



Research article

RNAi-mediated knockdown of VDR surprisingly suppresses cell growth in Jurkat T and U87-MG cells

Zeinab Shirvani-Farsani^a, Mehrdad Behmanesh^{b,*}^a Department of Cellular and Molecular Biology, Faculty of Life Sciences and Technology, Shahid Beheshti University G.C., Tehran, IR Iran^b Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, IR Iran

ARTICLE INFO

Keywords:

Neuroscience
Cell biology
Immunology
Genetics
Molecular biology
U87-MG cells
Jurkat T cells
Apoptosis
VDR
shRNA
Multiple sclerosis

ABSTRACT

Vitamin D receptor (VDR) is a nuclear receptor for 1,25-Dihydroxyvitamin D₃. VDR is expressed in many types of cells and involved in different biological processes such as immunity and inflammation. In addition, the role of VDR has been indicated in different diseases including multiple sclerosis (MS). In this study, we investigated the effects of VDR knockdown on growth, apoptosis, cell cycle, and some inflammatory gene expressions in Jurkat and U87-MG cell lines.

The cell lines were transfected with plasmids encoding short hairpin RNA specific to VDR mRNA. Next, growth, apoptosis, and cell cycle were evaluated using MTT assay and annexin VDR along with flowcytometry. Then the mRNA expression of some genes was determined by real-time PCR at 24 h and 48 h after transfection.

The cell growth and apoptosis of VDR-shRNA transfected Jurkat T cells and U87-MG cells were surprisingly changed compared with those in control cells. The expression of IL-10, NF- κ B, TGF- β 1, TGF- β R I, and TGF- β R II in two cell lines transfected with VDR-shRNA was significantly changed compared to control cells.

VDR showed a new unexpected function to control cell growth in vitro. In addition, while VDR knocking down in two different cell lines of U87-MG and Jurkat cells had different effects on NF- κ B and TGF- β expression levels, its effects on cell growth and apoptosis were similar. This may suggest that these two different cell lines can show similar anti-proliferative effects by different downstream signalling pathways. Therefore, these data may be useful to design novel diagnostic and therapeutic methods for diseases such as MS.

1. Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) [1] which has a higher incidence at higher latitudes due to low exposure to sunlight and hence lower levels of vitamin D₃ (25(OH)D₃) [2]. Low levels of this vitamin have also been associated with higher susceptibility to different infections. Therefore, vitamin D plays an important role in immune regulation [3]. The receptor of vitamin D is vitamin D receptor (VDR) [4]. VDR is a transcriptional regulator belonging to the super family of nuclear receptors, which has interaction with specific DNA sequences [5]. This transcription factor is mainly distributed in cytoplasm, where it interacts with biologically active form of vitamin D, 1,25(OH)₂D₃, heterodimerizes with retinoid X receptor (RXR), and then translocates to nucleus. Then, in conjunction with several transcription factors, it interacts with vitamin D response elements (VDREs). Depending on the target genes, the VDR/RXR heterodimers induce or repress gene transcription (based on presence of

co-activator or co-repressors) [6]. VDREs are found in the regulatory region of many genes such as osteocalcin, osteopontin, calbindin-D28K, calbindin-D9K, p21WAF1/CIP1, NF- κ B, TGF- β 2, and vitamin D 24-hydroxylase [7]. VDR is expressed in many types of tissues and cells, such as the kinds of cells in the immune systems and cancer [8]. Studies have reported that vitamin D/VDR signaling regulates innate and adaptive immunity [9, 10]. For instance, vitamin D enhances IL-10 expression in dendritic cells [11] and induces it in T cells [12]. Further, vitamin D supplementation leads to increased IL-10 mRNA levels in vivo [13]. Also, vitamin D/VDR blocks NF- κ B activation pathway [14]. Indeed, IL-10 and NF- κ B are two regulators of several processes in immunity and inflammatory responses [15, 16]. Other downstream targets of VDR include components of the TGF- β signaling pathway. TGF- β s are the members of cytokines superfamily which interact with TGF- β receptors to regulate differentiation, cell growth and death, angiogenesis, immune response, and inflammation [17]. In addition to VDR roles in the immune system and inflammation, it has some functions in physiological and

* Corresponding author.

E-mail address: behmanesh@modares.ac.ir (M. Behmanesh).<https://doi.org/10.1016/j.heliyon.2019.e02837>

Received 4 May 2019; Received in revised form 9 August 2019; Accepted 11 November 2019

2405-8440/© 2019 Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

neurological development as well as protection against apoptosis [18]. Earlier studies demonstrated that VDR induces apoptosis and inhibits cell growth in the presence of its ligand, vitamin D [19].

Although a number of studies have investigated the probable roles of VDR in immunity, inflammation, neurobiology of MS, some molecular mechanisms of VDR in T cells and neuronal cells have remained controversial and unknown. Therefore, in this study, we analyzed the effects of VDR knockdown on cell growth, apoptosis, and cell cycle in the human primary glioblastoma cell line (U87-MG cell line) and human T-cell leukaemia cell line (Jurkat cell line). Furthermore, we indicate that the down-regulation of VDR can alter the expression of some genes involved in inflammatory processes. Based on the previous studies, we assumed that VDR down-regulation in glioblastoma cell line and human T cell line would induce cell growth. However, we observed that silencing VDR expression inhibited cell growth indicating a novel role of VDR in inducing cell growth of neuronal cells and lymphocytes. In addition, we hypothesized that if VDR down-regulation would lead to enhanced apoptosis in these two cell lines, then in future studies, it could be used as a therapeutic target in MS.

2. Materials and methods

2.1. Cell culture

In this study, Jurkat E-6 and U87-MG cell lines were used. These cell lines were cultured in RPMI-1640 medium (Cat. 11879020 Invitrogen, USA), supplemented with 10% fetal bovine serum (Cat. 16000044, Invitrogen, USA), 2 mM glutamine, 100 U/I penicillin and 100 U/I streptomycin. The cells were grown at 37 °C in an air/5% CO₂ atmosphere at constant humidity.

2.2. VDR knockdown by short hairpin (sh) RNA vectors

In order to knock-down VDR, Jurkat T and U87-MG cells were transfected with four pGFP-V-RS shRNA clones targeting human VDR transcripts [shRNA1 (GI379488), shRNA2 (GI379489), shRNA3 (GI379490), shRNA4 (GI379491)] and a scrambled non-targeting control (TR30013) (OriGene Technologies, Inc. USA). Lipofectamine 2000 (Cat. 11668-019, Invitrogen) was used as a transfection reagent. Following the transfection, the cells emitted green light under fluorescent microscopy. Before transfection, 0.1 μM 1,25(OH)₂D₃ (cat. D1530, Sigma-Aldrich) was added into the culture. The final concentration of vitamin D (0.1 μM) was specified from different dilutions in primary experiments.

2.3. RNA extraction and real-time PCR

Total RNA was extracted from cells and treated with DNase I (Sigma) at 37 °C for 30 min cDNA was synthesized using the Revert Aid™ Reverse Transcriptase (Cat. EP0441, Fermentas, Canada) with 3 μg of purified total RNA, oligo dT, and random hexamer primers (MWG, Germany) in a total volume of 20 μl reaction mixture, according to the manufacturer's instructions. For analyzing gene expression and testing the knockdown efficiency, qPCR was performed on Applied Biosystems 7500 Real Time PCR System (Applied Biosystem/MDS SCIEX, Foster City, CA, USA), with 10 μl of SYBR Green master mix (Cat. #RR430S Takara, Shiga, Japan), 10 ng cDNA, and 200 nM of forward and reverse primers up to final reaction volumes of 20 μl, according to the manufacturer's instructions. The primer sequences for qPCR are presented in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference. Fold expression values were calculated using the 2^{-ΔΔCt} method. All experiments were performed in duplicate.

2.4. Determination of cell growth

An MTT assay was performed to detect the effect of VDR silencing on normal and transfected Jurkat E-6 and U87-MG cells' growth. These cells

Table 1
Primer list of qRT-PCR.

Gene names	RefSeq number	Primer sequences
GAPDH	NM_002046.5	F: 5-CCATGAGAAGTATGACAAC-3 R: 5-GAGTCCTTCCACGATACC-3
IL-10	NM_000572.2	F: 5-CCCAGACATCAAGGCGCATGTG-3 R: 5-GTAGATGCCCTTCTCTTGGAGC-3
TGF-β1	NM_000660.5	F: 5-ACACATCAGAGCTCCGAGAAGCG-3 R: 5-AGCAGCCGGTTGCTGAGGTATCG-3
TGF-β2	NM_001135599.2	F: 5-ATCTAGGGTGGAAATGGATACACG-3 R: 5-TGGTTAGAGGTTCTAAATCT-3
TGF-β RI	NM_004612.2	F: 5-GGAGAAGTTTGGAGAGGAAAGT-3 R: 5-ACCAGAGCTGAGTCCAAGTACC-3
TGF-β RII	NM_001024847.2	F: 5-GCCTCCATTTGGTTCCAAGGT-3 R: 5-GTCTCACACACCATCTGGATGC-3
VDR	NM_000376.2	F: 5-GATGAGGAAGTGCAGAGGAAGC-3 R: 5-CTCCCTCCACCATCATTCACAC-3
NF-KB	NM_003998.3	F: 5-TACTCTGGCGCAGAAATTAGGTC-3 R: 5-ACTGTCTCGGAGCTCGTCTATTG-3

were seeded in 96-multiwell plates at a density of 2 × 10⁴ per well and incubated for 24 and 48 h after shRNA transfection. Next, 20 ml of 5 mg/ml MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Cat. 11465007001, Roche Applied Science, Indianapolis, IN, USA) solution was added to each well. After 4 h of incubation, the medium was removed from the wells, and 150 ml of solubilization solution (Roche Applied Science, Indianapolis, IN, USA) was added to each well. Color intensity was measured at 490 nm by an enzyme linked immunosorbent assay plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Flow cytometric apoptosis assay

The level of apoptosis in Jurkat E-6 and U87-MG cells was measured using the Annexin V Apoptosis Detection Kit FITC (Cat. 88-8005, eBioscience, Inc., San Diego, CA, USA) at 24 and 48 h following transfection according to the manufacturer's instructions. The cells were examined using FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA) and Quest software (BD Biosciences).

2.6. Flow cytometric cell cycle analyses

The cells were harvested through trypsinization 24 and 48 h after transfection, washed four times with PBS. Next, RNase A (100 mg/ml) was added and cells were stained with 50 μg/ml propidium iodide (PI; Cat. 88-8005, eBioscience, Inc, San Diego, CA, USA). The samples were incubated for an additional 30 min. Then, the cell cycle analysis was performed by using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). The cell cycle distribution in different phases was interpreted using Quest software (BD Biosciences).

2.7. Statistical analysis

In the current study, all experiments were assayed in duplicate and repeated at least three times in separate experiments. Statistical analyses were performed using statistical analysis software (GraphPad Software, La Jolla, CA, USA and SPSS Version 13.0 software, SPSS, Chicago, IL, USA). All data were expressed as mean ± standard deviation (SD). Statistical analysis of the data was carried out using either an unpaired, 2-tailed Student's t-test or one-way ANOVA test. The differences were considered to be significant when P < 0.05.

3. Results

3.1. VDR mRNA expression after shRNA transfection

Quantitative PCR revealed that the VDR transcript expression was reduced in VDR shRNA-transfected groups. In Jurkat T cells, the

reduction of the VDR mRNA expression at 24 and 48 h following transfection was 77 % and 71% respectively ($P < 0.0001$) (Fig. 1, A and B), while in U87-MG cells it was 74% and 76% respectively ($p = 0.0002$ and $p = 0.0005$) (Fig. 1, C and D).

3.2. Suppression of Jurkat T and U87-MG cell growth

The effect of VDR silencing on the cell growth after transfection with the shRNA is displayed in Fig. 2. At 24 and 48 h post-transfection, the cell growth of VDR-shRNA transfected Jurkat T cells was significantly decreased by 48% ($P < 0.05$) compared with that in the Scramble-shRNA cells and in the untransfected cells (Fig. 2, A). Also, there was a difference in the cell growth among VDR-shRNA transfected U87-MG cells, Scramble-shRNA cells, and the untransfected cells (22%, $P < 0.05$) (Fig. 2, B).

3.3. Silencing VDR induces apoptosis of Jurkat T and U87-MG cells

Flow cytometry analysis revealed that suppression of VDR significantly enhanced cell apoptosis in Jurkat T cells (Fig. 3, A-C) as well as in U87-MG cells (Fig. 4, A-C), compared with Scramble-shRNA cells and the untransfected cells (control).

In Jurkat T cells, VDR-shRNA treatment promoted cell apoptosis by approximately 17%, while Scramble-shRNA cells and the untransfected cells only showed 1% and 2 % cell apoptosis respectively (Fig. 3, D, $p < 0.01$). In U87-MG cells, VDR-shRNA treatment induced nearly 15% cell apoptosis compared to 9 % and 10% cell apoptosis in two other groups (Fig. 4, D, $p < 0.05$).

3.4. Analysis of cell cycle following VDR down regulation

Cell cycle analysis using a flow cytometer indicated that following transfection with VDR-shRNA, the percentage of Jurkat T and U87-MG cells in all the phases was not significantly changed compared with Scramble-shRNA cells and the untransfected cells ($P > 0.05$) (data not

shown).

3.5. Gene expression analysis after VDR silencing

In this study, the expression of IL-10, NF-KB, TGF- β 1, TGF- β 2, TGF- β R I, and TGF- β R II mRNAs was measured in Jurkat T and U87-MG cells at 24 h and 48 h post-transfection.

Analysis of qRT-PCR showed that the expression of IL-10 (0.06 fold, $p = 0.004$), NF-KB (0.02 fold, $p = 0.001$), TGF- β 1 (0.1 fold, $p = 0.008$), TGF- β R I (0.15 fold, $p = 0.012$), and TGF- β R II (0.18 fold, $p = 0.015$) in Jurkat T cells transfected with VDR-shRNA significantly diminished compared to scramble-shRNA cells and the untransfected cells (Fig. 5, A), but no significant difference was observed in these cells for TGF- β 2 ($p = 0.079$).

Additionally, the expression of NF-KB (111 fold, $p < 0.0001$), TGF- β 1 (8 fold, $p < 0.001$), TGF- β 2 (12 fold, $p < 0.001$), TGF- β R I (90 fold, $p < 0.001$), and TGF- β R II (145 fold, $p < 0.001$) in U87-MG cells after transfection with the VDR-shRNA, was significantly increased compared with Scramble-shRNA cells and the untransfected cells (Fig. 5, B), but this difference was not significant for IL-10 ($p = 0.082$).

Also, the expression of these mRNAs was measured in Jurkat T and U87-MG cells before and after calcitriol (1, 25-Dihydroxyvitamin D3) treatment (without shRNA). In Jurkat T cells, the expression of IL-10 ($p = 0.0057$) and TGF- β 1 ($p = 0.0038$) significantly enhanced after calcitriol treatment, but no significant difference was observed in these cells for TGF- β 2 ($p = 0.085$), NF-KB ($p = 0.071$), TGF- β R I ($p = 0.091$), and TGF- β R II ($p = 0.066$) (Fig. 5, C). While in U87-MG cells, the expression of TGF- β 1 (8 fold, $p < 0.001$) and TGF- β 2 (12 fold, $p < 0.001$) was significantly reduced after vitamin D treatment, but this difference was not significant for IL-10 ($p = 0.101$), NF-KB ($p = 0.095$), TGF- β R I ($p = 0.121$) and TGF- β R II (145 fold, $p < 0.001$) (Fig. 5, D).

4. Discussion

Vitamin D receptor (VDR) is a ligand dependent transcription factor,

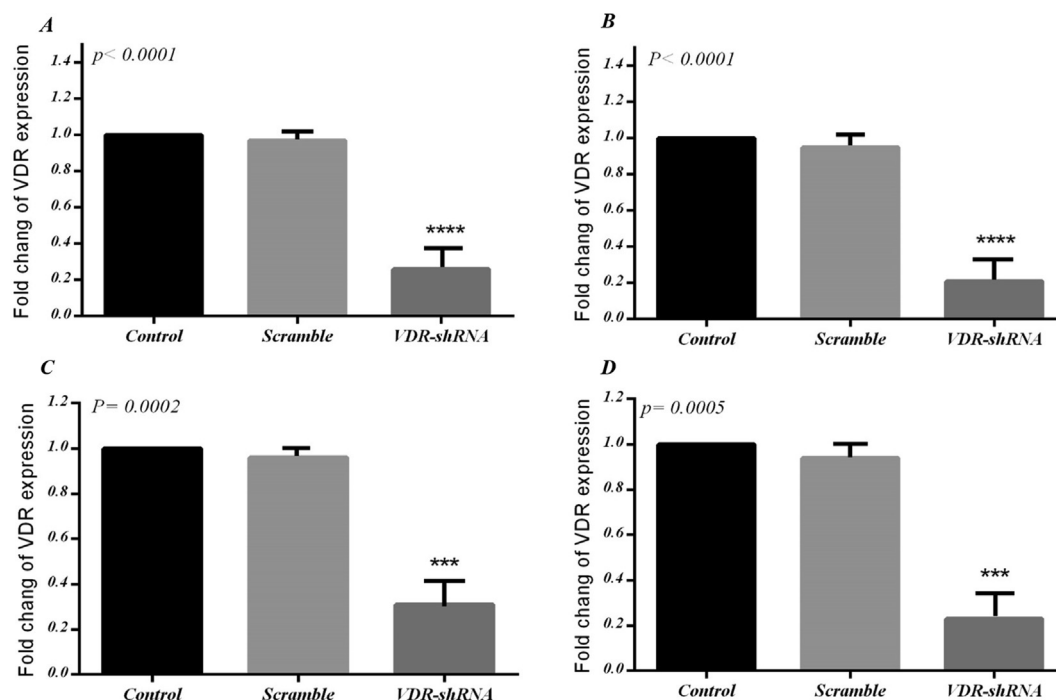


Fig. 1. Analysis of levels of VDR mRNA in Jurkat E-6 cells transfected with VDR-shRNA compared to scramble-shRNA cells and the untransfected cells at 24 h (A) and 48 h (B) after transfection; Analysis of VDR mRNA expression in U87-MG cells transfected with VDR-shRNA compared to Scramble-shRNA cells and the untransfected cells at 24 h (C) and 48 h (D) after transfection; Levels of VDR mRNA were analyzed by Real-Time PCR. mRNA fold change was presented. There are two replicates for each knock-down.

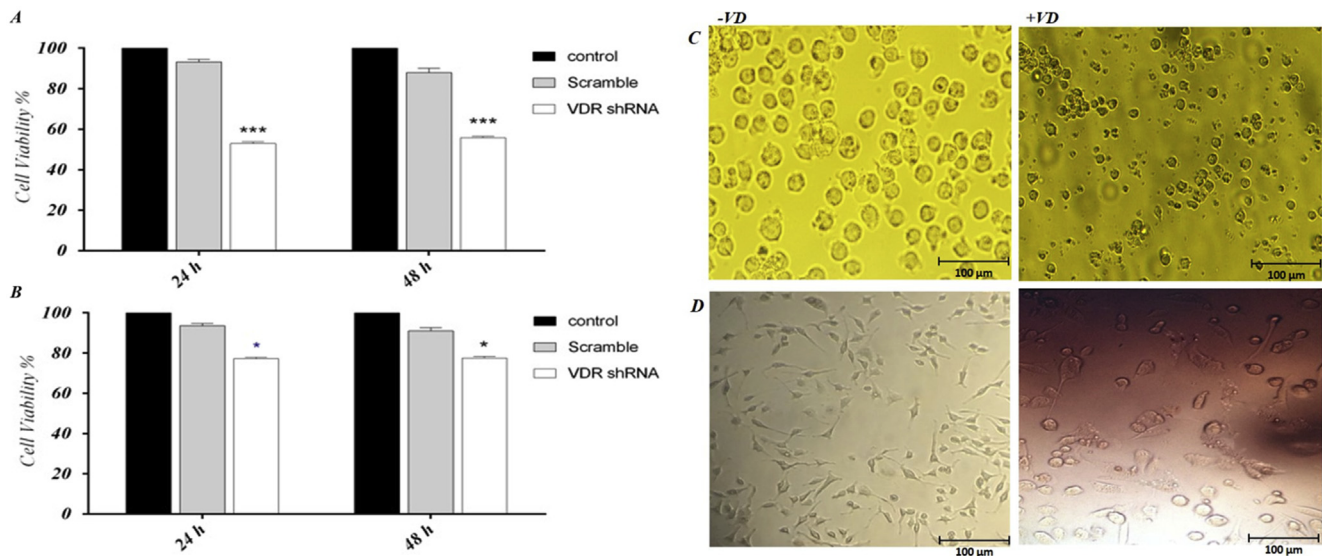


Fig. 2. VDR down-regulation reduces cell growth. The cell growth of Jurkat T cells (A) and U87-MG cells (B) was inhibited at 24 h and 48 h after transfection with VDR-shRNA compared to scramble-shRNA cells and the untransfected cells (controls). An MTT assay was used for cell growth measurement. Representative microscope images of Jurkat T cells (C) and U87-MG cells (D) before (left side) and after calcitriol treatment (right side). Cell viability reduced when cells treated with calcitriol. The results shown are from at least two biological replicates. -VD = without vitamin D; +VD = with vitamin D (1,25-Dihydroxyvitamin D3 or calcitriol).

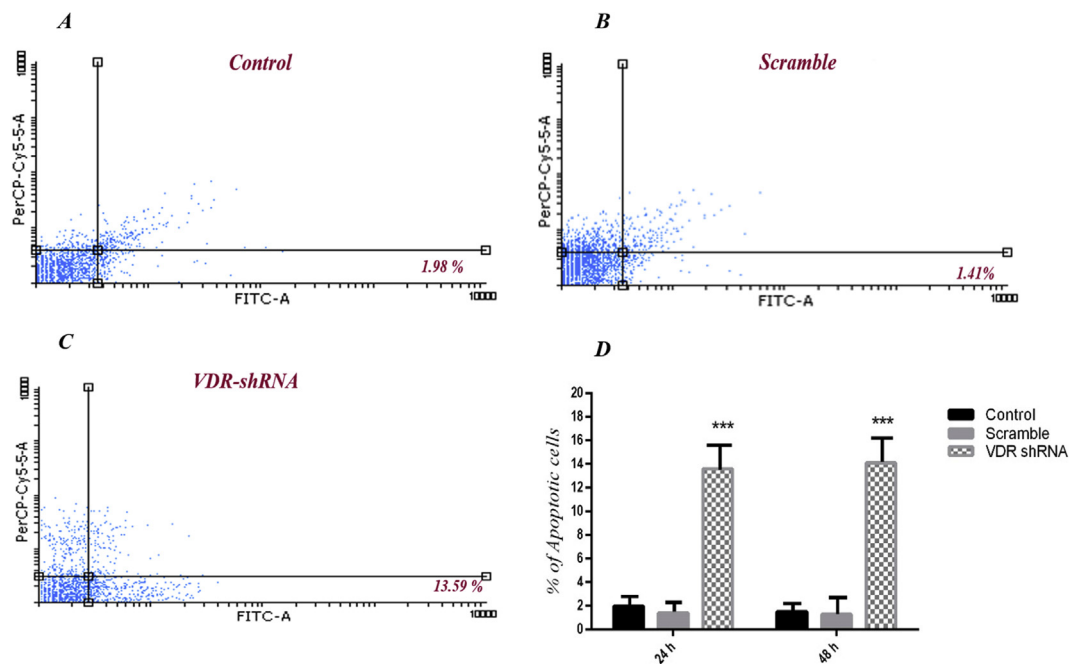


Fig. 3. FITC-Annexin V analysis of cell apoptosis. Jurkat T cells were untransfected (control) (A), transfected with scramble-shRNA (B) or transfected with VDR-shRNA (C). The percentage of apoptotic cells in Jurkat T cells (D) was presented at 24 h and 48 h after transfection. Left lower quadrant: live cells; Left upper quadrant: dead cells; Right lower quadrant: early apoptotic cells; Right upper quadrant: late apoptotic cells. The results shown are from at least two biological replicates.

which belongs to the superfamily of nuclear receptors [20]. Studies have suggested that VDR is expressed in a wide range of tissues and cell types, and has very different functions in several processes such as cell proliferation and differentiation, apoptosis, immunity, and inflammation [21, 22]. Here, we have evaluated the effects of VDR knock down on cell growth, apoptosis, and cell cycles of two originally different cell line, U87-MG cell line and Jurkat cell line. We decided to use U87-MG cells because this is a glioblastoma (astrocytoma) cell line originally derived from human brain [23]. Also, Jurkat is a type of T lymphocytes. T cells are one of cells in the immune system that their disruption have been reported in MS pathogenesis [24]. Previous studies have demonstrated

that 1,25(OH)2D3 has anti-proliferative effects by its receptor, VDR, on immune and inflammatory cells [25].

However, there is no evidence of the effects of VDR in U87-MG and Jurkat cell lines. Our analysis revealed that silencing VDR using shRNA reduced the expression levels of VDR-mRNA dramatically in both Jurkat T cells, and U87-MG cell line. Diminished VDR-mRNA levels subsequently decreased cell growth significantly and enhanced apoptosis in both cells ($P < 0.05$), but it had no significant effect on cell cycle.

There is inadequate evidence about the function of VDR in the absence of vitamin D. In breast cancer cell, unliganded VDR has proven to control breast cancer cell growth [26] and in skin cells, it is necessary for

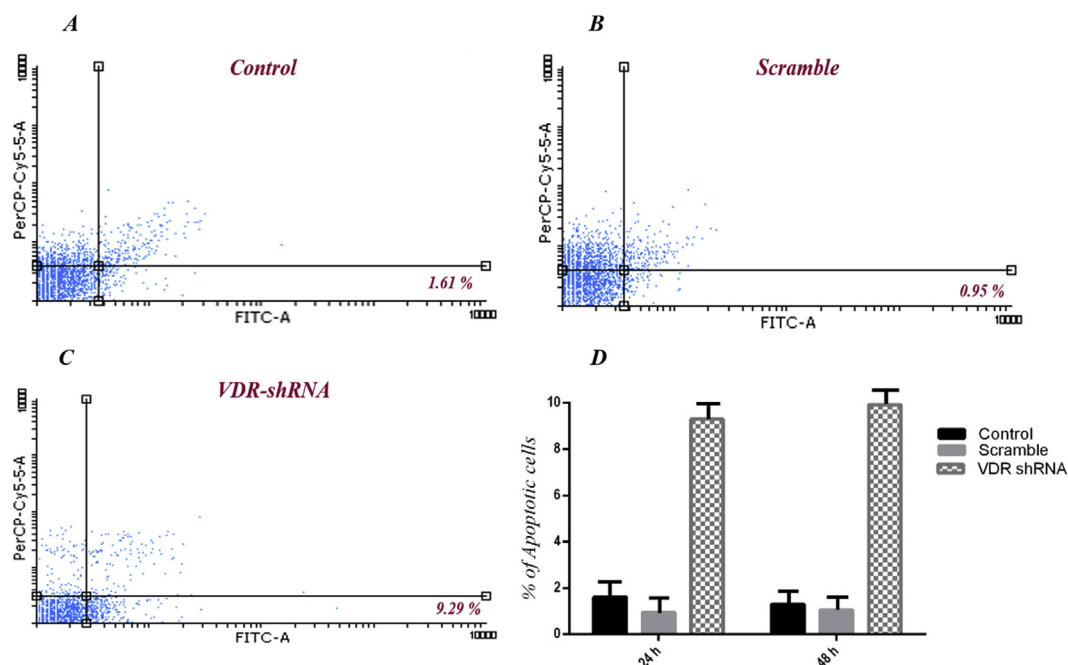


Fig. 4. FITC-Annexin V analysis of cell apoptosis. U87-MG cells were untransfected (control) (A), transfected with scramble-shRNA (B) or transfected with VDR-shRNA (C). The percentage of apoptotic cells in U87-MG cells (D) was presented at 24 h and 48 h after transfection. Left lower quadrant: live cells; Left upper quadrant: dead cells; Right lower quadrant: early apoptotic cells; Right upper quadrant: late apoptotic cells. The results shown are from at least two biological replicates.

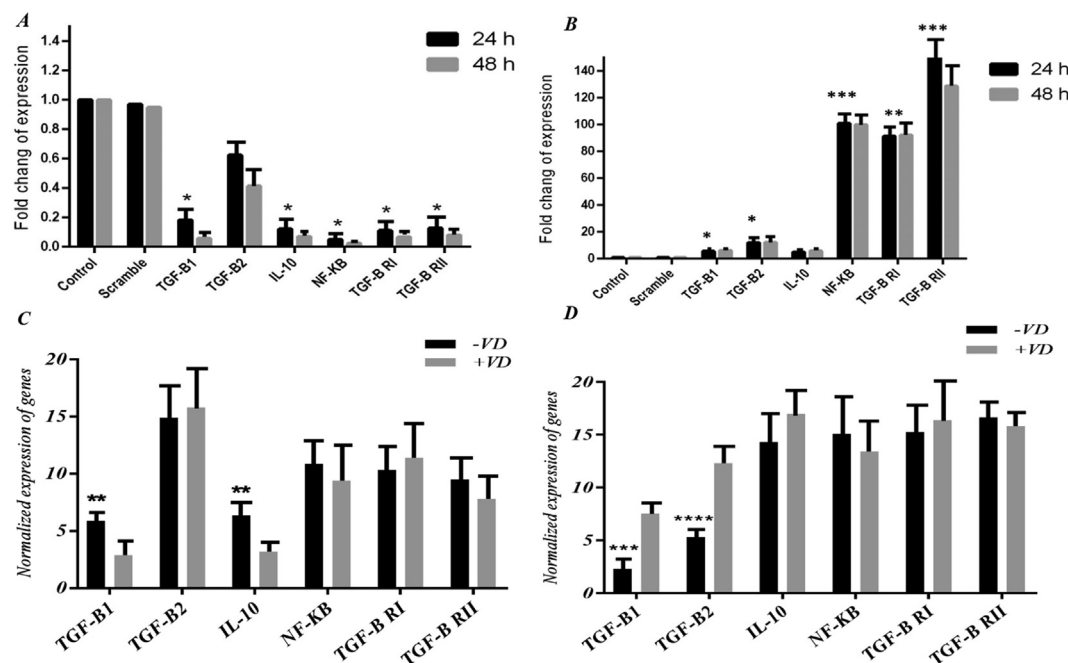


Fig. 5. Analysis of levels of mRNA expression in cell cultures following transfection with culture medium alone (control), scramble-shRNA, or VDR-shRNA at 24 h and 48 h after transfection or without transfection; (A) Down-regulation of IL-10, NF-κB, TGF-β1, TGF-β R I and TGF-β R II in Jurkat T cells after VDR knockdown; (B) Up-regulation of NF-κB, TGF-β1, TGF-β2, TGF-β R I and TGF-β R II in U87-MG cells after VDR silencing; (C) Up-regulation of IL-10 and TGF-β1 in Jurkat T cells after calcitriol treatment; (D) Down-regulation of TGF-β1 and TGF-β2 in U87-MG cells after calcitriol treatment; The mRNA levels were analyzed by Real-Time PCR and mRNA expression was normalized with GAPDH. The results shown presented are from at least two biological replicates; -VD = without vitamin D; +VD = with vitamin D (1,25-Dihydroxyvitamin D3 or calcitriol).

the function of keratinocyte cells [27]. Our silencing VDR results propose a different function of VDR in U87-MG and Jurkat cell growth.

Conversely, the study of Salomon et al. (2014) in T98G, a glioblastoma multiform cell line, found that reduction in VDR levels by shRNA significantly increased the survival of T98G cells [28]. Elsewhere, the

same results were obtained concluding that VDR has a negative effect on cancer and cancer cell lines and reduces their proliferation [21], but our study does not support this evidence. Trivedi et al (2017) suggested that in the absence of vitamin D, the cytoplasmic VDR induced breast cancer cell growth [26]. Indeed, a protein known as PTPH1 (protein tyrosine

phosphatase 1) which is a specific phosphatase for mitogen-activated protein kinase (MAPK), controls breast cancer cell growth by binding to VDR in the cytoplasm and stimulate it [29]. Therefore, the same mechanism may partially cause the VDR to promote growth in Jurkat and U87-MG cells in the absence of its ligand.

Our data demonstrate that VDR knock down enhanced apoptosis in Jurkat and U87-MG cells. This effect of VDR on apoptosis is presumably mediated by repression of the Wnt/ β -catenin signaling pathway [30]. Wnt proteins regulate several processes including immunity, neuronal development, stem-cell proliferation, and tumorigenesis [31]. Furthermore, Wnt is a positive regulator of myelin gene expression in the myelination process [32].

Vitamin D deficiency is common in patients with MS [2, 3, 33]. Although several recent studies have indicated the relationship between vitamin D deficiency and increased risk of MS, molecular mechanisms and pathways have remained controversial. Therefore, other investigations are necessary for explaining them. Meanwhile, vitamin D signaling is mediated by binding 1,25(OH) $_2$ D $_3$ to VDR. The expression of VDR has been reported in most immune cells as well as in neuronal cells [34]. Silencing VDR expression in the types of cells such as U87-MG and Jurkat T cells may help to elucidate the precise function of VDR in immune and neuronal cells, as well as in MS pathogenesis.

Although, in our previous studies we demonstrated that the expression of some inflammatory genes including VDR [35], IL-10 [13], TGF- β R I, TGF- β 2, and TGF- β R II [36] changed after supplementation with Vitamin D in PBMCs (peripheral blood mononuclear cell) from MS patients, to further clarify VDR function in U87-MG and Jurkat cell lines and elucidate molecular mechanisms of VDR, we evaluated the expression levels of NF- κ B, IL-10, TGF- β 1, TGF- β R I, TGF- β 2, and TGF- β R II at mRNA levels in the mentioned cell lines by Real-Time PCR. In U87-MG cells, VDR knockdown dramatically increased NF- κ B, TGF β 1, TGF β 2, TGF β R I, and TGF β R II mRNA levels. IL-10 expression level did not change significantly after VDR disruption.

On the other hand, in Jurkat cell line we found that NF- κ B, IL-10, TGF β 1, TGF β R I, and TGF β R II mRNA levels dropped significantly after VDR inhibition while TGF β 2 expression was not significantly altered after transfection.

Previous studies have shown that NF- κ B, TGF β 1, TGF β 2, TGF β R I, TGF β R II, and IL-10 are involved in immunomodulation and inflammation [37, 38, 39]. On the other hand, other studies have shown that VDR has immunomodulatory, pro-differentiative, and anti-proliferative effects, and VDR activation results in the activation of downstream gene in immune cells [40, 41]. Thus, we expect that VDR inhibition activates various signaling pathways involved in immunomodulation and inflammation such as NF- κ B, TGF- β , and so on. For example, VDR along with TGF- β mediates the expression of IL-10 and increases the generation of IL-10-producing CD4 $^+$ T cells [42].

Expression profiling results for U87-MG cell line have been concordant with this fact, but apoptosis and growth assay results indicate that activation of NF- κ B and TGF- β signaling pathways cannot lead to proliferation, and conversely induce apoptosis in these cells. Therefore, based on these results, we conclude that VDR inhibition causes apoptosis by other potential alternative pathways in this cell line. For instance, Yuan et al (2018) have reported that VDR activation leads to the activation of the ERK1/2 pathway in U87-MG cells which may result in apoptosis reduction [43].

In Jurkat cell line transfected with VDR shRNA, cell growth was reduced significantly while, apoptosis was enhanced after the treatment. VDR knocking down has dramatic impacts on the reduction in expression level of NF- κ B, TGF β 1, TGF β R I/II, and IL-10. Our results indicated that VDR inhibition can diminish NF- κ B and TGF β signaling pathways directly or indirectly in Jurkat cells, which is not consistent with previous studies in other cell lines [44, 45, 46]. For example, IL-10 locus has a VDR-binding site in its promoter that VDR directly binds to this element and increased IL-10 expression [13]. On the other hands, there are several indirect pathways, which have indicated how VDR influences on

the levels expression of IL-10 [13]. Also, studies of Zhang et al. (2008) suggested that nongenomic activation of a VDR/PI3K/Akt survival pathway could have anti-apoptotic effects on osteoblast cell line [47], so silencing the VDR could increase apoptosis; all these results are consistent with our findings. Furthermore, study of mice with VDR deficiency did not manifest any increase of sporadic tumorigenesis [48]. On the other hand, the controversial results about the roles of VDR in apoptosis and the effects of VDR-knock down on cell fate require further studies.

However, one limitation of this study is that the effects of VDR down-regulation on the expression of genes involved in apoptosis, cell growth, and cell cycle have not been investigated and these analyses are suggested for future studies.

5. Conclusion

In conclusion, these results indicate that VDR can play a fundamental role to regulate lymphocytes and neuronal cell growth without its ligand, vitamin D. However further studies are necessary to understand the function of VDR in the mentioned cells. Further, while VDR knocking down in two different cell lines of U87-MG and Jurkat cells had different effects on NF- κ B and TGF- β expression levels, its effects on cell growth and apoptosis were similar. This may suggest that these two different cell lines can show similar anti-proliferative effects by different downstream signaling pathways. Taken together, this information may be useful to design novel diagnostic and therapeutic methods for diseases associated to vitamin D deficiency.

Declarations

Author contribution statement

Z. Shirvani-Farsani: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Behmanesh: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Funding statement

The Iran National Science Foundation and the department of research affairs of Tarbiat Modares University provided the funding for this work.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

- [1] J.F. Kurtzke, et al., Epidemiology of multiple sclerosis in US veterans. 4. Age at onset, *Neuroepidemiology* 11 (4-6) (1992) 226–235.
- [2] C. Pierrot-Deseilligny, J.C. Souberbielle, Is hypovitaminosis D one of the environmental risk factors for multiple sclerosis? *Brain* 133 (Pt 7) (2010) 1869–1888.
- [3] A.A. Ginde, J.M. Mansbach, C.A. Camargo Jr., Association between serum 25-hydroxyvitamin D level and upper respiratory tract infection in the third national health and nutrition examination survey, *Arch. Intern. Med.* 169 (4) (2009) 384–390.
- [4] E. Baroni, et al., VDR-dependent regulation of mast cell maturation mediated by 1,25-dihydroxyvitamin D $_3$, *J. Leukoc. Biol.* 81 (1) (2007) 250–262.
- [5] M. Guzey, S. Kitada, J.C. Reed, Apoptosis induction by 1 α ,25-dihydroxyvitamin D $_3$ in prostate cancer, *Mol. Cancer Ther.* 1 (9) (2002) 667–677.
- [6] J.W. Pike, M.B. Meyer, K.A. Bishop, Regulation of target gene expression by the vitamin D receptor - an update on mechanisms, *Rev. Endocr. Metab. Disord.* 13 (1) (2012) 45–55.

- [7] P. Bortman, et al., Antiproliferative effects of 1,25-dihydroxyvitamin D3 on breast cells: a mini review, *Braz. J. Med. Biol. Res.* 35 (2002) 01–09.
- [8] L. Chen, et al., Transcriptional profiling of $\gamma\delta$ T cells identifies a role for vitamin D in the immunoregulation of the V γ 9V δ 2 response to phosphate-containing ligands, *J. Immunol.* 174 (10) (2005) 6144–6152.
- [9] T. Berge, et al., The multiple sclerosis susceptibility genes TAGAP and IL2RA are regulated by vitamin D in CD4+ T cells, *Genes Immun.* 17 (2) (2016) 118–127.
- [10] B. Naghavi Gargari, et al., Vitamin D supplementation up-regulates IL-6 and IL-17A gene expression in multiple sclerosis patients, *Int. Immunopharmacol.* 28 (1) (2015) 414–419.
- [11] L. Adorini, Intervention in autoimmunity: the potential of vitamin D receptor agonists, *Cell. Immunol.* 233 (2) (2005) 115–124.
- [12] F.J. Barrat, et al., 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 (5) (2002) 603–616.
- [13] Z.S. Farsani, M. Behmanesh, M.A. Sahraian, Interleukin-10 but not transforming growth factor-beta1 gene expression is up-regulated by vitamin D treatment in multiple sclerosis patients, *J. Neurol. Sci.* 350 (1-2) (2015) 18–23.
- [14] Y. Chen, et al., Vitamin D receptor inhibits nuclear factor kappaB activation by interacting with IkappaB kinase beta protein, *J. Biol. Chem.* 288 (27) (2013) 19450–19458.
- [15] K.W. Moore, et al., Interleukin-10 and the interleukin-10 receptor, *Annu. Rev. Immunol.* 19 (2001) 683–765.
- [16] G. Bonizzi, M. Karin, The two NF-kappaB activation pathways and their role in innate and adaptive immunity, *Trends Immunol.* 25 (6) (2004) 280–288.
- [17] F. Isabel, et al., TGF-beta signaling in cancer treatment, *Curr. Pharmaceut. Des.* 20 (17) (2014) 2934–2947.
- [18] D.L. Mason, et al., Nervous system autoantibodies and vitamin D receptor gene polymorphisms in hemodialysis patients, *Hemodial. Int. Int. Symp. Home Hemodial.* 17 (1) (2013) 3–11.
- [19] S.S. Jensen, et al., Inhibitory effects of 1alpha,25-dihydroxyvitamin D(3) on the G(1)-S phase-controlling machinery, *Mol. Endocrinol.* 15 (8) (2001) 1370–1380.
- [20] S. Nagpal, S. Na, R. Rathnachalam, Noncalcemic actions of vitamin D receptor ligands, *Endocr. Rev.* 26 (5) (2005) 662–687.
- [21] L. Vuolo, et al., Vitamin D and cancer, *Front. Endocrinol.* 3 (2012) 58.
- [22] M. Mangin, R. Sinha, K. Fincher, Inflammation and vitamin D: the infection connection, *Inflamm. Res.* 63 (10) (2014) 803–819.
- [23] M.J. Clark, et al., U87MG decoded: the genomic sequence of a cytogenetically aberrant human cancer cell line, *PLoS Genet.* 6 (1) (2010), e1000832.
- [24] Y. Cheng, et al., Diversity of immune cell types in multiple sclerosis and its animal model: pathological and therapeutic implications, *J. Neurosci. Res.* 95 (10) (2017) 1973–1983.
- [25] M. Di Rosa, et al., Vitamin D3: a helpful immuno-modulator, *Immunology* 134 (2) (2011) 123–139.
- [26] T. Trivedi, et al., The vitamin D receptor is involved in the regulation of human breast cancer cell growth via a ligand-independent function in cytoplasm, *Oncotarget* 8 (16) (2017) 26687–26701.
- [27] T. Huet, et al., A vitamin D receptor selectively activated by gemini analogs reveals ligand dependent and independent effects, *Cell Rep.* 10 (4) (2015) 516–526.
- [28] D.G. Salomon, et al., Vitamin D receptor expression is associated with improved overall survival in human glioblastoma multiforme, *J. Neuro Oncol.* 118 (1) (2014) 49–60.
- [29] H.Y. Zhi, et al., PTPH1 cooperates with vitamin D receptor to stimulate breast cancer growth through their mutual stabilization, *Oncogene* 30 (14) (2011) 1706–1715.
- [30] Y. Zheng, et al., Loss of the vitamin D receptor in human breast and prostate cancers strongly induces cell apoptosis through downregulation of Wnt/ β -catenin signaling, *Bone Res.* 5 (2017) 17023.
- [31] K. Maiese, Novel applications of trophic factors, Wnt and WISP for neuronal repair and regeneration in metabolic disease, *Neural Regen. Res.* 10 (4) (2015) 518–528.
- [32] M. Tawk, et al., Wnt/beta-catenin signaling is an essential and direct driver of myelin gene expression and myelinogenesis, *J. Neurosci.* 31 (10) (2011) 3729–3742.
- [33] S.V. Ramagopalan, et al., Multiple sclerosis: risk factors, prodromes, and potential causal pathways, *Lancet Neurol.* 9 (7) (2010) 727–739.
- [34] F.M. Alharbi, Update in vitamin D and multiple sclerosis, *Neurosciences (Riyadh, Saudi Arabia)* 20 (4) (2015) 329–335.
- [35] Z. Shirvani-Farsani, et al., The expression of VDR mRNA but not NF-kappaB surprisingly decreased after vitamin D treatment in multiple sclerosis patients, *Neurosci. Lett.* 653 (2017) 258–263.
- [36] Z. Shirvani-Farsani, et al., Vitamin D levels in multiple sclerosis patients: association with TGF-beta2, TGF-betaRI, and TGF-betaRII expression, *Life Sci.* 134 (2015) 63–67.
- [37] S. Sanjabi, et al., Anti- and pro-inflammatory roles of TGF- β , IL-10, and IL-22 in immunity and autoimmunity, *Curr. Opin. Pharmacol.* 9 (4) (2009) 447–453.
- [38] S.S. Iyer, G. Cheng, Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease, *Crit. Rev. Immunol.* 32 (1) (2012) 23–63.
- [39] M. Schuliga, NF-kappaB signaling in chronic inflammatory airway disease, *Biomolecules* 5 (3) (2015) 1266–1283.
- [40] K.W. Colston, et al., Vitamin D receptors and anti-proliferative effects of vitamin D derivatives in human pancreatic carcinoma cells in vivo and in vitro, *Br. J. Canc.* 76 (8) (1997) 1017–1020.
- [41] M.G. Anderson, et al., Expression of VDR and CYP24A1 mRNA in human tumors, *Cancer Chemother. Pharmacol.* 57 (2) (2006) 234–240.
- [42] J.H. Chang, et al., 1,25-Dihydroxyvitamin D3 inhibits the differentiation and migration of T(H)17 cells to protect against experimental autoimmune encephalomyelitis, *PLoS One* 5 (9) (2010), e12925.
- [43] J. Yuan, et al., Vitamin D receptor activation influences the ERK pathway and protects against neurological deficits and neuronal death, *Int. J. Mol. Med.* 41 (1) (2018) 364–372.
- [44] D. Padua, J. Massague, Roles of TGFbeta in metastasis, *Cell Res.* 19 (1) (2009) 89–102.
- [45] X. Dolcet, et al., NF-kB in development and progression of human cancer, *Virchows Arch.* 446 (5) (2005) 475–482.
- [46] R.S. Muraoka, et al., Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases, *J. Clin. Invest.* 109 (12) (2002) 1551–1559.
- [47] X. Zhang, L.P. Zanello, Vitamin D receptor-dependent 1 alpha,25(OH)2 vitamin D3-induced anti-apoptotic PI3K/AKT signaling in osteoblasts, *J. Bone Miner. Res.* 23 (8) (2008) 1238–1248.
- [48] D.K. Panda, et al., Targeted ablation of the 25-hydroxyvitamin D 1alpha -hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction, *Proc. Natl. Acad. Sci. U. S. A.* 98 (13) (2001) 7498–7503.