VDR PRIMARY TARGETS BY GENOME-WIDE TRANSCRIPTIONAL PROFILING

Alternative:

1,25-dihydroxyvitamin D3 primary targets by genome-wide transcriptional profiling and ChIP-Seq analysis of H3K4 trimethylation

Genome-wide transcriptional profiling and H3K4 trimethylation analysis after 1,25-dihydroxyvitamin D3 treatment

Frauke Goeman[1]*, Francesca De Nicola[2], Paolo D'Onorio De Meo[3], Berardino Elmi[1], Tiziana Castrignanò[3], Graziano Pesole[4], Sabrina Strano[5], Giovanni Blandino[1], Maurizio Fanciulli[2] and Paola Muti[6]*

[1]Translational Oncogenomics Unit, Regina Elena National Cancer Institute, 00144 Rome, Italy

[2] Regina Elena National Cancer Institute, 00144 Rome, Italy

[3] HPC CINECA, Via dei Tizii, 6. 00185 Rome, Italy.

[4] Institute of Biomembranes and Bioenergetics of the National Research Council and Department of Biosciences, Biotechnology and Biopharmaceutics, University of Bari, 70125 Bari, Italy

[5] Molecular Chemoprevention Unit, Regina Elena National Cancer Institute, 00144 Rome, Italy

[6] Department of Oncology, Juravinski Cancer Center, McMaster University Hamilton, Hamilton, Ontario, Canada

*corresponding authors

Correspondence to: Frauke Goeman and Paola Muti, email: goeman@ifo.it, muti@mcmaster.ca

ABSTRACT

1a,25-dihydroxyvitamin D3 (1α,25(OH)2D3) is reported to mediate a variety of antitumor activities like controlling cellular differentiation, proliferation and angiogenesis. Moreover, it is well established that it plays an important role in immunomodulation. Most of its biological effects are exerted via its nuclear receptor which acts as a transcriptional regulator. Here, we carried out a genome-wide investigation of the primary transcriptional targets of 1α,25(OH)2D3 in breast epithelial cells using RNA-Seq and ChIP-Seq. We identified targets involved in adhesion, growth regulation, angiogenesis, actin cytoskeleton regulation, hexose transport, inflammation and immunomodulation, apoptosis, endocytosis and signaling. Furthermore, we found several transcription factors to be regulated by 1α,25(OH)2D3 that subsequently amplify and diversify the transcriptional output driven by 1α,25(OH)2D3 leading finally to a growth arrest of the cells.

INTRODUCTION

1α,25(OH)2D3 which is the most active product of vitamin D synthesis, is well known to be the main regulator of calcium homeostasis and is therefore critical in bone mineralization [1]. However, recent results revealed a broad spectrum of activities beyond vitamin D's calcemic effects. Epidemiological studies indicate that vitamin D insufficiency could have an etiological role in various human cancers. Preclinical research indicates that 1α,25(OH)2D3, also known as calcitriol, or vitamin D analogues might have potential as anticancer agents because their administration has anti-proliferative effects, can activate apoptotic pathways and inhibit angiogenesis. Indeed, altered expression and function of proteins crucial in vitamin D synthesis and catabolism have been observed in many tumor types. Several epidemiological observations have shown an association between low serum 25(OH)D3

levels and increased risk for colorectal, breast and prostate cancers [2, 3]. In addition, the risk for breast cancer recurrence, and mortality in women with early-stage breast cancer was shown to be inversely correlated with low serum 25-hydroxyvitamin D levels [4]. However, clinical studies using vitamin D as chemopreventive agent are still controversial [5-7]. Further trials using an optimal dose range of vitamin D are needed to assess the preventive and therapeutic effect of vitamin D in breast cancer development.

The majority of the biological effects of 1a,25-dihydroxyvitamin D3 are exerted through its cognate nuclear receptor, the vitamin D receptor (VDR). VDR is a member of the nuclear receptor superfamily which in most cases heterodimerises on its DNA response element with another nuclear receptor superfamily member, the retinoic X receptor (RXR). Upon ligand binding VDR is able to activate or repress the transcription of its target genes depending on the type of response element (RE) [8]. In addition to the classical genomic pathway involving intracellular receptors there are also evidences for rapid, nongenomic effects of 1α,25(OH)2D3 via signaling cascades [9].

Interestingly, recent results showed an intense interplay between VDR and the tumor suppressor protein p53. All p53 family members are able to upregulate the VDR expression whose level is crucial for a therapeutic response to 1α,25(OH)2D3. In contrast, mutant His175 p53 can modulate differentially subsets of VDR target genes, inhibiting thereby apoptosis and turning 1α,25(OH)2D3 into a cytoprotective agent [10].

In order to determine the molecular genetic events underlying the broad physiological activities of 1α,25(OH)2D3 we performed differential expression profiling. In particular, we were interested to investigate the global transcriptional signature of 1a,25-dihydroxyvitamin D3 in breast cancer cells with a mutant p53 background. Therefore, we used SKBr3 breast epithelial cells that are inhibited in their growth after prolonged treatment with 1α,25(OH)2D3 although they are harboring endogenously mutant p53R175H. We conducted

Commentato [u1]: What kind of mutation is this one? Can it be representative of other mutations? In other words can we generalize the finding also to other p53 mutations?

RNA-Seq and ChIP-Seq experiments for trimethylated H3K4 (H3K4me3). Trimethylation in histone H3 lysine 4 is a mark of genes transcriptionally active, located in the promoter region.

Thus, we studied genome-wide the early effects of 1a,25(OH)2D3 on transcription and H3K4me3 histone modification.

RESULTS

To discover genome-wide genes differentially regulated after 1a,25(OH)2D3 treatment, we performed RNA-Seq analysis. We conducted early time point studies to focus on the primary VDR targets. Therefore, we treated breast epithelial SKBr3 cells for 3h and 6h with 100nM 1a,25-dihydroxyvitamin D3, respectively or with vehicle alone. We extracted total RNAs, and validated them with known target genes. After the generation of cDNA libraries we tested whether the libraries reflect the initial RNA (Figure 1) and subjected them subsequently to Illumina DNA sequencing. The RNA-Seq data included 28 million total reads for the vehicle treated cells (2.8Gb), 30 and 29 million reads for the 3 and 6hrs treated samples (3Gb and 2.9Gb, respectively). Of the total number of reads, 58-59% was successfully mapped to known mRNA genes, 3% to annotated ncRNAs. We detected 13898-13971 transcripts out of 18748 genes annotated in RefSeq (13898 transcripts in vehicle treated, 13944 transcripts in cells treated for 3h with 1α,25(OH)2D3 , and 13971 transcripts in cells treated for 6h with 1α,25(OH)2D3).

After 3hrs of 1a,25-dihydroxyvitamin D3 treatment we found 111 genes to be differentially regulated, 88 genes were significantly upregulated and 23 downregulated (Table 1) with respect to the control. After 6h the number of regulated genes increased to 318 (Table 2) with 241 genes being upregulated and 77 genes downregulated. Therefore, the majority of the differentially regulated genes were upregulated in agreement with previous reports [17, 18].

Furthermore, we investigated the genomic distribution of histone H3 trimethylated at lysine 4, a mark for active promoters, before and after 1a,25-dihydroxyvitamin D3 treatment [19-21]. We performed ChIP-Seq experiments after 2h of 1α,25(OH)2D3 treatment or vehicle as control to precede the subsequent accumulation of mRNA. Globally the profiles of trimethylated H3K4 are not changing between 1a,25(OH)2D3 treated or vehicle treated cells (Figure 2). Trimethylated H3K4 is enriched around the Transcriptional Start Sites (TSS) of genes, displaying a smaller pre-TSS and a larger post-TSS peak. A significant drop of trimethylated H3K4 near the TSS is typically for a nucleosome depletion of active transcribed genes [22, 23]. Instead, a subset of genes whose expression was upregulated after 1α,25(OH)2D3 treatment, show already after 2h of treatment a significant increase in trimethylated H3K4 at their transcriptional start site (Figure 3). CYP24A1, a well-known direct VDR target gene that is responsible for the metabolization of 1α,25(OH)2D3 into a less active form, is after 3h already 581-fold upregulated which augments after 6h to 2221-fold. 84 of the upregulated genes after 3hrs were also found to be upregulated after 6h (Figure 4). Interestingly, Pscan, a software tool that determines if in a subset of co-regulated genes transcription factor binding sites are over-represented [14] shows the RXRA::VDR motif only in the upregulated gene fraction to be significantly over-represented (Figure 5A). In the genes upregulated after 3h of 1α,25(OH)2D3 treatment we found an enrichment of genes involved in the insulin signaling pathway such as FOS, SLC2A4 and INSR. After 6h we identified also the ETS pathway (FOS, ETS2, NCOR2, CSF1R), the CD40 (DUSP1, IKBKG, NFKBIA) and the TNFR2 pathway (DUSP1, IKBKG, NFKBIA) to be significantly overrepresented (Figure 6). Looking at the KEGG pathways we found the ErbB signaling pathway to be enriched in the genes upregulated after 3hrs of 1α,25(OH)2D3 treatment (CDKN1A, HBEGF, AREG) (Figure 7). p21 (CDKN1A) is a well-known direct VDR target that is upregulated in SKBr3 cells and might be one of the factors mediating the cell cycle arrest occurring in SKBr3 cells after vitamin D treatment [24-26]. Amphiregulin (AREG)

displays another gene previously identified as a 1α,25(OH)2D3 target upregulated in HNSCC cells [27, 28]. As mentioned before 1a,25-dihydroxyvitamin D3 is thought to play an important role in breast cancer onset and prevention and it was shown to have an antiproliferative effect in SKBr3 cells [24]. In accordance with this we identified the KEGG pathway “Pathways in cancer” to be enriched after 6h of vitamin D exposure. 15 genes fall into this category, 4 of these are already differentially regulated after 3h of treatment (Figure 8). The majority of these genes is upregulated like FOS, CDKN1A, RASSF5, CASP3, PPARD, IKBKG, NFKBIA, CSF3R, EGLN2, TRAF4, CSF1R, while only 4 are downregulated (ITGA6, KITLG, CDK6, DAPK1). FOS, CDKN1A, PPARD and TRAF4 were already after 3h significantly upregulated. Another interesting functional group enriched by vitamin D was “cell adhesion molecules (CAM)”, which is composed of genes that were found to be differentially regulated after 6h, therefore representing the early but not immediate early group of responding genes. Among those were integrins like ITGAL, CDH5 and ITGA6 and syndecans like SDC1 and SDC3. Also genes involved in actin cytoskeleton regulation like VAV3, ARHGEF6, IQGAP2, PDGFC, BDKRB1, BDKRB2, SLC9A1, and phospholipase C epsilon (PLCE) are regulated by 1α,25(OH)2D3. Gene ontology analysis using the biological function modus reveals the differential expression of several genes involved in metabolic processes like phosphate metabolic process, steroid metabolic process, nucleic acid metabolic, and hexose transport, process like SLC2A10, SLC2A4, EDN1, STXBP4 and PPARD that are involved in the glucose metabolism (Figure 9). Another functional category enriched after 1α,25(OH)2D3 treatment included genes associated with regulation of apoptosis and proliferation such as the pro-apoptotic CASP3 and anti-apoptotic SERPINB9 which were both upregulated. SERPINB9 was also shown to be upregulated in normal mammary associated fibroblasts after 1α,25(OH)2D3 treatment [29]. Cyclin dependent kinase 6 (CDK6), cyclin G2 (CCNG2) and growth factors like KITLG and PDGFC were downregulated by 1α,25(OH)2D3. On the other hand we found also growth

factors like HBEGF, EPGN and AREG to be upregulated. We also observed modulation of several genes associated with the immune system like cytokines (CLCF1, IL12A) or cytokine binding proteins (IL1RL1, PRLR, IL4R, CX3CR1, CSF3R, and CSF1R), all being upregulated after vitamin D exposure. The list of regulated genes contains a number of previously identified primary VDR targets like the insulin-like growth factor-binding protein 3 IGFBP3 [30], the intestinal calcium ion channel gene TRPV6 [31], semaphorin 3B (SEMA3B) [32], the inhibitor of NF-κB signaling NFKBIA [29, 33-35], SLC4A7, the dual specificity protein phosphatase 1 DUSP1 [29], and B-cell lymphoma 6 protein BCL6 [28]. Gene ontology analysis revealed furthermore a role of vitamin D in the regulation of signaling cascades. Several genes involved in small GTPase mediated signaling transduction were upregulated (RAB43, RAB37, ARFRP1, RAB4B, RIN2, IQGAP2, RHOC, RAB20, RASD2) or vice versa downregulated (ARHGEF3, PLCE1, VAV3, RGL3, ABCA1, RAB27B). Another functional category and pathway identified to be enriched after 1α,25(OH)2D3 treatment was endocytosis. Most genes participating in this function were upregulated (ADRB2, NPC1, MYO7A, STXBP1, RIN2, MERTK, BIN1, RIN3, VLDLR), while DLG4 and ABCA1were downregulated.

Among the primary vitamin D targets are also various proteins known to regulate transcription. They are of particular interest because they will subsequently initiate a whole cascade of transcriptional changes leading to a broad change in transcription contributing as secondary effectors to the anti-proliferative action of 1α,25(OH)2D3. Krueppel like factor KLF4, a repressing transcription factor, is already after 3h of vitamin D treatment downregulated. The special AT-rich sequence-binding protein-1 SATB1 makes part of the early but not immediate early responding genes, being downregulated after 6h. Instead, Homeobox protein TGIF1 and T-cell leukemia homeobox 1 (TLX1) are upregulated after 3h and 6h of 1α,25(OH)2D3 treatment, respectively. B-cell lymphoma 6 protein BCL6 is already

after 3h 2-fold upregulated, while ID4 is only after 6h of 1α,25(OH)2D3 treatment significantly downregulated. Interstingly, analyzing the differentially regulated transcripts with Pscan reveals a strong over-representation of the Klf4 motif in the genes upregulated by vitamin D (Figure 5B).

DISCUSSION

1a,25-dihydroxyvitamin D3 exerts several anti-carcinogenic effects on breast cancer cells including: induction of growth arrest, apoptosis and inhibition of angiogenesis. Most of its actions are mediated via its receptor, the vitamin D receptor, and the genomic pathway. To identify the primary targets of 1α,25(OH)2D3 in breast cancer cells we performed differential expression analysis treating breast epithelial cancer cells SKBr3 for short periods, 3h and 6h, which is a time frame well suited for the detection of primary targets [36]. The RNA-Seq technology used herein permitted us to detect all possible transcripts in an unbiased manner. Furthermore we conducted ChIP-Seq experiments for trimethylated lysine 4 of histone H3. Trimethylated H3K4 is a mark for active transcription. . Most of the 88 genes being upregulated after 3h of 1α,25(OH)2D3 treatment 23 showed after 2h an increase in H3K4me3 occupancy. VDR was shown to bind to a subset of genes also in the absence of ligand, recruiting co-repressors and chromatin modifying enzymes such as histone deacetylases (HDACs). Upon binding of 1α,25(OH)2D3, the conformation of the receptor changes and favors the binding of co-activators that have histone acetylase activity or are complexed with proteins harboring such activity [37, 38]. Here we show that 1α,25(OH)2D3 leads to an increase of trimethylated H3K4 in a subset of promotors of early upregulated genes. VDR was shown to interact with Menin which co-activates VDR in the presence of the ligand 1α,25(OH)2D3. Menin is part of complexes (MLL1 and MLL2) that possesses histone

methyltransferase activity directed at lysine 4 of histone H3, especially trimethylation [39, 40]. Therefore, activated VDR might recruit histone methyltransferases to the promoters of its target genes leading to an increase of trimethylated H3K4.

After 3h of 1α,25(OH)2D3 treatment we detected a strong upregulation of the catabolic enzyme 24-hydroxylase (CYP24A1) which is responsible for the hydroxylation of 1α,25(OH)2D3 to 1,24,25-trihydroxyvitamin D3 and therefore the inactivation of calcitriol. It is often overexpressed in malignant cells [41] and its expression is independently prognostic of poor survival [42, 43]. Likewise, in breast cancer it is often upregulated [44]. The strong upregulation that we detected is therefore a typical feature of cancer cells, limiting to a certain extent the antiproliferative action of 1α,25(OH)2D3. The software tool Pscan, that scans a set of sequences (e.g. promoters) from co-regulated genes searching for transcription factor binding motifs significantly over- or under-represented [14] revealed the VDRE motif in the upregulated but not in the downregulated set of genes to be significantly enriched. So far, there are only a few genes characterized that are directly repressed by the ligand bound vitamin D receptor. The PTH and the CYP27B1 genes are reported to contain an E-box like motif distinct from the classical DR3 type response element. In the absence of 1α,25(OH)2D3 it is bound by the VDR-interacting repressor (VDIR) and co-activators. Upon ligand binding VDR interacts with VDIR, releases the coactivators and recruits corepressors to the target genes. In line with this, we did not find an over-representation of the VDR motif in the downregulated genes. The enrichment of the VDR motif in the upregulated genes is rather modest probably due to the fact that many VDREs are very distant from the promoter and Pscan considers only the nearby promoter regions -950 until +50bp [17, 45]. A number of genes that were regulated by vitamin D in SKBr3 cells participate in the category of immune response like cytokines and cytokine receptors. The interaction of vitamin D with the immune system has been recognized since many years. It is involved in the immune responses to

infection and aberrant inflammatory responses associated with autoimmune disease [46, 47]. Recent data shows that inflammation is a critical part of carcinogenesis in the colon, liver, and stomach [48] and it has been proposed to be a part of the etiology of prostate cancer [49]. In this light, the immune system becomes an important cell target for limiting cancer. One of the cytokines upregulated by 1α,25(OH)2D3 was IL12A. Il12A is one of the two subunits of interleukin 12 which is described to possess antitumor activity and to be currently used in several clinical trials [50, 51]. The IL1RL1 or T1/ST2 receptor that was upregulated in our cell system but also in squamous carcinoma cells after vitamin D treatment, is linked to apoptosis and suppression of anchorage-independent growth and malignancy [52-54].

Treatment of SKBr3 cells with 1a,25-dihydroxyvitamin D3 leads to a cell cycle arrest [24]. The downregulation of several growth factors and the upregulation of the IGF binding protein 3 (IGFBP3) could be one of the mechanisms of vitamin D action to limit the cell growth. IGFBP3, which is released into the medium, binds to IGF1 and 2 that in turn are not able to bind to their corresponding receptors and stimulate proliferation [55]. SATB1, a genome organizer protein, is downregulated in SKBr3 cells after 1α,25(OH)2D3 treatment. In previous studies it was shown to be a transcription factor that regulates more than 1000 genes related to cellular growth and metastasis and its overexpression in SKBr3 cells leads to a more aggressive phenotype promoting growth and metastasis. Furthermore, its expression in breast cancer is inversely correlated with prognosis [56]. Instead, bradykinin receptor B1 (BDKRB1) was upregulated by 1α,25(OH)2D3 and was previously shown to correlate with good prognosis in woman with estrogen receptor (ER)-negative breast tumors [57]. Among the differentially regulated genes after 1α,25(OH)2D3 treatment were several that are involved in endocytosis. Defective endocytosis is one of the hallmarks of cancer, leading to an aberrant disassembly of signaling and adhesion complexes [58]. Stambolsky et al. discovered recently that mutant p53 protein physically interacts and cooperates with the

vitamin D receptor. It increases its nuclear localization and modulates the transcriptional response of ligand bound VDR [10]. Furthermore, this interaction is transforming vitamin D into an antiapoptotic agent. Nevertheless, SKBr3 cells, that harbor mutant p53, stop growing after prolonged treatment with 1a,25-dihydroxyvitamin D3. Therefore, it is able to bypass the gain of function mutant p53 activity. The expression of ID4 is positively controlled by mutant p53R175H which in turn stabilizes pro-angiogenic factors IL-8 and GRO-α increasing hereby the angiogenic potential of cancer cells [59]. Incubation with 1a,25-dihydroxyvitamin D3 instead leads to a significant downregulation of ID4 after 6h. Kruppel-like factor 4 (KLF4) is a key transcriptional regulator of cell differentiation and proliferation and an altered expression of KLF4 has been reported in a number of human malignancies. In breast tumor it was found to be frequently upregulated and a prognostic factor and marker of a more aggressive phenotype [60, 61]. Recent studies showed that KLF4 is one of the factors that reprogram differentiated cells to iPS and that its expression is upregulated by mutant gain of function p53 [62, 63]. Therefore, vitamin D is able to counteract two important mediators of the gain of function p53 protein, ID4 and Klf4. In our study we identified several VDR target genes that might confer the antiproliferative capacity of 1a,25-dihydroxyvitamin D3. Among those the transcriptional regulators SATB1 and Klf4 are of special interest because they could initiate a whole cascade of transcriptional events leading finally to the activation of a growth inhibitory program. Pscan revealed o strong over-representation of the Klf4 binding motiv in the upregulated genes in line with the downregulation of this transcriptional repressor. Further analysis will elucidate their crucial role in mediating the antitumor capacity of 1a,25-dihydroxyvitamin D3 and their involvement in the evasion of the gain of function mutant p53 activity.

MATERIALS AND METHODS

Cell culture. SKBr3 breast cancer cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2mM L-glutamine and Pen/Strep. The cells were treated for 3 or 6hrs for the RNA-Seq, and 2h for the ChIP-Seq with 100nM 1a,25-dihydroxyvitamin D3 or vehicle as control.

Quantitative PCR. Quantitative PCR was carried out with SYBR green PCR Master Mix, gene specific primers and the StepOne Real Time (Applied Biosystems). Oligonucleotides employed in this study were: CYP24A1-F GAAAGAATTGTATGCTGCTGTCACA, CYP24R GGGATTACGGGATAAATTGTAGAGAA, CDKN1A-F CTGGAGACTCTCAGGGTCGAA, CDKN1A-R GCGGATTAGGGCTTCCTCTT and RPL19-F CGGAAGGGCAGGCACAT, RPL19-R GGCGCAAAATCCTCATTCTC for normalization.

RNA-Seq. Total RNA from exponentially growing SKBr3 cells was isolated using miRNeasy (Qiagen, Valencia, CA, USA). Induction of canonical VDR target gene expression was confirmed by qPCR, and RNA quality was verified using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA; RNA 6000 Nano kit). All RNAs used for subsequent library preparation had an RNA integrity number greater than 9.0. RNA libraries for sequencing were generated according to the standard Illumina TruSeq RNA sample preparation protocol using 2µg total RNA as starting material. The resulting library was controlled qualitatively with the High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA) and quantitatively with real-time analysis employing a SYBR Green quantitative PCR (qPCR) protocol with specific primers complementary to adapter sequences. Therefore, only the adapter-ligated fragments that are appropriate for sequencing will be quantified. Based on the qPCR quantification, libraries were normalized to 1 nM and denatured by using 0.1 N NaOH. Cluster amplification of denatured templates was carried

out according to manufacturer protocol (Illumina, Inc., San Diego, CA, USA). Sequencing was performed on a Genome Analyzer IIx (Illumina) in paired-end mode, sequencing from each side 51 bp.

ChIP-Seq. Chromatin immunoprecipitation was performed as described earlier [11] with minor changes. Cells were cross-linked for 10 min with 1% formaldehyde. The fixation was stopped by adding 0.125M glycine for 5 min to the cells. Nuclei were prepared by incubation with 5 mM Pipes (pH 8.0), 85 mM KCl, 0.5% NP40 plus protease inhibitors. Subsequently, the nuclei are resupended and lysed in 1% SDS, 10 mM EDTA, 50 mMTris-HCl (pH 8.0). The resulting chromatin was fragmented to a size range of 100-350bp by sonication. The chromatin was diluted 1:10 with 0.01% SDS, 1.2 mM EDTA, 16.7 mM Tris, 1.1% Triton X-100 and 167 mM NaCl. Protein G Dynabeads (Invitrogen, Carlsbad, CA) were washed with PBS/BSA [5mg/ml] and incubated over night at 4°C with the following antibodies: 5 µg histone H3 tri methyl K4 (Abcam, ab1012) or no antibody as negative control. The following day the beads were washed, resuspended in 100µl PBS/BSA[5mg/ml] and incubated with the chromatin over night at 4°C. After several washing steps with buffer A [0.1% SDS, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8, 1% Triton X-100 and 150 mM NaCl], buffer B [0.1% SDS, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8, 1% Triton X-100 and 500 mM NaCl] and TE, the immune-bound chromatin was eluted by 100 mM NaHCO3 and 1% SDS. Cross-linking was reversed by addition of NaCl to a final concentration 200 mM, RNA was removed by 10 µg of RNase A and subsequent incubation at 65°C overnight. Proteins were digested by adding EDTA pH 8 and Tris-HCl pH 6.5 to a final concentration of 10 mM and 40 mM, respectively and 20 µg proteinase K. The samples were incubated at 42°C for 2h. The DNA is recovered by phenol/chloroform purification using Phase Lock Gel (Eppendorf) and ethanol precipitation. The quantity of the immunoprecipitated material was determined by PicoGreen (Invitrogen, Carlsbad, CA).

10ng of the immunoprecipitated chromatin was used to prepare the libraries for sequencing following the manufacturer's instructions including DNA end repairing, adaptor ligation, and amplification. Fragments of about 100-180 bp (without linkers) were isolated from agarose gel and used for sequencing using the Illumina GA IIx. (36bp, 21-26 Mio quality-filtered and uniquely aligned reads per sample).

Data analysis.

For each sample generated by the Illumina platform, a preprocess step for quality control has been performed to assess sequence data quality and to discard low quality reads. The reference genome used for both analyses is the human genome assembly hg18. All gene and transcript annotation data, such as transcription start site positions, came from UCSC database (http://genome.ucsc.edu).

ChIP-Seq. ChIP-Seq fastq filtered files were mapped to the reference genome using Bowtie [12], using default parameters and allowing up to 2 mismatches. Only uniquely mapped reads were kept. The peaks were identified using MACS version 1.4 [13]. The setting for peak calling was tsize=36, bw=150, mfold=32, p-value at least e-5. In order to identify statistically significant enrichment regions for H3K4me3, non-treated DNA input control libraries served as negative control. Significant peaks are called using a fold discovery rate (FDR) less than 1%. A TSS plot was generated using the HTSeq package (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html). To reveal the modulation of trimethylated H3K4 after 1a,25(OH)2D3 treatment the qualified peaks are compared between treated and non-treated control samples. Fold-enrichment was calculated for peaks that have overlapping genome positions and that are located within 5,000bp of the Transcriptional Start Site (TSS). The analysis of transcription factor binding motifs overrepresented in promoters identified to be regulated by calcitriol was performed with Pscan using the Jaspar database and the regions spanning -950bp until +50bp of the transcriptional start sites [14].

RNA-Seq. RNA-Seq short-reads were processed first with the NEUMA tool [15], which makes use again of Bowtie for mapping. This step determined raw counts and FVKM (fragments per virtual kb per million) values for profiling on gene expression. The differential expression was determined with the DEGseq R package [16]. Statistics are obtained with a MA-plot-based method (random sampling model) (Supplementary Figure 1). All genes listed are filtered with p-value < 0.001. Genes having at least 0.58 fold-change for the log2-transformed expression values between 1a,25(OH)2D3-induced and non-induced control samples were defined to be differentially expressed. Gene ontology analysis, KEGG pathway analysis and pathway analysis using the BIOCARTA database have been carried out with DAVID (http://david.abcc.ncifcrf.gov/).

ACKNOWLEDGEMENTS

REFERENCES

1. Sutton AL and MacDonald PN: Vitamin D: more than a "bone-a-fide" hormone. Mol Endocrinol. 2003;17: 777-91.
2. Deeb KK, Trump DL, and Johnson CS: Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. Nat Rev Cancer. 2007;7: 684-700.
3. Lopes N, Paredes J, Costa JL, Ylstra B, and Schmitt F: Vitamin D and the mammary gland: a review on its role in normal development and breast cancer. Breast Cancer Res. 2012;14: 211.
4. Shao T, Klein P, and Grossbard ML: Vitamin D and breast cancer. Oncologist. 2012;17: 36-45.
5. Chlebowski RT: Vitamin D and breast cancer: interpreting current evidence. Breast Cancer Res. 2011;13: 217.
6. Ingraham BA, Bragdon B, and Nohe A: Molecular basis of the potential of vitamin D to prevent cancer. Curr Med Res Opin. 2008;24: 139-49.
7. Lazzeroni M, Gandini S, Puntoni M, Bonanni B, Gennari A, and DeCensi A: The science behind vitamins and natural compounds for breast cancer prevention. Getting the most prevention out of it. Breast. 2011;20 Suppl 3: S36-41.
8. Haussler MR, Whitfield GK, Haussler CA, Hsieh JC, Thompson PD, Selznick SH, Dominguez CE, and Jurutka PW: The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J Bone Miner Res. 1998;13: 325-49.

9. Falkenstein E, Tillmann HC, Christ M, Feuring M, and Wehling M: Multiple actions of steroid hormones--a focus on rapid, nongenomic effects. Pharmacol Rev. 2000;52: 513-56.
10. Stambolsky P, Tabach Y, Fontemaggi G, Weisz L, Maor-Aloni R, Siegfried Z, Shiff I, Kogan I, Shay M, Kalo E, Blandino G, Simon I, Oren M, and Rotter V: Modulation of the vitamin D3 response by cancer-associated mutant p53. Cancer Cell. 2010;17: 273-85.
11. Goeman F, Fontemaggi G, and Blandino G: ChIP-on-chip to Identify Mutant p53 Targets. Methods Mol Biol. 2013;962: 211-26.
12. Langmead B, Trapnell C, Pop M, and Salzberg SL: Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009;10: R25.
13. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W, and Liu XS: Model-based analysis of ChIP-Seq (MACS). Genome Biol. 2008;9: R137.
14. Zambelli F, Pesole G, and Pavesi G: Pscan: finding over-represented transcription factor binding site motifs in sequences from co-regulated or co-expressed genes. Nucleic Acids Res. 2009;37: W247-52.
15. Lee S, Seo CH, Lim B, Yang JO, Oh J, Kim M, Lee B, and Kang C: Accurate quantification of transcriptome from RNA-Seq data by effective length normalization. Nucleic Acids Res. 2011;39: e9.
16. Wang L, Feng Z, Wang X, and Zhang X: DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. Bioinformatics. 2010;26: 136-8.
17. Heikkinen S, Vaisanen S, Pehkonen P, Seuter S, Benes V, and Carlberg C: Nuclear hormone 1alpha,25-dihydroxyvitamin D3 elicits a genome-wide shift in the locations of VDR chromatin occupancy. Nucleic Acids Res. 2011;39: 9181-93.
18. Pike JW: Genome-wide principles of gene regulation by the vitamin D receptor and its activating ligand. Mol Cell Endocrinol. 2011;347: 3-10.
19. Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, Hawkins RD, Barrera LO, Van Calcar S, Qu C, Ching KA, Wang W, Weng Z, Green RD, Crawford GE, and Ren B: Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat Genet. 2007;39: 311-8.
20. Sutherland H and Bickmore WA: Transcription factories: gene expression in unions? Nat Rev Genet. 2009;10: 457-66.
21. Vermeulen M, Mulder KW, Denissov S, Pijnappel WW, van Schaik FM, Varier RA, Baltissen MP, Stunnenberg HG, Mann M, and Timmers HT: Selective anchoring of TFIID to nucleosomes by trimethylation of histone H3 lysine 4. Cell. 2007;131: 58-69.
22. He HH, Meyer CA, Shin H, Bailey ST, Wei G, Wang Q, Zhang Y, Xu K, Ni M, Lupien M, Mieczkowski P, Lieb JD, Zhao K, Brown M, and Liu XS: Nucleosome dynamics define transcriptional enhancers. Nat Genet. 2010;42: 343-7.
23. Jiang C and Pugh BF: Nucleosome positioning and gene regulation: advances through genomics. Nat Rev Genet. 2009;10: 161-72.
24. Muti P, Benassi B, Falvo E, Santoro R, Galanti S, Citro G, Carrubba G, Blandino G, and Strano S: Omics underpins novel clues on VDR chemoprevention target in breast cancer. OMICS. 2011;15: 337-46.
25. Saramaki A, Banwell CM, Campbell MJ, and Carlberg C: Regulation of the human p21(waf1/cip1) gene promoter via multiple binding sites for p53 and the vitamin D3 receptor. Nucleic Acids Res. 2006;34: 543-54.
26. Saramaki A, Diermeier S, Kellner R, Laitinen H, Vaisanen S, and Carlberg C: Cyclical chromatin looping and transcription factor association on the regulatory regions of the p21 (CDKN1A) gene in response to 1alpha,25-dihydroxyvitamin D3. J Biol Chem. 2009;284: 8073-82.
27. Akutsu N, Lin R, Bastien Y, Bestawros A, Enepekides DJ, Black MJ, and White JH: Regulation of gene Expression by 1alpha,25-dihydroxyvitamin D3 and Its analog EB1089 under growth-inhibitory conditions in squamous carcinoma Cells. Mol Endocrinol. 2001;15: 1127-39.

28. Lin R, Nagai Y, Sladek R, Bastien Y, Ho J, Petrecca K, Sotiropoulou G, Diamandis EP, Hudson TJ, and White JH: Expression profiling in squamous carcinoma cells reveals pleiotropic effects of vitamin D3 analog EB1089 signaling on cell proliferation, differentiation, and immune system regulation. Mol Endocrinol. 2002;16: 1243-56.
29. Campos LT, Brentani H, Roela RA, Katayama ML, Lima L, Rolim CF, Milani C, Folgueira MA, and Brentani MM: Differences in transcriptional effects of 1alpha,25 dihydroxyvitamin D3 on fibroblasts associated to breast carcinomas and from paired normal breast tissues. J Steroid Biochem Mol Biol. 2012;133C: 12-24.
30. Matilainen M, Malinen M, Saavalainen K, and Carlberg C: Regulation of multiple insulin-like growth factor binding protein genes by 1alpha,25-dihydroxyvitamin D3. Nucleic Acids Res. 2005;33: 5521-32.
31. Meyer MB, Watanuki M, Kim S, Shevde NK, and Pike JW: The human transient receptor potential vanilloid type 6 distal promoter contains multiple vitamin D receptor binding sites that mediate activation by 1,25-dihydroxyvitamin D3 in intestinal cells. Mol Endocrinol. 2006;20: 1447-61.
32. Sutton AL, Zhang X, Dowd DR, Kharode YP, Komm BS, and Macdonald PN: Semaphorin 3B is a 1,25-Dihydroxyvitamin D3-induced gene in osteoblasts that promotes osteoclastogenesis and induces osteopenia in mice. Mol Endocrinol. 2008;22: 1370-81.
33. Cohen-Lahav M, Shany S, Tobvin D, Chaimovitz C, and Douvdevani A: Vitamin D decreases NFkappaB activity by increasing IkappaBalpha levels. Nephrol Dial Transplant. 2006;21: 889-97.
34. Torri A, Beretta O, Ranghetti A, Granucci F, Ricciardi-Castagnoli P, and Foti M: Gene expression profiles identify inflammatory signatures in dendritic cells. PLoS One. 2010;5: e9404.
35. Wu S, Xia Y, Liu X, and Sun J: Vitamin D receptor deletion leads to reduced level of IkappaBalpha protein through protein translation, protein-protein interaction, and post-translational modification. Int J Biochem Cell Biol. 2010;42: 329-36.
36. Carlberg C and Seuter S: A genomic perspective on vitamin D signaling. Anticancer Res. 2009;29: 3485-93.
37. Carlberg C, Dunlop TW, Saramaki A, Sinkkonen L, Matilainen M, and Vaisanen S: Controlling the chromatin organization of vitamin D target genes by multiple vitamin D receptor binding sites. J Steroid Biochem Mol Biol. 2007;103: 338-43.
38. Seuter S, Pehkonen P, Heikkinen S, and Carlberg C: The gene for the transcription factor BHLHE40/DEC1/stra13 is a dynamically regulated primary target of the vitamin D receptor. J Steroid Biochem Mol Biol. 2013;136: 62-7.
39. Dreijerink KM, Mulder KW, Winkler GS, Hoppener JW, Lips CJ, and Timmers HT: Menin links estrogen receptor activation to histone H3K4 trimethylation. Cancer Res. 2006;66: 4929-35.
40. Dreijerink KM, Varier RA, van Nuland R, Broekhuizen R, Valk GD, van der Wal JE, Lips CJ, Kummer JA, and Timmers HT: Regulation of vitamin D receptor function in MEN1-related parathyroid adenomas. Mol Cell Endocrinol. 2009;313: 1-8.
41. Albertson DG, Ylstra B, Segraves R, Collins C, Dairkee SH, Kowbel D, Kuo WL, Gray JW, and Pinkel D: Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. Nat Genet. 2000;25: 144-6.
42. Chen G, Kim SH, King AN, Zhao L, Simpson RU, Christensen PJ, Wang Z, Thomas DG, Giordano TJ, Lin L, Brenner DE, Beer DG, and Ramnath N: CYP24A1 is an independent prognostic marker of survival in patients with lung adenocarcinoma. Clin Cancer Res. 2011;17: 817-26.
43. Zhang Q, Kanterewicz B, Shoemaker S, Hu Q, Liu S, Atwood K, and Hershberger P: Differential response to 1alpha,25-dihydroxyvitamin D(3) (1alpha,25(OH)(2)D(3)) in non-small cell lung cancer cells with distinct oncogene mutations. J Steroid Biochem Mol Biol. 2012.
44. Townsend K, Banwell CM, Guy M, Colston KW, Mansi JL, Stewart PM, Campbell MJ, and Hewison M: Autocrine metabolism of vitamin D in normal and malignant breast tissue. Clin Cancer Res. 2005;11: 3579-86.

45. Ramagopalan SV, Heger A, Berlanga AJ, Maugeri NJ, Lincoln MR, Burrell A, Handunnetthi L, Handel AE, Disanto G, Orton SM, Watson CT, Morahan JM, Giovannoni G, Ponting CP, Ebers GC, and Knight JC: A ChIP-seq defined genome-wide map of vitamin D receptor binding: associations with disease and evolution. Genome Res. 2010;20: 1352-60.
46. Hewison M: Vitamin D and immune function: autocrine, paracrine or endocrine? Scand J Clin Lab Invest Suppl. 2012;243: 92-102.
47. Hewison M: Vitamin D and immune function: an overview. Proc Nutr Soc. 2012;71: 50-61.
48. Coussens LM and Werb Z: Inflammation and cancer. Nature. 2002;420: 860-7.
49. Haverkamp J, Charbonneau B, and Ratliff TL: Prostate inflammation and its potential impact on prostate cancer: a current review. J Cell Biochem. 2008;103: 1344-53.
50. Colombo MP and Trinchieri G: Interleukin-12 in anti-tumor immunity and immunotherapy. Cytokine Growth Factor Rev. 2002;13: 155-68.
51. Yuzhalin AE and Kutikhin AG: Interleukin-12: clinical usage and molecular markers of cancer susceptibility. Growth Factors. 2012;30: 176-91.
52. Haga Y, Yanagisawa K, Ohto-Ozaki H, Tominaga S, Masuzawa T, and Iwahana H: The effect of ST2 gene product on anchorage-independent growth of a glioblastoma cell line, T98G. Eur J Biochem. 2003;270: 163-70.
53. Sandler U, Devary O, Braitbard O, Ohana J, Kass G, Rubinstein AM, Friedman ZY, and Devary Y: NEROFE--a novel human hormone-peptide with anti-cancer activity. J Exp Ther Oncol. 2010;8: 327-39.
54. Wang TT, Tavera-Mendoza LE, Laperriere D, Libby E, MacLeod NB, Nagai Y, Bourdeau V, Konstorum A, Lallemant B, Zhang R, Mader S, and White JH: Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. Mol Endocrinol. 2005;19: 2685-95.
55. Fleet JC: Molecular actions of vitamin D contributing to cancer prevention. Mol Aspects Med. 2008;29: 388-96.
56. Han HJ, Russo J, Kohwi Y, and Kohwi-Shigematsu T: SATB1 reprogrammes gene expression to promote breast tumour growth and metastasis. Nature. 2008;452: 187-93.
57. Esseghir S, Reis-Filho JS, Kennedy A, James M, O'Hare MJ, Jeffery R, Poulsom R, and Isacke CM: Identification of transmembrane proteins as potential prognostic markers and therapeutic targets in breast cancer by a screen for signal sequence encoding transcripts. J Pathol. 2006;210: 420-30.
58. Mosesson Y, Mills GB, and Yarden Y: Derailed endocytosis: an emerging feature of cancer. Nat Rev Cancer. 2008;8: 835-50.
59. Fontemaggi G, Dell'Orso S, Trisciuoglio D, Shay T, Melucci E, Fazi F, Terrenato I, Mottolese M, Muti P, Domany E, Del Bufalo D, Strano S, and Blandino G: The execution of the transcriptional axis mutant p53, E2F1 and ID4 promotes tumor neo-angiogenesis. Nat Struct Mol Biol. 2009;16: 1086-93.
60. Foster KW, Frost AR, McKie-Bell P, Lin CY, Engler JA, Grizzle WE, and Ruppert JM: Increase of GKLF messenger RNA and protein expression during progression of breast cancer. Cancer Res. 2000;60: 6488-95.
61. Pandya AY, Talley LI, Frost AR, Fitzgerald TJ, Trivedi V, Chakravarthy M, Chhieng DC, Grizzle WE, Engler JA, Krontiras H, Bland KI, LoBuglio AF, Lobo-Ruppert SM, and Ruppert JM: Nuclear localization of KLF4 is associated with an aggressive phenotype in early-stage breast cancer. Clin Cancer Res. 2004;10: 2709-19.
62. Cordani N, Pozzi S, Martynova E, Fanoni D, Borrelli S, Alotto D, Castagnoli C, Berti E, Vigano MA, and Mantovani R: Mutant p53 subverts p63 control over KLF4 expression in keratinocytes. Oncogene. 2011;30: 922-32.
63. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, and Yamanaka S: Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131: 861-72.

FIGURE LEGENDS

Figure 1 Quality control of the RNA-Seq library.

A Quantitative RT-PCR (qRT-PCR) analysis of CYP24A1 and CDKN1A mRNA expression in SKBr3 cells treated with 1a,25-dihydroxyvitamin D3 (1α,25(OH)2D3, vitD) or vehicle for the indicated time points. **B** qRT-PCR of CYP24A1 and CDKN1A mRNA expression after the RNA-Seq library preparation. mRNA levels were normalized to the amount of RPL19 transcript. Values obtained in the absence of 1a,25-dihydroxyvitamin D3 were set arbitrarily as one and the fold activation obtained after 1α,25(OH)2D3 treatment is indicated. The error bars indicate the deviation of the mean.

Figure 2 Global H3K4me3 occupancy around the Transcriptional Start Site (TSS).

Distance distribution of H3K4 trimethylation around the TSS before (Ctrl) and after 1α,25(OH)2D3 treatment (vitD). The input is shown for comparison.

Figure 3 Occupancy of trimethylated H3K4 at 1α,25(OH)2D3 induced gene promoters.

Raw ChIP-Seq data from five representative genomic regions showing the occupancy of histones modified at H3K4me3 (active epigenetic mark) before (Ctrl) and after 2h of 1α,25(OH)2D3 treatment (vitD). The 1α,25(OH)2D3 inducible peaks are highlighted in grey.

Figure 4 Venn diagram showing the number of genes up- (**A**) or downregulated (**B**) after 3h and 6h of 1α,25(OH)2D3 treatment, respectively. Using RNA-Seq analysis we determined the genes differentially regulated after 100nM 1α,25(OH)2D3 treatment vs control.

Figure 5 VDR and Klf4 motifs are over-represented after 1α,25(OH)2D3 treatment.

Pscan output file using the -950bp until +50bp promoter regions and the Jaspar database. The over-representation of VDR (**A**) and Klf4 binding sites (**B**) in the up- and downregulated genes after 100nM 1α,25(OH)2D3 treatment.

Figure 6 BIOCARTA pathways significantly enriched after (**A**) 3h or (**B**) 6h of 100nM 1α,25(OH)2D3 treatment in SKBr3 cells.

Figure 7 KEGG pathways significantly enriched for all upregulated genes after 3h of 100nM 1α,25(OH)2D3 treatment in SKBr3 cells.

Figure 8 KEGG pathways significantly enriched for all genes differentially regulated after 6h of 100nM 1α,25(OH)2D3 treatment in SKBr3 cells.

Figure 9 Gene ontology terms significantly enriched in all genes differentially regulated after 3h and 6h of 100nM 1α,25(OH)2D3 treatment in SKBr3 cells.

Table 1 List of genes differentially regulated after 3h of 100nM 1α,25(OH)2D3 treatment.

Table 2 List of genes differentially regulated after 6h of 100nM 1α,25(OH)2D3 treatment.

Table 3 List of genes upregulated after 3h of 100nM 1α,25(OH)2D3 treatment and their corresponding enrichment in H3K4me3 modification within 5,000bp flanking the TSS.

Supplementary Figure 1 Summary report graphs generated by DEGseq.

The statistical analysis for differential gene expression profiles after (**A**) 3h or (**B**) 6h of 1α,25(OH)2D3 treatment was performed with the DEGseq package. Five plots have been generated by DEGseq with the MARS method (MA-plot-based method with Random Sampling model). From left to right, from top to bottom, the graphs generated by this method are histograms of the (log2) number of reads per gene for the samples treated with vehicle alone (minus) or treated with 1α,25(OH)2D3 respectively, boxplots of the (log2) number of

reads per gene, scatterplots comparing the (log2) number of reads per gene for the treated and not treated samples, and the MA-plot of the number of reads per gene comparing again treated and not treated samples. Each point in the graph represents an individual gene. The red points in the last graph (MA-plot) are the genes identified as differentially expressed.