

# 9-cis-Retinoic Acid and 1,25-dihydroxy Vitamin D<sub>3</sub> Improve the Differentiation of Neural Stem Cells into Oligodendrocytes through the Inhibition of the Notch and Wnt Signaling Pathways

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## What's Known

- Differentiation of oligodendrocyte precursor cells into oligodendrocytes can be improved by inhibiting signaling pathways such as Wnt and Notch.
- Despite studies confirming the positive effects of 9-cis-retinoic acid and 1,25(OH)<sub>2</sub>D<sub>3</sub> on differentiation, the inhibitory effects of these compounds on signaling pathways have yet to be investigated.

## What's New

- Two distinct mechanisms for the differentiation of neural stem cells into oligodendrocytes by 9-cis-retinoic acid and 1,25(OH)<sub>2</sub>D<sub>3</sub> are recommended.
- 9-cis-retinoic acid and 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibit the Notch and Wnt signaling pathways through the downregulation of the HES5 transcription factor and the LRP6 co-receptor, respectively.

## Abstract

**Background:** Differentiating oligodendrocyte precursor cells (OPCs) into oligodendrocytes could be improved by inhibiting signaling pathways such as Wnt and Notch. 9-cis-retinoic acid (9-cis-RA) and 1,25-dihydroxyvitamin D<sub>3</sub> (1,25[OH]<sub>2</sub>D<sub>3</sub>) can ameliorate oligodendrogenesis. We investigated whether they could increase oligodendrogenesis by inhibiting the Wnt and Notch signaling pathways.

**Methods:** Cortical neural stem cells were isolated from 14-day-old rat embryos and cultured using the neurosphere assay. The cells were treated in 4 different conditions for 1 week: the negative control group received only the basic fibroblast growth factor, the positive control group received only T<sub>3</sub> without growth factors, the RA group was treated with 9-cis-RA, and the Vit D<sub>3</sub> group was treated with 1,25(OH)<sub>2</sub>D<sub>3</sub>. The effects of 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> on the level of the myelin basic protein (MBP) and the gene expression of the SOX10, MBP gene, HES5, and LRP6 were studied using flow cytometry and real-time PCR. The data were analyzed using one-way ANOVA with GraphPad Prism. A P value less than 0.05 was considered significant.

**Results:** The mRNA expressions of the SOX10, MBP, and MBP gene were significantly increased in the treated groups compared with the negative control group; the increase was similar in the 9-cis-RA group and the positive control group. Furthermore, 9-cis-RA significantly decreased the expression of the HES5 gene, a Notch signaling pathway transcription factor, and 1,25(OH)<sub>2</sub>D<sub>3</sub> significantly reduced the expression of the LRP6 gene, a Wnt signaling pathway co-receptor.

**Conclusion:** It seems that 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> are good candidates to improve the differentiation of OPCs into oligodendrocytes.

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**Keywords** • Cell differentiation • Oligodendrocyte precursor cells • 9-cis-retinoic acid • 1,25-dihydroxy vitamin D<sub>3</sub> • Receptors, Wnt • Notch signaling pathway

## Introduction

Multiple sclerosis is a demyelinating disorder which causes severe disability in patients due to loss of myelin sheaths around

axons as a result of the injury or death of adult oligodendrocytes.<sup>1</sup> During the development of glial cells, oligodendrocytes differentiate from oligodendrocyte precursor cells (OPCs) through the involvement of special intrinsic transcription factors such as oligodendrocyte transcription factor 2 (OLIG2), Nkx-2.2, and Sry-related HMG-Box gene 10 (SOX10); in addition, some inhibitory signaling pathways including Wnt and Notch, which are the result of their final performance, play critical roles in OPC differentiation into oligodendrocytes and the myelination process.<sup>2-5</sup> The Wnt signaling pathway is initiated by binding the Wnt family proteins to a cell-surface receptor, Frizzled, and a co-receptor, low-density lipoprotein receptor-related protein 5/6 (LRP5/6). Activating this pathway through the suppression of the myelin basic protein (MBP) inhibits the differentiation of oligodendrocytes.<sup>6-8</sup> Notch is another crucial signaling pathway that represses the differentiation of oligodendrocytes through the activation of the transcription factor HES family BHLH transcription factor 5 (HES5), which binds to the SOX10 gene promoter and suppresses its expression.<sup>2,9</sup>

Despite the inhibitory signaling pathways such as Wnt and Notch, endogenous OPC differentiation is enhanced by 9-cis-retinoic acid (9-cis-RA) and 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] as has been shown in recent research for remyelination therapies.<sup>10,11</sup> 9-cis-RA is an important metabolite of retinoids that controls many biological events such as immunomodulation, proliferation, and differentiation via binding to the retinoid X receptor (RXR).<sup>12</sup> Moreover, it has been found that the RXR signaling pathway induces the maturation of OPCs and eventually results in remyelination.<sup>13,14</sup> Furthermore, it has been demonstrated that poor vitamin D intakes and low blood levels of vitamin D metabolites are associated with increased risk of multiple sclerosis.<sup>1</sup> Recently, it has been reported that the vitamin D<sub>3</sub> receptor (VDR) is expressed in neural stem cells (NSCs) and 1,25(OH)<sub>2</sub>D<sub>3</sub> increases NSC differentiation into oligodendrocytes.<sup>15</sup>

For all the studies confirming the positive effects of 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> on the differentiation of oligodendrocytes, the inhibitory effects of these components on signaling pathways have yet to be thoroughly explored. The present study, therefore, sought to examine the effects of 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> on the inhibition of the Wnt and Notch signaling pathways in oligodendrogenesis.

## Materials and Methods

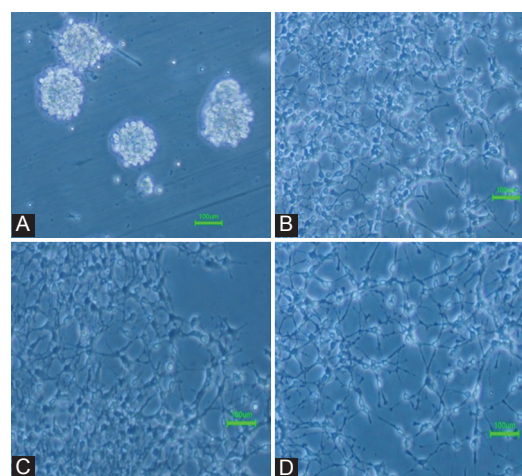
### Neurosphere Assay

NSCs were harvested from the cortex of E14 rat embryos and expanded by using the

neurosphere assay.<sup>16</sup> Briefly, the pregnant rats were anesthetized and 8 to 10 embryos were removed. Next, their brain cortex was dissected and cut into small pieces. The dissected tissues were dissociated into single cells, and a pool was prepared and resuspended in a complete NSC medium, containing DMEM/F12 (GIBCO, Carlsbad, CA, USA), 1% N2, and 2% B-27 (GIBCO, Carlsbad, CA, USA) supplements. Subsequently, the cells were plated at  $2 \times 10^5$  cells/mL in 5 mL of the complete medium, supplemented with 10 ng/mL of the basic fibroblast growth factor (bFGF) (Sigma-Aldrich, MO, USA) and 2  $\mu$ g/mL of heparin (Sigma-Aldrich, MO, USA). T25 flasks were incubated at 37 °C and 5% CO<sub>2</sub> for 5 to 7 days to generate neurospheres. Subsequently, the shape and appearance of the neurospheres were studied (figure 1). All the protocols of the study were approved by the Institutional Animal Ethics Committee of Shiraz University of Medical Sciences (Shiraz, Iran) (IR.SUMS.REC). The animals were kept in a temperature-controlled condition (24±2 °C) with a 12-hour light/dark alternating cycle and free access to water and standard diet.<sup>17</sup>

### Alamar Blue Assay

A cell viability test using the Alamar blue reagent was carried out to evaluate the cytotoxicity of the 9-cis-RA (Sigma-Aldrich, MO, USA) and 1,25(OH)<sub>2</sub>D<sub>3</sub> (Sigma-Aldrich, MO, USA). Appropriate cell density was assessed by coating microtiter wells with 200  $\mu$ g/mL of poly-D-lysine and then adjoining a serial density of the cells ( $5-30 \times 10^3$  cells/mL) to each well. Next, 10% Alamar blue (Sigma-Aldrich, MO, USA) was added and absorbance was measured at



**Figure 1:** Differentiation and morphological maturation of the oligodendrocyte precursor cells in different culture conditions after 7 days. A) Negative control; B) Positive control; C) Vitamin D<sub>3</sub> group; D) Retinoic acid group. Scale bar: 100  $\mu$ m.

590 nm after 2 hours. Subsequently, the cells at an optimal density were seeded in microtiter wells with different concentrations of 9-cis-RA (0.1, 0.5, 1, 5, 10, and 20  $\mu$ M) and 1,25(OH)<sub>2</sub>D<sub>3</sub> (12.5, 25, 50, 100, 200, and 400 nM) for 7 days. Finally, the optimal concentration of the mentioned compounds was measured using the IC50 curve.

#### Differentiation Assay

The in vitro differentiation of the NSCs into oligodendrocytes was induced by seeding a single cell suspension at a density of  $5 \times 10^3$  cells/L on 24-well plates coated with poly-D-lysine (Sigma-Aldrich, MO, USA). First, the cells were cultured in a complete medium, including the bFGF (10 ng/mL) and the platelet-derived growth factor (30 ng/mL, Sigma-Aldrich, MO, USA) for 24 hours, which was then replaced with a growth factor-free medium and treated in 4 different conditions for 1 week.<sup>5</sup> There were 4 groups in the present study. A negative control group, which received only the bFGF; a positive control group, which received only 30 ng/mL of T<sub>3</sub> (Sigma-Aldrich, MO, USA); a group treated with 20  $\mu$ M of 9-cis-RA; and a group treated with 200 nM of 1,25(OH)<sub>2</sub>D<sub>3</sub>.

#### Flow Cytometry

The expression of the MBP was evaluated by removing the media and then adding a trypsin-EDTA solution (GIBCO, Grand Island, NY, USA) to the plates in order to detach the cells after the cells had grown sufficiently in the groups. The enzyme activity of trypsin was counteracted by subjoining a soybean trypsin inhibitor (Sigma-Aldrich, MO, USA) and centrifuging the cells. First, the cells were fixed with a 2% paraformaldehyde solution for 15 minutes and then they were washed 3 times with phosphate-buffered saline.<sup>5</sup> Subsequently, the cells were stained with a diluted rabbit anti-MBP antibody (Abcam, UK, 1:500) overnight in a refrigerator. Afterward, the cells were rewashed and incubated with a diluted Alexa-Fluor 488 goat anti-rabbit secondary antibody (Eugene, OR, USA, 1:1000) for 45 minutes in the dark. Finally, the cells were resuspended in 300  $\mu$ L of phosphate-buffered saline and flow cytometry was carried out using a FACSCalibur System. The data were collected and analyzed using the

FlowJo 7.6 software. According to flowcytometry histograms, the percentage of the positive cells that expressed the MBP was calculated and then the data were entered into the GraphPad Prism 6.01 software and quantitatively compared between the groups.

#### RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted using the bioZOL reagent (BioFlux, Japan). Contamination was eliminated from the samples by treating total RNA (2  $\mu$ g) with a deoxyribonuclease (Fermentas, Burlington, Canada). Complementary DNA (cDNA) was prepared by using a reverse transcriptase in 20  $\mu$ L of the final reaction volume (Fermentas, Burlington, Canada). Real-time PCR was performed using the ABI real-time PCR 7500 system. Primers for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH), HES5, LRP6, SOX10, and MBP genes were designed using the AlleleID software. Their specificity was checked by Primer-BLAST at the NCBI database. The sequences of the specific primers utilized for gene expression in the current study and their fragment sizes (base pair [bp]) are listed in table 1. The PCR reaction components included 2  $\mu$ L of cDNA, 1  $\mu$ L of both forward and reverse primers, and 12.5  $\mu$ L of SYBR Green Master Mix (Amplicon, Denmark) in a total volume of 25  $\mu$ L. PCR cycling parameters were done in 40 cycles as follows: initial denaturation at 95°C for 15 minutes, denaturation at 95°C for 15 seconds, annealing at 55 to 60°C for 1 minute, and extension at 72°C for 30 seconds. The efficiency of the real-time PCR reaction was calculated from the slope of the standard curve. The effects of 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> on the expression levels of the SOX10, MBP, HES5, and LRP6 genes were investigated by determining the relative amounts of their transcripts from the  $\Delta$ Ct and 2<sup>- $\Delta\Delta$ CT</sup> formula. The GAPDH gene was regarded as the internal normalization control.

#### Immunofluorescence Staining

Immunofluorescence staining was performed with specific antibodies against beta-tubulin and Glial fibrillary acidic protein (GFAP), which are protein markers for neuron and astrocyte,

**Table 1:** Primer sequences used for quantitative real-time polymerase chain reaction

Primer	Forward	Reverse	Size (base pair)
SOX10	5'-CTGAACGAGACAAG-3'	5'-AACAACTCTTCGTCCGTAC-3'	198
GAPDH	3'-CTTGTCTCGTTCAG-5'	3'-CGAAGGTGGAAGAGTGAGT-5'	112
MBP	5'-CGGCTCACAAGGGATTCAAGG-3'	5'-AGGGCAGGATTCGGGAAGG-3'	145
HES5	3'-GCTCGCCAATCGCCTCCAG-5'	3'-ACGCATCTTCTCCACCACCG-5'	115
LRP6	3'-GTAGTTGGAGGCTTGGAGGAT-5'	3'-TAATGGCTTCTTCGCTGACG-5'	91

respectively, in order to ensure that the cells in the studied groups were differentiated into oligodendrocytes.

#### Statistical Analysis

All the experiments were done in triplicate. The data were analyzed with the GraphPad Prism software (version 6.01, San Diego, CA, USA) using one-way ANOVA, followed by the Tukey post-hoc test, after the normality and homogeneity of the variance test were checked. The values were represented as mean  $\pm$  standard error of the mean (SEM), and a P value less than 0.05 was considered the level of significance between the groups.

## Results

#### Determining the Optimal Concentrations of 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> for the Treatment of the NSCs

The nontoxic concentrations of 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> were evaluated using the Alamar blue cell viability assay. Based on our results, the optimal concentrations of 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> were 20  $\mu$ M and 200 nM, respectively, for 1 week's treatment.

#### Morphological Changes in the Cells

The differentiation and morphological maturation of the OPCs in the different culture conditions are depicted in figure 1. According to the images, while the process of the differentiation of the cells was similar in the positive control, RA, and vitamin D<sub>3</sub> groups, the process was different in the negative control group.

#### Effects of 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> on the mRNA Expression of the SOX10 and MBP Genes

The oligodendroglial differentiation potential of 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> in the treated OPCs was determined by examining the changes in the mRNA expression of the SOX10 and MBP genes. As is illustrated in figure 2, the expression level was increased significantly in all the groups compared to the negative control group and also the expression level was significantly different between the RA and vitamin D<sub>3</sub> groups (P<0.001). Interestingly, the expression level was increased significantly only in the RA group, similar to the positive control group, which revealed that 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> had remarkable effects on OPC maturation. However, 9-cis-RA probably played a more prominent role in the differentiation process.

#### Effects of 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> on the Expression of the MBP

GFAP<sup>+</sup> and beta-tubulin<sup>+</sup> cells were not observed in the positive control, RA, and vitamin D<sub>3</sub> groups. Accordingly, our results showed that the differentiated cells were specifically oligodendrocytes producing myelin cells. (Data are not shown.) Then, the level of the MBP in the different groups was assessed via flow cytometry to further confirm the results obtained at the mRNA level. As is shown in figures 3 and 4A, the amount of the MBP was significantly different in the positive control, RA, and vitamin D<sub>3</sub> groups compared to the negative control group (P<0.001). Our findings demonstrated that although the amount of the protein was increased in all the treated groups, it was relatively high in the RA group compared to the vitamin D<sub>3</sub> group. Interestingly, the protein level in the RA group was similar to that of the positive control group treated with T<sub>3</sub>.

#### Effects of 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> on the Notch Signaling Pathway

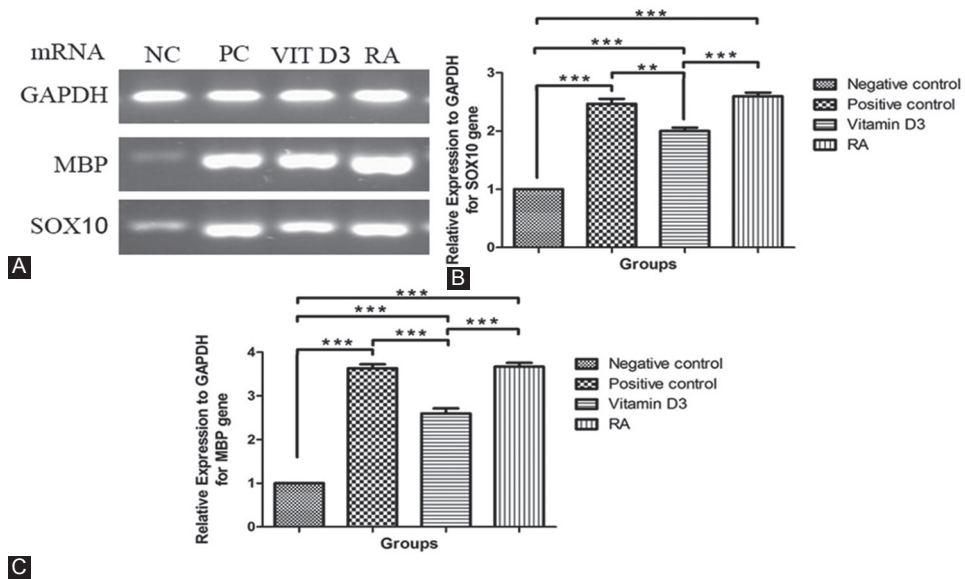
Treatment with 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> showed that the mRNA expression level of HES5 was significantly reduced in the positive control and RA groups compared to that of the negative control and vitamin D<sub>3</sub> groups (P<0.001) (figures 2A and 4B, 4C). Moreover, there was no significant difference between the positive control and RA groups. These data suggested that RA might be able to increase the differentiation of oligodendrocytes through the suppression of the Notch signaling pathway via the inhibition of HES5 expression.

#### Effects of 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> on the Wnt Signaling Pathway

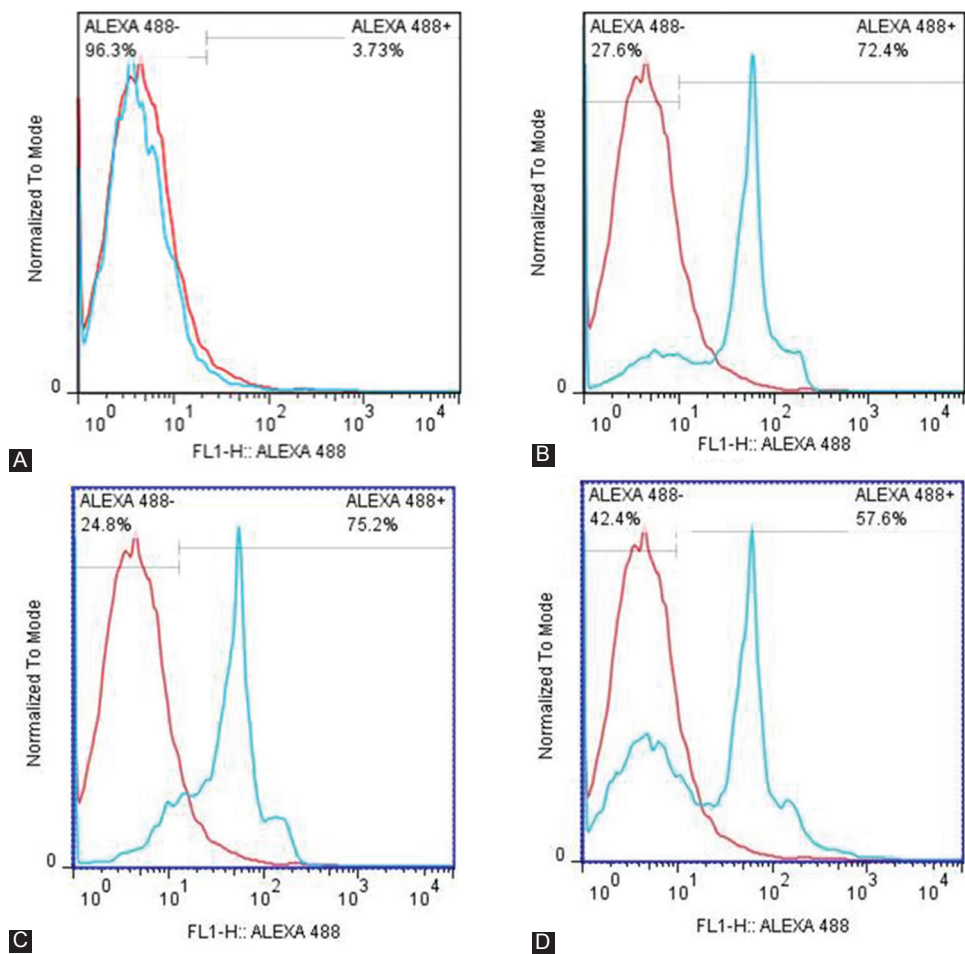
As is demonstrated in figures 2A and 4B, 4D, the mRNA expression level of the LRP6 gene was significantly decreased only in the positive control and vitamin D<sub>3</sub> groups compared to the negative control group (P<0.001). The expression level found in the positive control and vitamin D<sub>3</sub> groups was not significantly different. These findings suggested that 1,25(OH)<sub>2</sub>D<sub>3</sub> might enhance oligodendrocyte differentiation by repressing the Wnt signaling pathway through the downregulation of the LRP6 gene.

## Discussion

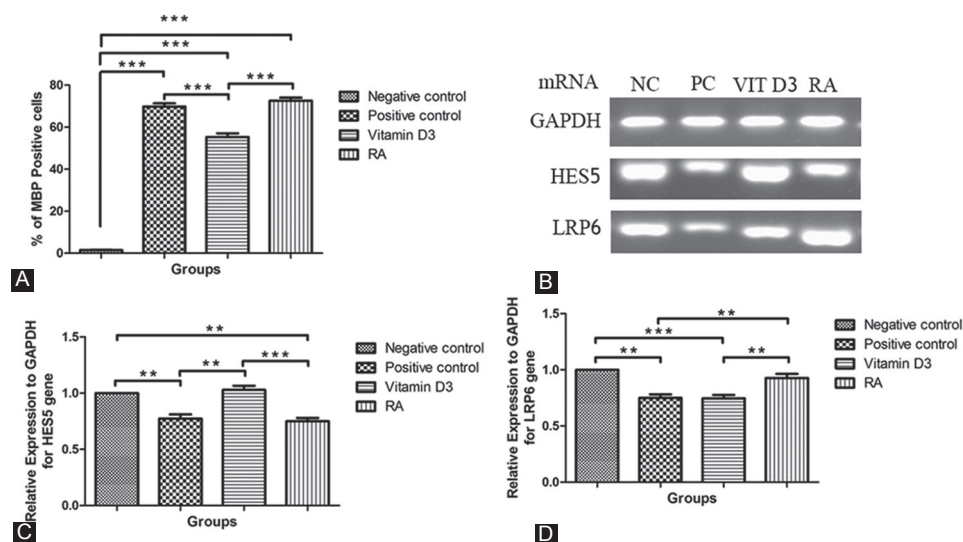
The current study for the first time showed that 9-cis-RA might induce the differentiation of NSCs into oligodendrocytes via more or less the same mechanisms as T<sub>3</sub>, while 1,25(OH)<sub>2</sub>D<sub>3</sub> was



**Figure 2:** Effects of 9-cis-retinoic acid (9-cis-RA) and 1,25-dihydroxyvitamin D3 [1,25(OH)<sub>2</sub>D<sub>3</sub>] on the mRNA expression of the Sry-related HMG-Box gene 10 (SOX10) and myelin basic protein (MBP) genes in the different groups. A) Real time-polymerase chain reaction (RT-PCR) data: NC, Negative control; PC, Positive control; Vit D<sub>3</sub>, Group treated with 1,25(OH)<sub>2</sub>D<sub>3</sub>; RA, Group treated with retinoic acid. B and C) RT-PCR analyses for the SOX10 and MBP genes, respectively (\*\*P<0.01 and P<0.001 \*\*\*indicate significant differences between the groups.) Data are given as mean±SEM, n=3.



**Figure 3:** Representative flow cytometry histograms, showing the expression of the myelin basic protein (MBP) in the different culture conditions after 7 days. A) Negative control; B) Positive control; C) Group treated with retinoic acid (RA); D) Vitamin D<sub>3</sub> group



**Figure 4:** Effects of 9-cis-retinoic acid (9-cis-RA) and 1,25-dihydroxyvitamin D3 [1,25(OH)<sub>2</sub>D<sub>3</sub>] on the mRNA expression of the HES family BHLH transcription factor 5 (HES5) and low-density lipoprotein receptor-related protein 6 (LRP6) genes as well as the myelin basic protein (MBP) in the different treated groups; A) Percentage of the MBP positive cells in the different treated groups; B) Real-time polymerase chain reaction (RT-PCR) data: NC, Negative control; PC, Positive control; Vit D3, Group treated with 1,25(OH)<sub>2</sub>D<sub>3</sub>; RA, Group treated with retinoic acid (RA); C and D) RT-PCR analyses for the HES5 and LRP6 genes, respectively (\*\*P<0.01 and P<0.001\*\*\* indicate significant differences between the groups). Data are given as mean±SEM, n=3.

less potent in oligodendrocyte differentiation compared to 9-cis-RA and evidently acted through different mechanisms.

According to our results, after we treated the NSCs with both 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub>, the expression level of SOX10, the late marker of OPC maturation, was upregulated and there was a rise in the expression level of the MBP. Moreover, the effects of RA treatment were almost identical to those in the positive control group, whereas, vitamin D<sub>3</sub> treatment was not as potent as RA treatment. Likewise, previous research has demonstrated that 9-cis-RA activates the RXR signaling pathway to improve OPC maturation in vitro and also remyelination in vivo.<sup>9,13</sup> Yang et al.<sup>14</sup> reported that the injection of exogenous RA in their study was able to accelerate OPC differentiation into oligodendrocytes through the RXR-γ signaling pathway. Further, the RXR, which has an important role in the nuclear receptor family, is involved in regulating the expression of target genes through the heterodimer with other nuclear receptors such as peroxisome proliferator-activated receptors (PPARs) and the VDR.<sup>18,19</sup> Moreover, it has been demonstrated that PPARs play a role in oligodendroglial differentiation via the upregulation of the OLIG1 and OLIG2 genes.<sup>20</sup> Except for the RXR-VDR complex, transcription can be activated through PPARs even when an RXR ligand agonist like 9-cis-RA exists alone.<sup>3,18</sup> This mechanism may explain why 9-cis-RA exhibits a better differentiation potential by comparison with 1,25(OH)<sub>2</sub>D<sub>3</sub>.

However, other studies have shown that 9-cis-RA can assist the differentiation of NSCs into neurons.<sup>21</sup> So far, the molecular mechanisms whereby different conditions of RA affect the differentiation of NSCs have remained unclear.<sup>21,22</sup> Oligogenic versus neurogenic outcomes could be attained depending on the concentrations of 9-cis-RA used in different studies or cross talk with several signaling pathways.<sup>21-24</sup>

Regarding the effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> on oligodendrogenesis, it has been reported that signaling through the VDR-RXR heterodimer may influence OPC differentiation and the VDR agonists may accelerate OPC differentiation.<sup>25</sup> In addition, it has been shown that 1,25(OH)<sub>2</sub>D<sub>3</sub> increases the expression of the VDR and accelerates the differentiation of NSCs into oligodendrocytes after 14 days.<sup>14</sup> Nonetheless, the low potent effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> in comparison to 9-cis-RA may have been due to the inappropriate concentrations of 1,25(OH)<sub>2</sub>D<sub>3</sub>, the time of incubation, or the less involvement of the signaling pathways.

From another point of view, the Wnt and Notch signaling pathways have a prominent role in managing the proliferation and differentiation of stem cells.<sup>26</sup> To the best of our knowledge, the effects of 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> on the Wnt and Notch inhibitory signaling pathways in OPC differentiation have not been studied yet. Apropos the Wnt and Notch pathways, literature shows that both have a negative role

in the differentiation of OPCs in patients with multiple sclerosis.<sup>6,27</sup> Interestingly in the present study, we found that 9-cis-RA inhibited the Notch signaling pathway in the OPC lineage cells by downregulating the HES5 gene, while it exerted no effect on the Wnt signaling pathway.

In concordance with our results, it has been shown that the RXR signaling pathway can be involved in gene regulation through coordination with proteins that interact with nuclear receptors.<sup>18</sup> Moreover, the nuclear receptor corepressor is well-known protein in association with the RXR receptors.<sup>28</sup> Recently, it has been reported that the Notch signaling pathway can be activated via the recombination binding protein for the immunoglobulin kappa J region-mediated transcription, which co-operates with the nuclear receptor corepressor 2 protein.<sup>29-31</sup> This may explain why RA acts more through the Notch signaling pathway.

In line with this finding, it has been shown that all-trans RA can repress neurosphere growth and induce the differentiation of glioblastoma stem cells as evidenced by the downregulation of HES2, HEY1, and HEY2, which are the targets of the Notch signaling pathway.<sup>32</sup> Conversely, Young et al.<sup>33</sup> used RNA sequencing and showed that long-term in vitro treatment with 9-cis and all-trans RA increased the expression of the negative regulators of oligodendrocyte differentiation such as HES5. Furthermore, Green and colleagues<sup>34</sup> demonstrated that the use of RA receptor agonists in osteogenic cultures was able to augment the expression of the Wnt antagonist, secreted frizzled-related protein 4, and ultimately lead to the inhibition of this signaling pathway.

Our findings also demonstrated that 1,25(OH)<sub>2</sub>D<sub>3</sub> suppressed the Wnt signaling pathway through inhibiting the expression of the LRP6 gene. In agreement with our results, it has been found that 1,25(OH)<sub>2</sub>D<sub>3</sub> influences the binding site of the VDR inside the LRP5 gene locus and, thus, increases the expression of LRP5 and improves the differentiation of osteoblast cells.<sup>35</sup> Moreover, numerous studies have reported that 1,25(OH)<sub>2</sub>D<sub>3</sub> counteracts the Wnt signaling pathway by attenuating the transcription of β-catenin or it can translocate it from the nucleus to the plasma membrane in colon cancer cells.<sup>36,37</sup> In disagreement, it has been reported that 1,25(OH)<sub>2</sub>D<sub>3</sub> plays a role in the upregulation of the Wnt pathway, which enhances the differentiation of hair follicle and bone formation in keratinocytes and osteoblasts, respectively.<sup>38</sup>

Based on our results, 1,25(OH)<sub>2</sub>D<sub>3</sub> has no effect on the expression level of the HES5 gene

and, therefore, the Notch signaling pathway. Nevertheless, only a few studies have explored the relationship between 1,25(OH)<sub>2</sub>D<sub>3</sub> and the Notch signaling pathway.<sup>38</sup> Treatment with vitamin D<sub>3</sub> in rodent osteoblasts induces the transcription factor HES1, the downstream effector of the Notch pathway, which increases the expression of osteopontin and consequently cooperates in bone remodeling.<sup>39</sup> In addition, 1,25(OH)<sub>2</sub>D<sub>3</sub> downregulates the RNA levels of the Notch ligands in RWPE1 non-tumorigenic prostate cells, while in human keratinocyte cells the Notch ligands are not effective.<sup>40</sup> To the best of our knowledge, it seems that the association between RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> and the Notch and Wnt signaling pathways is uncertain and depends on the type of cells.

In the past decade, the investigation and recognition of factors that regulate the timing of oligodendrocyte differentiation were issues of great interest.<sup>25</sup> Although the exact molecular mechanisms of differentiation have hitherto remained unknown, it has been revealed that the process is controlled by transcription factors, signaling pathways, and their interactions.<sup>2</sup> According to our findings, 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> improve the differentiation of OPCs through the inhibition of the Notch and Wnt signaling pathways in vitro, respectively.

First and foremost among the limitations of the present study is its incubation time for the treated cells, which unfortunately did not allow them to survive for more than a week. The interplay between 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> and their differentiation potential in other signaling pathways that inhibit remyelination should be further explored in future studies. What is also needed for further in vivo assays is an experimental autoimmune encephalomyelitis model of multiple sclerosis. Unfortunately, material constraints precluded us from performing the abovementioned experiments.

## Conclusion

The results of the present study demonstrated that 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> played effective roles in the development of NSCs into oligodendrocytes. Moreover, 9-cis-RA suppressed the Notch signaling pathway via the downregulation of the HES5 gene, while 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited the Wnt signaling pathways via the downregulation of the LRP6 gene. Given their inhibitory function in the mentioned signaling pathways, 9-cis-RA and 1,25(OH)<sub>2</sub>D<sub>3</sub> seem to be good candidates for the improvement of OPC differentiation into oligodendrocytes.

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**Conflict of Interest:** None declared.

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