Deciphering the calcitriol-induced transcriptomic response in keratinocytes: presentation of novel target genes

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Abstract

Numerous studies to date have been aimed at unraveling the large suite of calcitriol ($1\alpha,25$ -dihydroxyvitamin D_3) response genes in diverse tissues including skin, where this hormone is involved in regulating keratinocyte proliferation, differentiation, permeability barrier formation, innate immunity promotion, antimicrobial peptide production, and wound healing. However, the various approaches differ considerably in probed cell types, scale, throughput, and statistical reliability and do, of note, not reveal much overlap. To further expand our knowledge on presently elusive targets and characterize the extent of fragmentation of existing datasets, we have performed whole-transcriptome microarray examinations of calcitriol-treated human primary keratinocytes. Out of 28 869 genes investigated, we uncovered 86 differentially expressed (67 upregulated and 19 down-regulated) candidates that were functionally clustered into five annotation categories: response to wounding, protease inhibition, secondary metabolite biosynthesis, cellular migration, and amine biosynthetic processes. A complementary RTq-PCR study of 78 nominees selected thereof demonstrated significant differential expression of 55 genes (48 upregulated and seven downregulated) within biological replicates. Our hit list contains nine previously authenticated targets (16.36%, proof of concept) and 46 novel genes (83.6%) that have not yet been explicitly described as being differentially regulated within human primary keratinocytes. Direct vitamin D receptor response element predictions within the regulatory promoter regions of 50 of the RTq-PCR-validated targets agreed with known biological functionality and corroborated our stringent data validation pipeline. Altogether, our results indicate the value of continuing these kinds of gene expression studies, which contribute to an enhanced comprehension of calcitriol-mediated processes that may be dysregulated in human skin pathophysiology.

Key Words

- calcitriol signaling
- calcitriol response genes
- primary keratinocytes
- gene expression profiling

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Introduction

Metazoan epidermis consists of four distinguishable layers containing primarily keratinocytes at distinct stages of development (Bikle *et al.* 2007, Poumay & Coquette 2007). Following their initial proliferation in the epidermal bottom stratum basale, the majority of daughters undergo a process of (terminal) differentiation accompanied by characteristic morphological alterations as well as changes in biochemical markers as they continuously migrate upward to the outmost cornified, enucleated stratum corneum skin surface (Lu *et al.* 2005, Carlberg & Seuter 2007, Bikle 2010). A central regulator of keratinocyte (and also of other normal vs malignant cell types) growth and differentiation is hereby calcitriol (1 α ,25-dihydroxy-vitamin D₃) (McLane *et al.* 1990, Svendsen *et al.* 1997, Cianferotti *et al.* 2007), classically recognized for its role in Ca⁺⁺ equilibrium maintenance essential for bone integrity. Calcitriol not only differentially affects expression of certain epidermal differentiation markers (Su *et al.* 1994, Lutzow-Holm *et al.* 1995, Bikle *et al.* 2002, Xie *et al.* 2002) but also governs permeability barrier formation (Oda *et al.* 2009). It protects keratinocytes from apoptosis (Lee & Youn 1998, Diker-Cohen *et al.* 2003) and has gained attention for its role in innate immune defense (Wang *et al.* 2004, Schaubert *et al.* 2007). Remarkably, keratinocytes comprise the only cell population that possesses the entire metabolic equipment for autonomous synthesis of calcitriol from the 7-dehydrocholesterol precursor both *in vitro* (Lehmann *et al.* 2001) and *in vivo* (Lehmann *et al.* 2003). Thus, skin represents both the sites of production of calcitriol and is also the target organ for this potent, hormonally active secosteroid (Ebert *et al.* 2006, Norman 2006, Reichrath *et al.* 2007, Schmuth *et al.* 2007). Genomic control of primary calcitriol response genes is hereby achieved by attachment of ligand-bound vitamin D receptor (VDR)/retinoid X receptor (RXR) heterodimers to vitamin D response elements (VDREs) located within respective promoter regions (Rastinejad *et al.* 1995, Shaffer & Gewirth 2002, Carlberg 2003, Carlberg & Seuter 2007). Binding of calcitriol to VDR triggers the release of corepressor proteins (Polly *et al.* 2000, Carlberg 2004, Privalsky 2004, Carlberg & Seuter 2007, Reichrath *et al.* 2007) through a conformational change within the VDR ligand-binding domain. A subsequent recruitment of coactivators collectively mediates local chromatin remodeling and builds a stable bridge to the basal transcriptional machinery (Hermanson *et al.* 2002, Eelen *et al.* 2006, Carlberg & Seuter 2007). In addition, calcitriol can also mediate 'rapid responses' that are elicited by putative

receptor(s) located near or associated with the plasma membrane and its caveolae compartments (Boyan *et al.* 2006). These effects occur within minutes after hormone administration, which precludes a mechanism involving changes in gene expression, but appears instead to direct an increase in second messengers that can, ultimately, affect transcription through cross talk with other signal transduction cascades (Wali *et al.* 1990, Losel & Wehling 2003). Evidently, calcitriol target gene activation constitutes a multistep process, permitting keratinocytes to finely adjust their physiological responses to calcitriol (Darimont *et al.* 1998, Rosenfeld & Glass 2001).

The last few years have witnessed the introduction of global oligonucleotide and cDNA microarrays as increasingly appreciated, powerful instruments to reproducibly examine drug- and disease-induced steady-state snapshots of the (differential) expression of thousands of targets in massively parallel fashion (Kawasaki 2006, Auer *et al.* 2009). Together, with complementary chromatin immunoprecipitation (ChIP and sophisticated modifications developed thereof, such as genome-wide ChIP-chip hybridization of resultant chromatin fragments on microarrays or massively parallel ChIP-seq sequencings) and *in silico* screens for VDREs, they have largely rationalized the entirety of nuclear hormone receptor superfamily research (Tavera-Mendoza *et al.* 2006, Carlberg & Seuter 2009, Carlberg & Heinäniemi 2010, Meyer *et al.* 2010, Pike *et al.* 2010), which previously relied on the transfection of 'indirect' reporter gene chimeras (Lemon & Freedman 1996). To date, in-depth deciphering of calcitriol-triggered transcriptional changes has been achieved in both primary cells and established (tumor) cell lines derived from multiple tissues. Each of these studies has resulted in the uncovering of a far from exhaustive, barely overlapping list of (merely moderate- to high-ranking quality) response genes implicated in diverse biological processes. Only two studies have actually been devoted to either immortalized (Lu *et al.* 2005) or primary human keratinocytes (Moll *et al.* 2007). In our endeavor to further expand our knowledge on calcitriol target genes, we have applied a global whole-transcriptome microarray to delineate alterations within hormone-induced transcriptomic changes and have confirmed the results with complementary RTq-PCR and *in silico* promoter scanning. In doing so, we have been able to verify nine candidates previously described as differentially regulated in human keratinocytes to connect more than 30 additional targets reported from diverse other (tumor) tissue-derived lines

with primary keratinocyte functioning and to define 13 potentially new, likely keratinocyte-specific genes. A graphical overview of our experimental design and outcomes is shown in Fig. 1.

Materials and methods

Cultivation of primary keratinocytes from human foreskin tissue

Clinically normal (uninflamed, sunlight-protected) human foreskin tissue obtained after informed consent from six individuals aged between 4 and 12 years in the course of routine surgery (phimosis, religious ritual; Children's Hospital, SALK/PMU, Salzburg, Austria) was incubated for 2 min in 100% ethanol to eliminate any bacterial contamination, neutralized in Dulbecco's PBS (Sigma-Aldrich), aseptically sliced into small pieces, and incubated at 4 °C overnight in Dispase solution

(2.24 units, GIBCO brand, Invitrogen GmbH) diluted 1:1 in buffer A (30 mM HEPES, 10 mM glucose, 3 mM KCl, 130 mM NaCl, and 1 mM Na₂HPO₄, 1× phenol red, pH 7.4) to gently disaggregate the epidermis from the underlying dermis. As described previously (Bikle *et al.* 1986, Trost *et al.* 2010), primary keratinocytes were harvested from separated foreskin epidermis via incubation with repeated vigorous shaking for 15 min in trypsin-EDTA (PAA Laboratories GmbH, Pasching, Austria) diluted 1:10 in buffer A at 37 °C. The resultant epidermal cell suspension was neutralized with fetal bovine serum (PAA Laboratories, 1:10 in buffer A) and centrifuged for 5 min at 206 *g* (Varifuge 3.0 RS, Heraeus Instruments, Hanau, Germany). The cell pellet was then resuspended in 5 ml defined serum-free SFM medium (kit) containing L-glutamine, EGF (5 µg/l), and bovine pituitary extract (50 mg/l) and a calcium chloride concentration of 0.09 mM (Gibco, Invitrogen GmbH) and filtered through a 40 µm cell strainer (BD Biosciences, Heidelberg,

Figure 1

Schematic illustration of our experimental strategy (plus resultant data) applied for the identification of differentially expressed calcitriol targets within primary human keratinocytes.

Germany). Cells in suspension were centrifuged again, seeded in 75 cm² flasks (TPP, Trasadingen, Switzerland), and incubated in SFM medium at 37 °C, 5% CO₂. Cells were subsequently passaged until passage three (~3 weeks) in keratinocyte-specific SFM medium formulated to inhibit fibroblast growth. For calcitriol exposure, the hormone (Sigma–Aldrich) was solubilized in 100% ethanol, diluted to the desired concentration in culture medium, and administered to the cells, growing at a confluency of about 70–80%, at a final concentration of supraphysiological 200 nM for 16 h (individuals 1, 2, and 3: microarray, RTq-PCR) vs more physiological 1 nM for 8 h (individuals 4, 5, and 6: RTq-PCR). Mock controls (ethanol vehicle) were analogously treated.

RNA isolation from cultivated primary keratinocytes

Total RNA from cells grown to ~80% confluence was extracted with an RNeasy Midi Kit (Qiagen GmbH) according to the manufacturer's instructions, resuspended in DEPC-treated nuclease-free water, and quantified spectrophotometrically (Ultraspec 2000, Amersham Pharmacia) at 260 nm (Oender *et al.* 2008). Resultant preparations were appraised as being suitable for subsequent DNA microarray hybridization only if samples were of sufficient yield, exhibited intact bands corresponding to the 18S and 28S rRNA subunits as determined by agarose gel electrophoresis in formaldehyde-supplemented 1× MOPS running buffer, and simultaneously displayed no spurious peaks or RNA degradation artifacts on the u.v. absorption spectrum. Portions of the total RNA samples were stored (long term) at –80 °C.

Whole-transcript Affymetrix microarray assessment

Technical triplicate aliquots of total RNA per treatment condition were forwarded to the Kompetenzzentrum Fluoreszente Bioanalytik (KFB, Regensburg, Germany) for use in probing a high-precision, whole-transcript Human Gene 1.0 ST array (Affymetrix, Santa Clara, CA, USA). The entire experimental pipeline from sense target labeling to hybridization, washing, array scanning, and final raw data capture was performed as part of a comprehensive, commercial solution including standard Affymetrix protocols, reagents, and instrumentation meeting-certified performance criteria. Returned raw data were checked for quality, background-adjusted, and quantile-normalized and analyzed by the linear model for microarray data (LIMMA) statistical method. Normalized log₂ intensity values were rank-ordered; fold-changes in gene expression

were calculated; and *P* values, a statistical measure of consistency between paired groups, were determined using Student's *t*-test.

Functional clustering of differentially expressed genes

To more thoroughly characterize the resultant set of genes, we employed the bioinformatics resource DAVID version 6.7 (<http://david.abcc.ncifcrf.gov/>) to annotate candidates according to their involvement in diverse (redundant) cellular processes, pathways, or components by controlled vocabulary gene ontology (GO) assignment (Ashburner *et al.* 2000). DAVID (Sherman *et al.* 2007, Huang da *et al.* 2009) permits systematic identification of biological themes significantly accumulated within particular (differentially expressed) gene collections at nominal *P* < 0.05 with the transcript population comprising the Affymetrix Human Gene 1.0 ST arrays as background. Explicit categorization was achieved using a modified Fisher exact probability test, in the course of which the significance (*P*) for each assembled group was calculated as the negative log-transformed enrichment score (ES; Hosack *et al.* 2003).

Confirmatory RTq-PCR

RefSeq accession IDs of differentially expressed genes were subjected to Batch Entrez analysis (<http://www.ncbi.nlm.nih.gov/sites/batchentrez>) for exposing mRNA sequences. These were forwarded to the WIBR UTR extractor (<http://jura.wi.mit.edu/bioc/tools/utrs>) to excerpt coding regions and pasted into the Primer 3 program (<http://frodo.wi.mit.edu/primer>) to generate automatically designed (and manually controlled) oligonucleotides with similar properties (uniform GC content/melting temperature, optimal length, and similar amplicon size) to guarantee accurate results. Forward and reverse primer stocks (100 pmol/μl) were purchased in 96-well format from Invitrogen GmbH. After a 20-min digestion of total human RNA preparations from three different individuals with DNase I (deoxyribonuclease kit, Sigma–Aldrich) at room temperature, 1.5 μg pure RNA was reverse transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the supplier's instructions. Resultant cDNA served as the template for subsequent RTq-PCR in plate format using a GoTaq qPCR Master Mix (Promega Corporation, Madison, WI, USA) plus a CFX96 apparatus (Bio-Rad, Hercules, CA, USA). Each run profiled the expression of differentially expressed genes in relation to seven unregulated housekeeping transcripts (*B2M*, *HPRT1*,

RPL13A, *GAPDH*, *ACTB*, *ANXA1*, and *TUBB*) serving as internal references for data normalization, determination of experimental variance, and optimal fold-change cutoff estimation. Two built-in intron-specific probes for *KRT14* intron 1 and *BEST1* intron 2 were used for assessing possible contamination with genomic DNA. All reactions were carried out in a final volume of 25 μ l. Product quality was checked by post-PCR melting curve analysis. Fold inductions were calculated according to a mathematical model described by Pfaffl (2001) employing the formula $2^{-(\Delta\Delta Ct)}$ (where $\Delta\Delta Ct$ is $\Delta Ct_{\text{calcitriol}}$ minus $\Delta Ct_{\text{ethanol}}$, ΔCt designates $Ct_{\text{sample}} - Ct_{\text{housekeeping-pool}}$, and Ct denotes the cycle at which the threshold is crossed). A fold-change cutoff of 1.8 was implemented to record the final list of statistically ascertained (homoscedastic *t*-test, $P < 0.05$) differentially expressed calcitriol target genes in human primary keratinocytes.

In silico screening for (putative) VDR response elements

Genomic sequences of differentially expressed genes located within a distance of approximately -3000 to $+150$ bps from their respective transcription start site (TSS) were directly inferred from Genomatix's annotated EIDorado (version 08-2011, built on NCBI build 37)/Gene2Promoter database or manually downloaded from Cold Spring Harbor Laboratory's mammalian promoter database (version 05-2005, NCBI build 35) (Xuan *et al.* 2005). Promoter regions were interactively screened in both orientations for (predicted) theoretical nuclear hormone receptor superfamily transcription factor (VDR-RXR heterodimer)-binding sites using optimized, fixed similarity thresholds via MatInspector 8.0 (Genomatix, Ann Arbor, MI, USA) with its embedded weighted nucleotide distribution matrix family concept (library version 8.4, 06-2011) (Cartharius *et al.* 2005). 'Core similarity' is herein defined as the maximally conserved positions. 'Matrix similarity' (perfect match: score of 1.00, acceptable match: >0.80) additionally takes into account all bases over the entire matrix distance, with mismatches in conserved positions decreasing the score more than discrepancies in less conserved domains.

Exemplary electrophoretic mobility agarose gel shift assay

The promoter fragments (100–200 bp) harboring central MatInspector predicted (bipartite) DR3-type VDREs for *CRABP2* (forward: 5'-CAAGCCCCCTCTGAGTACG-3', reverse: 5'-GATGCTCAGGGCTCGTGTAT-3'), *OLFML3*

(5'-TCCCAGTGGTAAGGAAGTCAG-3', 5'-TTGCTTGAAA-GAGGCCAGAT-3'), *BNC2* (5'-GTCAAGCATGCCTGT-GAAGA-3', 5'-GGCCCTCCTTATTTTCAGTCC-3'), *TMEM91* (5'-CCACGATCACTTCACTGCAC-3', 5'-GCCTTAAC-CAAGGACCTTCC-3'), and *KRT19* (5'-CTGCTGTGAG-GATCCAGTGA-3', 5'-GTCTGGGGAGGGACTTTGTA-3') were PCR amplified from genomic DNA isolated from cultured human keratinocytes (Gentra Puregene, Qiagen GmbH) with the Primer3-designed oligonucleotides listed in brackets. The VDR DNA-binding domain (DBD, AA 16–125) (Sone *et al.* 1991) was shuttled into recombinational cloning compatible destination vector pDEST 17 (Life Technologies), transformed into *Escherichia coli* BL21 (DE3) cells, expressed via 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) induction, and then purified as recombinant hexahistidine-tagged fusion protein by nickel affinity chromatography following common procedures. To investigate binding of VDR-DBD to amplified promoters, 600 ng of respective PCR products were incubated with recombinant DBD in 100 mM Tris buffer in a total reaction volume of 20 μ l for 5 min and subsequently separated on a 1% agarose gel in $1 \times$ TAE buffer. Samples were visualized for apparent electrophoretic mobility shifts in relation to a previously described osteopontin DR3 repeat (Hijiya *et al.* 1994) positive control (5'-CGCAGAGCATTTCATCTAA-3', 5'-GGTTCTGAATCCGCTGTGT-3') plus four analogously treated negative control DNA fragments (designated as C7, D1, A12 and G8) containing no definite VDRE. Band intensities were densitometrically quantified via ImageJ software (<http://rsbweb.nih.gov/ij/docs/index.html>).

Results

Global transcriptional profiling uncovers known and novel calcitriol response genes in human primary keratinocytes

To develop a comprehensive picture of calcitriol-mediated changes in gene expression patterns, total RNA isolated from proliferating primary keratinocytes treated with 200 nM calcitriol for 16 h was subjected to a commercially available microarray hybridization service (Dalma-Weiszhausz *et al.* 2006). Before definite sample submission, an aliquot of RNA was pretested in a miniature scale RTq-PCR analysis to ensure that a calcitriol response could be detected at the level of mRNA induction as judged by an increase in *CYP24A1* expression (Anderson *et al.* 2003) (data not shown). The interrogated Affymetrix human 1.0 ST gene chip platform offers coverage of roughly the entire

transcriptome wherein each of the 28 869 annotated constituents is cumulatively represented by ~26 distinct perfect match-only designed probes spread across the entire length of the genes (including ~20 000 generic background/hybridization controls), resulting in high intra-platform consistency, robustness, and reproducibility (Shi *et al.* 2006, Sarmah & Samarasinghe 2011). A preliminary, unbiased, preprocessed, normalized raw data summary identified 86 probe sets as differentially expressed (67 upregulated and 19 downregulated genes with fold changes of 1.8 or greater) under calcitriol exposure compared with reference conditions. For subsequent RTq-PCR validation, this list was reduced to a cohort of 78 promising candidate calcitriol response genes, employing only those genes whose pattern of expression was consistent in all three independently performed technical replicates and across all probe sets that detect the same gene.

Functional clustering suggests enrichment of genes involved in response to wounding, secondary metabolite biosynthesis, protease inhibition, cellular migration, and amine biosynthetic processes

We next assigned biological themes to our authenticated, calcitriol-modulated genes by taking advantage of the web-accessible DAVID knowledgebase, an integrated high-throughput data mining environment (Sherman *et al.* 2007, Huang da *et al.* 2009). Employing pre-adjusted 'medium' default parameter settings, five annotation categories appeared to be significantly enriched among our identified cohort of 78 genes. These clusters encompass response to wounding/inflammatory response (ES 2.58), protease inhibition (ES 2.01), secondary metabolite biosynthesis, transport, and catabolism (ES 2.2), cellular migration (ES 1.44), and amine biosynthetic processes (ES1.33).

The majority of microarray-identified targets can be reproduced in RTq-PCR analysis using biological replicate samples

Differential expression patterns of the candidate 200 nM/16 h calcitriol response genes in primary human keratinocytes isolated from three different individuals was separately assessed by RTq-PCR analysis. The RTq-PCR analysis permits identification of induction or repression as relative fold changes in transcript levels compared with seven internal unregulated reference housekeeping gene transcripts (Walker 2001, Dallas *et al.* 2005, Provenzano &

Mocellin 2007). RTq-PCR data from all three individuals were integrated, and 55 candidates were definitively authenticated. We found that 48 of these were congruently upregulated (Fig. 2) and seven were downregulated (Fig. 3). Although mean interindividual variation, the percentaged relative s.d. across individuals in the calcitriol-stimulated vs ethanol-treated control group, was experimentally determined to be ~10% (Table 1), which would encourage selection of a fold change cutoff of 1.1, we chose a more stringent selection. Only genes with a fold change of at least 1.8 and $P < 0.05$ were considered to be explicit 'hits'. As highlighted in Figs 2 and 3, mean fold changes over three biological and technical replicates in all tested cases autonomously verified our earlier findings. Additionally, we performed RTq-PCR analyses on primary keratinocytes isolated from another set of three individuals, which were stimulated with a physiological dose of 1 nM calcitriol for 8 h using the same 78 probe panel. As depicted in Figs 2 and 3, though the fold changes are not as high, all genes, except *ABHD3*, are regulated in the same directions as upon stimulation with 200 nM for 16 h. However, with a fold change of at least 1.8, 23 genes were congruently upregulated and two were downregulated. Mean interindividual variation was again determined to be ~10% (Table 1).

Comparison of differentially regulated targets to current literature indicates the necessity for ongoing gene expression profiling studies

Based on an in-depth literature survey (Pubmed database) of currently available expression profiling studies investigating differential calcitriol-mediated gene expression in diverse cell and tissue types, our final list of 55 candidate calcitriol-regulated genes includes 46 candidates whose responsiveness has, to the best of our knowledge, not been observed previously in human primary keratinocytes, and nine genes (*NET1*, *GOS2*, *IL1RL1*, *CD14*, *KLK6*, *KLK13*, *SERPINB1*, *SEMA3B*, and *DUSP10*) previously identified as calcitriol-regulated genes in human primary keratinocytes. In addition, as can be perceived from Fig. 1, 33 candidates have previously been related to calcitriol in diverse (tumor-derived) cell lines, and 13 candidates (*BNC2*, *CALB2*, *IGFL3*, *CYP4F3*, *FETUB*, *DHRS9*, *HCAR3*, *KRT19*, *OLFML3*, *TMEM91*, *CRABP2*, *CYP4B1*, and *TMEM63C*) have not previously been identified as being regulated by calcitriol. Because overlap between the various individual studies is limited, ongoing efforts will be necessary for fully elucidating all calcitriol response genes.

GenBank	Symbol	Gene description	MA Fold	RTq-PCR	
				200 nM/16 h Fold	1 nM/8 h Fold
NM_005502	ABCA1	ATP-binding cassette, sub family A (ABC1), member 1	2.7	4.3	1.7
NM_138340	ABHD3	Abhydrolase domain containing 3	2.0	1.8	–2.5
NM_005100	AKAP12	A kinase (PRKA) anchor protein 12	2.3	8.8	1.7
NM_017637	BNC2	Basonuclin 2	1.9	4.6	1.5
NM_016289	CAB39	Calcium binding protein 39	1.9	3.2	1.5
NM_007088	CALB2	Calbindin 2	1.9	6.2	1.3
NM_000591	CD14	CD14 molecule	11.2	218.7	
NM_014143	CD274	CD274 molecule	3.1	3.1	
NM_001784	CD97	CD97 molecule	2.1	4.2	1.9
NM_001785	CDA	Cytidine deaminase	1.9	2.7	1.6
NM_018413	CHST11	Carbohydrate (chondroitin 4) sulfotransferase 11	2.0	3.6	1.5
NM_001307	CLDN7	Claudin 7	2.0	2.9	1.2
NM_024734	CLMN	Calmin (calponin-like, transmembrane)	2.6	2.5	1.9
NM_020361	CPA6	Carboxypeptidase A6	2.4	ND	ND
NM_000782	CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1	66.5	ND	ND
NM_000777	CYP3A5	Cytochrome P450, family 3, subfamily A, polypeptide 5	2.1	4.0	3.3
NM_021187	CYP4F11	Cytochrome P450, family 4, subfamily F, polypeptide 11	2.6	–	1.7
NM_000896	CYP4F3	Cytochrome P450, family 4, subfamily F, polypeptide 3	2.2	4.4	2.7
NM_005771	DHRS9	Dehydrogenase/reductase (SDR family) member 9	3.3	4.4	2.2
NM_007207	DUSP10	Dual specificity phosphatase 10	5.2	6.9	1.6
NM_024580	EFTUD1	Elongation factor Tu GTP binding domain containing 1	2.9	3.6	2.4
NM_018208	ETNK2	Ethanolamine kinase 2	2.5	4.0	1.8
NM_052966	FAM129A	Family with sequence similarity 129, member A	1.9	2.1	1.7
NM_015714	G0S2	G0/G1switch 2	3.0	6.9	1.9
NM_000402	G6PD	Glucose-6-phosphate dehydrogenase	1.9	–	2.4
NM_019593	GPCPD1	Hypothetical protein KIAA1434	2.3	2.6	
NM_001945	HBEGF	Heparin-binding EGF-like growth factor	1.9	–	1.3
NM_177551	HCAR2	Hydroxycarboxylic acid receptor 2	2.3	ND	ND
NM_006018	HCAR3	Hydroxycarboxylic acid receptor 3	2.1	4.5	1.3
NM_002165	ID1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	1.8	3.6	1.6
NM_207393	IGFL3	IGF-like family member 3	2.2	4.0	1.9
NM_000576	IL1B	Interleukin 1, β	2.1	–	1.2
NM_003856	IL1RL1	Interleukin 1 receptor-like 1	4.4	45.3	
NM_000216	KAL1	Kallmann syndrome 1 sequence	1.8	ND	ND
NM_015990	KLHL5	Kelch-like 5 (<i>Drosophila</i>)	1.9	–	1.2
NM_015596	KLK13	Kallikrein-related peptidase 13	2.8	10.7	2.3
NM_002774	KLK6	Kallikrein-related peptidase 6	9.2	21.6	23.3
NM_002276	KRT19	Keratin 19	2.0	3.4	1.6
NM_001031749	LYPD5	LY6/PLAUR domain containing 5	2.4	–	1.8
NM_173803	MPV17L	MPV17 mitochondrial membrane protein-like	1.9	–	
NM_000433	NCF2	Neutrophil cytosolic factor 2	2.4	2.7	1.2
NM_005863	NET1	Neuroepithelial cell transforming 1	2.3	3.9	1.4
NM_003489	NRIP1	Nuclear receptor interacting protein 1	2.1	2.1	1.3
NM_020190	OLFML3	Olfactomedin-like 3	2.8	7.3	1.2
NM_002564	P2RY2	Purinergic receptor P2Y, G-protein coupled, 2	2.2	4.5	2.0
NM_006474	PDPN	Podoplanin	2.0	3.1	2.1
NM_001134438	PHLDB2	Pleckstrin homology-like domain, family B, member 2	1.8	ND	ND
NM_012417	PITPNC1	Phosphatidylinositol transfer protein, cytoplasmic 1	1.8	ND	ND
NM_000930	PLAT	Plasminogen activator, tissue	2.1	–	1.6
NM_032726	PLCD4	Phospholipase C, δ 4	2.0	3.6	2.0
NM_015993	PLLP	Plasma membrane proteolipid (plasmolipin)	1.9	2.9	2.5
NM_001017368	RFFL	Ring finger and FYVE-like domain containing 1	1.8	ND	ND
NM_003003	SEC14L1	SEC14-like 1 (<i>Saccharomyces cerevisiae</i>); SEC14-like 1 pseudogene	2.0	2.1	
NM_004636	SEMA3B	Semaphorin 3B	2.0	11.9	4.2
NM_030666	SERPINF1	Serpin peptidase inhibitor, clade B (ovalbumin), member 1	6.9	17.9	1.7
NM_004170	SLC1A1	Solute carrier family 1, member 1	1.9	62.6	3.6
NM_152697	SLC44A5	Solute carrier family 44, member 5	2.1	–	2.5
NM_003615	SLC4A7	Solute carrier family 4, sodium bicarbonate cotransporter, member 7	2.2	1.9	1.2
NM_007231	SLC6A14	Solute carrier family 6 (amino acid transporter), member 14	2.7	–	1.4
NM_014467	SRPX2	Sushi-repeat-containing protein, X-linked 2	1.8	–	1.5
NM_003238	TGFB2	Transforming growth factor, β 2	3.0	6.0	1.7
NM_000361	THBD	Thrombomodulin	2.0	3.9	2.3
NM_022164	TINAGL1	Tubulointerstitial nephritis antigen-like 1	2.0	2.9	1.5
NM_024943	TMEM156	Transmembrane protein 156	3.5	26.3	7.0
NM_001042595	TMEM91	Transmembrane protein 91	2.4	6.0	2.2
NM_003330	TXNRD1	Thioredoxin reductase 1; hypothetical LOC100130902	2.8	3.7	1.6
NR_015379	UCA1	Urothelial cancer associated 1 (non-protein coding)	3.4	ND	ND

Figure 2

Catalog of candidates upregulated in human primary keratinocytes in response to 200 nM calcitriol treatment compared with response to 1 nM calcitriol. Although 67 genes were demonstrated to be upregulated within our global microarray study, only the most promising were selected for subsequent RTq-PCR confirmation. Genes that demonstrated ≥ 1.8 -fold induction and $P < 0.05$ within our 200 nM RTq-PCR approach are highlighted in light gray. Genes that have previously been identified as

(putative) calcitriol targets in various cell lines are portrayed in bold. New targets (to the best of our knowledge neither described in single-gene nor in genome-wide approaches) fulfilling our stringent criteria are shown in dark gray. ND, not determined (not explicitly chosen for RTq-PCR confirmation); –, not listed due to a fold-change < 1.8 or statistically insignificant in 200 nM/16 h RTq-PCR.

GenBank	Symbol	Gene description	MA	RTq-PCR	
			Fold	200 nM/16 h	1 nM/8 h
NM_133265	AMOT	Angiomotin	-1.8	ND	ND
NM_153000	APCDD1	Adenomatosis polyposis coli down-regulated 1	-2.0	–	-1.4
NM_003986	BBOX1	Butyrobetaine (γ), 2-oxoglutarate dioxygenase (γ -butyrobetaine hydroxylase) 1	-2.3	–	1.1
NM_016352	CPA4	Carboxypeptidase A4	-2.9	-3.3	-1.5
NM_001878	CRABP2	Cellular retinoic acid binding protein 2	-2.4	-3.0	-1.3
NM_004887	CXCL14	Chemokine (C-X-C motif) ligand 14	-4.7	-5.6	-1.6
NM_000779	CYP4B1	Cytochrome P450, family 4, subfamily B, polypeptide 1	-1.9	-2.9	-1.1
NM_004948	DSC1	Desmocollin 1	-2.0	–	-1.2
NM_001942	DSG1	Desmoglein 1	-2.3	–	-1.3
NM_014375	FETUB	Fetuin B	-2.2	-4.3	-1.4
NM_006121	KRT1	Keratin 1	-3.0	–	
NM_000421	KRT10	Keratin 10	-2.1	–	
NM_018192	LEPREL1	Leprecan-like 1	-1.8	-2.7	-3.2
NM_001102469	LIPN	Lipase, family member N	-1.8	–	-2.2
NM_014033	METTL7A	Methyltransferase like 7A	-2.6	–	-1.1
NM_002974	SERPINB4	Serpin peptidase inhibitor, clade B (ovalbumin), member 4	-2.0	–	-1.3
NM_205841	SPINK6	Serine peptidase inhibitor, Kazal type 6	-1.8	ND	
NM_020431	TMEM63C	Transmembrane protein 63C	-2.4	-4.8	
NM_003810	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	-2.0	–	-1.1

Figure 3

Catalog of candidates downregulated in human foreskin-derived primary keratinocytes in response to 1 nM vs 200 nM calcitriol exposure. For detailed color code description, refer to the caption of Fig. 2.

The majority of validated calcitriol response genes contain (predicted) VDR binding elements within their promoter regions

To evaluate whether both induced and repressed candidates represent direct, i.e. primary, VDR targets, their promoter regions were analyzed for the presence of (putative) response elements, an essential prerequisite for positioning calcitriol-bound VDR–RXR heterodimers close to the basal transcription machinery (Toell *et al.* 2000, Shaffer & Gewirth 2002). The internet-based software tool MatInspector employed for this purpose accurately calculates theoretical nuclear hormone receptor superfamily binding preferences, which are expressed as positional weight matrices (Cartharius *et al.* 2005). The nucleotide frequency templates applied comprise DR3 (random expectation: 0.14 matches per 1000 bps), bipartite DR3 (random expectation: 0.01 matches per 1000 bps), DR4 (random expectation: 0.07 matches per 1000 bps), and DR5 (random expectation: <0.01 matches per 1000 bps) arrangements. In this analysis, of our 55 RTq-PCR-validated candidates, 50 – including some established constituents for which definite VDREs have already been characterized – were found to explicitly contain at least one response element occurring without positional bias

relative to the TSS (Fig. 4). As highlighted in Fig. 1, the majority of genes carry either (bipartite) DR3 (63.5%) or DR4 (27.3%) motifs. As speculative binding sites are not sufficient to indicate definite functionality, we performed *in vitro* electrophoretic mobility agarose gel shift assays using a combination of predicted, PCR-amplified promoter regions of some randomly selected differentially expressed calcitriol response genes, plus recombinantly expressed and purified hexahistidine-tagged VDR DBD. Figure 5 shows the obtained results for *CRABP2*, *OLFML3*, *BNC2*, *TMEM91*, and *KRT19* in relation to an osteopontin-positive control DR3 containing PCR product (47.5% shift under our experimental conditions, as visible by a densitometrically quantified decrease in the amount of input DNA upon VDR DNA-BD addition, leading to a smear-like displacement) and four analogously treated negative controls not containing any VDREs. We observed definite gel shifts for *CRABP2* (33.5% shift), *OLFML3* (33.5%), and *BNC2* (31.9%) promoters, whereas *TMEM91* (14.9%) and *KRT19* (10.45) lingered considerably inferior. This test set, although to be refined and further extended in follow-up approaches, is a preliminary incidence of definite biological functionality of a realistic amount of predicted response elements *in vitro*.

Table 1 Interindividual variation of gene expression investigated by RTq-PCR. The mean interindividual variance (IV), the percentage relative s.d. across individuals in the calcitriol-stimulated or ethanol-treated control group, was experimentally determined to be ~10% for the 200 nM calcitriol/16 h and the 1 nM calcitriol/8 h configurations. The data therefore are considered to be highly reliable

Symbol	IV (%)			
	200 nM/16 h		1 nM/8 h	
	Calcitriol	EtOH	Calcitriol	EtOH
ABCA1	2.4	3.2	10.4	9.8
ABHD3	3.9	3.1	16.5	12.6
AKAP12	14.8	6.7	16.7	16.7
APCDD1	7.7	5.2	7.5	9.2
BBOX1	5.2	1.7	4.1	12.9
BNC2	7.0	12.7	4.7	7.9
CAB39	4.2	8.5	9.2	15.2
CALB2	10.2	2.4	7.2	4.9
CD14	15.8	9.7		
CD274	6.0	6.5	8.7	9.9
CD97	4.2	3.1	8.7	6.8
CDA	4.5	9.2	6.8	12.5
CHST11	7.3	7.9	4.5	7.5
CLDN7	9.6	11.4	14.9	13.9
CLMN	8.1	4.5	3.3	7.8
CPA4	3.6	7.9	13.5	19.1
CRABP2	3.5	6.5	13.3	8.8
CXCL14	31.1	43.6	15.4	15.7
CYP24A1	2.0			
CYP3A5	0.7	8.8	13.8	12.3
CYP4B1	7.3	5.8	5.6	1.6
CYP4F11	13.9	21.6	8.2	4.9
CYP4F3	9.6	9.4	1.5	3.2
DHR59	6.7	4.0	4.9	7.1
DSC1	22.6	15.2	14.0	17.7
DSG1	26.6	18.7	18.3	22.2
DUSP10	4.8	2.9	3.6	3.9
EFTUD1	2.9	8.0	15.2	12.3
ETNK2	6.6	3.3	3.3	3.0
FAM129A	9.4	11.4	19.4	17.6
FETUB	6.7	4.7	9.8	9.2
G0S2	12.3	11.4	46.5	59.7
G6PD	32.3	20.6	5.5	6.0
GPCPD1	11.1	2.3		
HBEGF	17.5	13.5	3.1	7.1
HCAR3	2.5	2.4	0.4	3.1
ID1	3.2	18.1	4.2	5.9
IGFL3	30.3	1.2	25.1	25.4
IL1B	31.8	32.0	8.5	9.6
IL1RL1	13.8	16.0		
KLHL5	3.7	4.4	10.1	15.9
KLK13	8.3	6.2	2.3	4.3
KLK6	21.8	14.1	3.4	1.7
KRT1	7.3	6.4		
KRT10	19.5	23.7	16.1	13.9
KRT19	63.4	15.2	23.9	15.6
LEPREL1	5.1	15.7	17.1	36.2
LIPN	5.4	3.3	3.5	16.7

Table 1 Continued

Symbol	IV (%)			
	200 nM/16 h		1 nM/8 h	
	Calcitriol	EtOH	Calcitriol	EtOH
LYPD5	7.9	18.9	32.2	30.3
METTL7A	9.9	9.6	4.8	3.8
MPV17L	5.2	10.5		
NCF2	4.8	9.9	2.8	4.8
NET1	24.2	6.5	23.2	25.5
NRIP1	0.7	1.6	14.0	16.7
OLFML3	13.9	2.9	16.6	14.4
P2RY2	4.6	3.4	3.9	4.3
PDPN	25.9	10.6	27.1	18.2
PLAT	0.7	10.2	5.7	2.6
PLCD4	5.7	8.5	5.7	5.2
PLCXD2	0.7	3.4	3.3	5.9
PLLP	4.0	4.0	6.4	11.8
SEC14L1	3.1	4.7		
SEMA3B	7.5	12.1	7.0	4.9
SERPINB1		22.8	8.1	7.4
SERPINB4	5.0	26.1	12.8	15.2
SLC1A1	6.1	7.3	16.0	13.7
SLC44A5	4.7	1.7	9.5	5.4
SLC4A7	10.4	4.5	8.7	11.5
SLC6A14	7.7	6.9	0.9	3.9
SRPX2	11.5	4.1	12.5	14.1
TGFB2	9.9	13.5	5.4	9.9
THBD	10.1	6.3	9.0	7.6
TINAGL1	3.1	6.6	7.2	9.5
TMEM156	2.9	4.6	5.4	9.2
TMEM63C	2.0	10.9		
TMEM91	4.3	10.0	10.0	8.4
TNFSF10	8.7	14.6	5.4	7.0
TXNRD1	23.5	13.3	13.1	14.5
Mean IV (%)	10.2	9.7	10.4	11.6

Discussion

Apart from a large assortment of single-gene investigations, several medium-scale to HTP global transcriptomic studies have been performed for deciphering (classical) calcitriol target genes and concomitant pathways (summarized in Fig. 6). The most important examples encompass primary, immortalized, or malignant prostate (Krishnan *et al.* 2004, Peehl *et al.* 2004, Kovalenko *et al.* 2010, Wang *et al.* 2011), colon (Wood *et al.* 2004), squamous cell carcinoma (Akutsu *et al.* 2001, Lin *et al.* 2002, Wang *et al.* 2005), myelocytic/monocytic leukemia (Savli *et al.* 2002, Suzuki *et al.* 2006, Heikkinen *et al.* 2011), ovarian (Zhang *et al.* 2005), coronary artery smooth muscle (Wu-Wong *et al.* 2006), (pre-) adipocytic (Sun *et al.* 2008), lymphoblastoid (Ramagopalan *et al.* 2010), or breast cancer (Swami *et al.* 2003, Kriebitzsch *et al.*

Gene	Matrix family	From	To	Anch or	Matrix sim.	Strand	Consensus sequence
ABCA1	DR4	276	300	288	0.809	+	gagaGAGGagatggaagggcagggga
	DR3	512	536	524	0.858	+	cactttgggagggtGAGGgcagagg
	DR3	1297	1321	1309	0.862	-	tttgggaggccaagGTGGgcagctc
	DR3	2432	2456	2444	0.877	-	gggggtgaggggaagGCGGacagtcc
ABHD3	Bipartite DR3	1479	1503	1491	0.831	+	ccagcctGCTCacatggtgaaacc
AKAP12	DR3	2323	2347	2335	0.854	+	ttcccgggggaagGGGGccggcta
	Bipartite DR3	2639	2663	2651	0.819	-	aatgatgGATCaccaaggcaggag
	DR4	2771	2795	2783	0.821	+	acggGAGGcggccgagggccgggtg
	DR4	2917	2941	2929	0.817	+	ggggGAGGggggcgaggctaagag
BNC2	Bipartite DR3	4249	4273	4261	0.800	-	tcattggGCTCcatcatctcatcat
	DR5	5842	5866	5854	0.829	+	aagggtggtcagtggcAGGAtagtc
CAB39	DR4	2730	2754	2742	0.802	+	acgcGAGGcagagaggggaacgcc
	DR4	2819	2843	2831	0.808	-	cggtGAGGgcccagcggggcaggcg
CALB2	Bipartite DR3	325	349	337	0.858	+	tagaaagGTTCaagaggttaata
	DR3	576	600	588	0.877	+	gtttaagggtcactGTGGccaccct
	DR3	734	758	746	0.871	-	tgtgttgggtgagaGAGGcatccc
	Bipartite DR3	1295	1319	1307	0.821	-	gtgggcaGATCacgaggtaagaga
CD14	Bipartite DR3	2989	3013	3001	0.812	+	ccgaagaGTTCacaagtgtgaagcc
CD274	DR3	627	651	639	0.919	-	ggggcagggtcttgGAGGtcaacat
	Bipartite DR3	1291	1315	1303	0.821	+	attcataGATCaccagccacta
	Bipartite DR3	1735	1759	1747	0.816	-	ctttgaGCTCacatagtagaata
	DR3	2127	2151	2139	0.891	+	cacttgagggtcagGAGTtcgagaa
CDA	Bipartite DR3	1702	1726	1714	0.809	-	cagccaaGATCacatagggcagaac
	Bipartite DR3	2061	2085	2073	0.885	+	aactgtgGTTcagggaggtcaagt
CHST11	DR3	184	208	196	0.854	+	gagggtcaggagatcGAGAccatcct
	Bipartite DR3	1344	1368	1356	0.905	+	ggtgagaGTTcacttggtgacctg
	DR4	1505	1529	1517	0.812	+	gttgGAGGgtggggaggggtgcagt
	DR3	2423	2447	2435	0.918	-	gagtcgggggttaaaGAGGgtgact
CLDN7	DR3	108	132	120	0.854	+	aagggtcaggagatcGAGAccatcct
	DR4	924	948	936	0.832	+	ttggGAGGtcagggtgggtgggat
	DR4	1397	1421	1409	0.831	+	ctgaGAAGtatgaggaggttgagg
	DR5	2218	2242	2230	0.818	-	agcaggggtgagggtcAGGTcagagt
CLMN	DR4	544	568	556	0.842	+	tggaGAAGtctctgaggtgcacatg
	Bipartite DR3	692	716	704	0.877	+	actgctgGCTCaaaaagtgtagagc
	Bipartite DR3	1450	1474	1462	0.806	-	aagggtggGATCagtggaagcagaag
	DR5	1726	1750	1738	0.805	-	atggagggtccccaAGGAcagacc
CYP3A5	Bipartite DR3	1892	1916	1904	0.800	+	aactggaGCTCcatctgttaagtga
CYP4F3	DR3	2149	2173	2161	0.901	-	tagcgggggacaatGAGGgtccta
	DR4	3012	3036	3024	0.814	+	gagaGAGGgttgtgtgggacaaggt
DHRS9	DR4	106	130	118	0.820	-	gtgaGAAGtattataggggaatta
	DR4	1094	1118	1106	0.816	+	atcGAAGtcaatgtaggatgagtt
DUSP10	DR4	368	392	380	0.804	-	agggGGGgtgggcagggggtggggc
	DR4	583	607	595	0.861	+	agggGAAgtgagggtgggatgagcc
	DR3	2274	2298	2286	0.861	-	ccgtcccggacaggGAGGcccttca
EFTUD1	DR3	341	365	353	0.854	+	aaagtcaggagatcGAGAccatcct
	DR3	1422	1446	1434	0.953	+	gagggtcaggagatGAGGccaacat
ETNK2	DR3	5	29	17	0.866	-	ctgcctgggtggcaGAGGgtctcag
	DR4	492	516	504	0.811	+	cagaagccagctctgaGTTcagact
	DR4	544	568	556	0.893	-	gaggGAGGttttgcaaggacaagag
	DR3	691	715	703	0.868	+	cagggtgggaaactGAGGccagag
	DR4	1466	1490	1478	0.813	+	ttgaGAGGcggaggaggggtgatca
	DR4	2945	2969	2957	0.800	+	cgggGGCgtcccgggccaccgc
	DR4	3078	3102	3090	0.867	-	gggaGTGtagaggaggggccaggg
FAM129A	DR3	547	571	559	0.861	-	gagatcaggagatGAGAccatcct
G0S2	DR3	118	142	130	0.936	-	agacacaggggaaaGAGGtctttg
	Bipartite DR3	2488	2512	2500	0.824	-	ggcaatgGATCagagaggagaaggc

Figure 4 (continued)

GPCPD1	DR5	1737	1761	1749	0.824	–	gtggaggttacagtgAGCTgagatc
	DR3	2846	2870	2858	0.864	+	ctgagaagggcagcGGGGacacggg
HCAR3	Bipartite DR3	33	57	45	0.831	–	ccagcctGCTCaacatggtgaaac
ID1	Bipartite DR3	1705	1729	1717	0.866	+	tattaggGCTCagagagggcatgcg
	Bipartite DR3	2925	2949	2937	0.864	–	ggggcggGCTCagaacgttcaaaag
IGFL3	DR5	2801	2825	2813	0.801	–	tttgaggtgactagtAGGGcaccagg
IL1RL1	Bipartite DR3	734	758	746	0.844	+	aaagctgGTTCaatgagcttagatt
KLK13	DR5	147	171	159	0.804	–	gctctgggtgaagtGAGGTCaaggga
	DR5	211	235	223	0.807	–	tgagggggtgagtgccAGGTcaaatt
	DR4	443	467	455	0.826	+	ctgtGAGGtgggagaagtcagggt
	DR4	452	476	464	0.821	+	gggaGAAgtccagggtgggagaaga
	DR5	476	500	488	0.821	–	ctcaaggtgaagaaAGGTcactgt
	Bipartite DR3	507	531	519	0.818	–	tggtgggGCTGagctgggtcatacc
	DR4	685	709	697	0.802	–	gagGAGgtatctagggtataagtt
	DR5	975	999	987	0.830	–	aagtggctcaagacAGGAaagggt
	DR3	1672	1698	1684	0.880	–	cagaggggggtgaggGAGTccacggg
	DR4	2115	2139	2127	0.827	+	ggtGAAgttagggaaggcatgga
	DR5	2144	2168	2156	0.801	+	agcggggtagggggaAGGAaagggg
	DR4	2342	2366	2354	0.800	+	gagaGGGgaggccaggaccagga
	DR3	2376	2400	2388	0.865	+	tgagctgggggaggGAGTtcagggg
	DR3	2503	2527	2525	0.878	+	cggaggagggcaggGAGGggagaga
	Bipartite DR3	3049	3073	3061	0.804	–	gggaggcGATCactagggccagggg
	DR3	3061	3085	3073	0.893	–	aggccaagggtcaggGAGGcgtcac
KLK6	DR4	424	448	436	0.835	+	gagaGAGGaggcagagggatagggc
	DR3	513	537	525	0.860	+	gtgctgaggtcagttGGGgaccaa
	DR4	1094	1118	1106	0.841	–	ctggGAGtagcagagggatctggg
	DR3	2227	2251	2239	0.904	+	ctcctggggacacaGAGGtcggcag
	Bipartite DR3	2857	2881	2869	0.877	+	ggtcaggGCTCaccaagagcagagc
KRT19	DR4	155	179	167	0.824	–	cagaGATGgaactggaggicaagct
	DR3	761	785	773	0.874	+	ctctgaagggtaggGAGCtcagaac
	Bipartite DR3	1452	1476	1464	0.891	+	ggacgggGCTCactggggacaggaa
	Bipartite DR3	2387	2411	2399	0.831	+	ccagcctGCTCaacatggtgaaacc
	DR5	2834	2858	2846	0.809	+	cctctggtctctgggAGGgagggga
NCF2	DR5	701	725	713	0.803	+	ttggcagtcagattaAGGAagattc
	DR3	1750	1774	1762	0.857	+	gaggggaggccaggGAGGccgaatc
	Bipartite DR3	2586	2610	2598	0.808	+	cactctaGCTCacgggttcaattg
NET1	DR3	929	953	941	0.887	–	gcaaagagggttaagGAGGgtatgaa
	Bipartite DR3	2689	2713	2701	0.829	–	ggaataaGTTcagtaggacaactt
	Bipartite DR3	2825	2849	2837	0.867	–	aagctggGTTCaagtaggacagaaa
NRIP1	Bipartite DR3	997	1021	1009	0.827	+	attgggaGTTcagtaggaatagtgt
OLFML3	Bipartite DR3	579	603	591	0.848	–	aaagcagGCTCagaatggccagaac
	Bipartite DR3	2128	2152	2140	0.886	–	gaggaggtGTTcagtaggttcacact
P2RY2	DR3	49	73	61	0.875	+	tggcctgggtcttcGAGGaccatg
	DR3	867	891	879	0.891	–	atcgctgggttcaGAGGccatcaa
	DR4	2385	2409	2397	0.813	+	gagGCGGtggaccagagagaagag
	Bipartite DR3	2404	2428	2416	0.832	+	gaagagaGGTAaagcagctcagat
PLCD4	DR3	2791	2815	2803	0.932	+	ggattggggtcaatGAGGgctggca
	Bipartite DR3	3076	3100	3088	0.849	–	gctgtggGATCaggaaagcccagcct
PLL	DR3	146	170	158	0.866	–	atttctgggttagGTTGGTcaagga
	Bipartite DR3	1563	1587	1575	0.807	–	caccagaGTTcagtagttgagacc
	Bipartite DR3	2167	2191	2179	0.865	–	gaggaggGCTCactcagaagatggg
SEC14L1	Bipartite DR3	44	68	56	0.857	+	gctgattGGTcagggaggaaatcat
	DR4	209	233	221	0.840	+	aggaGCAgttagggagggtcagaa
	DR4	548	573	560	0.800	+	gggtGAGGtgcagtagccaagat
	DR5	1599	1623	1611	0.800	–	ttgggagtttagggcAGGAaagg
	DR3	1708	1732	1720	0.854	–	gaggtcaggagatcGAGAccatcct
	DR4	2555	2579	2567	0.817	–	aaggGAAgtttcgggagggtcaaaa

Figure 4 (continued)

SEMA3B	DR4	301	325	313	0.853	—	ccgcGAAGGtgaagtgggctatgc
	DR3	884	908	896	0.882	—	taggggagggagagGAGGgaggag
	DR4	1546	1570	1558	0.816	+	cggtCGAGctgtgagaaggcagccag
	DR3	1579	1603	1591	0.862	—	cttctcagggttctaGAGGtctcca
	Bipartite DR3	1958	1982	1970	0.841	+	aaggccaGCTCagcgaggccaaggag
	DR3	2634	2658	2646	0.851	—	tgtgaaagggggacaGAGTgcaca
SERPINB1	DR3	2908	2932	2920	0.851	—	gcccctaagggaagGAGGctggggg
	DR3	3051	3075	3063	0.802	—	gctcagggtgctcaggGTTcagcgg
	DR3	1357	1381	1369	0.879	+	agtgcacaggaccctGAGGgcagaat
SLC1A1	DR3	2433	2457	2445	0.850	+	ggtgactggattatGAGGgcacatt
	DR3	1563	1587	1575	0.858	—	gaggtcaggagattGAGAccatcct
SLC4A7	DR3	2232	2256	2244	0.803	+	atgctccagggccctGTGGTcacaga
	DR4	198	222	210	0.806	+	gagtcGAAGagaccagggatcaaat
TGFB2	Bipartite DR3	2196	2220	2208	0.829	—	tctgataGTTTcaaaagctcaaaa
	DR3	2554	2578	2566	0.854	+	aggagcaggagagagGAGGgagctgg
THBD	Bipartite DR3	28	52	40	0.841	+	ggtgaagGCTCacaagtgcacatgg
	DR5	1243	1267	1255	0.827	+	caggggggtcgcgctGAGGgagggcg
	DR4	1398	1422	1410	0.809	+	ctggCGAGctgtgcggcgagcagtc
	DR3	1656	1680	1668	0.855	—	gggttaagggttagGAGCacagagt
	DR4	2091	2115	2103	0.811	+	tgtGGGGtgcaagaagccaccatcc
TINAGL1	Bipartite DR3	20	44	32	0.806	+	cggaaggGCTCagagggggaagtct
	DR3	189	213	201	0.854	+	aaggtcaggagatcGAGAccatcct
	DR5	438	462	450	0.824	+	ctaggagtcactaggAGGCaaagg
	Bipartite DR3	1655	1679	1667	0.811	+	gactaagGCTCactagggglaaagg
	Bipartite DR3	2428	2452	2440	0.832	+	aacaaggGATCaaagggtgcagagg
TMEM156	DR3	2435	2459	2447	0.866	+	gatcaaaagggtgcaGAGGccacccc
	DR3	54	78	66	0.862	+	aaaactggggcgggGAGGccagggtg
	DR4	107	131	119	0.870	+	tggGAGGtgagggtgggatgattg
	DR4	679	703	691	0.836	—	tagcGAAGtcaagtaagggtctaaa
TMEM91	DR3	246	270	258	0.894	—	tttggagggtctctGAGGtcaggag
	DR4	821	845	833	0.806	—	gatgGTGGtgacctggggccaagaa
	DR4	1456	1480	1468	0.842	—	ggcaGAAGtgcagtgggccgagat
	DR4	1494	1518	1506	0.802	—	tcggGAGGctgaggcaggacaatca
TXNRD1	DR3	1660	1684	1672	0.830	+	atgaccctgttcaaggGGTCattcg
	Bipartite DR3	1669	1693	1681	0.842	+	gttcattGGTCattccgtagactaa
CPA4	DR3	2281	2305	2293	0.858	—	gaggtcaggagattGAGAccatcct
CRABP2	Bipartite DR3	18	42	30	0.837	—	ccgctggGATCataaggagagga
	DR4	873	897	885	0.835	+	cagtcGAAGggaaggaggggcagcag
	DR3	977	1001	989	0.870	—	tgctgtagglaagGAGGgggatta
	DR3	1134	1158	1146	0.862	—	ggcagcagggtcagGAGGtctctga
	Bipartite DR3	2031	2055	2043	0.801	+	ttttacaGCTCaggatggtgacacc
	DR3	2519	2543	2531	0.824	+	ctggatccagttcaggGTTCaatgg
CXCL14	DR3	104	128	116	0.865	—	ctaactgggtgaggGAGGgggcttg
FETUB	DR3	1803	1827	1815	0.809	—	gaaataactgtccaaGGTCacaga
	DR4	2650	2674	2662	0.827	—	tggcGAGGctgtgtaagggcactgc
	Bipartite DR3	126	150	138	0.852	—	agggagaGATCagacaggtgagaca
	DR4	1111	1135	1123	0.829	+	ccgtCCAGtggaagaggttgaattt
	Bipartite DR3	3059	3083	3071	0.843	+	caggatgGCTCagttgtggagctg

Figure 4

In silico prediction of putative VDR–RXR heterodimer binding sites within the promoter regions of RTq-PCR-validated calcitriol target genes. Columns specify applied matrix concepts, relative position/orientation with respect to the relevant TSS, calculated statistics, and matching consensus motifs. Marked in dark gray are the predicted (bipartite) DR3 elements that have been analyzed for definite recombinant VDR–DBD binding in agarose gel

shift assays. Nucleotides highlighted in black within the ‘consensus sequence’ column exhibit a high informational content, i.e. the matrix reveals high conservation (consensus index values > 60) at this position. Nucleotides given in capital letters denote the core sequence routinely exploited by MatInspector.

2009, Vanoirbeek *et al.* 2009) (derived) cell lines. To date, only two studies have been truly devoted to ‘healthy’ skin cells. The first was accomplished by Lu *et al.* (2005) who profiled differential expression among 12 600 constituents in HPV immortalized keratinocytes compared with

‘normal’ human keratinocytes. The second study, by Moll *et al.* (2007), applied a set of 2135 sequence verified ESTs (explicitly enriched in transcription factors, signal transducers, and disease effectors) to identify calcitriol-governed genes within foreskin-derived primary

Figure 5

Electrophoretic mobility shift assays demonstrating the *in vitro* functionality of five MatInspector-predicted VDR-binding sites within some novel calcitriol response genes. Shown are PCR-amplified promoter fragments (denoted as input) for *CRABP2*, *OLFML3*, *BNC2*, *TMEM91*, and *KRT19* in relation to osteopontin (OPN)-positive control and four different negative control PCR products designated as C7, D1, A12, and G8. Shown are the

differences before (assumed as a 100% PCR band intensity) and after addition of recombinant VDR-DBD. Actual shifts (obvious decreases in the amount of input DNA, leading to a smear-like appearance) in promoter (PCR band) intensity following DBD incubation are marked by arrows. Values calculated after ImageJ quantification are given below.

keratinocytes. However, it is apparent from our literature survey that the basic strategies not only employed vastly diverse cell lines derived from numerous tissue models but also vary in application of in-house-prepared vs commercially available microarrays. Gene sets range from fairly restricted to genome spanning. There is significant variation in underlying sample labeling protocols, hybridization conditions, imaging quantification, and data filtering and interpretation (set cutoff values). Differences in detected differential regulation ranged from the per mill interval to as much as 7% (Moll *et al.* 2007) or 11.1% (Farach-Carson & Xu 2002) of the investigated genes. These obvious disparities might, to a certain extent, be due to the fact that early efforts often lacked biological sample replication and statistical normalization necessary to eliminate false-positive artifacts. Because of cell-type and tissue-specific phenomena – tumor cells might exhibit reduced vitamin D responses, for instance, by displaying altered nuclear corepressor levels (Khanim *et al.* 2004) – or transient responses (Zhang *et al.* 2005, Carlberg & Seuter 2009, Carlberg 2010), these parameters not only complicate direct mutual comparative assessments but might explain the limited agreement between the resultant catalogs of experimentally detected calcitriol targets (Tavera-Mendoza *et al.* 2006). Nonetheless, from the published studies, the number of reliable (primary) response genes can be estimated to range somewhere between 250 (Palmer *et al.* 2003, Carlberg 2010) and 1000 (Lin *et al.* 2002), not taking into account the plethora of secondary, delayed constituents that might contribute to the observable broad physiological actions. In one way or another, recognized targets appear implicated in cell cycle

progression/arrest, transcriptional regulation, DNA replication, DNA repair, growth/proliferation, differentiation, apoptosis/survival, oxidative stress defense, immunity, barrier function, vitamin D metabolism, cell communication, lipid metabolism, cytoskeletal organization, cell adhesion, extracellular matrix constitution, membrane transport, and cytosolic Ca^{++} /redox homeostasis, etc. – all are inherent properties of both skin homeostasis and pathogenesis (White 2004, Tavera-Mendoza *et al.* 2006). Except for *CYP24A1*, an established marker of (metabolically time frame limited) calcitriol activity (Swami *et al.* 2003, Peehl *et al.* 2004, Wood *et al.* 2004, Zhang *et al.* 2005), only a handful of candidates have been identified by multiple research groups. Thus far, the available data fail to delineate the precise distribution, diversity, and entirety of affected targets, emphasizing the necessity to continue calcitriol-mediated gene expression studies if we are to be able to explain the entire spectrum of calcitriol-provoked activities.

In the current report, we present a substantially expanded ‘hit list’ of 55 specific calcitriol response genes that are differentially expressed upon calcitriol exposure within human primary keratinocytes isolated from three different individuals. Included are nine (16.36%) calcitriol response genes previously known from human primary keratinocytes, as well as 46 (83.6%) novel calcitriol response genes (33 of which have, parenthetically, previously been described in other settings) whose definite modulation was independently confirmed and statistically assessed. Our chosen calcitriol concentrations (1 nM/200 nM) and stimulation times (8 h/16 h) hereby represent a literature-based compromise between a 2- and

Test system	Employed array platform	Obtained results	Overlap to our study	Reference
Squamous cell carcinoma lines	Atlas Array with 588 genes (Clontech), GF211 Named Human 4000+ filters (Research Genetics, Huntsville, AL, USA)	38 upregulated targets, including multiple growth/transcription factors, adhesion molecules, as well as kinases	None	Austuk <i>et al.</i> (2001)
	Affymetrix 6800+ HuGene FL human gene oligonucleotide chips	Expression profiling with the low-calcemic analog EB1089 identified 89 up- and 63 downregulated targets. No evidence for gene-specific differences in efficacy to calcitriol was identified. Genes control signaling, G protein-coupled receptor function, redox balance, adhesion, cell cycle progression, steroid metabolism, and immune system functions	CD14↑, SEC14L1↑, NET1↑, DUSP10↑, G0S2↑, TXNRD1↑, TGFB2↑	Lin <i>et al.</i> (2002)
	Affymetrix Hu133A 35 000+ oligonucleotide arrays, combined with screens for consensus DR3 and ER6 elements	Of 913 identified genes, 734 were induced and 179 repressed. VDREs lying within -10 to +5 kb relative to the 5' TSS could be assigned to 65% of them	ABCA1↑, CD14↑, CAB39↑, CD274↑, CD97↑, CDA↑, CHST11↑, CLMN↑, NRIP1↑, CXCL14↓, ABHD3↑, CYP3A5↑, DUSP10↑, CPA4↓, EFTUD1↑, ETNK2↑, G0S2↑, KLK13↑, KLK6↑, NET1↑, P2RY2↑, SEMA3B↑, PLLP↑, SEC14L1↑, TGFB2↑, IL1RL1↑, SLC4A7↑, SERPINB1↑, SLC1A1↑, TXNRD1↑, CYP24A1↑, AKAP12↑, APCDD1↓, G6PD↑, HBEGF↑, KLHL5↑	Wang <i>et al.</i> (2005)
LnCaP prostate cancer cells	Stanford University in-house-established array with 23 078 constituents (among them 18 433 sequenced clones)	18 upregulated genes (with maximum increases mostly seen at 24 h), four downregulated genes. Putative targets appear to be involved in growth regulation, DNA repair, differentiation, cell-matrix interactions, membrane transport, and inhibition of metastasis	None	Krishnan <i>et al.</i> (2004)
	Nimblegen human HG18-4plex whole-genome microarray, Agilent human miRNA v3 (Sanger release 12.0) microarray	Gene list containing 1127 entities that are modulated by either testosterone (326), calcitriol (825), or both compounds additively (280). Affected biological processes encompass mitosis, DNA repair/recombination, elevation of cytosolic Ca ²⁺ , as well as phosphoinositide-mediated signaling	CD14↑, CD274↑, CLMN↑, CYP24A1↑, CYP3A5↑, KLK6↑, AKAP12↑, TGFB2↑, EFTUD1↑, SEC14L1↑, ABHD3↑, PLLP↑, DSC1↓, METTL7A↓, G6PD↑, HBEGF↑	Wang <i>et al.</i> (2011)
Normal, immortalized, or malignant prostate epithelial cells	Affymetrix U133 Plus 2.0 GeneChip covering over 47 000 transcripts plus splice variants. Four analyses per time and dose	1571, 1816, and 3566 transcript level alterations at 6, 24, and 48 h at a 5% false discovery rate. 288 genes were similarly targeted at all time intervals (182 up-, 80 downregulated)	G0S2↑, ABCA1↑, CD14↑, TINAGL1↑, CDA↑, CPA4↓, CYP24A1↑, CLMN↑, PDPN↑, TXNRD1↑, CAB39↑, CD97↑, CLDN7↑, CHST11↑, KLK6↑, SLC4A7↑, DUSP10↑, NET1↑, EFTUD1↑, THBD↑, PLLP↑, GPCPD1↑, ETNK2↑, ID1↑, FAM129A↑, P2RY2↑, NRIP1↑, PLCD4↑, CXCL14↓, SEC14L1↑, TGFB2↑, SEMA3B↑, SERPINB1↑, SLC1A1↑, LEPREL1↓, AKAP12↑, HBEGF↑, KLHL5↑, IL1B↑, CYP4F11↑, G6PD↑, APCDD1↓, LYPD5↑, SLC44A5↑, METTL7A↓	Kovalenko <i>et al.</i> (2010)
	Array composed of 24 192 spots with 23 078 elements containing DNA. 18 433 clones were sequenced to verify their identity. Over 14 000 spots represented uncharacterized ESTs	48/38 genes upregulated and 24/39 genes downregulated after 6/24 h of calcitriol exposure in normal prostatic epithelial cells. 27/37 genes upregulated vs 14/139 genes downregulated in prostate cancer-derived epithelial cells	CYP24A1↑, DUSP10↑, TXNRD1↑, TGFB2↑, NET1↑, AKAP12↑, IL1RL1↑, NRIP↑	Peehl <i>et al.</i> (2004)
	Array containing 2000 cancer-related constituents representing 18 functional families (in-house spotted in duplicates on four grids)	51 upregulated (cell adhesion molecules, kinases, steroid receptors, growth factors, cytokines, and transcription factors) and 19 reliably downregulated genes	CYP24A1↑, TGFB2↑, TXNRD1↑	Swami <i>et al.</i> (2003)
MCF-7 and MDA MB 231 breast cancer cell lines	Affymetrix human genome U133 Plus 2.0 microarray	Cluster containing 142 genes (involved in lipid metabolism, apoptosis, proliferation, transcription, cytoskeleton organization, immune response, ECM maintenance, transport, or ubiquitination) upregulated by both calcitriol and a superagonistic 17-methyl-D-ring WY1112 analog	CYP24A1↑, TGFB2↑, CD14↑, CD97↑, DUSP10↑, KLK6↑	Vanoir beek <i>et al.</i> (2009)

Figure 6 (continued)

Monocytic/ promyelocytic leukemia cells	Atlas hematology filters containing 406 genes (Clontech Laboratories)	43 differentially expressed genes (18 upregulated and 25 downregulated candidates)	CD14[↑] , <i>IL1B[↑]</i>	Savli <i>et al.</i> (2002)
	IntelliGene HS Human Expression CHIP (Takara Biomedicals, Inc., Tokyo, Japan) with 16 600 genes	583, 852, and 667 genes with more than a twofold intensification (many correlated with differentiation-associated changes toward monocyte/macrophage lineages) and 1403, 655, and 1645 genes with a two fold decrease after 24, 48, or 72 h	CDA[↑] , CD14[↑] , SERPINB1[↑] , NCF2[↑] , <i>G6PD[↑]</i> , <i>IL1B[↑]</i>	Suzuki <i>et al.</i> (2006)
	Genome-wide ChIP-Seq mapping of VDR-binding sites, Sentrix Human-6 v2 Expression Bead chips (Illumina, San Diego, CA, USA)	Ligand treatment revealed 638 calcitriol target genes enriched in GO categories associated with immunity and signaling. Of 408 upregulated genes, 72% showed VDR binding within 400 kb of their TSS; 43% of 239 downregulated candidates showed VDR binding within 400 kb of their TSS	CD14[↑] , CD97[↑] , CLMN[↑] , THBD[↑] , EFTUD1[↑] , PDPN[↑] , DUSP10[↑] , G0S2[↑] , NCF2[↑] , P2RY2[↑] , TXNRD1[↑] , NRIP[↑] , <i>HBEGF[↑]</i> , <i>TNFSF10[↓]</i>	Heikkinen <i>et al.</i> (2011)
Ovarian cancer OVCAR3 cell line	Initial Affymetrix U95Av2 vs U133A (containing 22 283 probe sets) gene chips	Catalog of 58 genes upregulated (implemented in cell cycle, apoptosis, signaling, tumor suppression, and transcription); 38 genes downregulated	CYP24A1[↑] , DUSP10[↑] , KLK6[↑] , <i>G6PD[↑]</i>	Zhang <i>et al.</i> (2005)
Coronary artery smooth muscle cells	Affymetrix U133Av2 microarray with more than 22 000 probe sets	Using a cutoff value of 2, 116 genes were found to be significantly upregulated and 60 to be downregulated. Top functional DAVID clusters encompass GO categories like signal transduction, cell communication, growth, or differentiation	TGFB2[↑] within the differentiation/proliferation specific GO categorization	Wu-Wong <i>et al.</i> (2006)
Colon carcinoma cell line	Affymetrix HG-U95Av2 cDNA array harboring 12 625 distinct sequences (corresponding to about 11 300 unique GenBank accession numbers)	Only 12 genes, among them two previously known candidates, demonstrated statistically significant changes in expression of two fold or greater	CYP24A1[↑]	Wood <i>et al.</i> (2004)
	Affymetrix HG-U95A array with >12 665 features for individual known genes and ESTs	81 upregulated and 20 downregulated genes after 4-h exposure, with only little overlap to the list retrieved after 48 h. Genes play roles in transcription, cell adhesion, DNA synthesis, redox status, translation, cytoskeleton, adhesion, apoptosis, signaling, metabolism, and transport	G0S2[↑]	Palmer <i>et al.</i> (2003)
Primary vs immortalized epidermal keratinocytes	Non-redundant, small-scale set of 2135 pre selected, sequence-verified EST clones. Two in-house-spotted nylon membrane filter arrays per time point	86/43 positively and 50/1 negatively regulated targets after 12/24 h. Genes are involved in intracellular signaling, cell cycle, transcription, metabolism, growth, constitution of ECM, cytoskeleton, apoptosis, immune response, as well as DNA repair	CYP24A1[↑] , NET1[↑] , G0S2[↑] , IL1RL1[↑]	Moll <i>et al.</i> (2007)
	Duplicate Affymetrix HG-U133A GeneChips (about 12 600 genes)	82 genes were upregulated and 16 were downregulated. Many candidates appear implemented in immune response, differentiation, and proliferation	CD14[↑] , KLK6[↑] , KLK13[↑] , SERPINB1[↑] , SEMA3B[↑] , DUSP10[↑] , IL1RL1[↑] (as far as accessible on the web)	Lu <i>et al.</i> (2005)
Human (pre-) adipocytes	Affymetrix HG-U133A array	237 vs 360 candidates differentially responding to either calcitriol or a calcitriol/cortisone combination. Targets are involved in angiogenesis, cell cycle, proliferation, inflammation, or oxidative stress management	CYP3A5[↑] , EFTUD1[↑] , NRIP[↑] , SLC1A1[↑] , SLC4A7[↑] , PLAT[↑]	Sun <i>et al.</i> (2008)
Lymphoblastoid cell lines	ChIP-seq defined genome-wide map of VDR occupation, Affymetrix GeneChip Human Exon 1.0 ST Array	Identification of 2776 genomic positions occupied by VDR (enriched near autoimmune and cancer-associated genes) plus 229 candidates with significant changes in gene expression (226 upregulated, three downregulated)	CAB39[↑] , CD274[↑] , CD97[↑] , CLMN[↑] , DUSP10[↑] , EFTUD1[↑] , SERPINB1[↑] , TMEM156[↑] , CYP24A1[↑]	Ramagopalan <i>et al.</i> (2010)

Figure 6

Medium-scale to HTP genome-wide microarray studies examining differential expression of human calcitriol targets. Individual columns depict employed cell lines, applied commercial vs in-house-established array platforms, obtained results, and evident (if any) overlap to our present work. The only two previous approaches definitely focusing on

keratinocytes are highlighted in gray. Concentration and induction times typically ranged around 30–50 nM calcitriol and 4, 6, 12, 24, 36, 48, or 72 h. ↑ and ↓ denote comparable fold-changes (log₂) or gene expression ratios as published within the respective studies. Previously reported candidate genes that were not confirmed in this study are shown in italics.

6-h interval (Heikkinen *et al.* 2011) and a 24- to 72-h range (Suzuki *et al.* 2006) for obtaining reasonable up- or downregulation of genes that can still be considered as primary targets (Lin *et al.* 2002, Carlberg 2010). From our in-house-performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay that may – under applied experimental conditions – reflect the number of viable, proliferating cells by measuring the activity of cellular enzymes reducing the respective MTT dye, we can parenthetically estimate that 62.91% of primary keratinocytes are metabolically active at the 200 nM calcitriol dose (half maximal effective concentration EC_{50} of 908.8 nM, [Supplementary Figure 1](#), see section on supplementary data given at the end of this article). Consistent with both high(er)-quality single-gene approaches and with earlier microarray studies performed in diverse (tumor tissue derived) cell types, our results reveal fewer genes downregulated than upregulated (Vanoirbeek *et al.* 2009). Employing a RTq-PCR fold-change cutoff of 1.8 or greater, we expect our data to correlate with results from complementary test arrangements. However, several acknowledged targets, including APCDD1, G6PD, HBEGF, KLHL5, DSC1, METTL7A, IL1B, CYP4F11, TNFSF10, LYPD5, or SLC44A5, fell through our rigorous, unbiased *post hoc* data filtering, which distinguishes verifiable findings from false-positive fluctuations. Our RTq-PCR confirmation of 55 differentially expressed calcitriol response genes at 200 nM/16 h, as well as the demonstration of comparable tendencies of gene regulation plus the confirmation of at least 45% of the genes as calcitriol targets using 1 nM calcitriol for 8 h, nonetheless affirms the potency of our streamlined experimental design while simultaneously underscoring the legitimacy of our 46 novel (among them 13 entirely new) candidates. Because we measured changes in gene expression at only one concentration of calcitriol at a single time point for microarray analysis and reevaluated only 78 of the 86 original candidates derived from the whole-transcriptome microarray chip at two concentrations, our confirmed list of regulated genes likely reflects an underestimate of the total number of calcitriol targets in primary keratinocytes.

Lastly, our RTq-PCR-evaluated data have been complemented by literature-inspired (Wang *et al.* 2005, Carlberg & Heinäniemi 2010), weighted nucleotide distribution matrix-based predictions that assign (near) consensus nuclear hormone receptor superfamily response elements (Cartharius *et al.* 2005). As the affinity of a monomeric VDR molecule to a single-core binding motif is not sufficient to allow optimal DNA binding,

transcriptional control of primary calcitriol targets is preferentially achieved by association of VDR–RXR heterodimers to the major groove of classical DR3-type (i.e. spaced by three intervening nucleotides) repeats of the hexamer sequence RGKTS_A (where R is A or G, K is G or T, and S is C or G) (Shaffer & Gewirth 2002, Carlberg *et al.* 2007). Furthermore, effective linkage has also been observed on DR6, DR5, and DR4 VDREs as well as on cis-acting everted repeat structures with seven to nine intervening nucleotides, arrangements that are not decidedly included in the exploited prediction profile (Toell *et al.* 2000). Using weighted nucleotide distribution matrix-based *in silico* predictions, we have been able to uncover putative direct VDR-binding sites within the promoter regions of 50 (90.9%) of our RTq-PCR-confirmed candidates. Of the 13 entirely new calcitriol target genes identified by our study, 11 reveal variable numbers plus combinations of response elements, with (bipartite) DR3s actually prevailing. Definite recombinant VDR-DBD binding to (bipartite) DR3 repeats could be confirmed for three out of five randomly selected candidates genes and thereby provides us with both a level of further validation and a putative mechanism of regulation. Nevertheless, definite VDRE uncovering does not signify unambiguous response element functioning *in vivo*, as VDRE may be covered by nucleosomes (especially elements in locations isolated from transcription factor ‘modules’), while maintenance of responsiveness may, on the other hand, not demand strict binding site conservation (Toell *et al.* 2000, Carlberg & Seuter 2009).

In conclusion, our study suggests that the role of calcitriol in human primary keratinocytes is more complex than perhaps formerly anticipated. It also illuminates how continuing, sophisticated endeavors can significantly contribute to a further description of novel calcitriol targets – information that is indispensable for our enhanced understanding of underlying (regulatory) pathways and associated disease phenotypes at the molecular level. We nevertheless have to acknowledge some apparent restrictions of this study, namely its absolute *in vitro* nature and its probably limited representativeness of biological data. A better, physiologically more relevant, complementation for future corroboration of this first overall impression on novel calcitriol response genes could be to isolate keratinocytes from patients treated with different calcitriol doses and align with existing *in vitro* data. Although whole-transcriptome microarrays were admittedly performed with RNA isolated from only one single individual, the majority of differentially regulated calcitriol target genes could nonetheless be confirmed in a complementary RTq-PCR setup

averaged over three/six individuals. Because calculated interindividual variation has been determined to lie in the 10% range, our combined data represent a consistent compromise – even if several literature-described targets fell through our rigorous data filtering. Also, the specific physiological engagement of proposed candidates in calcitriol-mediated cellular activities remains to be appraised in the follow-up assays, such as RNA interference/knockdown approaches or yeast two-hybrid (Y2H) systems that could depict elementary reaction partner networks. In the long run, the ‘hit list’ presented here, compiled with (eventually re-validated) small-scale to HTP experimental results, could guide the prioritization of new therapeutic targets. As well, this list could serve as a test system for revealing distinct molecular actions (e.g. with respect to differences in gene expression or in induction levels) of synthetic vitamin D analogs synthesized with the goal of improving the biological (calcemic) profile of the natural substance for therapeutic applications, as for example, in hyperproliferative skin pathologies such as psoriasis or various cancer types (Holick 2003).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-11-0191>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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