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# VDR primary targets by genome-wide transcriptional profiling

Frauke Goeman[a,*], Francesca De Nicola[b], Paolo D'Onorio De Meo[c], Matteo Pallocca[a], Berardino Elmi[b], Tiziana Castrignanò[c], Graziano Pesole[d], Sabrina Strano[e], Giovanni Blandino[a], Maurizio Fanciulli[b], Paola Muti[f,**]

[a] Translational Oncogenomics Unit, Italian National Cancer Institute "Regina Elena", 00144 Rome, Italy
[b] Laboratory of Epigenetic, Molecular Medicine Area, Italian National Cancer Institute "Regina Elena", 00144 Rome, Italy
[c] HPC CINECA, Via dei Tizii, 6 00185 Rome, Italy
[d] Institute of Biomembranes and Bioenergetics of the National Research Council and Department of Biosciences, Biotechnology and Biopharmaceutics, University of Bari, 70125 Bari, Italy
[e] Molecular Chemoprevention Unit, Italian National Cancer Institute "Regina Elena", 00144 Rome, Italy
[f] Department of Oncology, McMaster University, Main Street West Hamilton, ON L8S 4K1, Canada

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ABSTRACT

There is growing evidence that 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3) plays a role in breast cancer prevention and survival. It elicits a variety of antitumor activities like controlling cellular differentiation, proliferation and angiogenesis. 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 cancer cells using RNA-Seq technology. We identified early transcriptional targets of 1α,25(OH)2D3 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. Moreover, we could show that 1α,25(OH)2D3 elevates the trimethylation of histone H3 lysine 4 at several target gene promoters. Our present transcriptomic analysis of differential expression after 1α,25(OH)2D3 treatment provides a resource of primary 1α,25(OH)2D3 targets that might drive the antiproliferative action in breast cancer epithelial cells.



## 1. 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 evidence 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 [2]. 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

*Abbreviations:* 1α,25(OH)2D3, 1α,25-dihydroxyvitamin D3; ChIP, chromatin immunoprecipitation; ChIP-Seq, ChIP-sequencing; DAVID, Database for Annotation, Visualization and Integrated Discovery; FVKM, fragments per virtual kb per million; log2fc, log2 fold change; H3K4me3, trimethylation at lysine 4 of histone H3; HNSCC, head and neck squamous cell carcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes; MARS, MA-plot-based method with random sampling model; qPCR, quantitative PCR; qRT-PCR, quantitative RT-PCR; RE, response element; RNA-Seq, RNA-sequencing; TSS, transcriptional start site; VDR, vitamin D receptor; VDRE, vitamin D response element.

* Corresponding author. Tel.: +39 0652662879; fax: +39 0652662880.
** Corresponding author. Tel.: +1 9055272299x42606; fax: +1 9055752639.
*E-mail addresses:* frauke.goeman@gmail.com, fraukegoeman@yahoo.it (F. Goeman), denicola@ifo.it (F. De Nicola), p.donoriodemeo@cineca.it (P.D.D. Meo), matteo.pallocca@gmail.com (M. Pallocca), beradiel87@gmail.com (B. Elmi), t.castrignano@cineca.it (T. Castrignanò), graziano.pesole@biologia.uniba.it (G. Pesole), strano@ifo.it (S. Strano), gblandino@activep53.eu (G. Blandino), fanciulli@ifo.it (M. Fanciulli), muti@mcmaster.ca (P. Muti).

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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 1α,25(OH)2D3 are exerted through its cognate nuclear receptor, the vitamin D receptor (VDR). Invasive mammary tumor cell lines deriving from VDR knockout mice demonstrated that VDR is necessary for the antiproliferative activity of 1α,25(OH)2D3 *in vitro* [8]. Furthermore, knockout mice show increased chemical carcinogenesis in mammary, epidermis and lymphoid tissue [2]. VDR is a member of the nuclear receptor superfamily which frequently heterodimerizes on its DNA response element with another nuclear receptor superfamily member, the retinoic X receptor. Upon ligand binding VDR is able to activate or repress the transcription of its target genes depending on the type of response element (RE) [9]. 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 [10].

Interestingly, recent evidence 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 [11].

In order to determine the molecular genetic events underlying the broad physiological activities of 1α,25(OH)2D3 we performed genome-wide differential expression profiling via deep sequencing analysis. In particular, we were interested to investigate the global transcriptional signature of 1α,25(OH)2D3 in breast cancer cells with a mutant p53 background. Therefore, we used SKBr3 breast cancer epithelial cells that are inhibited in their growth after prolonged treatment with 1α,25(OH)2D3 although they are harboring endogenously the conformational mutant p53R175H which is one of the most frequent mutations in breast cancer. Moreover, we conducted ChIP-Seq analyses 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 1α,25(OH)2D3 on transcription and H3K4me3 histone modification.

## 2. Methods

### 2.1. Cell culture

SKBr3 breast cancer cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and penicillin/streptomycin. The cells were treated for 3 or 6 h for the RNA-Seq, and 2 h for the ChIP-Seq with 100 nM 1α,25(OH)2D3 or vehicle as control.

### 2.2. Quantitative PCR

RNA was reverse transcribed with the random hexamer method (M-MLV reverse transcriptase, Life Technologies, Carlsbad, CA, USA). Quantitative PCR (qPCR) 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, *CYP24A1*-R GGGATTACGGGATAAATTGTAGAGAA, *CDKN1A*-F CTGGAGACTCTCAGGGTCGAA, *CDKN1A*-R GCGGATTAGGGCTTCCTCTT and *RPL19*-F CGGAAGGGCAGGCACAT, *RPL19*-R GGCGCAAAATCCTCATTCTC for normalization.

### 2.3. 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 2 μg 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 qPCR protocol with specific primers complementary to adapter sequences. Therefore, only the adapter-ligated fragments that are appropriate for sequencing were quantified. Based on the qPCR quantification, libraries were normalized to 1 nM and denatured by using 0.1N 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.

### 2.4. ChIP-Seq

Chromatin immunoprecipitation was performed as described earlier [12] with minor changes. Cells were cross-linked for 10 min with 1% formaldehyde. The fixation was stopped by adding 0.125 M 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 resuspended and lysed in 1% SDS, 10 mM EDTA, 50mMTris-HCl (pH 8.0). The resulting chromatin was fragmented to a size range of 100–350 bp 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 [5 mg/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 100 μl PBS/BSA[5 mg/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 $NaHCO_3$ 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 2 h. 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 or original input 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. (36 bp, 21–26 Mio quality-filtered and uniquely aligned reads per sample).



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