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Altered Gene Expression Profile in the Kidney of Vitamin D Receptor Knockout Mice

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Abstract The kidney is a primary target organ of the vitamin D endocrine system, and both vitamin D-deficiency and vitamin D receptor (VDR) ablation lead to impaired renal functions. As an initial step to understand the molecular basis underlying the renal dysfunctions resulted from VDR inactivation, we used DNA microarray technology to search for changes in the gene expression profile in the kidney of VDR knockout mice. Three independent DNA microarray experiments were performed using Affymetrix GeneChips, which included two replicate comparisons between VDR null and wild-type littermates, and a third comparison between 1,25-dihydroxyvitamin D_3-treated and vehicle-treated wild-type mice. Based on the assumption that VDR inactivation and vitamin D stimulation cause opposite changes in the expression of vitamin D target genes, we identified 95 genes that displayed the same changes in the two VDR-null/wild-type comparisons but an opposite change in the third assay, of which 28 genes were up-regulated and 67 were down-regulated in VDR null mice. These genes can be divided into several functional categories involved in vitamin D and steroid metabolism, calcium metabolism and signaling, volume and electrolyte homeostasis, signal transduction, transcriptional regulation, cell adhesion, metabolism, immune response, and other functions. These data provide a basis for further investigations into the molecular bases underlying the physiological abnormalities associated with VDR- and vitamin D-deficiency. J. Cell. Biochem. 89: 709–719, 2003.

Key words: vitamin D; vitamin D receptor; DNA microarray; kidney

The kidney and the vitamin D endocrine system are very closely related. The kidney is not only a primary vitamin D target organ, but also critically involved in vitamin D metabolism. It is well known that 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], the hormonal form of vitamin D, is almost exclusively synthesized in the kidney, which is also important for $1,25(OH)_2D_3$ catabolism [Holick, 1996]. As such, the two key enzymes in vitamin D metabolism, 25-hydroxyvitamin D 1α-hydroxylase and 24-hydroxylase, are predominantly regulated by $1,25(OH)_2D_3$ itself [Chen et al., 1993; Takeyama et al., 1997]. Other key functions of the kidney, such as calcium and phosphate transport, water and electrolytes excretion, and blood pressure control, are also regulated by $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ regulation of renal calcium reabsorption is an important mechanism for the maintenance of calcium homeostasis in the body [Bushinsky, 1999]. $1,25(OH)_2D_3$ functions as a negative endocrine regulator of the renin–angiotensin system and plays an important role in the regulation of electrolyte, extracellular volume, and blood pressure homeostasis [Li et al., 2002; Li, 2003]. These physiologically important regulations of renal functions by $1,25(OH)_2D_3$ have largely been confirmed by studies of genetically mutant mice lacking the 1α-hydroxylase or the vitamin D receptor (VDR) [Li et al., 1997; Yoshizawa et al., 1997; Dardenne et al., 2001; Panda et al., 2001]. For instance, VDR knockout mice accumulated a high level of $1,25(OH)_2D_3$ in the serum [Yoshizawa et al., 1997] due to the lack of both feedback suppression of 1α-hydroxylase and stimulation of 24-hydroxylase by $1,25(OH)_2D_3$.

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VDR knockout mice had increased urinary calcium excretion and developed hypocalcemia and rickets, in large part due to the drastic reduction of renal calbindin-D9k expression [Li et al., 2001]. The mutant mice also developed hypertension due to the over-stimulation of the renin–angiotensin system [Li et al., 2002].

These are a few examples in which the functional abnormalities of the kidney resulted from VDR ablation have been directly linked to changes in some key vitamin D target genes. However, the global impact of VDR inactivation on renal gene expression remains unclear. Given the key roles that vitamin D plays in the functions of the kidney, we hypothesized that VDR inactivation causes renal dysfunctions through multiple pathways. We have tested this hypothesis by DNA microarray analyses. Here we report the identification of nine distinct functional categories with altered gene expression profiles associated with VDR inactivation. Our data confirm some previous findings, and also reveal novel information regarding the role of the vitamin D endocrine system.

MATERIALS AND METHODS

Animals and Treatment

The use of animals in this study was approved by the Institutional Animal Care and Use Committee of the University of Chicago. Wild-type ($VDR^{+/+}$) and VDR-null ($VDR^{-/-}$) mice were produced through breeding of $VDR^{+/-}$ mice, and identified by PCR with tail genomic DNA as the templates [Li et al., 1997]. Mice were fed the standard rodent chow and housed in a barrier facility with 12 h dark/light cycle. Total RNAs were extracted from freshly dissected kidneys of 3-month-old $VDR^{+/+}$ and $VDR^{-/-}$ mice, using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). For $1,25(OH)_2D_3$ treatment, 3-month-old $VDR^{+/+}$ mice were injected intraperitoneally with two doses of 50 pmole of $1,25(OH)_2D_3$ (dissolved in 5% ethanol–95% propylene glycol) at 7 am and 7 pm. Injection of the vehicle served as controls. Sixteen hours after the last injection, the mice were sacrificed and total kidney RNAs were isolated. In this study, two pairs of kidney RNAs isolated from $VDR^{-/-}$ and $VDR^{+/+}$ littermates and one pair of kidney RNAs isolated from $1,25(OH)_2D_3$-treated and vehicle-treated $VDR^{+/+}$ littermates were used for microarray analyses.

Target Preparation

Target preparation protocol was largely based on the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Inc., Santa Clara, CA) with minor modifications. Briefly, 10 μg of total RNA was used to synthesize double-stranded cDNA using Superscript Choice System (Invitrogen Life Technologies). First strand cDNA synthesis was primed with a T7-(dT_{24}) oligonucleotide. From 3 μg of log phase gel-purified cDNA, biotin-labeled antisense cRNA was synthesized using BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY). After precipitation with 4 M lithium chloride, 20 μg cRNA was fragmented in fragmentation buffer (40 mM Tris-acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc) for 35 min at 94°C and then hybridized to Affymetrix Arrays for 16 h at 60 rpm in a 45°C Affymetrix Hybridization Oven 640. The arrays were washed and stained with streptavidin phycoerythrin in Affymetrix Fluidics Station 400 using the Affymetrix GeneChip protocol, and then scanned using the Affymetrix Agilent GeneArray Scanner.

Data Analysis

Original data were analyzed using Affymetrix Microarray Suite Version 5.0 (MAS 5.0) with the following parameters: Alpha1 = 0.04, Alpha2 = 0.06, Tau = 0.015, and Global scaling target signal = 500. The analysis was carried out in three steps. First, the scatter plot of signal intensity from each compared groups was visually examined to ensure the data quality for comparison. Second, genes with signal intensity below 100 intensity units, which represents noise, were excluded. Finally, genes were identified that were up-regulated (or down-regulated) in the two $VDR^{-/-}$ versus $VDR^{+/+}$ comparisons but down-regulated (or up-regulated) in the $1,25(OH)_2D_3$-treated vs. vehicle-treated $VDR^{+/+}$ comparison in a magnitude of the following specified threshold in at least one of the comparisons: (a) $\geq \pm 3.5$ fold if the highest signal intensity (χ) in the six hybridizations was within the range of $100 \leq \chi < 200$; (b) $\geq \pm 1.9$ fold if $200 \leq \chi < 400$; (c) $\geq \pm 1.6$ fold if $400 \leq \chi < 800$; and (d) $\geq \pm 1.4$ fold if $\chi \geq 800$. These signal-dependent thresholds were empirically established by analyzing 65 replicate chip–chip comparisons (Li, unpublished observations). Genes that passed those filtrations

were considered as being significantly changed between the compared samples.

Cloning of cDNA Probes

cDNA probes were cloned by RT-PCR. Briefly, total kidney RNAs (5 μg) were reverse-transcribed by MMLV reverse transcriptase (Invitrogen Life Technologies) using an oligo-dT primer. The RT product was then subject to PCR amplification using specific primers synthesized according to the published cDNA sequences (Table I). The PCR products were gel purified, cloned into pSK(+) vector, and sequenced to confirm the identity before being used as probes in Northern blot analyses.

Northern Blot

Northern blot analyses were carried out as described previously [Li et al., 2001]. Briefly, total RNA was separated on 1.2% agarose gels and transferred onto nylon membranes. The membranes were hybridized with a specific ^{32}P-labeled cDNA probe. After hybridization, the mRNAs were detected by autoradiography and quantitated with a PhosphorImager (Molecular Dynamic, Sunnyvale, CA). Hybridization with 36B4 cDNA probe served as internal loading control.

RESULTS AND DISCUSSION

Experimental Strategy and Overall Analysis

The main purpose of this study was to identify genes whose expression patterns were altered, directly or indirectly, in association with VDR inactivation. In order to verify independently the microarray results, six hybridizations were performed in this study, which consisted of two independent comparisons between $VDR^{-/-}$ and $VDR^{+/+}$ kidneys and one comparison between $1,25(OH)_2D_3$-treated and vehicle-treated $VDR^{+/+}$ kidneys. Figure 1 shows the scatter plots of the signal intensity in the comparison between $VDR^{-/-}$ and $VDR^{+/+}$ kidneys (Fig. 1A) and between $1,25(OH)_2D_3$-treated and vehicle-treated $VDR^{+/+}$ kidneys (Fig. 1B). Based on the assumption that VDR inactivation and $1,25(OH)_2D_3$ stimulation should have opposite effects on the expression of a vitamin D target gene, genes that were changed consistently in the same direction in the two $VDR^{-/-}$ and $VDR^{+/+}$ comparisons but in the opposite direction in the $1,25(OH)_2D_3$-treated and vehicle-treated $VDR^{+/+}$ comparison were identified by computer analyses (Table II). This strategy allowed a verification of the results from two opposite directions.

Of the 12,422 genes analyzed on the Affymetrix MU74Av2 chips, 9,681 genes passed the first filtration at the cutoff signal intensity of 100, and approximately 1% of these genes were significantly changed under the second filtration condition. Table II lists 95 genes with known functions, of which 28 genes were up-regulated and 67 were down-regulated in $VDR^{-/-}$ mice. Thus, under normal conditions, the majority of vitamin D target genes in the kidney are stimulated by $1,25(OH)_2D_3$. The altered EST sequences were excluded in Table II, as their identities and functions are unknown. Figure 2 shows some representative Northern blot analyses that compared $VDR^{-/-}$ and $VDR^{+/+}$ RNAs. All the Northern blot analyses that we performed were able to confirm the microarray results. However, as commonly seen in microarray studies, the fold change in mRNA expression revealed by Northern blot was not

TABLE I. PCR Primers Used for Cloning cDNA Probes

Gene	Accession number	PCR primers
H^+/K^+-ATPase	U17282	Forward 5′-GCTGAGAGCGACATCATGCACC-3′ Reverse 5′-TCTGGCACATCTCGATGCTGATG-3′
Band-3	X02677	Forward 5′-CCTTTGACGAGGAGAATGGCCTGG-3′ Reverse 5′-GCTCAGGTACGCGCTGTGTGGC-3′
Prostaglandin EP3 receptor	D10204	Forward 5′-AATCACCACGGAGACGGCCATCC-3′ Reverse 5′-CAGACCCAGGGAAACAGGTACTGC-3′
Prostaglandin F receptor	D17433	Forward 5′-TGCTCTCCGCATGGCAACGTGG-3′ Reverse 5′-GGTGTGTCTGACCAGTGTTCTCC-3′
Prostaglandin D synthetase	AB006361	Forward 5′-CCTCGCCTCCAACTCAAGCTGG-3′ Reverse 5′-CAGCTCGTCCTTCAGAGTCTG-3′
ER-81	L10426	Forward 5′-TTGCGAAGAGGAGCAGAATGGATGG-3′ Reverse 5′-ACGACGGGGTGCCTTGCTTGACGG-3′

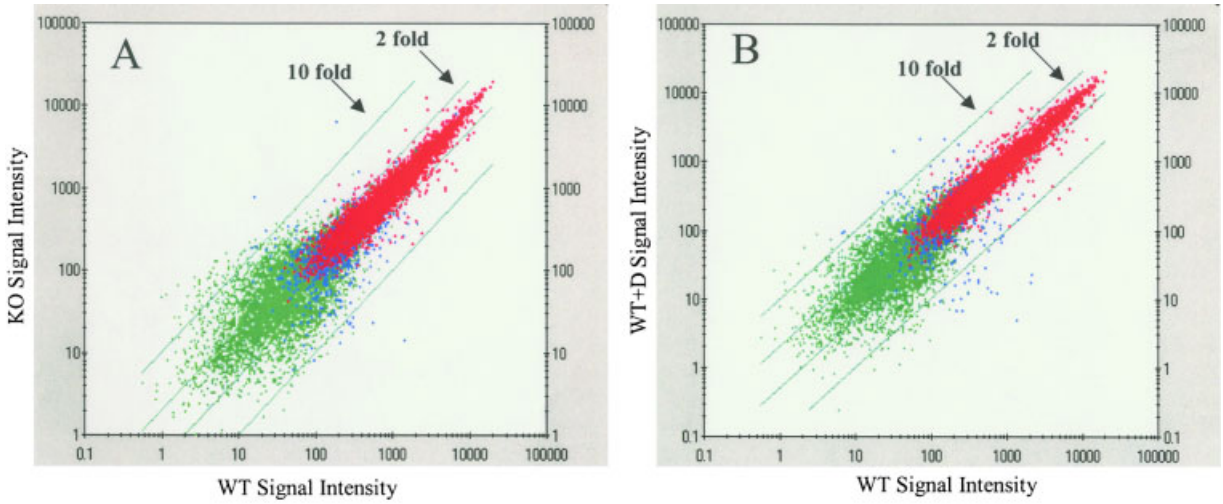

Fig. 1. Representative scatter plots of the microarray data. Red dots: present–present plots; Green dots: absent–absent/marginal plots; Blue dots: present–absent/marginal plots. KO, VDR knockout; WT, wild-type; WT + D, 1,25(OH)$_2$D$_3$-treated WT. **A**: KO versus WT. **B**: WT + D versus WT. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

always exactly the same as what the microarray assay had predicted, but the direction of change in gene expression was always identical in both assays.

Based on their functions, the altered genes were grouped into several categories, which included steroid and vitamin D metabolism, calcium homeostasis, volume and electrolyte homeostasis, cellular signaling, transcriptional factors, metabolism, cell adhesion, immune response, and others (Table II).

Vitamin D Metabolism

As expected, VDR was undetectable in VDR$^{-/-}$ mice. After 1,25(OH)$_2$D$_3$ treatment, VDR expression was increased by 40% in VDR$^{+/+}$ mice (data not shown). Increased VDR expression by 1,25(OH)$_2$D$_3$ has been reported previously [Strom et al., 1989]. Consistent with previous findings [Takeyama et al., 1997], the two key cytochrome P450 enzymes that are involved in vitamin D metabolism, 25-hydroxyvitamin D 1α-hydroxylase and 24-hydroxylase, were changed dramatically in opposite directions in VDR$^{-/-}$ mice, which largely accounted for the dramatic elevation in serum 1,25(OH)$_2$D$_3$ levels seen in VDR$^{-/-}$ mice. The microarray data revealed that vitamin D is also involved in the regulation of other cytochrome P450 genes involved in the metabolism of steroids, retinoids, and fatty acids (e.g., Cyp7b1, P450RA, Cyp4B1, and P450 15α).

Calcium Metabolism and Signaling

The vitamin D endocrine system plays a critical role in calcium homeostasis. Thus it was not surprising that many genes involved in calcium metabolism and signaling were altered in VDR$^{-/-}$ mice. All but one gene in this group were down-regulated. As we showed previously, calbindin-D9k, the cytosolic calcium-binding protein important for transcellular calcium transport, was drastically reduced in VDR$^{-/-}$ mice, which was believed to cause impaired renal calcium reabsorption, leading to the development of sustained hypocalcemia in VDR$^{-/-}$ mice [Li et al., 2001]. Interestingly, the calcium-sensing receptor was also significantly down-regulated in VDR$^{-/-}$ mice and up-regulated in 1,25(OH)$_2$D$_3$-treated VDR$^{+/+}$ mice. The receptor is expressed on the basal membrane along the nephron and plays an important role in the regulation of calcium reabsorption [Brown et al., 1998]. Regulation of calcium-sensing receptor by 1,25(OH)$_2$D$_3$ has been controversial [Rogers et al., 1995; Brown and Hebert, 1996], and our finding here confirmed a recent report that 1,25(OH)$_2$D$_3$ stimulates calcium-sensing receptor gene transcriptionally [Canaff and Hendy, 2002].

VDR inactivation also affected other calcium-binding proteins and calcium channels. For instance, chromogranin B is a high-capacity low-affinity calcium-binding protein coupled

TABLE II. Changes in Gene Expression in the Kidney of Vitamin D Receptor-Null Mice

Gene	Accession number	Fold change KO/WT #1	KO/WT #2	WT + D/WT	Function
Vitamin D and steroid metabolism					
25-hydroxyvitamin D3 1alpha-hydroxylase	AB006034	27.9	34.3	−7.5	Vitamin D metabolism
Cyp7b1	U36993	3.7	1.3	−1.1	Steroid metabolism
P450RA	Y12657	3.5	1.7	−1.3	Retinoic acid metabolism
Vitamin D-24-hydroxylase	D89669	−36.8	−128.0	6.1	Vitamin D metabolism
Retinol binding protein 2	X74154	−11.4	−3.7	1.7	Retinol metabolism
CYP4B1	D50834	−4.6	−2.5	1.1	Procarcinogen, fatty acid metabolism
P450 15alpha	M19319	−1.9	−1.3	1.1	Testosterone 15α-hydroxylation
Calcium metabolism and signaling					
Capacitative calcium entry channel Trp4	AF011543	4.0	4.3	−3.5	Calcium entry
Calcium binding protein D-9k	AF028071	−9.2	−8.0	2.3	Calcium binding
Ryanodine receptor	D38216	−6.5	−2.6	1.1	Intracellular Ca^{2+} release channels
Chromogranin B	X51429	−4.6	−1.7	3.5	Intracellular calcium storage
Parvalbumin	X59382	−3.7	−1.6	1.6	Calcium binding
FKBP51	U16959	−2.0	−2.0	1.1	Calcineurin inhibition
Growth factor regulated calcium channel	AB021665	−1.7	−1.2	1.5	Calcium metabolism
Calcium sensing receptor	AF022252	−1.4	−3.0	2.1	Extracellular calcium sensor
Calpactin I light chain	M16465	−1.2	−1.7	1.6	Calcium effector
Calcium-binding protein Cab45	U45977	−1.1	−1.4	3.5	Calcium binding
Volume and electrolyte homeostasis					
K^+ channel beta-1 subunit	AF033003	2.8	4.0	−3.5	K^+ channel
Gastric H(+)-K(+)-ATPase alpha subunit	U17282	2.5	3.7	−1.6	Cation transportor
Renin (Ren-1-d)	M32352	1.9	3.2	−1.5	Angiotensin production
Band 3 protein	X02677	1.9	1.7	−1.2	Anion exchanger
Prostaglandin E receptor EP3 subtype	D10204	1.7	1.5	−1.4	Prostaglandin E_2 signaling
Prostaglandin F receptor	D17433	1.7	1.4	−1.2	Prostaglandin F_2 signaling
Band 3-related protein	J04036	1.2	1.3	−1.1	Anion exchanger
Cation channel HAC3	AJ225124	1.2	1.9	−1.8	Cation channel
Inward rectifier potassium channel (IRK1)	AF021136	−2	−3.0	1.6	K^+ channel
Natriuretic peptide receptor A	L31932	−1.7	−2.8	1.9	Volume regulation
Organic cationic transporter-like 1	U52842	−1.2	−1.6	1.4	Neutrient transportor
Solute carrier family 19	L23755	−1.2	−1.4	1.3	Sodium/hydrogen exchanger
Signal transduction					
Bone morphogenetic protein 2 (BMP-2)	L25602	7.0	2.6	−2.5	Bone formation
Insulin-like growth factor-1 (IGF-1)	X04480	4.3	2.3	−1.5	Growth factor
GTP binding protein Gz subunit alpha	AF056973	2.6	2.1	−1.1	Cell signaling
Neurotrophin 3	X53257	2.0	4.6	−2.5	Neurotrophic factor
SH3-domain binding protein	AB016835	1.7	1.7	−1.9	Regulation of tyrosine kinase
Sonic hedgehog	X76291	1.4	2	−1.6	Neural development
GNA-14	M80631	−16	−3.0	1.1	Guanine nucleotide-binding protein
IRG-47	M63630	−6.5	−4.9	1.2	Guanine nucleotide-binding protein
CDC7-related kinase	AB019388	−2.8	−3.5	1.7	Cell division
Tyrosine kinase Emb & EMt	L10627	−2.0	−3.0	1.6	Cell signaling
Glutamate receptor beta 2	D10054	−2	−1.7	2.6	Glutamate-gated ion channel
Neuropilin-1	D86949	−1.6	−3.5	1.5	Transmembrane receptor
Cholecystokinin A receptor	D85605	−1.5	−1.4	1.4	Cell signaling
Transcriptional regulation					
Homeobox gene S8	X52875	5.3	4.9	−2.0	Embryogenesis/ development
TNF-response element binding protein	AF010600	4.6	1.7	−1.2	TNF transcriptional regulation
TFIIH subunit p62	AJ002366	3.5	1.7	−2.6	Subunit of TFIIH complex
Transcription factor 17 (Kid-1)	L77247	2.1	3.7	−3.0	Renal tubule transcriptional repressor
Homeobox gene C5	U28071	2.1	2.6	−2.1	Development
Ikaros	L03547	−2.8	−1.9	1.2	T lymphocyte maturation
ER81	L10426	−2.6	−9.2	1.5	ETS family transcriptional factor
E2F3	AF015948	−2.5	−2.8	1.9	Transcriptional activator
HNF-3/forkhead homolog 4	L13204	−1.7	−2.3	1.7	Unknown
Metabolism					
Prostaglandin D synthetase	AB006361	3.7	2.8	1	Prostaglandin D2 synthesis
Protein phosphatase 1 binding protein PTG	U89924	2.6	2.0	−1.9	Glycogen metabolism
Carboxypeptidase A	J05118	2.5	2.1	−2.0	Protein metabolism
Alpha-amylase isozyme	X02578	−25.0	−3.7	1.4	Carbohydrate metabolism

(*Continued*)

TABLE II. (*Continued*)

Gene	Accession number	Fold change KO/WT #1	KO/WT #2	WT + D/WT	Function
Mpgc60 protein	Y11505	−8.0	−2.5	2.3	Trypsin inhibitor
Serine protease inhibitor 2.4	X69832	−4.3	−6.5	1.1	Protein metabolism
Serine protease	M13226	−2.6	−2.1	1.4	Protein metabolism
Threonine synthase	X04603	−2.6	−3.2	1.2	Biosynthesis
Serine protease inhibitor 2	D00725	−2.5	−3.0	1.6	Protein metabolism
Lecithin cholesterol acyltransferase	J05154	−2.1	−2.1	1.4	Lipoprotein metabolism
Testicular condensing enzyme	AF016712	−2.0	−2.3	2.1	Lipid metabolism
Glutathione-S-transferase	L06047	−1.9	−2.8	1.4	Thiols metabolism
Alpha-2,3-sialytransferase	D28941	−1.6	−1.6	1.1	Biosynthesis
Cell adhesion					
Sperm adhesion molecule-1 (Spam-1)	U33958	−4.9	−3.2	1.6	Sperm–egg interactions
Desmocollin 2	L33779	−4.3	−12.1	2.5	Desmosome formation
CNR gene	AB008183	−2.5	−2.3	1.5	Cadherin-related neuronal receptor
Sialoadhesin	Z36293	−2.0	−2.5	7.5	Macrophage lectin-like receptor
Endothelial ligand for L-selectin	M93428	−1.9	−2.6	1.6	Leukocyte/lymphocyte adhesion
Lymph node homing receptor (Ly-22)	M36058	−1.9	−2.3	2.3	Lymphocyte trafficking
Bystin	AF007802	−1.5	−5.7	3.2	Embryo implantation
CD44 antigen	U57611	−1.5	−4.0	1.7	Adhesion/signal transduction
Ninjurin	U91513	−1.3	−1.5	1.4	Homophilic adhesion molecule
Immune response					
Domesticus IgG variable region	Z22111	−16.0	−10.6	5.3	Immune response
BPS3.26 immunoglobulin heavy chain	AF036737	−5.3	−2	5.3	Immune response
Anti-DNA antibody Ig kappa chain	U30629	−4.9	−8.6	2.5	Immune response
Ig active kappa-chain V-region	M13284	−4.0	−2.8	2.6	Immune response
Immunoglobulin kappa light chain	AF044077	−4.0	−2.0	1.7	Immune response
Immunoglobulin light chain V region	AB017349	−3.7	−1.9	1.2	Immune response
Macrophage migration inhibitory factor	L39357	−2.8	−5.7	3.5	Regulation of inflammation
Others					
Transthyretin	D00073	2.6	2.5	1.2	Thyroxine transporter
Modified polytropic viruses	M17327	2.5	1.7	−1.4	Unknown
Keratin 19	M36120	−13.9	−4.0	1.2	Filament formation
Pituitary tumor transforming gene (Pttg)	AF071209	−8.0	−3.7	1.9	Unknown
TECK	AJ249480	−6.5	−5.3	1.3	Thymocyte development
Small proline-rich protein 2A	AJ005559	−4.6	−3.0	1.2	Adaptive barrier function
Collagen VIII alpha-1 gene	X66976	−4.6	−4.9	1.1	Extracellular matrix
Melatonin receptor 1A	U52222	−3.7	−1.7	1.2	Rhythmic regulation
GDF-8	U84005	−3.5	−2.8	2.1	Regulation of muscle growth
Heat shock protein, Hsp25	L07577	−3.5	−1.6	1.1	Stress response
Tektin	AF081947	−2.5	−2.0	2.1	Ciliary protein
Villin	M98454	−1.9	−1.4	1.3	Actin regulation
Resistance-associated macrophage protein 1	L13732	−1.9	−4.0	1.4	Self-defense
Angiogenin	U22516	−1.7	−1.9	1.6	Angiogenesis
NAD(P)H menadione oxidoreductase 1	U12961	−1.3	−1.2	2.1	Menadione detoxification

Genes selected were present in at least one of the three studied groups and met the signal-dependent thresholds in at least one of the three comparisons. KO, VDR knockout; WT, wild-type; WT + D, $1,25(OH)_2D_3$-treated WT.

with IP3 receptor/calcium channel and involved in intracellular calcium storage [Yoo, 2000], parvalbumin is a calcium-binding protein thought to buffer free calcium inside the cells [Pauls et al., 1996], ryanodine receptor is an intracellular membrane channel involved in calcium release [Fill and Copello, 2002], and Trp4, the only gene that was up-regulated in $VDR^{-/-}$ mice in this category, is a calcium entry channel thought to link to calcium oscillation [Torihashi et al., 2002; Wu et al., 2002]. The physiological effects of these changes remain to be determined.

Volume and Electrolyte Homeostasis

Another important physiological function of the kidney is to regulate the homeostasis of extracellular volume, electrolytes, and blood pressure. We have recently shown that $1,25(OH)_2D_3$ functions as a negative endocrine regulator of the renin–angiotensin system, and $VDR^{-/-}$ mice developed hypertension due to the deregulated elevation of renin and angiotensin II production [Li et al., 2002]. Here the microarray analysis confirmed the up-regulation of renin gene expression in $VDR^{-/-}$ kidney.

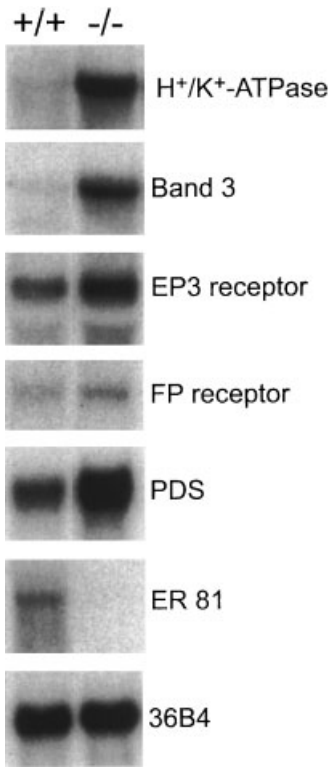

Fig. 2. Representative Northern blots confirming the microarray results. Total RNAs (20 μg/lane) isolated from 3-month-old VDR knockout (–/–) and wild-type littermates (+/+) were hybridized with cDNA probes as indicated. Hybridization with 36B4 probe served as internal loading control.

Interestingly, the natriuretic peptide receptor A (NPR-A) was significantly down-regulated in $VDR^{-/-}$ mice and up-regulated by $1,25(OH)_2D_3$ treatment. NPR-A is the receptor for atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) with guanylyl cyclase activity [Drewett and Garbers, 1994], and mediates the natriuretic, diuretic, and vasorelaxant effects of ANP and BNP, which lowers blood pressure [Levin et al., 1998]. NPR-A ablation led to hypertension and cardiac hypertrophy [Oliver et al., 1997]. These findings suggest that vitamin D may function as a blood pressure-lowering hormone by suppressing the renin–angiotensin system as well as stimulating the natriuretic peptide system.

Another interesting observation was that prostaglandin EP3 receptor, F receptor, and prostaglandin D synthetase (listed in Metabolism in Table II) were increased in $VDR^{-/-}$ kidneys, indicating that vitamin D may play some roles in regulating prostaglandin signaling. There are four receptors (EP1–EP4) for prostaglandin E_2, a major metabolite of arachidonate in the kidney that modulates renal hemodynamics and water and salt excretion [Breyer and Breyer, 2000]. PGE_2 has been shown to stimulate renin production and induce hypertension [Hockel and Cowley, 1979; Jensen et al., 1996]. The EP3 receptor is highly expressed in the kidney and considered to be a constrictor receptor [Breyer and Breyer, 2000]. Thus, increased EP3 receptor is consistent with the fact that $VDR^{-/-}$ mice are hypertensive [Li et al., 2002]. The prostaglandin F receptor is the receptor for $PGF_{2\alpha}$ and highly expressed in the ovary and kidney [Sugimoto et al., 1994]. $PGF_{2\alpha}$ is implicated in such physiological functions as reproduction and calcium metabolism. Finally, prostaglandin D synthetase is the enzyme for PGD_2 biosynthesis, and intriguingly, an increase in this enzyme has been associated with hypertension and renal failure in recent studies [Melegos et al., 1999; Hirawa et al., 2002].

VDR inactivation also resulted in altered expression of several ion channels and transporters involved in sodium and potassium transport. For instance, the beta-1 subunit of votage-gated K^+ channel [Butler et al., 1998] and the gastric H^+-K^+-ATPase α subunit, which was originally isolated from stomach [Mathews et al., 1995], later found in the kidney [DuBose et al., 1995], and thought to be involved in hydrogen and potassium transport in the collecting ducts [Kraut et al., 2001], were dramatically increased in $VDR^{-/-}$ mice. The band 3 and band 3-related protein, which function as the anchor for the erythrocyte cytoskeleton and as a plasma membrane anion antiporter [Kopito and Lodish, 1985], were also up-regulated. The effects of these changes on renal functions are unclear.

Signal Transduction

Another group of genes that were altered by VDR ablation were involved in cellular signal transduction. They include growth factors (e.g., bone morphogenetic protein (BMP)-2, insulin-like growth factor (IGF)-1, neurotrophin 3, sonic hedgehog), membrane receptors (e.g., CNR8, glutamate receptor beta 2, neuropilin-1, and cholecystokinin A receptor), and intracellular signaling molecules (e.g., Gzα, SH3-domain binding protein, GNA-14, IRG-47, and a tyrosine kinase). Vitamin D is known to regulate bone formation via mineral homeostasis [Li et al., 1998]. However, recent studies demonstrated that $1,25(OH)_2D_3$ directly suppressed

bone formation, and the mechanism was unknown [Tanaka et al., 2001; Inoue et al., 2002]. In this regard, it is particularly interesting that BMP-2, IGF-1, and sonic hedgehog were significantly up-regulated in $VDR^{-/-}$ mice and suppressed by $1,25(OH)_2D_3$. BMP-2, a member of TGF-β family, is a major stimulator of bone formation [Wozney et al., 1988], and IGF-1 and sonic hedgehog also play critical roles in skeletogenesis and osteoblast differentiation [Chiang et al., 1996; Zhao et al., 2000; Bikle et al., 2001; Yakar et al., 2002; Yuasa et al., 2002]. Thus, it might be possible that $1,25(OH)_2D_3$ suppresses bone formation by suppressing BMP-2, IGF-1, and sonic hedgehog. Generally speaking, growth factors have multiple functions, and some signaling molecules are shared by multiple pathways. Thus, alteration of this group of proteins may have multiple effects.

Transcriptional Regulation

The microarray data revealed that the expression of a number of transcriptional factors was altered by VDR inactivation. Genes up-regulated in $VDR^{-/-}$ mice included two homeobox genes, S8 and C5, TNF-response element binding protein, and p62 subunit of TFIIH, a multisubunit protein complex that plays an essential role in nucleotide excision repair and transcription of protein-coding genes [Coin and Egly, 1998]. Kid-1, a zinc finger-containing transcriptional repressor highly expressed in kidney and eyes [Witzgall et al., 1993; Brady et al., 1997], was also increased. A recent study suggested that Kid-1 plays an important role during the differentiation of the proximal tubule [Witzgall et al., 1998]. The down-regulated transcriptional factors included Ikaros, ER-81, E2F3, and HNF-3/forkhead homolog 4. E2F3 is a member of E2F transcriptional factors, which interact with the retinoblastoma protein (pRB) and are involved in differentiation, development, and apoptosis [Muller et al., 2001]. ER-81 is a member of ETS transcriptional factors and highly expressed in the brain and the kidney [Monte et al., 1994]. It has been shown recently that ER-81 controls the formation of functional connections between sensory afferents and motor neurons [Arber et al., 2000], but its role in the kidney is unknown. The alterations in the expression pattern of many transcriptional factors in $VDR^{-/-}$ mice suggest that some target genes identified in this study may be indirectly regulated by $1,25(OH)_2D_3$ through these transcriptional factors.

Metabolism

This group includes genes involved in different metabolic pathways. In addition to prostaglandin D_2 synthesis mentioned above, genes involved in protein metabolism, carbohydrate metabolism, glycogen metabolism, and lipid and cholesterol metabolism were altered in $VDR^{-/-}$ mice.

Cell Adhesion

The genes involved in cell adhesion were all down-regulated in $VDR^{-/-}$ mice. Spam-1 is important for sperm–egg interaction and implicated in the sperm dysfunction [Deng et al., 1997]. Spam-1 has been detected in the kidney [Sun et al., 1998], but its physiological function in the kidney is unclear. Desmocollin 2 and CNR gene are two cadherin-related genes involved in the formation of desmosome and neuronal networks, respectively [Lorimer et al., 1994; Kohmura et al., 1998]. Interestingly, vitamin D also seems to be involved in the regulation of interactions of macrophages, leukocytes, and lymphocytes with haematopoietic and lymphoid tissues, since sialoadhesin, a lectin-like receptor expressed in macrophages and involved in cellular interactions of macrophages [Crocker et al., 1991], L-selectin endothelial ligand, a mucin-like glycoprotein expressed in lymph nodes [Lasky et al., 1992], and the lymph node homing receptor Ly-22 [Siegelman et al., 1990] were all down-regulated in $VDR^{-/-}$ mice.

Immune Response

A number of immunoglobulin genes were down-regulated in $VDR^{-/-}$ kidney. Since kidney itself is not involved in immunoglobulin synthesis, it is possible that these changes are due to the difference in the number of lymphocytes present in the $VDR^{+/+}$ and $VDR^{-/-}$ kidneys. The only one protein that may be synthesized by kidney cells is macrophage migration inhibitory factor (MIF), which is expressed ubiquitously in various cells and involved in inflammatory responses to tissue damage [Shimizu et al., 1999].

Other Miscellaneous Genes

These are the genes that were not included in the above categories, and most of them are

down-regulated in $VDR^{-/-}$ mice. The physiological impacts of these changes are unclear.

Summary

As a key vitamin D target organ, the physiological functions of the kidney are heavily regulated by $1,25(OH)_2D_3$. In the present study, we identified an altered gene expression profile in the kidney of $VDR^{-/-}$ mice that covers multiple functional categories, which strongly reinforces the notion that vitamin D is an endocrine hormone of multiple functions. It should be emphasized that, since VDR ablation causes multiple physiological changes such as hypocalcemia and secondary hyperparathyroidism, the alteration in gene expression may be attributed to direct vitamin D regulation, to changes of upstream genes (e.g., transcriptional factors), or to other physiological, cellular, and molecular abnormalities associated with VDR ablation. Therefore, not all of the altered genes should be considered as direct vitamin D target genes. The main purpose of this study is to uncover changes in the gene expression profile in the kidney associated with VDR inactivation. The DNA microarray data confirm some early findings, and also reveal new information regarding the role of the vitamin D endocrine system. For instance, one interesting observation is that vitamin D may regulate electrolyte, volume, and blood pressure homeostasis by targeting the renin–angiotensin system as well as the natriuretic peptide and prostaglandin signaling pathways. Recent studies from many groups have begun to uncover the physiological impacts resulted from the changes in gene expression in $VDR^{-/-}$ mice. Further investigations are needed to fully understand the molecular bases underlying the renal abnormalities as a consequence of VDR ablation, and the microarray data presented in this study may have provided a foundation for such investigations.

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