domain protein (CIPP), FK506 binding protein 4, and calmodulin.

CIPP was found to interact with several ion channels including the acidic sensing ion channel ASIC3 and the inward rectifying potassium channel Kir [13, 14]. CIPP is a PDZ domain-containing protein that contains four PDZ domains. Interestingly, TRPV5 contains both in the amino and carboxyl-termini type I (S/T-*X*-V/L) PDZ-interacting domains, that are similar to the carboxyl-terminal sequence of the ASIC3 protein [15].

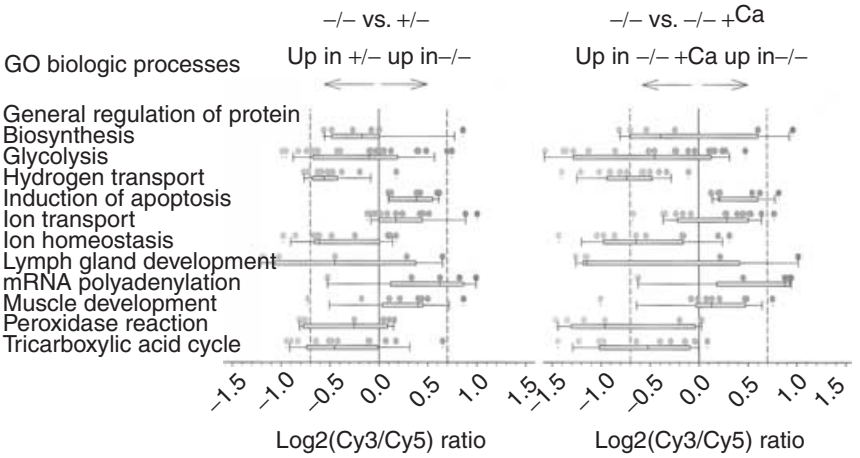


**Fig. 5. A subset of regulated functional gene groups based on Gene Ontology (GO) with differential regulation due to 1α-hydroxylase (1α-OHase) inactivation or $Ca^{2+}$ supplementation.** Displayed GO groups contained at least five genes, had either a mean overall ratio of at least 0.3 or had at least 20% of the genes changed significantly in both comparisons. Box plots indicate the quartiles of the ratios.

PDZ-containing proteins can indeed influence the cell surface expression of ion channel proteins by, for instance, affecting their insertion, endocytosis, and recycling [16, 17].

The second transcript, FK506 binding protein 4 (FKBP4), has been implicated as an important regulation of ion channel activity. Of interest, the transient receptor potential-like channel (TRPL), a $Ca^{2+}$-permeable cation channel found in *Drosophila* photoreceptor cells that shares 25% homology and an identical structural topology with TRPV5, is modulated by FKBP4 [18]. Intriguingly, renal transplant recipients receiving the immunosuppressive drug FK506 (also known as tacrolimus), which is the substrate for FKBP4, had strongly reduced renal calbindin-$D_{28K}$ protein levels and increased urine $Ca^{2+}$ excretion [19, 20]. Furthermore, the treatment caused intratubular calcification [21]. The data suggest a link between the observed regulation of FKBP4 in the present study and the molecular regulation of $Ca^{2+}$ reabsorption in the distal part of the nephron.

The third interesting candidate regulated on the chip was calmodulin. Calmodulin is a major cellular sensor of $Ca^{2+}$ signalling and interacts with numerous proteins associated with cellular second messenger systems [22, 23]. Changes in intracellular $Ca^{2+}$ concentration regulate calmodulin in various distinct ways. First, at the cellular level, by directing its subcellular distribution. Second, at the molecular level, by promoting different modes of association with many target proteins. Third, by directing a variety of conformational states in calmodulin that result in target-specific activation. Fourth, the present study suggests an association between serum $Ca^{2+}$ levels and the expression of calmodulin. Calmodulin -dependent regulation of protein kinases illustrates a potential mechanism by which $Ca^{2+}$-sensing proteins can recognize and generate affinity as well as specificity for effectors in a $Ca^{2+}$-dependent manner. It has been demonstrated that calmodulin interacts with several $Ca^{2+}$ channels, including voltage-gated $Ca^{2+}$ channels [24], but also members of the TRP family [25, 26]. Niemeyer et al [26] identified a new calmodulin binding site in the carboxyl-terminus of the TRPV5 homolog TRPV6. These investigators showed that $Ca^{2+}$-dependent calmodulin-binding to TRPV6 facilitates channel inactivation, which was counteracted by protein kinase C (PKC)-mediated phosphorylation of this putative calmodulin binding site. Based on the observed genomic regulation of calmodulin it will be of interest to investigate whether this $Ca^{2+}$ sensor interacts with TRPV5. However, functional studies are required to study the role of calmodulin expression in regulating the $Ca^{2+}$ balance and in particular the activity of TRPV5. The afore-mentioned genes were reversely regulated by inactivation of the 1α-OHase gene and normalized in expression by dietary $Ca^{2+}$, which raises the attractive hypothesis that these putative candidates regulate $Ca^{2+}$ reabsorption in general and in particular TRPV5 activity, and thereby, restoring the hypocalcemia and subsequently other pathologic symptoms in the vitamin D–deficient mice.

In addition to the previously discussed reversely regulated genes, a subgroup of genes was regulated by dietary $Ca^{2+}$ independent of the vitamin D status. These genes were found to be up- or down-regulated by $Ca^{2+}$ in 1α -OHase$^{+/-}$ and 1α-OHase$^{-/-}$. An interesting candidate is MAPK (p70/p85 s6 kinase), an enzyme that has been implicated in many cellular signaling processes, including voltage-operated $Ca^{2+}$ channels [27]. Furthermore, cDNAs encoding proteins, which are involved in activation of MAPK, are coregulated like syntrophin. Anchoring of ion channels at specific subcellular sites is critical for signaling, but the mechanisms underlying channel localization and clustering are largely unknown. Molecules such as ankyrin and syntrophin, that bind ion channels, may be important to maintain a high channel density at the plasma membrane [28]. Gene regulation was studied in total kidney RNA, although active $Ca^{2+}$ reabsorption is restricted to the distal part of the nephron. The observed genomic regulation is, therefore, an averaged mRNA

difference measured in the whole kidney and studies with isolated tubules or cell lines originating from $Ca^{2+}$ transporting segments are necessary to confirm the specific genomic regulation of particular genes.

Apart from normalizing plasma $Ca^{2+}$ levels by $Ca^{2+}$ supplementation in 1α-OHase$^{-/-}$ mice, PTH levels were also normalized. $Ca^{2+}$-induced effects could, therefore, be secondary to a change in the circulating PTH levels. The third comparison of $Ca^{2+}$ supplementation in 1α-OHase$^{+/-}$ mice with normal $1,25(OH)_2D_3$ and PTH levels suggests that these genes are regulated by $Ca^{2+}$, irrespective of the $1,25(OH)_2D_3$ and PTH state.

## CONCLUSION

The present study demonstrated that high dietary $Ca^{2+}$ is an important regulator of $Ca^{2+}$ homeostasis in a vitamin D–deficient status. Using microarray analysis, we have demonstrated that dietary $Ca^{2+}$ normalizes a large part of the renal gene expression changes resulting from the absence of 1α-OHase. We also identified novel genes and biological pathways that are regulated in the $Ca^{2+}$-mediated rescue of 1α-OHase$^{-/-}$ mice. Subsequent studies on these potential targets will provide insight in the molecular rescue mechanisms of dietary $Ca^{2+}$ supplementation.

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