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Expression Profiling of Vitamin D Treated Primary Human Keratinocytes

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Abstract Vitamin D has attracted much attention by its ability to stop cell proliferation and induce differentiation, which became of particular interest for the treatment of cancer and psoriasis. We performed an expression profile of 12 hours and 24 hours 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) treated primary human keratinocytes, to determine the changes in gene expression induced by the steroid in order to improve our understanding of the biological activity of 1α,25$(OH)_2D_3$. This we expect to be useful for establishing a test system for vitamin D analogs or might open new therapeutic targets or uses for the hormone. For the filter array experiments a non-redundant set of 2135 sequence verified EST clones was used. The normalized raw data of 2 filters per time point were combined and subjected to SAM analysis to further increase the statistical significance. 86 positive and 50 negative genes were identified after 12 h. The numbers went down to 43 positive and 1 negative gene after 24 h of treatment. Fifteen genes are up-regulated over a longer period of time (12 h and 24 h). Results were verified by real-time PCR and/or Northern blots. Targets identified are involved in intracellular signaling, transcription, cell cycle, metabolism, cellular growth, constitution of the extracellular matrix or the cytoskeleton and apoptosis, immune responses, and DNA repair, respectively. Expression profiles showed an initial stop of proliferation and induction of differentiation, and resumed proliferation after prolonged incubation, most likely due to degradation of the hormone. J. Cell. Biochem. 100: 574–592, 2007.

Key words: expression profiling; primary human skin keratinocytes; 1,25$(OH)_2D_3$; vitamin D target genes

Since McCollum demonstrated the existence of a vitamin, which promotes calcium deposition, many more features have been attributed to vitamin D [McCollum et al., 1922]. Besides its traditionally known activities on calcium and phosphorus homeostasis, it has numerous non-calcaemic functions in the body. It acts as a strength-preserving agent in the aging musculoskeletal system [Montero-Odasso and Duque, 2005] and it helps to prevent several degenerative as well as autoimmune diseases. Furthermore it has pronounced effects on cell proliferation and differentiation [DeLuca, 2004; Holick, 2004a,b; Nagpal et al., 2005], as it has been shown to stop proliferation and induce differentiation of many cells including a wide variety of cancer cells or psoriatic cells.

Vitamin D is synthesized in the skin from 7-dehydrocholesterol by irradiation with ultraviolet light and is turned into its actual active form 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), by two successive steps of hydroxylation, one taking place in the liver and the second one in the kidney [reviewed by Jones et al., 1998]. Surprisingly it turned out that not only the conversion of 7-dehydrocholesterol to vitamin D takes place in the skin but the human skin is the only organ capable of performing all the steps to 1α,25$(OH)_2D_3$ [Lehmann et al., 2001, 2003; Schuessler et al., 2001]. Therefore, the human skin and in particular the keratinocytes are both a site of 1α,25$(OH)_2D_3$ synthesis and a target of it.

The physiological activities of 1α,25$(OH)_2D_3$ are mediated by the vitamin D receptor (VDR), a member of the nuclear hormone receptor family of Zn-finger transcription factors. VDR has been detected in most organs including the skin, small intestine, colon, bones, heart, brain,

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gonads, prostate, as well as activated T and B lymphocytes [DeLuca, 2004; Holick, 2004a].

There are however some actions of $1\alpha,25(OH)_2D_3$ that are too rapid to be explained via a transcriptional response through the VDR. The factor responsible for this non-genomic or membrane-associated rapid response is a protein recently termed $1,25D_3$-MARRS (membrane-associated, rapid-response steroid-binding) which has been isolated from chick intestinal epithelial cells [Nemere et al., 2004a,b] and it has been demonstrated that it is responsible for the activation of PKC by $1\alpha,25(OH)_2D_3$ in chondrocytes and osteoblasts from VDR knock-out mice [Boyan et al., 2006].

$1,25D_3$-MARRS, however, is thought not only to be responsible for the rapid response, but to elucidate long-lasting effects too like maintaining differentiated functions of intestinal epithelial cells [Rohe et al., 2005]. This demonstrates that the regulation of vitamin D target genes is a fairly complex process.

Since $1\alpha,25(OH)_2D_3$ shows very promising results in the treatment of certain forms of cancer and psoriasis, it is a major goal in molecular biology to investigate the underlying mechanisms and changes in gene expression which mediate these anti-proliferative effects. Thus, a number of expression profiling experiments have been performed to identify vitamin D target genes in several tumor cell lines such as LNCaP, which is a prostate tumor cell line [Krishnan et al., 2004], a colon carcinoma cell line [Wood et al., 2004], squamous carcinoma cells [Lin et al., 2002], hHL-60 cells, an acute myeloid leukemia cell line [Savli et al., 2002], breast cancer cells [Swami et al., 2003], different osteoblastic cell lines [Eelen et al., 2004], and kidney cells of VDR knock-out mice [Li et al., 2003]. So far no study on primary human keratinocytes has been published, and this is essential for improving our understanding of the biological activity of $1\alpha,25(OH)_2D_3$. In accordance with the incubation times used in the above mentioned studies, we analyzed the change of gene expression induced in primary human keratinocytes after 12 h and 24 h of treatment with $1\alpha,25(OH)_2D_3$, using a filter array containing 2135 sequence verified genes. Although there was a certain degree of overlap between the other arrays and our array, the majority of upregulated or downregulated genes were previously not identified as being regulated by $1\alpha,25(OH)_2D_3$.

The aim of this study is the identification of $1\alpha,25(OH)_2D_3$ target genes in primary human foreskin keratinocytes in order to improve our understanding of the biological activity of $1\alpha,25(OH)_2D_3$. This knowledge could help us to establish a test system for vitamin D analogs, or identify new therapeutic targets or applications for the hormone.

MATERIALS AND METHODS

Cell Culture and Treatments

Keratinocytes that were harvested from human foreskin by separating the dermis and epidermis were cultured in keratinocyte growth medium (modified MCDB 153 Clonetics Bio-Whittaker) supplemented with bovine pituitary extract, insulin (0.1 ng/ml), hydrocortisone, GA-100, 0.12 mM Ca^{2+} and streptomycin/penicillin at 37°C and 5% CO_2. Cells were grown in 75 cm^2 flasks to 70% confluence and treated either with ethanol (control) or 2×10^{-7} M $1\alpha,25(OH)_2D_3$ dissolved in ethanol. $1\alpha,25(OH)_2D_3$ was a kind gift from W. Reischl in the Department of Organic Chemistry in Vienna. RNA was isolated after 6 h, 12 h, and 24 h of treatment, respectively.

RNA Isolation

RNA was isolated using the acidic phenol-guanidinium-thiocyanate-chloroform extraction described in Sambrook and Russell, Molecular Cloning, A laboratory manual, CSH, 2001. The concentration of the isolated RNA was determined by spectrophotometry and the quality was checked on a 1.2% agarose gel containing 2.2 M formaldehyde (2.5% v/v).

Filter Array Production

For the array experiments an EST library containing 2135 sequence verified non-redundant clones, which had been selected from the non-redundant human UniGem V2.0 library (Incyte Genomics, Inc.) and the RZPD Unigene clone collection (http://www.rzpd.de) was amplified by PCR as previously described [Aberger et al., 2001]. PCR products were spotted onto nylon membranes (Hybond N+, Amersham Biosciences) with the MicroGridII (Biorobotics, UK) spotting robot. Each clone was printed in quadruplicate.

Membranes were hybridized with probes synthesized from total RNA obtained from

keratinocytes treated either with $1\alpha,25(OH)_2D_3$ or mock treatment, respectively. Twelve micrograms total RNA was reverse transcribed with SuperScript II (RNase H−) reverse transcriptase (Invitrogen) using 2 μg oligo dT_{23} primer, 70 μCi α^{33}PdCTP (3,000 Ci/mmol, Amersham Biosciences), 10 μl 3× labeling-buffer (for 200 μl: 120 μl 5× SuperScript buffer (Invitrogen), 3 μl dATP/dGTP/dTTP (100 mM each), 60 μl 0.1 M DTT, 0.8 μl 1 mM dCTP, 14.2 μl DEPC-ddH_2O), and 2 μl SuperScript II reverse transcriptase (Invitrogen). The labeled probe was purified using GFX columns (GFX PCR DNA and gel band purification kit, Amersham Pharmacia) according to the manufacturer's protocol. Radiolabeled probes were heated to 95°C for 5 min and arrays were hybridized for 48 h at 65°C in pre-warmed hybridization buffer (5× Denhardt's/5× SSC/1% SDS). After hybridization membranes were washed for 20 min at 65°C once in 2× SSC/0.1% SDS, twice in 0.2× SSC/0.1% SDS, and finally in 0.1× SSC. Filters were exposed for 2 days and scanned with a BAS-1800II (Fuji) phosphoimager. Images were analyzed using the AIDA Metrix suite (Raytest).

Data Analysis

Data were normalized for total signal intensity and statistically analyzed using SAM software (University of Stanford, [Tusher et al., 2001]). For significance analysis of microarrays (SAM) analysis, the fold change parameter was set to 2, such that only those genes that showed at least a twofold induction or repression in response to $1\alpha,25(OH)_2D_3$ were called significant. The median false detection rate (FDR) was 0 for all filters analyzed. Two independent filters were made for each time point, resulting in eight data points/clone. Filters were analyzed separately and in combination (two filters per time point) using SAM analysis. Only genes that are called significant by SAM on both filters were considered differentially expressed and included in Table II. Significant genes were clustered using gene ontologies (GOs see Fig. 1) and Swiss prot functions that are presented on the webpage http://bioinfo.weizmann.ac.il/cards/index.shtml.

Sequencing of cDNA Clones

Selected clones were picked and plasmid DNA was isolated using a GFX plasmid isolation kit (Amersham Biosciences) according to the manufacturer's instructions. cDNA sequences were determined by using an ABI PRISM™ dye terminator cycle sequencing ready reaction kit (Applied Biosystems) containing fluorochrome labeled ddNTPs. The reaction was carried out according to the manufacturer's instructions. Sequences were determined using an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

The obtained sequences were compared with the databases by Blast analysis (www.ncbi.nlm.nih.gov) and compared to the 2135 EST clone list used for filter array production.

Northern Blots

Ten micrograms of total RNA prepared from untreated human foreskin keratinocytes as well as RNA isolated from keratinocytes treated for 6 h, 12 h, and 24 h with $1\alpha,25(OH)_2D_3$ was separated on an 1.2% agarose gel (6.5% formaldehyde), transferred to GeneScreen Nylon membranes (DuPont, New England Nuclear, and now Perkin Elmer), and covalently crosslinked to the membrane by UV light. Probes for positive cDNA clones were prepared using random primed radioactive labeling reactions according to Feinberg and Vogelstein [Feinberg and Vogelstein, 1984]. Probes were purified using Sephadex G-50 columns. The probes were eluted in 1× NETS buffer (150 mM NaCl/10 mM Tris-Cl pH 8.1/1 mM EDTA/0.1% SDS) and 200 μl fractions were collected. The fractions from the first peak of radioactivity were pooled and added to the pre-hybridized membranes. Hybridization was carried out as described in Sambrook and Russell, Molecular Cloning, A Laboratory Manual, CSH, 2001. Membranes were exposed to X-ray films, analyzed with a BAS reader 1800 (Fuji) using BAS Reader 3.11 software and AIDA analyzer 2.11 software to determine the intensities of the probes. For normalization, a probe for the large ribosomal protein P0 (RPLP0) was used. In addition to self-made Northern blots, a commercially available Human 12-Lane MTN Blot from BD Biosciences was used according to the manufacturer's instructions.

Real-Time PCR

Single-stranded cDNA was synthesized with SuperScript II (RNase H−) reverse transcriptase (Invitrogen) according to the manufacturer's instructions. RNA templates were hydrolyzed using 250 mM NaOH. Real-time

PCR analysis was performed on a Rotor Gene 2000 (Corbett Research) using iQTM SYBR Green Supermix (BIO-RAD). Real-time PCR primers are shown in Table I. Cycling conditions were as follows: Cycle 1: 95°C, 360 s, then 47 cycles: 95°C, 60 s; 65°C, 15 s; 72°C, 30 s (acquiring on FAM and Sybr Green), and a final cycle of 72°C for 300 s. Absence of genomic DNA was confirmed by omitting reverse transcriptase during cDNA synthesis. Primer quality was checked by melting curves: reactions were heated from 50°C to 95°C with 12 s holds at each temperature. Large ribosomal protein P0 (RPLP0) was used as a reference for all analyses to control the amount of sample material [Martin et al., 2001]. Fold differences were calculated by a mathematical model described by Pfaffl [2001] using the formula: $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct$ is $\Delta Ct_{(1\alpha,25(OH)_2D_3)} - \Delta_{Ct_{(EtOH)}}$, Δ_{Ct} is $Ct_{(test\ gene)} - Ct_{(control\ gene)}$ and Ct is the cycle at which the threshold is crossed.

RESULTS

Filter Array Analysis

To identify differentially expressed genes in human primary foreskin keratinocytes after 12 h and 24 h of $1\alpha,25(OH)_2D_3$ treatment, we used a cDNA microarray containing 2135 preselected sequence verified human EST clones. Two arrays were hybridized for each time point and condition: mock-treated, 12 h and 24 h of $1\alpha,25(OH)_2D_3$ (2×10^{-7} M) treatment. The combined normalized raw data were subjected to SAM to calculate the statistical significance of upregulated or downregulated genes. The minimum fold change for the list of significant genes was set to 2, which means only those genes,

TABLE I. Primer Sequences for Real-Time PCR

Genbank ID	HUGO gene name		Sequence (5′–3′)	Amplicon (bp)
L13286	*CYP24*	Fwd	CCATCGCGTTTTGCCAGCGATAATA	185
		Rev	AGACAGAACAGGCTCCCAGGCCATT	
BC040643	*SORL1*	Fwd	AACTGGAGTGTGTGCTGCCTTTCAGGT	199
		Rev	ACAACCCATCCTCCCTCAACCCATTT	
M30703	*AREG*	Fwd	CGGTCTCCACTCGCTCTTCCAACAC	199
		Rev	GGTGTCATTGAGGTCCAATCCAGCA	
U02081	*NET1*	Fwd	AAGCCAATGACGTGTTCCACAAGCAG	171
		Rev	ATGCCCTCCTCTGGGCTGTGAGTTT	
Y00272	*cdc2*	Fwd	CAGAGCTTTGGGCACTCCCAATAATGA	187
		Rev	TTTGCCAGAAATTCGTTTGGCTGGA	
D13156	*PI 3 (SKALP)*	Fwd	CTGCCCAGGAATCAAGAAGTGCTGTG	183
		Rev	GCATCCTGAATGGGAGGAAGAATGGA	
AI271688	*CCNG2*	Fwd	ACTGCACTCGGCCTCTTTTCCCTTTTT	188
		Rev	TCCAGGCACCTACCCCTCAATCTCTTC	
D87258	*PRSS 11*	Fwd	ATGAGGACTCTGGGCTGCTGGAATAGG	197
		Rev	CCCAAGCTGGCAAGAAAAAGCACACT	
X65024	*XPC*	Fwd	CAGCTTCCCACCTGTTCCCATTTGA	199
		Rev	AGCTTGGCCTCGTCTCCCCTGAC	
M69199	***G0S2***	Fwd	GGGAGGGAGACGCAGTAGACAGAGACA	186
		Rev	CAAACTCCTTTGGTGGATGCTTGTGGT	
NM_000095	*COMP*	Fwd	GACGTGTGCCAGGACGACTTTGATG	192
		Rev	GTTCATTGTCTGCACGATCTCCCTTCC	
V01512	*FOS*	Fwd	CCAAGCCCTCAGTGGAACCTGTCA	173
		Rev	CAGAGGCTCCCAGTCTGCTGCATAGAA	
AA426586	*BMP6*	Fwd	CTCCTCCCCCAAAAACCCACCAA	183
		Rev	CCCCACTTCCCCGATTTCTGTTCTCT	
R72243	*OAS2*	Fwd	GCCGACAATGCAGACACCAGGAAG	195
		Rev	GGACCATCAAGGGAAGAATGGATGTGA	
N48949	*Nkx 2.2*	Fwd	CCCCTTCTACGACAGCAGCGACAAC	170
		Rev	TACATGGCCCCTTCCCCTTTCACTC	
NM_006945	*SPRR-2B*	Fwd	GCCAAAGTGTCCACCCAAGAGCAAGT	175
		Rev	GATCATCACAGGCAGGCCACAGGTTA	
NM_033307	*CASP4*	Fwd	CGAATATGGAGGCTGGACCACCTGA	197
		Rev	CAGCTCCATTCCTCGGAGGCAGA	
NM_005909	*MAP1B*	Fwd	CCCAAGTCCACCCCAATTAACTGAAGC	193
		Rev	ACACACGCTCACCCACGTGTTCCT	
NM_002467	*MYC*	Fwd	CTACGCAGCGCCTCCCTCCACT	170
		Rev	GGCGCTCCAAGACGTTGTGTGTTC	
NM_001002	*RPLP0 (ARP)*	Fwd	GGCACCATTGAAATCCTGAGTGATGTG	214
		Rev	TTGCGGACACCCTCCAGGAAGC	

Gene names written in bold are not approved by the HUGO gene nomenclature committee.
Cycling conditions were as follows: Cycle 1: 95°C, 360 s, then 47 cycles: 95°C, 60 s; 65°C, 15 s; 72°C, 30 s (acquiring on FAM and Sybr Green), and a final cycle of 72°C for 300 s. Absence of genomic DNA was assured by omitting reverse transcriptase during cDNA synthesis. Primer quality was checked by melting curves: reactions were heated from 50°C to 95°C with 12 s holds at each temperature.

whose expression changed by at least a factor 2 in response to $1\alpha,25(OH)_2D_3$ treatment on two independent filter sets for each time point (i.e., each clone was checked eight times for upregulation or downregulation) are listed in the Tables II and III. SAM assigns a score (SAM score d) to each gene on the basis of the change in expression relative to the standard deviation of repeated measurements [Tusher et al., 2001]. By assimilating a set of gene-specific *t*-tests, statistically significant changes in expression can be identified. Genes with scores greater than the threshold (delta, Δ) are considered to be significant. For combined analysis (two independent filters) of the 12 h time point, the delta value was 1.63 and for 24 h 2.16. The percentage of genes identified by chance was also calculated and called the false discovery rate (FDR). The false discovery rate (FDR) was 0% for each of the arrays. The *q*-value is the lowest FDR at which the gene is called significant. It is like the familiar *P*-value but adapted to the analysis of a large number of genes [Tusher et al., 2001]. The *q*-value for 12 h was 0.35 and for 24 h 0.63.

SAM revealed 86 significant positive genes (see Table II) and 50 significant negative genes (see Table III) after 12 h of $1\alpha,25(OH)_2D_3$ treatment. After 24 h of treatment fewer genes were differentially expressed, namely 43 significant positive genes (see Table II) and 1 significant negative gene (see Table III). Previous array studies, which used different methods and tissues showed significant variance ranging from as little as 0.09% [Krishnan et al., 2004] to as much as 11.1% [Farach-Carson and Xu, 2002] of the investigated genes. In primary human foreskin keratinocytes, approximately 7% (165 genes) of the 2135 EST clones investigated were differentially expressed in response to $1\alpha,25(OH)_2D_3$. As already mentioned previous expression profiles involving $1\alpha,25(OH)_2D_3$ (or analog) treatments were performed on either tumor cell lines or cells from VDR knock-out mice. Tumor cells might be expected to show reduced response to $1\alpha,25(OH)_2D_3$. Just recently, it has been shown that some tumor cell lines have elevated levels of nuclear corepressors, like SMRT, and thus, often display reduced sensitivity to $1\alpha,25(OH)_2D_3$ [Khanim et al., 2004]. This appears to be the case with the expression profiles of $1\alpha,25(OH)_2D_3$-treated LNCaP (0.09% target genes) [Krishnan et al., 2004] or colon carcinoma cells (0.1% target genes) [Wood et al., 2004]. Certainly one can expect less vitamin D target genes to be differentially expressed for VDR knock-outs, due to the missing nuclear receptor.

For almost all published expression profiles involving $1\alpha,25(OH)_2D_3$ treatment, different arrays were used. There is a very large heterogeneity ranging from oligonucleotide arrays to ESTs, as well as from glass slides to filter arrays, representing different genes. The majority of these studies used fluorescently labeled probes (Cy3 and Cy5). However, we decided to use radioactively labeled probes and ESTs due to better sensitivity. The 2135 EST clones used for this experiment were selected from two different libraries (the human UniGem V2.0 library and the RZPD Unigene clone collection) and specifically enriched in ESTs representing transcription factors, molecules involved in signal transduction, and genes involved in several diseases. With this bias, the set is thus most likely to contain more $1\alpha,25(OH)_2D_3$ targets than other arrays used in previous studies, explaining why the number of genes influenced in total is about 7%. In addition to that, this is the first study performed on primary human keratinocytes, which might be influenced by the hormone to a greater extent than tumor cell lines or other cell lines investigated previously, due to their known role in vitamin D response.

Reduced Number of Target Genes After 24 h Treatment

The fact that there are less genes upregulated after 24 h of treatment can be explained by the degradation of $1\alpha,25(OH)_2D_3$. As expected, *25(OH)D_3-24-hydroxylase (CYP24)*, the degradation enzyme of $1\alpha,25(OH)_2D_3$, was among the upregulated genes of the filter array.

In fact, real-time PCR and Northern blot analysis for *CYP24* demonstrated a massive induction of *CYP24* mRNA (more than 6,000-fold) after 24 h of $1\alpha,25(OH)_2D_3$ treatment (see Fig. 4). Even after only 6 h of $1\alpha,25(OH)_2D_3$ treatment we could already see a 940-fold induction of *CYP24* mRNA in human foreskin keratinocytes. Since we only used a single treatment, the concentration of $1\alpha,25(OH)_2D_3$ must have decreased dramatically after 24 h of incubation. The decreased amount of hormone might also be insufficient to induce nuclear receptor-independent changes in gene

TABLE II. List of Upregulated Genes

HUGO	Gene name	Gene ID	Score (d)	Fold change 12 h	Score (d)	Fold change 24 h
Metabolism/biosynthesis/catabolism						
CYP24A1	CYP24A1	U60699	20,803	4.60	20,169	6.91
ARSE	Arylsulfatase E (chondrodysplasia punctata 1)	X83573	2,352	3.53		
NELL1	nel (chicken)-like 1	D83017	2,817	3.40		
PPIF	Peptidylprolyl isomerase F (cyclophilin F)	M80254	2,516	2.80		
LPL	Lipoprotein lipase	X14390	6,632	2.72		
RPS6K1	Ribosomal protein S6 kinase, 70 kD, polypeptide 1	AA426590	2,540	2.61		
ENTPD2	CD39-like 1	U91510	2,254	2.50		
ATP9A	ATPase type IV, phospholipid-transporting (P-type),(putative)	AA620508	3,226	2.44		
GUK1	Guanylate kinase 1	T36282	2,678	2.20		
SQLE	Squalene epoxidase	AF098865	8,554	2.20		
FDFT1	Farnesyl-diphosphate farnesyltransferase 1	X69141	21,254	2.18		
CYP2C8	Cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase), polypeptide 8	M17398	2,806	2.07		
AUTS2	Homo sapiens KIAA0442 mRNA, partial cds	AB007902	6,930	2.00	29,722	3.12
RNF8	Ring finger protein (C3HC4 type) 8	AB014546			22,174	3.37
TM4SF5	Transmembrane 4 superfamily member 5	AF027204			17,354	3.01
SORL1	SorL1	BC040643			14,599	2.54
Signaling						
WSB2	Homo sapiens clone 23714 mRNA sequence	AA759282	2,780	3.68		
KRAS2	KRAS2 (V-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog)	AI050915	2,228	3.16		
PPP2R5D	Protein phosphatase 2, regulatory subunit B (B56), delta isoform	AB000634	2,704	3.05		
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog	AA433922	2,780	2.84		
RAPGEF2	KIAA0313 gene product	AL041825	2,293	2.76		
HIPK1	KIAA0630 protein	AI761800	2,708	2.67		
PIP5K2B	Phosphatidylinositol-4-phosphate 5-kinase, type II, beta	U85245	2,559	2.45		
NET1	Guanine nucleotide regulatory protein (oncogene)	U02081	40,263	2.35	12,034	2.62
PRKACA	Protein kinase, cAMP-dependent, catalytic, alpha	X07767	12,316	2.29	19,462	3.17
GNAI3	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	J03198	6,072	2.19	9,951	2.83
ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	L20688	5,404	2.13	17,424	2.39
TBC1D15	ESTs	AI082025	2,450	2.14		
AKAP8	A kinase (PRKA) anchor protein 8	Y11997	3,887	2.13		
IL22RA1	ESTs	AI660842	3,008	2.12		
ARHGAP4	Rho GTPase activating protein 4	U52112	3,066	2.09		
PITX2	Pitx 2 (Paired-like homeodomain transcription factor 2)	H13139	2,153	2.05		
ACVR2	Activin A receptor, type II	X62381	2,251	2.04		
PTPN12	Protein tyrosine phosphatase, non-receptor type 12	D13380			16,317	2.45
MAP3K7IP2	KIAA0733 protein	AI379462			9,877	2.11
PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide	U86453			10,449	2.00
Transcription factor/transcription						
RXRA	Retinoid X receptor alpha	AA865269	2,806	3.50		
CD8A	CD8 antigen, alpha polypeptide (p32)	M12824	2,312	3.35		
LOC340371	Tu translation elongation factor, mitochondrial	AI654590	2,472	3.22		
FOSL2	FOS-like antigen 2	AI088306	3,149	3.04		
LEF1	Lef 1 (Lymphoid enhancer-binding factor)	AI141511	2,358	3.04		
RARG	Retinoic acid receptor, gamma	M38258	2,678	3.01		
TIEG2	TGFβ inducible early growth response 2	AF028008	2,774	2.95		
M96	Putative DNA binding protein	AI582249	2,315	2.88		
SCARB1	CD36L1(CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 1)	AI091647	2,604	2.79		

(*Continued*)

TABLE II. (*Continued*)

HUGO	Gene name	Gene ID	Score (d)	Fold change 12 h	Score (d)	Fold change 24 h
NKX2-2	Nkx 2,2 (NK-2 (Drosophila) homolog B)	N48949	8,219	2.78	111,368	6.24
M96	Putative DNA binding protein	AF073293	2,495	2.56		
CREB3L4	Jumping translocation breakpoint	AI928081	2,743	2.41		
PMS1	Postmeiotic segregation increased (S, cerevisiae) 1	AI076038	3,123	2.38		
KIAA1404	ESTs	AA148582	2,154	2.34		
FBXL10	ESTs, weakly similar to KIAA1004 protein [*H. sapiens*]	AI088992	2,897	2.03		
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	V01512			24,168	2.84
DLX3	Dlx 3 (Distal-less homeo box 3)	AI080000			37,521	2.80
FOS	FOS(V-fos FBJ murine osteosarcoma viral oncogene homolog)	AI810067			18,121	2.39
BAPX1	Nkx 3,2 (Bagpipe homeobox (Drosophila) homolog 1)	AW073413			13,532	2.34
MYCN	v-myc avian myelocytomatosis viral related oncogene, neuroblastoma derived	M13241			3,528	2.34
DBP	D site of albumin promoter (albumin D-box) binding protein	D28468			13,104	2.10
Growth factors/receptors/cell growth						
BMP2	BMP2	AA436230	2,370	3.00		
EGFR	EGFR (epidermal growth factor receptor (avian erythroblastic leukemia viral (v-erb-b) oncogene homolog))	H80438	2,896	2.93		
EGFR	Epidermal growth factor receptor (avian erythroblastic leukemia viral (v-erb-b) oncogene homolog)	X00588	15,658	2.32	37,121	4.35
PRSS11	Protease, serine, 11 (IGF binding)	D87258	6,188	2.23	33,837	2.67
FGF7	Fibroblast growth factor 7 (keratinocyte growth factor)	AI075338	2,599	2.15		
AREG	Amphiregulin (schwannoma-derived growth factor)	M30703			30,867	3.66
TGFBR2	TGFBR2 (transforming growth factor, beta receptor II (70–80 kD)	AI206758			10,819	2.14
BMP6	Bone morphogenetic protein 6	AA426586			18,658	2.14
TGFα	Transforming growth factor, alpha	X70340			16,602	2.07
Cell cycle						
G0S2	Human G0S2 protein gene, complete cds	M69199	23,198	3.44	42,311	9.03
NOL1	Nucleolar protein 1 (120 kD)	X55504	2,209	3.39		
CCNG2	Cyclin G2	AI271688	19,691	3.31	8,839	3.64
APC	APC (adenomatosis polyposis coli)	T64816	2,673	2.80		
FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog	AI718238	2,325	2.80		
cdc2	Cell division cycle 2, G1 to S and G2 to M	Y00272	15,387	2.73	11,843	4.12
CCNG1	Cyclin G1	U53328	2,589	2.39		
TERF2	Telomeric repeat binding factor 2	AF002999	2,522	2.09		
IFRD1	Interferon-related developmental regulator 1	Y10313	2,031	2.08		
MAD2L1	MAD2 (mitotic arrest deficient, yeast, homolog)-like 1	AJ000186	10,288	2.04		
GADD45A	Growth arrest and DNA-damage-inducible, alpha	AI634658			8,159	2.61
CKS1B	cdc28 protein kinase 1	AI033892			23,597	2.08
ECM/cytoskeleton						
PI3	Protease inhibitor 3, skin-derived (SKALP)	D13156	30,589	2.95	9,533	3.11
COMP	COMP	NM_000095	5,410	2.29	40,673	4.03
LAMB3	Laminin, beta 3 (nicein (125 kD), kalinin (140 kD), BM600 (125 kD))	U17760			49,433	3.06
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	X07979			8,757	2.98
ITGA6	Integrin, alpha 6	X53586			16,670	2.82
LAMA3	Laminin, alpha 3 (nicein (150 kD), kalinin (165 kD), BM600 (150 kD), epilegrin)	X84900			38,368	2.60
TNIK	KIAA0551 protein	AB011123			30,113	2.47

Immune response						
IL1RL1	Interleukin 1 receptor-like 1	AB012701	5,555	3.25		
IL1A	Interleukin 1, alpha	M28983	2,410	3.13		
PDCD1	Programmed cell death 1	U64863	2,439	3.07		
OAS2	2-5A synthetase (2′-5′-oligoadenylate synthetase 2 (69–71 kD)	R72243	14,786	2.95	24,961	6.51
PAP	HIP (Huntingtin interacting protein 1) (pancreatitis-associated protein)	AI478673	2,271	2.36		
LY96	Homo sapiens MD-2 mRNA, complete cds	AB018549	2,995	2.03		
DNA repair						
PCNA	PCNA (proliferating cell nuclear antigen)	H62620	7,307	2.33		
FEN1	Flap structure-specific endonuclease 1	X76771	18,742	2.08		
XPC	Xeroderma pigmentosum, complementation group C	X65024			15,386	2.36
DDB2	Damage-specific DNA binding protein 2 (48 kD)	U18300			14,610	2.13
Apoptosis						
BNIP2	BCL2/adenovirus E1B 19 kD interacting protein 2	AI831130	2,980	2.23		
BIRC4	Apoptosis inhibitor 3	U45880	3,714	2.12		
CASP4	Caspase 4, apoptosis-related cysteine protease	U25804			22,505	2.08
Unknown						
BIRC3	ESTs	AI581499	2,751	3.31		
Unknown 1	ESTs	AI051950	2,922	2.86		
RAB3IL1	ESTs	AA971785	2,281	2.81		
GPP34	ESTs	AI094117	2,326	2.79		
AKIP	ESTs, weakly similar to alpha 1 [*H. sapiens*]	AI031800	2,158	2.67		
C16orf34	ESTs, Weakly similar to !!!! ALU SUBFAMILY SP WARNING ENTRY !!!! [*H. sapiens*]	AI962918	2,964	2.60		
RWDD1	ESTs, moderately similar to CGI-24 protein [*H. sapiens*]	AI080762	2,768	2.49		
SH2BP1	KIAA0155 gene product	D63875	2,146	2.36		
LOC441104	Homo sapiens mRNA from chromosome 5q21-22, clone:843Ex	AB002449	2,925	2.21		
DDX28	Homo sapiens chromosome 16 BAC clone CIT987SK-A-67A1	AI684870	3,278	2.21		
TNFRSF12A	ESTs, highly similar to HYPOTHETICAL PROTEIN KIAA0195 [*H. sapiens*]	AI827127	2,947	2.10		
Unknown 2	ESTs, weakly similar to cDNA EST EMBL:T00822 comes from this gene [C,elegans]	N72675	3,648	2.08		
Unknown 3	Homo sapiens clone DT1P1B6 mRNA, CAG repeat region	AI912004	3,019	2.07		
NOS2A	Nitric oxide synthase 2A (inducible, hepatocytes)	X73029	2,157	2.05		
Unknown	CAG repeat domain	AI536600			15,906	3.01
FJ11196	ESTs, weakly similar to LA PROTEIN HOMOLOG [D,melanogaster]	AI190690			15,316	2.03

Genes whose expression was increased by 1α,25-dihydroxyvitamin D_3 after 12 h or 24 h of treatment in human foreskin keratinocytes are clustered according to their gene ontologies. Genes are listed according to their fold of induction after 12 h of $1\alpha,25(OH)_2D_3$ treatment. They are clustered into 10 categories: growth factors/receptors/cell growth, transcription factor/transcription, cell cycle, metabolism/biosynthesis/catabolism, extracellular matrix/cytoskeleton, immune response, intracellular signaling, apoptosis, repair, and unknown function. After 12 h of treatment 86 genes were significantly upregulated, whereas after 24 h of treatment only 43 significant positive genes were identified by SAM analysis of two independent arrays (a total of eight measurements per clone). Fifteen clones showed prolonged upregulation, which means their mRNA was upregulated at both the 12 h and the 24 h time points of $1\alpha,25(OH)_2D_3$ treatment in human keratinocytes. Gene names written in bold are not approved by the HUGO gene nomenclature committee.

TABLE III. List of Downregulated Genes

HUGO	Gene name	Gene ID	Score (d)	Fold change 12 h	Score (d)	Fold Change 24 h
Metabolism/biosynthesis/catabolism						
MGC10204	ESTs, weakly similar to spliceosome associated protein 62 [M,musculus]	AI674922	−3,975	0.15		
RPS29	Ribosomal protein S29	AA715449	−16,170	0.23		
ATP5l	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit e	D50371	−24,490	0.29		
EIF4E	Eukaryotic translation initiation factor 4E	AI278425	−3,798	0.32		
RPL17	Ribosomal protein L17	X53777	−9,982	0.35		
LAPTM4A	Golgi4-transmem,Transp,MTP (Lysosomal-associated protein transmembrane 4 alpha)	R12846	−9,950	0.39		
TIMM14A	Pre-protein translocase	X97544	−9,060	0.39		
RPL35	Ribosomal protein L35	AI815757	−4,307	0.42		
ATP5O	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (oligomycin sensitivity conferring protein)	AA890495	−11,926	0.42		
RPL35	Ribosomal protein L35	AA305945	−4,840	0.40		
TBP	Proteasome (prosome, macropain) subunit, beta type, 1	AL031259	−3,686	0.44		
ATP6V1F	ATPase, vacuolar, 14 kD	D49400	−5,308	0.48		
STX8	Homo sapiens syntaxin 8 mRNA, complete cds	AF036715	−15,122	0.49		
NEDD8	Neural precursor cell expressed, developmentally downregulated 8	AI141545	−6,004	0.50		
Signaling						
MPZL1	ESTs	W63710	−2,965	0.10		
PLCG1	Phospholipase C, gamma 1 (formerly subtype 148)	*N22311*	−3,031	0.21		
BRD2	Female sterile homeotic-related gene 1 (mouse homolog)	*AA576562*	−3,785	0.24		
PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	U96180	−5,497	0.27		
TRIP10	Thyroid receptor interacting protein 10 (cdc42-interacting protein)	AL040320	−2,979	0.37		
PPP2CB	Protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform	AI954176	−3,842	0.46		
AREG	Amphiregulin (schwannoma-derived growth factor)	M30703	−3,301	0.47		
PPP1CB	Protein phosphatase 1, catalytic subunit, beta isoform	X80910	−15,039	0.49		
Transcription factors/transcription						
ZBTB26	ESTs	AI582359	−38,956	0.26		
PSMC3	Proteasome (prosome, macropain) 26S subunit, ATPase, 3	AA604188	−3,565	0.35		
TCEA1	Incyte EST	AI880413	−18,137	0.39		
MYC	Myc (v-myc avian myelocytomatosis viral oncogene homolog)	*AI093842*	−5,664	0.42		
MYCP	v-myc avian myelocytomatosis viral oncogene homolog	X00198	−6,699	0.43		
SCARB2	CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 2 (lysosomal integral membrane protein II)	D12676	−14,355	0.47		
NME2	Non-metastatic cells 2, protein (NM23B) expressed in TATA box binding protein (TBP)-associated factor, RNA	L16785	−9,854	0.48		
TAF9	Polymerase II, G, 32 kD	U21858	−5,847	0.48		
Cell cycle						
BUB3	BUB3 (budding uninhibited by benzimidazoles 3, yeast) homolog	AF047472	−3,987	0.03		
CCNH	Cyclin H	AA451817	−3,415	0.13		
CCNA1	Cyclin A1	U66838	−4,315	0.37		
MLH1	mutL (*E. coli*) homolog 1 (colon cancer, non-polyposis type 2)	U40978	−3,601	0.41		
ECM/cytoskeleton						
MAP1B	Microtubule-associated protein 1B	Y09836	−3,182	0.03		
SMPDL3B	*H. sapiens* mRNA for ASM-like phosphodiesterase 3b	AW015709	−2,969	0.20		
TIMP3	Tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	D45917	−3,131	0.24		
GAS2	Growth arrest-specific 2	U95032	−10,380	0.32		
PCDHGC3	Protocadherin 2 (cadherin-like 2)	AB011160	−8,891	0.45		
LGALS3BP	Lectin, galactoside-binding, soluble, 3 binding protein (galectin 6 binding protein)	L13210	−3,066	0.50		
SPRR-2B	Small proline-rich protein 2B	M21302			−20,424	0.32

Immune response				
HLA-G	HLA-G histocompatibility antigen, class I, G	AI123699	−3,716	0.14
HLA-E	MHC Class I HLA-E (major histocompatibility complex, class I, E)	H73887	−7,538	0.49
Apoptosis				
CASP4	Caspase 4, apoptosis-related cysteine protease	U25804	−7,133	0.38
BAG1	BCL2-associated athanogene	AF022224	−3,324	0.42
SERPINB2	Plasminogen activator inhibitor, type II (arginine-serpin)	M31551	−12,708	0.42
SMN1	Survival of motor neuron 1, telomeric	AI656334	−7,653	0.45
IER3	Immediate early response 3	AI185199	−7,121	0.47
SNN	152067 unk	H50906	−5,030	0.48
Unknown				
COPS5	JUN(=COP9 (constitutive photomorphogenic, Arabidopsis, homolog) subunit 5)	AI088509	−3,439	0.24
CROP	Acid-inducible phosphoprotein	AA411969	−3,031	0.35

Genes whose expression was decreased by 1α,25-dihydroxyvitamin D_3 after 12 h and 24 h of treatment in human foreskin keratinocytes are clustered according to their gene ontologies. Genes are listed according to their fold of repression after 12 h of 1α,25$(OH)_2D_3$ treatment. They are clustered into 10 categories: growth factors/receptors/cell growth, transcription factor/transcription, cell cycle, metabolism/biosynthesis/catabolism, extracellular matrix/cytoskeleton, immune response, intracellular signaling, apoptosis, repair, and unknown function. After 12 h of treatment 50 genes were significantly repressed, whereas after 24 h only a single gene downregulated. SAM analysis was performed for two independent filter arrays (a total of eight measurements per clone). Gene names written in bold are not approved by the HUGO gene nomenclature committee.

expression which might be contributing to the upregulation of some target genes after 12 h of 1α,25$(OH)_2D_3$ treatment. It is most likely that 1α,25$(OH)_2D_3$ degradation is responsible for the smaller amount of target genes detected after 24 h of incubation.

Correlation of Fold Induction Determined by Different Methods

In order to assess the reliability of the cDNA microarray, several clones were sequenced and the induction or repression was validated by real-time PCR (see Fig. 2A,B) and Northern blot analysis (see Fig. 3). Experiments were repeated three times at least and the values shown in Figures 2 and 3 as well as in Table IV are the means of at least four independent experiments. In general, the correlation between the filter experiments, real-time PCR, and Northern blot analysis was very high with only one major exception—*CYP24* (see Table IV). *CYP24* was among the highest upregulated mRNAs on the filter array, although the fold induction determined with this method was only 6.9-fold (see Table II). As already mentioned, real-time PCR and Northern blots demonstrated a far higher induction (up to 6,000-fold). This discrepancy is due to the fact that only a very limited amount of cDNA can be spotted on the filter, and therefore only a limited amount of probe can hybridize. Nevertheless, filter array analysis allows the efficient detection of upregulated or downregulated genes. Fold inductions are more accurate when determined by real-time PCR or Northern blot analysis, especially when it comes to massive inductions. A general trend for higher inductions seen with the real-time PCR compared to filters has been described by several authors [Savli et al., 2002; Krishnan et al., 2004; Wood et al., 2004].

To get an insight into the function of the differentially expressed genes, they were clustered according to their GOs and/or their functions as annotated at Swiss prot. They were divided into 10 categories: metabolism/biosynthesis/catabolism, signaling, transcription factor/transcription, growth factors/receptors/cell growth, cell cycle, extracellular matrix/cytoskeleton, immune response, DNA repair, apoptosis, and unknown function. Many genes are involved in multiple cellular processes but were placed under the category that applied most for them in the list of upregulated or

TABLE IV. Comparison of Filter Array Results and Real-Time PCR

Function	Name[a]	GenBank accession number	Expression level (fold change) cDNA array[b] 12 h	cDNA array[b] 24 h	Real-timePCR[c] 12 h	Real-timePCR[c] 24 h
Metabolism/catabolism	*CYP24*	U60699	4.6	6.9	2,641	6,317
	SORL1	BC040643	—	2.54	1.6	3.7
Signaling	*NET1*	U02081	2.4	2.6	3.0	2.5
Transcription/TFs	*Nkx2.2*	N48949	2.8	6.2	4.1	8.2
	FOS	V01512	—	2.8	2.7	4.1
	MYC	NM_002467	0.4	—	0.8	0.7
Cell growth/GFs	*AREG*	M30703	0.5	3.7	0.9	2.8
	PRSS11	D87258	2.2	2.7	2.1	2.7
	BMP6	AA426586	—	2.1	3.6	16.3
Cell cycle	***GOS2***	M69199	3.4	9.0	3.1	6.2
	CCNG2	AI271688	3.3	3.6	4.3	3.1
	cdc2	Y00272	2.7	4.1	4.6	7.4
ECM/cytoskeleton	*PI3*	D13156	2.9	3.1	2.3	2.7
	COMP	NM_000095	2.3	4.0	4.7	13.2
	MAP1B	Y09836	0.03	—	0.6	2.8
	SPRR-2B	M21302	—	0.3	0.4	0.2
Immune response	*OAS2*	R72243	2.9	6.5	1.6	5.4
DNA repair	*XPC*	X65024	—	2.4	1.5	6.9
Apoptosis	*CASP4*	U25804	0.4	2.1	1.5	2.7

Two arrays were hybridized for each time point and condition: mock-treated, 12 h and 24 h of $1\alpha,25(OH)_2D_3$ (2×10^{-7} M) treatment. The combined normalized raw data were subjected to significance analysis of microarrays (SAM) to calculate the statistical significance of upregulated or downregulated genes. The minimum fold change for the list of significant genes was set to 2. SAM analysis was performed for two independent filter arrays (a total of eight measurements per clone).
Fold changes shown calculated for real-time PCR experiments are the mean values of at least four independent experiments. *Large ribosomal protein P0 (RPLP0)* was used as a reference for all analyses to control the amount of sample material. Absence of genomic DNA was confirmed by omitting reverse transcriptase during cDNA synthesis.
[a]Gene names according to the Hugo gene nomenclation committee, gene names written in bold are not approved by the Hugo gene nomenclation committee.
[b]Values are the means of two independent array experiments.
[c]Values are the means of three independent real-time PCR experiments.

downregulated genes. The percentages shown in Figure 1A–C were calculated according to the number of genes, which fell into each cluster. Genes with multiple functions were calculated into the total for each cluster they belonged in, which means one gene can account for the percentage in more than one cluster (see Fig. 1A–C).

Fifteen genes are upregulated over a longer period of time than the rest, meaning that their expression is increased more than twofold after 24 h as well as after 12 h of $1\alpha,25(OH)_2D_3$ treatment (see Table II). The majority of these genes were involved in intracellular signaling processes (*NET1, protein kinase alpha, GNAI3, Rho GDP dissociation inhibitor) or cell cycle (cell division cycle 2, cyclin G2, and G0S2*). Prolonged effects could also be seen on extracellular matrix constitution and cell adhesion (*PI3*, *COMP*), and metabolism (*CYP24A1 and AUTS2*) (see Table II).

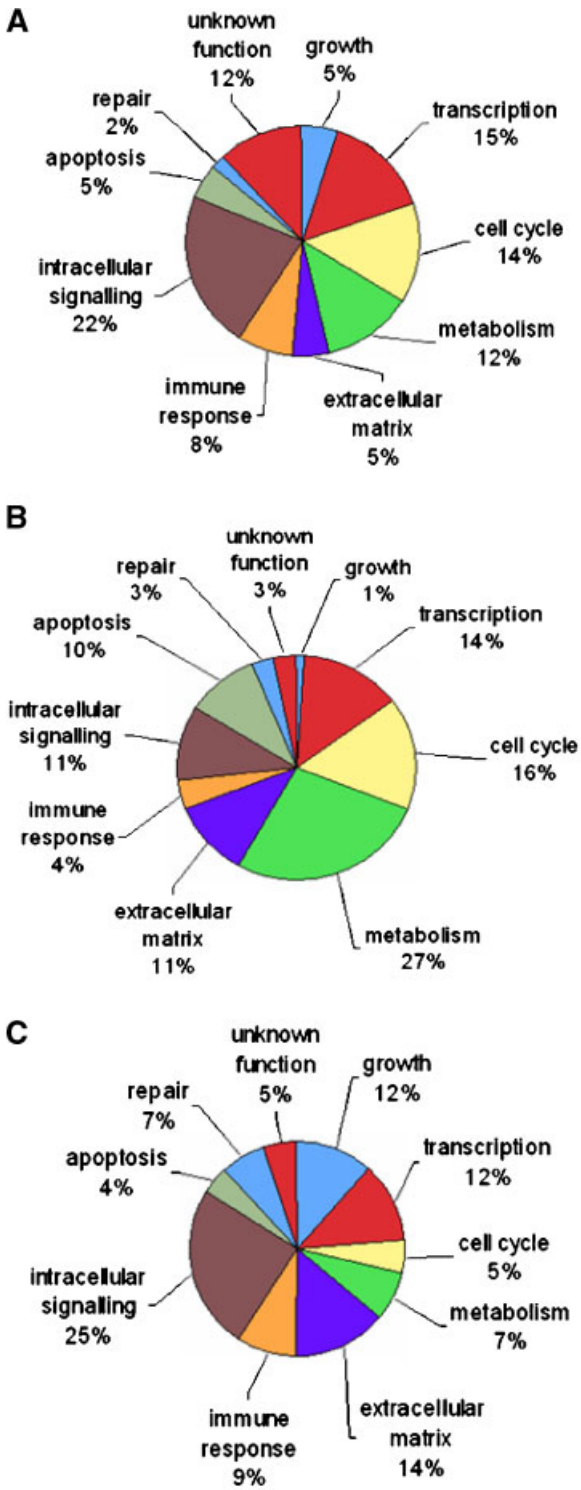
Since rather little is known about G0S2, a multiple tissue Northern, containing RNA from 12 different human tissues, was performed to get more information on the expression pattern of *G0S2*. The highest expression of *G0S2* mRNA was found in peripheral blood leucocytes, where it was first identified [Cristillo et al., 1997]. Expression was also high in heart and liver. Kidney and skeletal muscle also contained *G0S2* mRNA. Traces of the mRNA were detected in spleen and lung, but *G0S2* mRNA was undetectable in brain, colon, thymus, small intestine, or placenta (see Fig. 5). G0S2 expression is required to commit cells to enter the G1 phase of the cell cycle [Cristillo et al., 1997].

DISCUSSION

Of the 2135 sequence verified genes on the filter array 136 were differently expressed after 12 h of $1\alpha,25(OH)_2D_3$ treatment compared to untreated keratinocytes. Eighty-six of these have been upregulated and 50 have been repressed. After 24 h these numbers were reduced to 44 upregulated genes and one downregulated. All these genes are presented in Table II

(upregulated) and Table III (downregulated), respectively.

As we are most interested in the effects on cell proliferation and differentiation we discuss a few selected genes from this point of view.



Keratinocytes Respond With Initiation of Differentiation to 1α,25(OH)$_2$D$_3$ Treatment After 12 h of Incubation

In human skin c-Jun and c-Fos expression is restricted to non-proliferating, terminally differentiated cells of the stratum granulosum [reviewed in Angel et al., 2001]. This fits very well with the upregulation of c-Fos in response to 1α,25(OH)$_2$D$_3$ in primary human keratinocytes that we have observed (2.7-fold after 12 h and 4.1-fold after 24 h) (see Table IV). c-Fos is unable to form homodimers but together with c-Jun it constitutes AP-1. Functional synergism between AP-1 and Ets proteins has been found to mediate expression of the two keratinocyte terminal differentiation markers, the small-proline rich proteins, SPRR-1A and SPRR-3 [Sark et al., 1998; Sinha et al., 2000]. In contrast the expression of SPRR-2 is negatively regulated. Real-time PCR (see Fig. 2B) and filter array analysis (see Table III) both showed a downregulation of *SPRR-2B* mRNA. This downregulation points to the induction of c-Fos.

A transcription factor so far not reported in skin is Nkx2.2. This factor has been reported as important for the development of brain and pancreas. Nkx2.2 knock-out mice die within a few days of birth with severe hyperglycemia indicating an important role of Nkx2.2 in the terminal differentiation of pancreatic β-cells [Sussel et al., 1998]. This suggests a similar role in terminal differentiation of keratinocytes as Nkx2.2 is upregulated 4.1-fold after 12 h and 8.2-fold after 24 h of 1α,25(OH)$_2$D$_3$ treatment (see Fig. 2A and Table IV).

Furthermore retinoid X receptor alpha (RXRA) and most interestingly retinoic acid receptor gamma (RARG) were among the 20 upregulated transcription factors listed in Table II.

Fig. 1. Clustering analysis for genes influenced by 1α,25-dihydroxyvitamin D$_3$ in human foreskin keratinocytes. They are clustered into 10 categories: growth factors/receptors/cell growth, transcription factor/transcription, cell cycle, metabolism/biosynthesis/catabolism, extracellular matrix/cytoskeleton, immune response, intracellular signaling, apoptosis, repair, and unknown function. Genes with multiple functions were calculated separately for each cluster they fit into, which means one gene can account for the percentage in more than one cluster. **A**: Clustering analysis for genes upregulated after 12 h of 1α,25(OH)$_2$D$_3$ treatment. **B**: Clustering analysis for genes downregulated after 12 h of 1α,25(OH)$_2$D$_3$ treatment. **C**: Clustering analysis for genes upregulated after 24 h of 1α,25(OH)$_2$D$_3$ treatment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

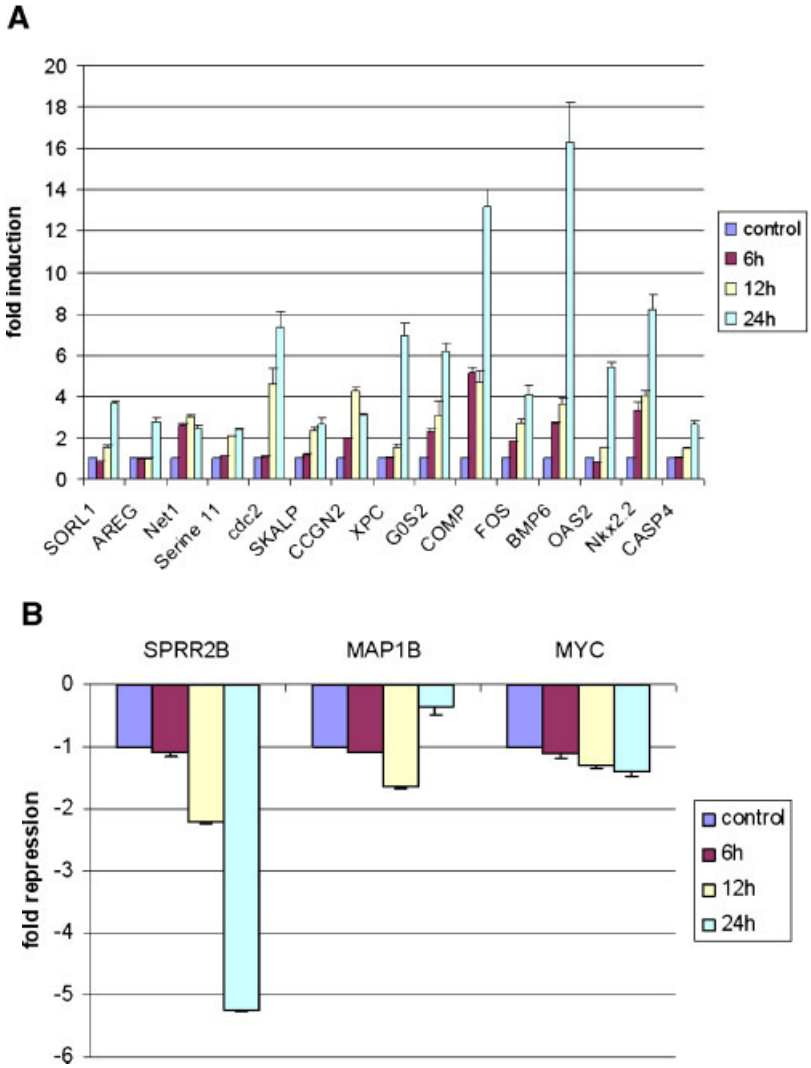
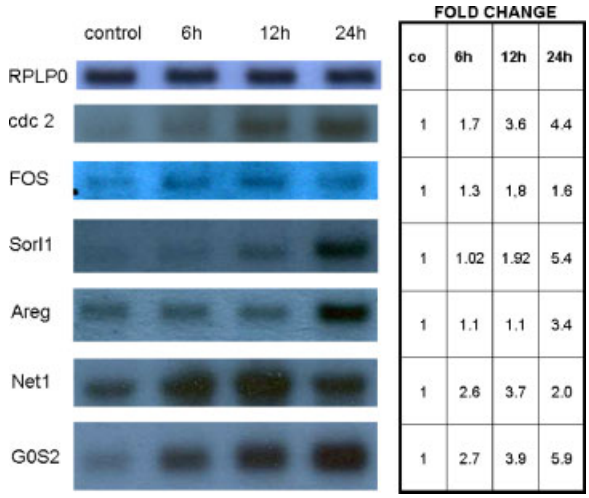


Fig. 2. Fold of induction determined by real-time PCR. RNA isolated from primary human foreskin keratinocytes after mock treatment, 6 h, 12 h, or 24 h of 1α,25-dihydroxyvitamin D_3 treatment was used for cDNA synthesis and subsequent real-time PCR. Absence of genomic DNA was confirmed by omitting reverse transcriptase during cDNA synthesis. *Large ribosomal protein P0 (RPLP0)* was used as a reference for all analyses to control for the amount of sample material [Martin et al., 2001]. In figure, (**A**) shows the fold mRNA induction determined by real-time PCR for selected clones, whereas (**B**) shows the fold mRNA repression of three selected genes. Gene identities are listed underneath the corresponding graphs. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

RXRA upregulation can be explained by the fact that this receptor is the heterodimeric partner of VDR and is necessary for transcriptional activation of target genes [for review see Jones et al., 1998]. RARG has been shown to mediate the growth inhibitory effect of all-trans retinoic acid and 9-cis retinoic acid on pancreatic adenoma cells [Pettersson et al., 2002].

Proliferation and differentiation are also interconnected with the control of the cell cycle. *G0S2*, *cdc2*, and *cyclin G2* were among the upregulated genes. Real-time PCR (see Fig. 2A) and Northern blot analysis (see Fig. 3) confirmed an approximately sixfold upregulation for *GOS2* (for comparison of real-time and array results see Table IV).

G0S2 expression is required to commit cells to enter the G1 phase of the cell cycle [Cristillo et al., 1997]. It was previously shown to be upregulated after treatment of squamous carcinoma cells with the vitamin D analog EB1089 [Lin et al., 2002]. For *cyclin G2* real-time PCR showed a peak of mRNA expression (4.3-fold) after 12 h of 1α,25$(OH)_2D_3$ treatment in human



FOLD CHANGE

co	6h	12h	24h
1	1.7	3.6	4.4
1	1.3	1.8	1.6
1	1.02	1.92	5.4
1	1.1	1.1	3.4
1	2.6	3.7	2.0
1	2.7	3.9	5.9

Fig. 3. Fold of induction determined by Northern blot analysis. RNA isolated from primary human foreskin keratinocytes after mock treatment, 6 h, 12 h, or 24 h of 1α,25-dihydroxyvitamin D_3 treatment was used to make Northern blots, which were then hybridized with specific probes for *cdc2*, *FOS*, *SorL1*, *AREG*, *NET1*, and *G0S2*. For normalization a specific probe for *large ribosomal protein P0 (RPLP0)* was used. Signal intensities were determined using a phosphoimager (BAS reader 1800, Fuji) and the fold induction that was determined is shown in the accompanying table. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

keratinocytes (see Fig. 2A). *Cyclin G2* mRNA expression oscillates through the cell cycle, peaks in the mid/late S phase and decreases during G2/M phase. It is presumed to be involved in negative growth regulation and in

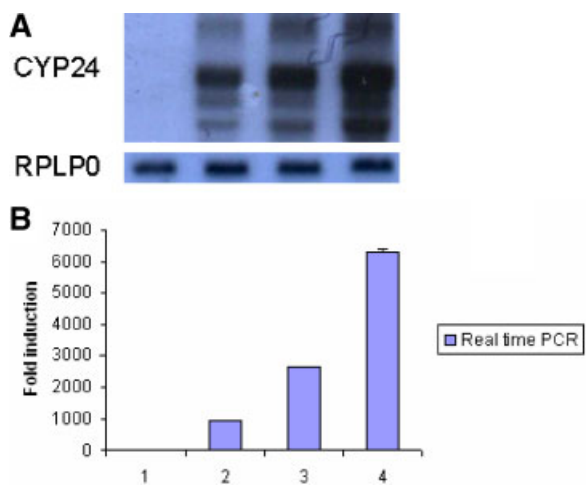


Fig. 4. Northern blot analysis for *CYP24* as well as for the control *Large ribosomal protein P0 (RPLP0)* are shown in (**A**). The real-time PCR results for *CYP24* are shown in a graphical view (**B**), again *large ribosomal protein P0 (RPLP0)* was used as a control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

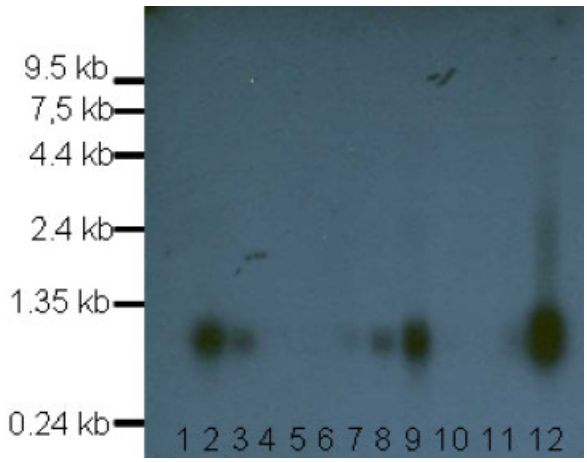


Fig. 5. Multiple tissue Northern blot (MTN; BD Biosciences) hybridized with a radiolabeled *G0S2* probe. MTN contains RNA isolated from the following human tissues: brain (**lane 1**), heart (**lane 2**), skeletal muscle (**lane 3**), colon (**lane 4**), thymus (**lane 5**), spleen (**lane 6**), kidney (**lane 7**), liver (**lane 8**), small intestine (**lane 9**), placenta (**lane 10**), lung (**lane 11**), and peripheral blood leukocyte (**lane 12**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

negative regulation of cell cycle progression [Bennin et al., 2002]. Cyclin G2 is also hypothesized to play a role in signal transduction [Horne et al., 1996]. In a previous study, after 24 h of 1α,25$(OH)_2D_3$ treatment, *cyclin G1* and *G2* were found to be upregulated in two human breast cancer cell lines MCF-7 and MDA MB231 [Swami et al., 2003]. Taken these data together they strongly suggest that upregulation of cyclin G2 is one way of 1α,25$(OH)_2D_3$ to downregulate proliferation.

According to the filter array analysis (see Table II) and real-time PCR (see Fig. 2A) *cdc2* mRNA levels are strongly increased in human primary foreskin keratinocytes by treatment with 1α,25$(OH)_2D_3$ (see Table IV). Northern blots also support this finding (see Fig. 3). Increased expression of cdc2 is often associated with cell cycle arrest in the G2 to M phase, but also with apoptotic processes [Wang et al., 2003]. cdc2 has been demonstrated to be a downstream effector of $\alpha_V\beta_3$ integrin. Caldesmon, a cytoskeleton-associated molecule, is phosphorylated by cdc2, which contributes to increased cell motility [Manes et al., 2003]. This points to a function of cdc2 during the migration of keratinocytes into upper, more differentiated layers of the epidermis, where they experience cell cycle arrest.

In addition to that some differentiation markers are also turned on, predominantly keratin 1 and keratin 10 (data not shown), as

well as protease inhibitor 3 (PI3), and serine protease 11 (see Table II and IV).

The downregulation of genes like c-myc, cyclin A, ribosomal proteins (S29, L17, and L35) also supports the growth inhibitory effect of $1\alpha,25(OH)_2D_3$ on keratinocytes.

The Situation After 24 h of $1\alpha,25(OH)_2D_3$ Treatment

The expression of the pro-differentiation transcription factor c-Fos increased from 2.7-fold after 12 h to 4.1-fold after 24 h as observed by real-time PCR and the expression of Nkx2.2 at an even higher level (8.2-fold) after 24 h supports the induction of differentiation by $1\alpha,25(OH)_2D_3$ (see Table IV).

A few other genes whose expression correlates well with a more differentiated phenotype are coding for serine protease 11 (PRSS11), PI3, and cartilage oligomeric matrix protein (COMP). Mature layers of the epidermis contain high levels of PRSS11 [De Luca et al., 2003]. PRSS11 overexpression inhibits cell growth and proliferation in vitro and in vivo, thus suggesting a possible role as a tumor suppressor [Baldi et al., 2002]. Upregulation of serine protease 11 (2.7-fold after 24 h) very likely contributes to the anti-tumor activity of $1\alpha,25(OH)_2D_3$.

PI3, showed an approximately threefold induction (Table IV). PI3 is often found to be upregulated in suprabasal, differentiated keratinocytes of psoriatic skin, in wound healing processes, and after UV irradiation [Pol et al., 2003]. Expression of *PI3* is downregulated by retinoic acid, a known inducer of keratinocyte proliferation. The observed upregulation of *PI3* mRNA in response to $1\alpha,25(OH)_2D_3$ treatment points to a connection to cornified envelop formation. Among the most strongly upregulated genes is *COMP* (13-fold by real-time PCR, see Fig. 2A). COMP is a large disulfide-linked pentameric, calcium-binding protein, which plays an important role in extracellular matrix assembly and matrix–matrix protein interactions [Di Cesare et al., 2002] and this way it influences differentiation. Its expression is enhanced by epidermal growth factor [Di Cesare et al., 2002] and TGFβ [Dodge et al., 1998]. The coiled-coil domain of COMP has been shown to bind both, vitamin A and $1\alpha,25(OH)_2D_3$, and is thus thought to act as a storage and delivery protein for signaling molecules relevant in cartilage tissue [Ozbek et al., 2002].

Also upregulated are genes involved in hormone metabolism: *CYP24* ($1\alpha,25(OH)_2D_3$), *arylsulfatase E* (sex hormones), and lipid catabolism (lipoprotein *lipase*). As CYP24 is by far the most strongly upregulated gene that we were able to detect (more than 6,000-fold after 24 h as estimated by real-time PCR) this has certainly consequences: The observed upregulation of *SorL1* mRNA (coding for a multifunctional endocytic receptor, Jacobson et al., 1996) after 24 h of $1\alpha,25(OH)_2D_3$ treatment in primary human keratinocytes (see Figure 2A and Table IV) might be a result of resumed proliferation after a major portion of the actual active hormone has been degraded by CYP24. Resumed proliferation is most likely also connected with the observed upregulation of the expression of growth factors: fibroblast growth factor 7 (FGF7), also termed keratinocyte growth factor (KGF), a major growth factor for keratinocytes, is upregulated twofold after 12 h of $1\alpha,25(OH)_2D_3$ treatment. Also upregulated are the major autocrine growth factor, *transforming growth factor alpha (TGFα)* (twofold, see Table II), as well as *amphiregulin* (*AREG*) (2.8-fold after 24 h, see Table IV, for Northern blot see Fig. 3). *AREG* is upregulated in response to $1\alpha,25(OH)_2D_3$ in Caco-2 cells, a human colon carcinoma cell line [Wood et al., 2004], squamous carcinoma cells [Lin et al., 2002], and breast cancer cell lines [Akutsu et al., 2001] where it inhibits cell growth [Shoyab et al., 1988].

Probably also contributing to the induction of differentiation is the TGFβ family member, *bone morphogentic protein 6 (BMP6)* which was found to be highly upregulated after 24 h of $1\alpha,25(OH)_2D_3$ (16.3-fold in real-time PCR, see Fig. 2A, for comparison with array see Table IV). BMP6 has been shown to induce growth arrest [McDonnell et al., 2001] and keratinocyte differentiation via induction of E2F-5, which in turn recruits histone deacetylases (HDACs) to turn off transcription of proliferation specific genes [D'Souza et al., 2001]. BMP6 is synthesized in suprabasal layers of the murine epidermis and induces the expression of keratin 1, a marker of differentiation [McDonnell et al., 2001]. $1\alpha,25(OH)_2D_3$ was previously seen to upregulate the expression of *BMP6* mRNA in human prostatic epithelial cells [Krishnan et al., 2004].

In addition 11 genes involved in cell cycle processes were downregulated in their expression,

among them *cyclin H* and *cyclin A1* (see Table III) correlating with the anti-proliferative effect of $1\alpha,25(OH)_2D_3$.

Finally we want to mention a few genes which are not involved in differentiation but show some remarkable features: One of the genes whose expression was upregulated 6.9-fold after 24 h of $1\alpha,25(OH)_2D_3$ treatment is *Xeroderma pigmentosum, complementation group C (XPC)* (see Fig. 2A, Table IV). XPC recognizes a variety of DNA damage [Wang et al., 2003], and mediates DNA excision repair [Shimizu et al., 2003]. Given the fact that UV light is needed to synthesize vitamin D from 7-dehydrocholesterol in the skin, it seems reasonable to protect the cells against UV damage by upregulating the DNA repair machinery.

Another interesting finding is the downregulation of *microtubule-associated protein 1B (MAP1B)* (see Fig. 2B and Table IV) in response to $1\alpha,25(OH)_2D_3$ treatment. Phosphorylated MAP1B co-localizes with neurofibrillary tangles, a common hallmark of Alzheimer's disease. MAP1B is proposed to act as an effector of cell death that is triggered by amyloid β deposition in neurodegenerative disorders. It has also been suggested that an increase in non-phosphorylated MAP1B at the hippocampus may be responsible for the cytoarchitectural abnormalities found in schizophrenia [Benitez-King et al., 2004]. This suggests that $1\alpha,25(OH)_2D_3$ might be helpful in treatment of mental illness and neurodegenerative disorders, like Alzheimer's disease or schizophrenia.

$1\alpha,25(OH)_2D_3$ also caused the downregulation of four anti-apoptotic genes (*BAG1*, *SERPINB2*, *SMN1*, and *IER3*) as well as a 2.7-fold upregulation of Caspase 4 (see Fig. 2A). Induction of *CASP4* expression in response to $1\alpha,25(OH)_2D_3$ treatment is one explanation for the anti-tumor activity observed for this hormone.

CONCLUSION

$1\alpha,25(OH)_2D_3$ is an extremely powerful substance influencing many biologically important processes like proliferation and differentiation. It has immune-modulatory activities and regulates mineral homeostasis. Human epidermal keratinocytes are the only cells capable of performing the complete synthesis of $1\alpha,25(OH)_2D_3$ from 7-dehydrocholesterol, but they are also massively influenced by the hormone.

Using mRNA from $1\alpha,25(OH)_2D_3$-treated human primary keratinocytes we screened a non-redundant set of 2135 sequence verified EST clones. Of this set, a total of 114 clones showed an at least twofold upregulation and 51 clones were downregulated after 12 h or 24 h of $1\alpha,25(OH)_2D_3$ treatment. The majority of these genes are involved in intracellular signaling, transcription, and cell cycle. But there is also a number of growth factors and proteins involved in metabolism, components of the extracellular matrix, as well as proteins involved in DNA repair, apoptosis, and immune response, which are regulated to a considerable extent (Tables II and III).

With the identification of this set of target genes for $1\alpha,25(OH)_2D_3$ in human primary keratinocytes we have taken the first step in elucidating the transcriptional network of $1\alpha,25(OH)_2D_3$ action. In the future yeast 2-hybrid screens will be necessary to identify reaction partners for the isolated genes. With an emerging regulatory network of $1\alpha,25(OH)_2D_3$ treatment response, it will be possible to considerably enhance the pharmaceutical applications of $1\alpha,25(OH)_2D_3$ and its analogs.

LIST OF ABBREVIATIONS

$1\alpha,25(OH)_2D_3$	$1\alpha,25$-dihydroxyvitamin D_3
VDR	vitamin D receptor
Zn-finger	zinc finger
RXR	retinoic X receptor
PKC	protein kinase C
$1,25D_3$-MARRS	$1,25D_3$-membrane-associated, rapid-response steroid-binding
SAM	significance analysis of microarrays
GOs	gene ontologies
CYP24	$25(OH)D_3$-24-hydroxylase
FAM	carboxyfluorescein
VDR	vitamin D receptor
GOs	gene onthologies
RXR	retinoid X receptor
RXRA	retinoid X receptor alpha
RARG	retinoic acid receptor gamma
EST	expressed sequence tag
RPLP0	large ribosomal protein P0
SorL1	sortilin-related receptor, L
NET1	neuroepithelial cell transforming gene 1
AP-1	activator protein 1 (jun and fos family)

AREG	amphiregulin
PRSS11	serine protease 11
PI3	protease inhibitor 3
COMP	cartilage oligomeric protein
XPC	xeroderma pigmentosum, complementation group C
CASP4	caspase 4
TF	transcription factor
GF	growth factor
IGF	insulin-like growth factors
PCR	polymerase chain reaction
EGFR	epidermal growth factor receptor
FGF	fibroblast growth factor
KGF	keratinocyte growth factor
TGFα	transforming growth factor alpha
BMPs	bone morphogenetic proteins
cdc	cell division cycle
CDKIs	cyclin-dependent kinase inhibitors
HDACs	histone deacetlyases
MAP1B	microtubule-associated protein 1B
MTN	multiple tissue northern
RPL0	ribosomal protein, large, P0
FDR	false discovery rate
BAG1	Bcl2-associated athanogene
SERPINB2	plasminogen activator inhibitor, type II (arginine-serpin)
SMN1	survival of motor neuron 1
IER3	immediate early response 3

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