Journal of Steroid Biochemistry & Molecular Biology 121 (2010) 142–145

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

The genes encoding cytokines *IL-2*, *IL-10* and *IL-12B* are primary 1α,25(OH)$_2$D$_3$ target genes☆

Juha M. Matilainen, Antti Räsänen, Petra Gynther, Sami Väisänen*

Department of Biosciences, University of Eastern Finland, FIN-70211 Kuopio, Finland

ARTICLE INFO

Article history:
Received 23 November 2009
Received in revised form 12 February 2010
Accepted 8 March 2010

Keywords:
Chromatin
Cytokine
VDR
Vitamin D response elements
Transcription

ABSTRACT

A number of studies have described the effects of 1α,25(OH)$_2$D$_3$ in immune system. Most of the known effects of 1α,25(OH)$_2$D$_3$ are indirect since only two functional VDREs that regulate transcription of cytokine gene has been reported until today. In this study we have examined a possibility of direct transcriptional regulation of *IL-2*, *IL-10* and *IL-12B* genes in activated Jurkat or THP-1 cells via liganded VDR by using gene expression analysis and chromatin immunoprecipitation assays. According to our data the *IL-2*, *IL-10* and *IL-12B* genes respond to 1α,25(OH)$_2$D$_3$ treatment by 3–6 h. In addition, all of these genes contain several genomic regions that recruit VDR in a ligand dependent fashion. These data suggest that the above cytokines are under direct transcriptional regulation by 1α,25(OH)$_2$D$_3$.

1. Introduction

The biological effects of the biologically most active form of vitamin D, 1α,25-dihydroxyvitamin D$_3$ (1α,25(OH)$_2$D$_3$) are mediated through the vitamin D receptor (VDR), which is a member of a superfamily of ligand-inducible nuclear receptors that control expression of their primary target genes in response to binding of steroidal or other lipophilic compounds. The binding of 1α,25(OH)$_2$D$_3$ to VDR changes the conformation of the ligand-binding domain of the VDR [1]. The changed conformation promotes the interaction of VDR with its heterodimeric partner, the retinoid X receptor (RXR) and modulates interactions between VDR and a number of different nuclear proteins, such as co-activators and co-repressors [2]. These interactions lead to the association of activating or repressing VDR complexes with specific genomic sequences (VDREs) that are located within the regulatory regions of primary 1α,25(OH)$_2$D$_3$ responding genes and ultimately modulate the expression of these genes.

In addition to the classic functions in controlling the mineral homeostasis and cell growth and differentiation, 1α,25(OH)$_2$D$_3$ has also role in immune response [3,4]. Repression of *IL-2* and *IL-12* cytokines in response to 1α,25(OH)$_2$D$_3$ treatment is well documented in different cells of the immune system as well as *in vivo* [5–11]. The effect of 1α,25(OH)$_2$D$_3$ to the production of cytokine *IL-10* has been reported to be opposite to that of *IL-2* and *IL-12B*, while it is induced by 1α,25(OH)$_2$D$_3$ in T and B cells [12–14]. However, there are also contradictory studies, that suggest that 1α,25(OH)$_2$D$_3$ treatment decreases the amount of IL-10 in cultured cells and living animals [8,10,15]. According to the present understanding, most of the effects of 1α,25(OH)$_2$D$_3$ on cytokine production appear to be indirect, since until today active vitamin D response elements (VDRE) have been identified only within *IL-10* gene [13,16]. In this study we have examined the possibility of direct transcriptional regulation of *IL-2*, *IL-10* and *IL-12B* genes via liganded VDR by using gene expression analysis and chromatin immunoprecipitation assays. According to our data the *IL-2*, *IL-10* and *IL-12B* genes contain several genomic regions that recruit VDR in a ligand dependent fashion.

2. Materials and methods

2.1. Cell culture

THP-1 human acute monocytic leukemia cells and Jurkat human T lymphocyte leukemia cells were maintained in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/ml streptomycin,

Abbreviations: 1α,25(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D$_3$; ChIP, chromatin immunoprecipitation; FBS, fetal bovine serum; RE, response element; RXR, retinoid X receptor; TSS, transcription start site; VDR, vitamin D receptor; VDRE, vitamin D response element.

☆ Special issue selected article from the 14th Vitamin D Workshop held at Brugge, Belgium on October 4–8, 2009.

* Corresponding author at: Department of Biosciences, University of Eastern Finland, P.O. Box 1627, FIN-70211 Kuopio, Finland. Tel.: +358 40 3553064; fax: +358 17 2811510.
E-mail addresses: Sami.Vaisanen@uku.fi, Sami.Vaisanen@uef.fi (S. Väisänen).

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.03.020

and 100 U/ml penicillin in a humified 95% air/5% CO_2 incubator. For experiments the cells were maintained in phenol red-free DMEM, supplemented with 5% charcoal-stripped FBS, 2 mM L-glutamine, 0.1 mg/ml streptomycin, and 100 U/ml penicillin. Prior to mRNA or chromatin extraction, THP-1 cells were first treated with 100 ng/ml LPS for 24 h, and Jurkat cells with 2 μg/ml phytohemagglutinin (PHA) (Sigma–Aldrich) and 50 ng/ml 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma–Aldrich) for 24 h. After that the cells were exposed to either solvent (ethanol, 0.1% final concentration) or to 10 nM 1α,25(OH)$_2$D$_3$ (kindly provided by Milan Uskokovic, Bioxell Inc, Nutley, USA). For mRNA stability studies the cells were first treated as above after which 1 μg/ml actinomycin D (Sigma–Aldrich) was added.

2.2. Total RNA extraction, cDNA synthesis, and real-time quantitative PCR

Total RNA was extracted using High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany), and cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. Real-time quantitative PCR was performed with LightCycler 480 apparatus (Roche) using TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA), with *RPLP0* serving as a control gene. PCR cycling conditions were: 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 60 s at 60 °C. Fold changes were calculated using the formula $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = \Delta Ct_{(stimulus)} - \Delta Ct_{(solvent)}$, and $\Delta Ct = Ct_{(target\ gene)} - Ct_{(RPLP0)}$. Ct is the cycle at which the threshold line is crossed. Two-tailed Student's *t*-tests were performed to calculate statistical significances between solvent treated and ligand treated samples.

2.3. ChIP assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously described [17]. The recovered chromatin solutions were diluted 1:10 (v/v) in ChIP dilution buffer, and incubated with 1 μg of antibody against VDR (sc-1008) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) at 4 °C overnight. The non-specific IgG (12-370) used as a control was from Upstate Biotechnology (Lake Placid, NY, USA). The immunocomplexes were collected with protein A agarose slurry (Millipore, Billerica, MA, USA) and washed as previously described. The samples were reverse cross-linked with 2 μl of proteinase K (Fermentas, Vilnius, Lithuania) overnight at 64 °C, after which phenol:chloroform extraction and ethanol precipitation were performed. ChIP samples were analyzed with real-time PCR by using SYBR Green dye and 2% agarose gel electrophoresis.

3. Results

3.1. Genes encoding cytokines IL-2, IL-10 and IL-12B respond rapidly to 1α,25(OH)$_2$D$_3$ treatment

We measured ligand-dependent mRNA expression of *IL-2* in Jurkat cells pretreated with PHA/TPA and expression of *IL-10* and *IL-12B* in THP-1 cells pretreated with LPS. According to our data, the expression of *IL-2* (Fig. 1A) and *IL-10* (Fig. 1B) decreased sig-

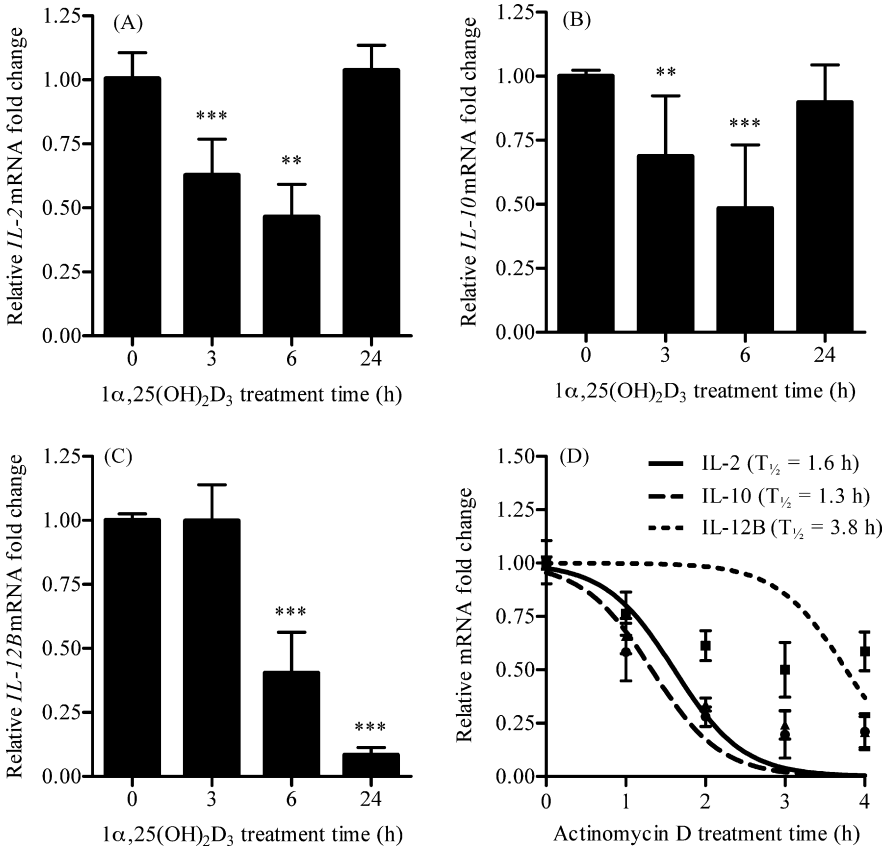

Fig. 1. Expression profiling of the human *IL-2*, *IL-10* and *IL-12B* genes in Jurkat or THP-1 cells. Quantitative real-time PCR was performed in order to study the relative mRNA expression levels of the *IL-2* in activated Jurkat cells (A) and IL-10 (B) and IL-12B (C) in activated THP-1 cells and their responsiveness to 1α,25(OH)$_2$D$_3$ over time. The cells were treated with 10 nM 1α,25(OH)$_2$D$_3$ for indicated times prior to the extraction of RNA. Stabilities of *IL-2*, *IL-10* and *IL-12B* mRNAs in Jurkat or THP-1 cells were studied by treating the cells with actinomycin D (1 μg/ml) prior to extraction of total RNA (D). Two-tailed Student's *t*-tests were performed using Prism4.0c software and *P*-values were calculated in reference to solvent treatments (*P = 0.01 to 0.05, **P = 0.001 to 0.01, ***P < 0.001). In each panel, n is at least 3. Error bars indicate S.D.

Table 1
PCR primers used in ChIP assays. Sequences and location relative to the TSS (+1) are shown.

Gene	Region	Location from TSS	Primer sequences
IL-2	23	−8744 bp to −8280 bp	5′-ATGGCTGGGTACTCCTC-3′ 5′-TTCTTTGCCGTGGGTTC-3′
	1	+1255 bp to +1647 bp	5′-TGCCAGGGTGAATCCAA-3′ 5′-TTCTACCACCCCCTAAAT-3′
IL-10	22	−7885 bp to −7271 bp	5′-CACATGCTCAGCCGCCCA-3′ 5′-GCACTCTTCACTTTCTACTTC-3′
	9	−1986 bp to −1432 bp	5′-CTAACACAGACACAGCCCAG-3′ 5′-GCAATAGCACCTGTCCTCC-3′
	2	+1006 bp to +1473 bp	5′-AGGGCATCAAAAAGACCGCAT-3′ 5′-TGGATCATCTCAGACAAGGC-3′
IL-12B	17	−6750 bp to −6297 bp	5′-ACCACACCTAAAACAGATGC-3′ 5′-ACCACCACTAACATACTTTG-3′
	13	−4512 bp to −3643 bp	5′-ACACTTCCACTCTTCTTTTG-3′ 5′-GTGATAGACAAGGCAGGTTT-3′
	5	−516 bp to −17 bp	5′-AGGACTAATGTGGAGGAAGG-3′ 5′-TGCTGTTGCTGGTACTGGAA-3′

nificantly already after 3 h ligand treatment. Interestingly, the repression of both *IL-2* and *IL-10* was temporal, since the lowest expression could be observed 6 h after onset of ligand treatment and after that the expression returned to basal level at 24 h. Instead, the 1α,25(OH)$_2$D$_3$ dependent repression of *IL-12B* expression could not be observed until 6 h after onset of ligand treatment, but then the repression was continuous and the strongest repression was reached 24 h after onset of ligand treatment (Fig. 1C). These results suggest that the above genes are primary 1α,25(OH)$_2$D$_3$ target genes and thus there should be at least one direct or indirect binding site for VDR within their promoter or coding regions.

The stabilities of *IL-2*, *IL-10* and *IL-12B* mRNAs were determined by treating the Jurkat or THP-1 cells with actinomycin D after PHA/TPA or LPS pretreatments, respectively (Fig. 1D). According to our results, the half-lifes of the mRNAs of *IL-2*, *IL-10* and *IL-12B* were 1.6 h, 1.3 h and 3.8 h, respectively.

3.2. Number of VDR binding sites within IL-2, IL-10 and IL-12B genes

In order to locate the genomic regions within *IL-2*, *IL-10* and *IL-12B* genes that recruit VDR in a ligand dependent fashion, we followed the same strategy as were used previously [17]. First we used *in silico* screening to locate all the putative VDREs and E-box elements. With all the genes above, regions from −10 kB to +2 kB were screened. The screening revealed a number of classic DR3-type and DR4-type VDREs as well as ER7, ER8 and ER9 elements. In addition, several E-box elements could be found (Fig. 2).

Next we used chromatin from formaldehyde cross-linked, PHA/TPA or LPS pretreated Jurkat or THP-1 cells, respectively, which had been treated with 1α,25(OH)$_2$D$_3$. The chromatin was precipitated with anti-VDR antibody and the eluted chromatin template was used for a ChIP scanning analysis (data not shown). Our ChIP data suggest that VDR associated ligand dependently at two regions within *IL-2* gene (Fig. 2A). *IL-10* had three regions that recruited VDR (Fig. 2B) and *IL-12B* had three VDR binding regions (Fig. 2C). The PCR primers used to amplify the genomic regions that recruited VDR are indicated in Table 1.

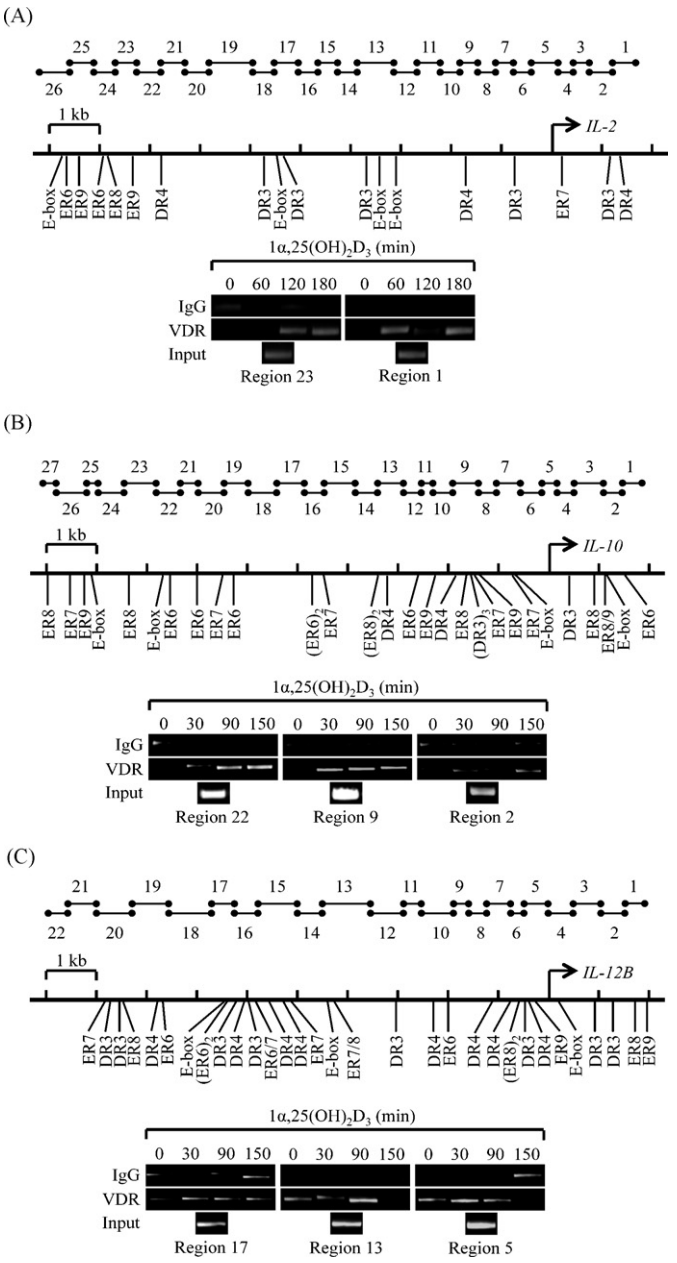

Fig. 2. *In silico* screening and ligand-dependent association of VDR to *IL-2* (A), *IL-10* (B) and *IL-12B* (C) genes in Jurkat or THP-1 cells. For ChIP analysis, chromatin was extracted from PHA/TPA or LPS pretreated Jurkat or THP-1 cells that had been treated for indicated time periods with 10 nM 1α,25(OH)$_2$D$_3$.

4. Discussion

Although there are number of studies on 1α,25(OH)$_2$D$_3$ functions in immune system, only two functional VDREs have been reported within any cytokine genes till today. In this study we utilized *in silico* screening and ChIP assays in order to locate genomic regions that recruit liganded VDR within *IL-2*, *IL-10* and *IL-12B* genes. Our mRNA expression assays suggested that *IL-2* and *IL-10* were significantly and ligand dependently repressed already 3 h after onset of 1α,25(OH)$_2$D$_3$ treatment. Due to the rapid and strong repression, it is unlikely that the observed ligand effect could be explained as a secondary or other indirect effect suggesting a direct transcriptional down regulation of these genes by 1α,25(OH)$_2$D$_3$. An interesting feature in the mRNA expression profiles of *IL-2* and *IL-10* was their temporality as both of them were strongly repressed 3 and 6 h after onset of ligand treatment, but then their expression returned back to basal level. Because the mRNA levels were not determined later than 24 h after onset of 1α,25(OH)$_2$D$_3$ treatment, we cannot exclude a possibility that one or both of these genes would be induced after longer ligand treatment times by secondary effects or other indirect ways. The possibility of direct

transcriptional regulation of *IL-2* and *IL-10* by $1\alpha,25(OH)_2D_3$ was supported by our *in silico* and ChIP scanning data which revealed genomic regions that both recruited liganded VDR and contained classic NR binding consensus sequences.

The repression of *IL-12B* was not visible until 6 h after onset of $1\alpha,25(OH)_2D_3$ treatment. However, the stability of *IL-12B* mRNA was more than two fold in comparison to that of *IL-2* or *IL-10*, which explains at least in part, the later repression than with *IL-2* and *IL-10*. Nevertheless, in the case of *IL-12B* the observed repression can be explained as secondary effect or indirect effect via repression of NF-kB transcription and inhibition of NF-kB binding to *IL-12B* promoter as was reported by D'Ambrosio et al. [7]. However, our ChIP results revealed three genomic regions within *IL-12B* promoter that recruited VDR in a ligand dependent fashion suggesting that $1\alpha,25(OH)_2D_3$ may directly regulate the transcription of *IL-12B*.

In conclusion, by using *in silico* screening and ChIP scanning we have found several VDR binding sites within *IL-2*, *IL-10* and *IL-12B* genes that recruit VDR ligand dependently. Whether these regions actually take part to the transcriptional regulation of above cytokines remains to be elucidated.

Acknowledgments

We would like to thank Mrs Hanna Eskelinen and Mrs Maija Hiltunen for help in cell culture. This work was supported by The Academy of Finland [grant number 00897].

References

[1] S. Väisänen, K. Juntunen, A. Itkonen, P. Vihko, P.H. Mäenpää, Conformational studies of human vitamin-D receptor by antipeptide antibodies, partial proteolytic digestion and ligand binding, Eur. J. Biochem. 248 (1997) 156–162.
[2] L. Xu, C.K. Glass, M.G. Rosenfeld, Coactivator and corepressor complexes in nuclear receptor function, Curr. Opin. Genet. Dev. 9 (1999) 140–147.
[3] H.F. Deluca, M.T. Cantorna, Vitamin D: its role and uses in immunology, FASEB J. 15 (2001) 2579–2585.
[4] C. Mathieu, L. Adorini, The coming of age of 1,25-dihydroxyvitamin D(3) analogs as immunomodulatory agents, Trends Mol. Med. 8 (2002) 174–179.
[5] M. Cippitelli, A. Santoni, Vitamin D3: a transcriptional modulator of the interferon-gamma gene, Eur. J. Immunol. 28 (1998) 3017–3030.
[6] I. Alroy, T.L. Towers, L.P. Freedman, Transcriptional repression of the interleukin-2 gene by vitamin D3: direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor, Mol. Cell. Biol. 15 (1995) 5789–5799.
[7] D. D'Ambrosio, M. Cippitelli, M.G. Cocciolo, D. Mazzeo, P. Di Lucia, R. Lang, F. Sinigaglia, P. Panina-Bordignon, Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene, J. Clin. Invest. 101 (1998) 252–262.
[8] L.A. Lyakh, M. Sanford, S. Chekol, H.A. Young, A.B. Roberts, TGF-beta and vitamin D3 utilize distinct pathways to suppress IL-12 production and modulate rapid differentiation of human monocytes into CD83+ dendritic cells, J. Immunol. 174 (2005) 2061–2070.
[9] A. Takeuchi, G.S. Reddy, T. Kobayashi, T. Okano, J. Park, S. Sharma, Nuclear factor of activated T cells (NFAT) as a molecular target for 1alpha,25-dihydroxyvitamin D3-mediated effects, J. Immunol. 160 (1998) 209–218.
[10] C.J. Bemiss, B.D. Mahon, A. Henry, V. Weaver, M.T. Cantorna, Interleukin-2 is one of the targets of 1,25-dihydroxyvitamin D3 in the immune system, Arch. Biochem. Biophys. 402 (2002) 249–254.
[11] X.P. Qi, P. Li, G. Li, Z. Sun, J.S. Li, 1,25-Dihydroxyvitamin D(3) regulates LPS-induced cytokine production and reduces mortality in rats, World J. Gastroenterol. 14 (2008) 3897–3902.
[12] F.J. Barrat, D.J. Cua, A. Boonstra, D.F. Richards, C. Crain, H.F. Savelkoul, R. de Waal-Malefyt, R.L. Coffman, C.M. Hawrylowicz, A. O'Garra, In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines, J. Exp. Med. 195 (2002) 603–616.
[13] G. Heine, U. Niesner, H.D. Chang, A. Steinmeyer, U. Zügel, T. Zuberbier, A. Radbruch, M. Worm, 1,25-Dihydroxyvitamin D(3) promotes IL-10 production in human B cells, Eur. J. Immunol. 38 (2008) 2210–2218.
[14] A. Boonstra, F.J. Barrat, C. Crain, V.L. Heath, H.F. Savelkoul, A. O'Garra, 1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells, J. Immunol. 167 (2001) 4974–4980.
[15] E. van Etten, O. Dardenne, C. Gysemans, L. Overbergh, C. Mathieu, 1,25-Dihydroxyvitamin D3 alters the profile of bone marrow-derived dendritic cells of NOD mice, Ann. N. Y. Acad. Sci. 1037 (2004) 186–192.
[16] L. Tavera-Mendoza, T.-T. Wang, B. Lallemant, R. Zhang, Y. Nagai, V. Bourdeau, M.R. Calderon, J. Desbarats, S. Mader, J.H. White, Convergence of vitamin D and retinoic acid signalling at a common hormone response element, EMBO Rep. 7 (2006) 180–185.
[17] M.M. Turunen, T.W. Dunlop, C. Carlberg, S. Väisänen, Selective use of multiple vitamin D response elements underlies the 1 alpha,25-dihydroxyvitamin D3-mediated negative regulation of the human CYP27B1 gene, Nucleic Acids Res. 35 (2007) 2734–2747.