

# Enhanced Differentiation of Stromal Cells and Embryonic Stem Cells with Vitamin D3

Mayada Alqaisi, Nasser Al-Shanti, Quiyu Wang, and William S. Gilmore

**Abstract**—*In-vitro* mouse co-culture of E14 embryonic stem cells (ESCs) and OP9 stromal cells can recapitulate the earliest stages of haematopoietic development, not accessible in human embryos, supporting both haemogenic precursors and their primitive haematopoietic progeny.  $1\alpha, 25$ -Dihydroxy-vitamin D3 (VD3) has been demonstrated to be a powerful differentiation inducer for a wide variety of neoplastic cells, and could enhance early differentiation of ESCs into blood cells in E14/OP9 co-culture. This study aims to ascertain whether VD3 is key in promoting differentiation and suppressing proliferation, by separately investigating the effects of VD3 on the proliferation phase of the E14 cell line and on stromal OP9 cells. The results showed that VD3 inhibited the proliferation of the cells in a dose-dependent manner, quantitatively by decreased cell number, and qualitatively by alkaline-phosphatase staining that revealed significant differences between VD3-treated and untreated cells, characterised by decreased enzyme expression (colourless cells). Propidium-iodide cell-cycle analyses showed no significant percentage change in VD3-treated E14 and OP9 cells within their G and S-phases, compared to the untreated controls, despite the increased percentage of G-phase compared to the S-phase in a dose-dependent manner. These results with E14 and OP9 cells indicate that adequate VD3 concentration enhances cellular differentiation and inhibits proliferation. The results also suggest that if E14 and OP9 cells were co-cultured and VD3-treated, there would be further-enhanced differentiation of ESCs into blood cells.

**Keywords**—Differentiation, embryonic stem cells, OP9 stromal cells,  $1\alpha, 25$ -dihydroxy-vitamin D3

## I. INTRODUCTION

EMBRYONIC STEM CELLS (ESCs), derived from the inner cell mass of the blastocyst stage of early mammalian embryos, have the potential to undergo unlimited self-renewal by being placed in specific culture conditions, either in vitro or in vivo [1]. Interestingly, once the cells are released from these conditions and placed in a differentiation-promoting environment (in vitro or in vivo), the cells differentiate into derivatives of all three primary germs: ectoderm, mesoderm and endoderm, and then into many different cell types in the body [2]. In the presence of a combination of growth factors, in-vitro mouse co-culture of E14 ESCs and OP9 stromal cells can recapitulate early haematopoietic development, supporting both haemogenic precursors and their primitive haematopoietic progeny [3]. The active form of VD3 is widely used to treat metabolic bone diseases such as rickets/osteomalacia, renal osteodystrophy and osteoporosis

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[4]. The differentiation of ESCs into osteoblasts is enhanced to 60% when exposed to VD3 [5]. As well as maintaining calcium homeostasis, and exerting a wider range of biological activities, including the regulation of cellular differentiation and proliferation [6], VD3 demonstrated to be a powerful differentiation inducer for a wide variety of neoplastic cells, including carcinoma cells of various origins, and acute myeloid leukaemia [7], and been shown to have pro-differentiation and anti-proliferative effects on keratinocytes [8] and prostate cancer cells [9]. The vitamin D receptor (VDR) is a transcription factor that mediates the actions of its ligand, VD3, which can promote monocyte/macrophage differentiation, and inhibit proliferation and cytokine production by activated T lymphocytes [10]. In vivo granulocyte-monocyte committed stem cells are stimulated into clonal proliferation by VD3 [11]. In different stages of differentiation in various types of cells, the effects of VD3 were examined using in-vitro techniques. Hiroshi et al., 2012 investigated the effect VD3 in three phases of C2C12 myoblasts: proliferating, differentiating and differentiated. They concluded that VD3 supplementation inhibited C2C12-myoblast proliferation, significantly decreased mRNA expression of neonatal myosin heavy chain (MHC), and increased gene expression of MHC isoforms in C2C12-myoblast differentiation for 8 days [12]. Systemic or locally produced VD3 may be important in modulating cell development processes such as haematopoiesis [13]. Physiological levels of VD3 were found to promote differentiation of CD34 haematopoietic progenitors, characterised by the induction of all the monomacrophagic immunophenotypic and morphological markers in a liquid-culture model [11]. Patients receiving treatment for acute myeloid leukaemia (AML) or acute lymphoblastic leukaemia (ALL) may have had limited exposure to sunlight and often experience gastrointestinal side-effects that may decrease their ability to maintain an adequate VD3 level [14].

## II. MATERIALS AND METHODS

### A. Inducing Proliferation of E14 Cells

In the present study, mouse ESCs (E14 cell line passages 1-36) were obtained from Dr. Quiyu Wang. Cells were induced to proliferate and expanded by thawing cell-stock vials at room temperature and then added to gelatinised T 75 flasks, at a density of  $2 \times 10^6$  per flask. They were in an undifferentiated state in a fully humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C in growth medium (GM) composed of 450ml Dulbecco's Modified Eagle's Medium (DMEM) with 10% Knockout Serum replacement, 5ml non-essential amino

acid solution, 5ml L-glutamine (200mM final), 5ml penicillin-streptomycin solution (10.000 units/ml penicillin and 10.000µg/ml streptomycin), 50µl leukaemia inhibitory factor (LIF), and 500 µl 2-Mercaptoethanol (50mM final) until 90% confluence was attained. The medium was changed every 2-3 days.

#### *B. Inducing Proliferation of OP9 Cells*

OP9 cells (ATTC, LGC standards, UK) were induced to proliferate and expanded by thawed cell stock vials at room temperature and added to a gelatinised T75 flask, at a density of  $2 \times 10^6$  per flask, in an undifferentiated state in a fully humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C in growth medium (GM), composed of 385ml alpha modification Minimum Essential Medium (No nucleosides) medium (αMEM) plus 2.2 g/L sodium bicarbonate at PH 7.2, Foetal Bovine Serum (HyClone), Heat-inactivated, 5ml non-essential amino acid solution (NEAA), 5ml L-glutamine (200mM final), 5ml penicillin-streptomycin solution (10.000 units/ml penicillin and 10.000µg/ml streptomycin), and 500 µl 2-Mercaptoethanol (50mM). The medium was changed every 2-3 days. OP9 cultures are never grown beyond 90% confluency as this would result in their differentiation into adipocytes.

#### *C. Cell Counting*

If you modify to assess the effects exerted by VD3 on cell proliferation of pluripotent OP9 stromal cells and pluripotent ESCs, after formation of confluent culture of T75 of OP9 cells flasks and T75 of ESC flasks, 6-well plates were pre-coated with 2ml of 0.1% gelatine (those containing OP9 cells were incubated overnight at 37°C, and those with ESCs were incubated for 45 minutes at room temperature). Excess gelatine was then aspirated. For cell plating, adherent cells on the T75 flasks were collected following trypsinisation, and were counted using a haemocytometer in the presence of trypan blue dye. Cells were then seeded at  $8 \times 10^4$  cells in 2ml GM per well of 6-well plates, with or without VD3, and incubated for up to 72 hours for cell counting. VD3 was added to the medium at concentrations of 1nM, 10nM, and 100nM respectively. The 6-well plates were incubated at 37°C in a fully humidified atmosphere of 5% CO<sub>2</sub> in air for up to 72 hours. Cell counting was performed by aspirating culture mediums of confluent 6-well plates, washing cells with PBS to remove any remaining serum, trypsinising with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) for 3-5 minutes at 37°C to detach cells adhered to the substrate, and re-suspending the cells in pre-warmed GM (in at least 1:1 ratio of trypsin:GM) to deactivate the action of the trypsin. ESCs were centrifuged at 750rpm for 5-min at RT. OP9 stromal cells were centrifuged at 850rpm for 7 minutes at RT. The supernatant was discarded, and cells were re-suspended in fresh GM in a known volume. For cell counting, cell suspension was prepared in a 1:1 dilution in trypan blue stain, and cells were counted with a haemocytometer. Viable cells exclude the trypan blue dye. However, dead cells absorb the dye, their membrane integrity being disrupted, and are

positively stained. The same steps above were performed on 6-well plates of E14 and OP9 cells, which were incubated for up to 72 hours to evaluate cell proliferation.

#### *D. Visual Analysis by Alkaline-Phosphatase Staining*

Alkaline phosphatase (AP) is a hydrolase enzyme responsible for dephosphorylating molecules such as proteins, nucleotides, and alkaloids under alkaline PH condition. In general, alkaline is located at the cell surface, and is linked to the cell membrane, thus is widely used as a stem cell membrane marker. Elevated expression of this enzyme is associated with undifferentiated pluripotent cells and stem cells [15]. To assess the effect of VD3 on cell proliferation of OP9 stromal cells and ESCs (E14), alkaline-phosphatase staining was used. The above procedure to prepare cells for counting was also carried out in preparation for alkaline-phosphatase staining. Then, after 48 hours of incubation, the medium was aspirated and the cells were washed twice with PBS and fixed with methanol for 10 minutes. Excess methanol was then aspirated. For cell staining, 0.0012g of Naphthol (Sigma, Steinheim, Germany) was added into 30 ml of Tris (pH 9.2), followed by the addition of 1200 µl of dimethylformamide (Sigma,UK) to dissolve the Naphthol, and then 0.0012 g of Fast Red Salt (Sigma,UK) was added to the solution immediately before use at RT. Sufficient staining solution was added to cover the plates (2-2.5ml) for 30 minutes. The stain was aspirated, and the plates were washed with distilled water. After drying, alkaline phosphatase colonies were counted. Undifferentiated cells appeared red or purple, whereas differentiated cells appeared colourless. The same steps above were performed on 6-well plates of E14 and OP9 cells, which were incubated for up to 72 hours to evaluate cell proliferation. The results were evaluated in the presence of a control. Optimum staining was obtained by developing the substrate in the dark.

#### *E. Visual Cell-Cycle Analysis by Using Flow Cytometry*

To analyse the effect of VD3 on the cell cycle distribution of OP9 stromal cells and ESCs (E14) in the proliferation phases, flow cytometry was used. The above procedure to prepare cells for counting and for alkaline-phosphatase staining was also carried out in preparation for cell-cycle analysis by using flow cytometry. For cell-cycle analysis, after 48 hours and trypsinisation and neutralisation, cells were harvested in 15ml-centrifuge tubes, then pelleted by centrifugation for 7 minutes at 850rpm for OP9 cells and for 5 minutes at 750rpm for E14 cells. The supernatant was discarded, and cells were washed with PBS and centrifuged. After centrifugation, the PBS supernatant was discarded and cells were fixed with 3ml of 75% ethanol, which was added in a drop-wise manner to the cell pellet while in vortex (ethanol allows PI to intercalate into double-standard DNA). Samples were stored at -20°C overnight before centrifugation and discarding of the ethanol supernatant. Cells were washed twice with PBS, and 1ml of PBS was retained with the cell pellet to enable re-suspension. Cells were mixed and transferred to 3 ml BD Falcon flow cytometry tubes (BD Biosciences, San

Jose, CA, USA). 50µl of ribonuclease A (RNase) and 10µL of PI were added to the cell solution. RNase treatment was necessary as PI is also able to bind to RNA molecules. After 30min incubation at room temperature, samples were stored at 4°C for flow cytometric analysis. The same steps above were performed on 6-well plates of E14 and OP9 cells, which were incubated for up to 72 hours to evaluate cell proliferation.

### III. STATISTICAL ANALYSIS

All experiments were repeated two times independently in duplicate, unless otherwise stated. Statistical analyses and significance of data were determined using GraphPad Prism version 5.0. Statistical significance for interactions between two paired groups was determined with a Paired t-test; when more than one factor was present, two-way ANOVA was performed to investigate significances of factors or interactions (comparisons of the anti-proliferative between controls and VD3-treated groups), when matched observations were investigated. All values below  $p < 0.05$  were considered significant.

### IV. RESULTS

#### A. Effect of VD3 on OP9 Stromal Cells in the Proliferation Phase, Using Cell Counting

The impact of VD3 on OP9 cells was determined through its action on cell proliferation. Cells were cultivated in a GM containing VD3 at concentrations of 1, 10 and 100nM for up to 72 hours incubation. Cell proliferation was assessed by cell counting. There was a significant decrease in the number of OP9 cell compared to the control, following treatment with VD3 and 48 hours of incubation in the two higher VD3 concentrations, of 10 and 100nM respectively (the mean cell numbers for the control vs. VD3 concentrations of 10nM and 100nM respectively were  $157.7/\text{ml} \pm 9.97$  vs.  $78 \pm 2.08/\text{ml}$  and  $52.33/\text{ml} \pm 13.16$ , after 48 hours of incubation;  $n=3$ , paired t-test,  $P < 0.05$ , Fig. 1). Similar results were obtained when the cells were incubated in VD3 at the three VD3 concentrations for 72 hours. In this case, there was a significant decrease in the number of OP9 cells after the incubation at all three VD3 concentrations, compared to the control (the mean cell numbers for the control vs. VD3 concentrations of 1nM, 10nM and 100nM respectively were  $286/\text{ml} \pm 15.52$  vs.  $139.66/\text{ml} \pm 12.54$ ,  $129.66/\text{ml} \pm 18.81$  and  $117.7/\text{ml} \pm 38.88$ , after 72 hours of incubation;  $n=3$ , paired t-test,  $P < 0.001$ , Fig. 1).

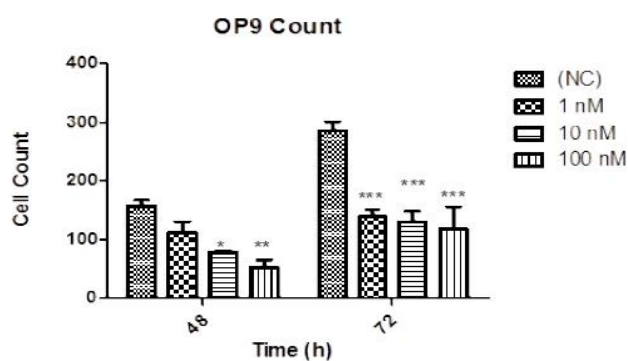


Fig. 1 Comparisons of the anti-proliferative effects of VD3 between the control and VD3-treated groups, showing significant decreases in OP9-cells number: after 48-hour incubation in 10nM ( $p < 0.01$ , represented by \*) and 100nM ( $p < 0.001$ , represented by \*\*) VD3 concentrations; after 72-hour incubation in 1nM ( $p < 0.0001$ , represented by \*\*\*), 10nM ( $p < 0.0001$ , represented by \*\*\*) and 100nM ( $p < 0.0001$ , represented by \*\*\*) of VD3-concentrations

#### B. Effect of VD3 on E14 Cells in the Proliferation Phase, Using Cell Counting

The impact of VD3 on E14 was also determined via cultivation and VD3 treatment, as before. Following VD3 treatment, a similar trend of results was observed in E14, as for OP9 cells above, there was a significant decrease in the number of OP9 cells after the incubation at all three VD3 concentrations, compared to the control (the mean cell numbers for the control vs. VD3 concentrations of 1nM, 10nM and 100nM respectively were  $66.66 \pm 8.21$  vs.  $43.67/\text{ml} \pm 0.66$ ,  $34.00 \pm 2.88$  and  $21.67/\text{ml} \pm 1.66$  after 48 hours of incubation;  $n=3$ , paired t-test,  $P < 0.05$ , Fig. 2). Similar results were obtained when the cells were incubated in VD3, compared to the control, at 72 hours at the different concentrations employed, there was a significant decrease in the number of OP9 cells after the incubation at all three VD3 concentrations, compared to the control (the mean cell numbers for the control vs. VD3 concentrations of 1nM, 10nM and 100nM respectively were  $132.6/\text{ml} \pm 2.33$  vs.  $113.3/\text{ml} \pm 7.02$ ,  $73.3 \pm 3.84/\text{ml}$  and  $63.3 \pm 3.05$  after 72 hours of incubation;  $n=3$ , paired t-test,  $P < 0.001$ , Fig. 2).

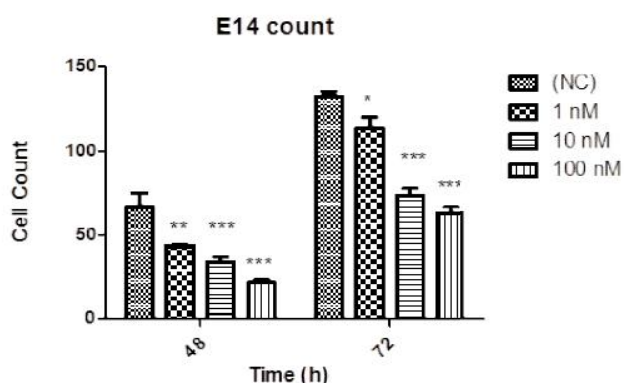


Fig. 2 Comparisons of the anti-proliferative effects of VD3 between the control and VD3-treated groups, showing significant decreases in E14-cells number: after 48-hour incubation in 1nM ( $p < 0.001$ , represented by\*\*), 10nM ( $p < 0.0001$ , represented by\*\*\*), and 100nM ( $p < 0.0001$ , represented by\*\*\*) VD3 concentrations; after 72-hour incubation in 1nM ( $p < 0.01$ , represented by \*), 10nM ( $p < 0.0001$ , represented by \*\*\*) and 100nM ( $p < 0.0001$ , represented by \*\*\*) of VD3 concentrations

*C. The Anti-Proliferation Effect of VD3 on OP9 Cells, Shown by Alkaline-Phosphatase Staining Assay*

The influence of VD3 on cellular proliferation and the extent of such an action (if any) were qualitatively investigated by the visual staining of alkaline phosphatase. Cells were cultivated in a GM containing VD3 at concentrations of 1, 10 and 100nM for up to 72 hours.

Alkaline phosphatase is associated with undifferentiated cells, and decreased expression of this enzyme is associated with differentiated cells. The specific cellular localisation of the staining inside cells was considered to be indicative of the presence of undifferentiated cells [15]. In contrast, in the absence of such staining (i.e. colourless cells), it was assumed that these cells have differentiated [16], based on earlier speculation that VD3 may promote differentiation. Due to the large area of colourless cells found, it is an indication that VD3 inhibited cell proliferation and increased cell differentiation. A reduction in the staining colour was manifest when comparing the effect of VD3 at 1nM, 10nM, and 100nM concentrations after both 48 and 72 hours of incubation, as compared to the control (Fig. 3 and 4, respectively).

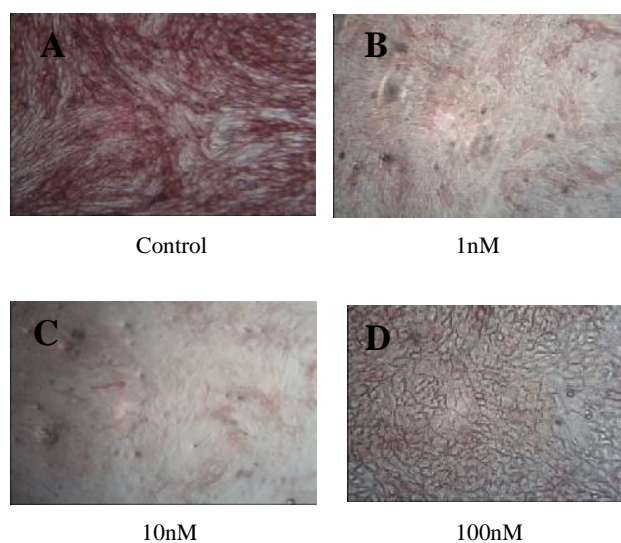


Fig. 3 The anti-proliferation effect of VD3 after 48-hour incubation on OP9 cells, shown by alkaline-phosphates staining assay. A reduction in the staining colour was manifest when comparing the effect of VD3 incubation at 1nM (B), 10nM (C) and 100nM (D) concentrations to the control (A)

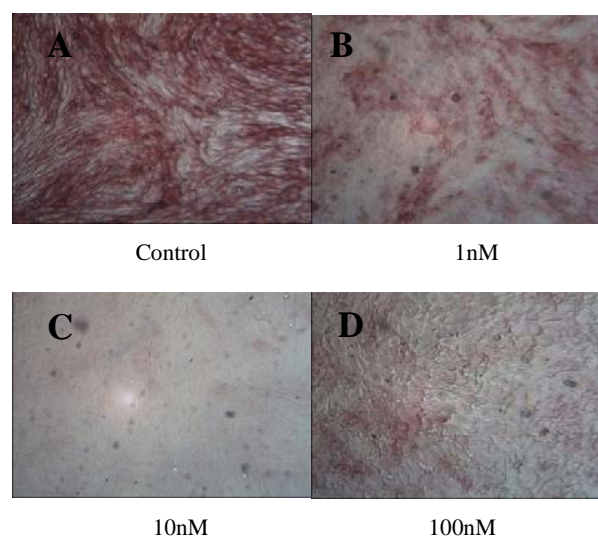


Fig. 4 The anti-proliferation effect of VD3 after 72-hour incubation on OP9 cells, shown by alkaline-phosphates staining assay. A reduction in the staining colour was manifest when comparing the effect of VD3 incubation at 1nM (B), 10nM (C) and 100nM (D) concentrations to the control (A)

*D. The Anti-Proliferation Effect of VD3 on E14 Cells, Shown by Alkaline-Phosphatase Staining Assay*

A similar staining pattern to that in OP9 cells, described above, was found in E14 cells when comparing the effect of VD3 at 1nM, 10nM and 100nM concentrations after both 48 and 72 hours of incubation, as compared to the control (Fig. 5 and Fig. 6).

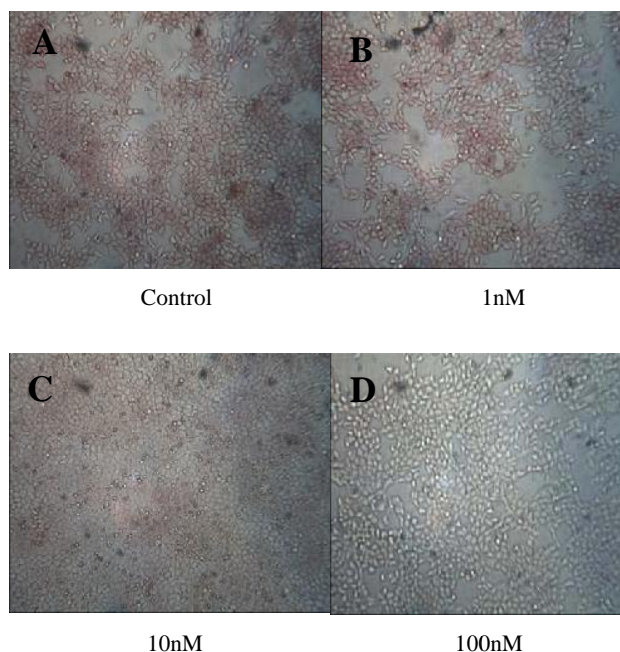


Fig. 5 The anti-proliferation effect of VD3 after 48-hour incubation on E14 cells, shown by alkaline-phosphates staining assay. A reduction in the staining colour was manifest when comparing the effect of VD3 incubation at 1nM (B), 10nM (C) and 100nM (D) concentrations to the control (A)

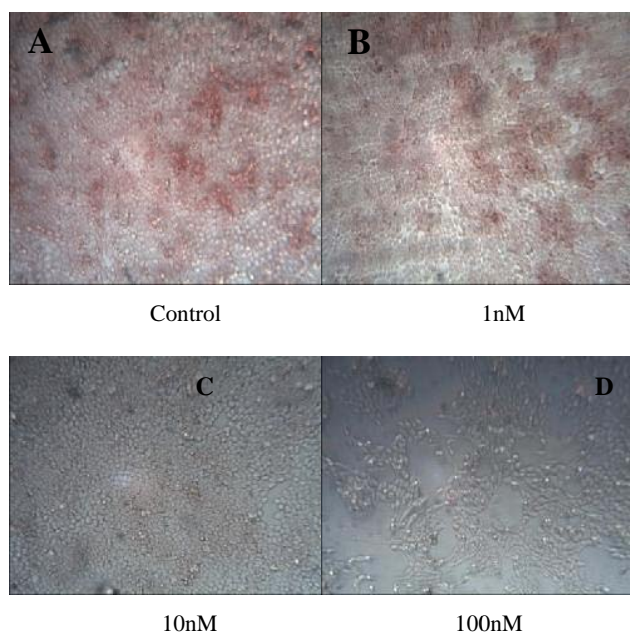


Fig. 6 The anti-proliferation effect of VD3 after 72-hour incubation on E14 cells, shown by alkaline-phosphates staining assay. A reduction in the staining colour was manifest when comparing the effect of VD3 incubation at 1nM (B), 10nM (C) and 100nM (D) concentrations to the control (A)

*E. The Effect of VD3 on Cell-Cycle Distribution of OP9 in the Proliferation Phase, Shown by Flow Cytometry*

To determine the specificity of cell counting and staining methods, and confirm the results obtained, cell-cycle analysis was performed using propidium iodide. There was no

significant change in the percentage of OP9 cells in the G and S- phases as compared to the control, in spite of the increased percentage in the G- phase coupled with a decrease in the percentage in the S- phase in OP9 cells at the respective VD3 concentrations of 1, 10 and 100nM after 48 hours of incubation. The fact that there was a significant increase in the percentage in the G-phase with respect to the S-phase following VD3 incubation at the two higher concentrations of 10 and 100nM, as compared to control, indicates that VD3 promotes cell differentiation and inhibits proliferation (at 10nM and 100nM VD3 concentrations respectively, the mean percentages of cells displaying the G-phase were  $74.02 \pm 8.88\%$  and  $75.49 \pm 16.73\%$ , and the S-phase were  $25.51 \pm 9.66\%$  and  $24.96 \pm 8.87\%$ , after 48 hours of incubation, n=3, paired t-test, P<0.01; for the control, the mean percentage of cells displaying the G-phase was  $58.74 \pm 11.78\%$ , and the S-phase was  $41.25 \pm 11.78\%$ ; Fig. 7). Similar results were obtained at the respective VD3 concentrations of 1, 10 and 100nM after 72 hours of incubation (at 100nM VD3 concentration, the mean percentage of cells displaying the G-phase was  $91.29 \pm 2.63\%$ , and the S-phase was  $9.03 \pm 2.92\%$ , after 72 hours of incubation, n=3, paired t-test, P<0.05; for the control, the mean percentages of cells displaying the G-phase was  $70.96 \pm 17.96\%$ , and the S-phase was  $30.60 \pm 18.96\%$ ; Fig. 8). Flow cytometry has been shown to be consistent with the other findings in this study, from cell counting and alkaline-phosphatase staining, that indicates VD3 to be a differentiation-inducer and a proliferation-inhibitor.

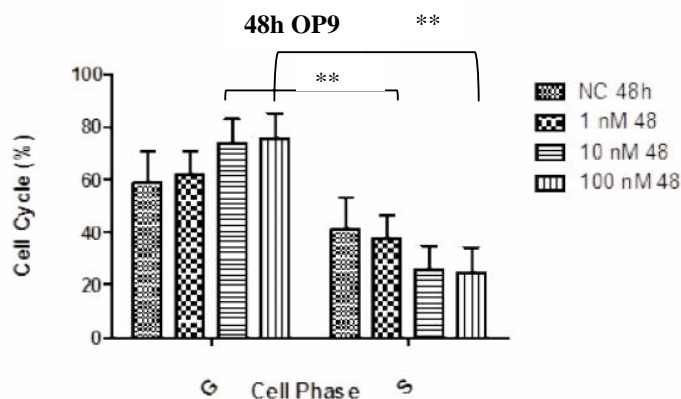


Fig. 7 The effect of VD3 treatment on the percentage of OP9 cells displaying either the G or S-phases of cell cycle using flow-cytometric analyses after 48-hour incubation. There was no significant change in the percentage of cells displaying either the G or S-phases as compared to controls, but there were significant increases in the percentages of cells displaying the G-phase with respect to the S-phase in 10nM and 100nM VD3 concentrations (p<0.01, represented by \*\*)

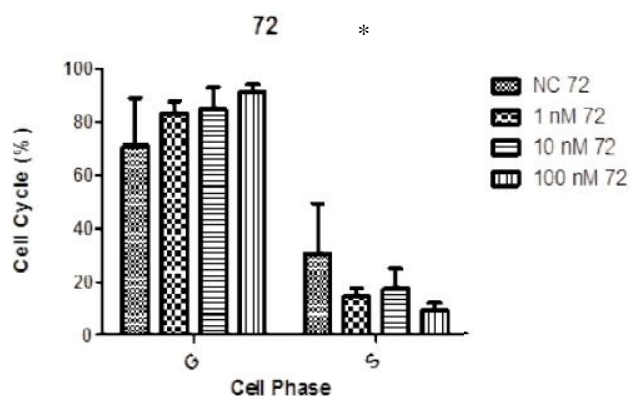


Fig. 8 The effect of VD3 treatment on the percentage of OP9 cells displaying either the G or S-phases of cell cycle using flow-cytometric analyses after 72-hour incubation. There was no significant change in the percentage of cells displaying either the G or S-phases as compared to controls, but there was a significant increase in the percentage of cells displaying the G-phase with respect to the S-phase in 100nM VD3 concentration ( $p < 0.05$ , represented by \*)

*F. The Effect of VD3 on Cell-Cycle Distribution of E14 in the Proliferation Phase, Shown by Flow Cytometry*

As in the case of OP9 cells, described above, there was no significant change in the percentages of E14 cells in the G and S- phases as compared to the control, in spite of the increased percentages in the G- phase coupled with a decrease in the percentages in the S- phase in E14 cells at the respective VD3 concentrations of 1, 10 and 100nM after both 48 hours and 72 hours of incubation. However, after both incubation periods, there were significant increases in the percentages in the G-phase with respect to the S-phase following VD3 incubation at 100nM VD3 concentration, as compared to control, indicating that VD3 promotes cell differentiation and inhibits proliferation (at 100nM VD3 concentration, the mean percentages of cells displaying the G-phase being  $48.69 \pm 15.05\%$ , and the S-phase being  $41.24 \pm 9.13$ , after 48 hours of incubation,  $n=3$ , paired t-test,  $P < 0.01$ ; for the control, the mean percentage of cells displaying the G-phase was  $21.97 \pm 2.28\%$ , and the S-phase was  $65.91 \pm 1.02\%$ , after 48 hours of incubation; at 100nM VD3 concentration, the mean percentages of cells displaying the G-phase being  $61.94 \pm 17.69\%$ , and the S-phase being  $17.77 \pm 15.77$ , after 72 hours of incubation,  $n=3$ , paired t-test,  $P < 0.05$ ; for the control, the mean percentage of cells displaying the G-phase was  $49.28 \pm 18.16\%$ , and the S-phase was  $40.94 \pm 14.02\%$ , after 72 hours of incubation; Fig. 9).

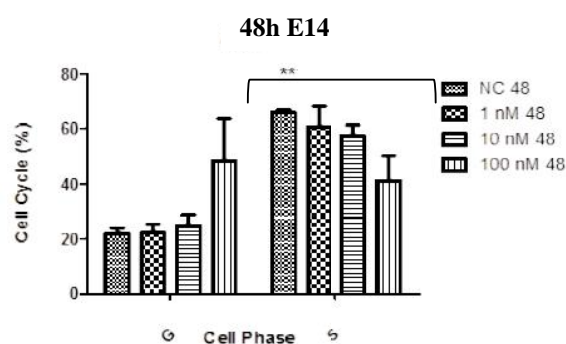


Fig. 9 The effect of VD3 treatment on the percentage of E14 cells displaying either the G or S-phases of cell cycle using flow-cytometric analyses after 48-hour incubation. There was no significant change in the percentages of cells displaying either the G or S-phases as compared to controls, but there was a significant increase in the percentage of cells displaying the G-phase with respect to the S-phase in 100nM VD3 concentration ( $p < 0.01$ , represented by\*)

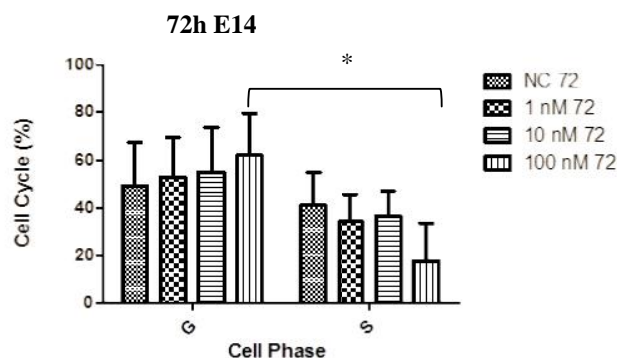


Fig. 10 The effect of VD3 treatment on the percentage of E14 cells displaying either the G or S-phases of cell cycle using flow-cytometric analyses after 72-hour incubation. There was no significant change in the percentages of cells displaying either the G or S-phases as compared to controls, but there was a significant increase in the percentage of cells displaying the G-phase with respect to the S-phase in 100nM VD3 concentration ( $p < 0.05$ , represented by \*)

V. DISCUSSION

The findings from the present study demonstrate that the proliferation phases of both OP9 stromal cells and embryonic stem cells (E14 cell line) were inhibited by the  $1\alpha, 25$ -Dihydroxyvitamin D3 (VD3). This study is the first demonstration of the anabolic effect of VD3 on mouse OP9 and E14 cells in vitro, suggesting, not for the first time, that there may be clinical effects from VD3 treatment. Previous in vitro studies using squamous cell carcinoma [17], prostate adenocarcinoma [18], cancer of the ovary [19], cancer of the breast [20] and cancer of the lung [21] showed significant anticancer effects with addition of VD3 or its analogues. An anti-proliferative activity of VD3 in tumour cells is cell cycle perturbation. It has been demonstrated that the effects of VD3

and its derivatives operate by means of the vitamin D receptor (VDR) regulating proliferation, apoptosis [22] and angiogenesis [23]. It was found that VD3-induced cell-cycle arrest by means of VDR in the proliferating C2C12 cells is indicated by the VD3 dose-dependent increase of VDR mRNA level [12]. Cell cycle progression is regulated by cyclins, and their association with cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs). In particular, p21 [24] and p27 [25] have been found to be inhibitors of G1 cyclin-dependent kinase. VD3 treatment inducing a G0/G1 phase arrest in squamous cell carcinoma cell lines [26] and also breast cancer MCF-7 cells [27] reportedly increased expressions of p21 and p27. The gene expressions of p21 and p27 were up-regulated by VD3 treatment in the C2C12 myoblast cell line [12]. VD3 inhibits the phosphorylation of retinoblastoma (pRb) and blocks progression of the cell cycle from G1 to S phase. Significant decreases in the amount of pRb and significant increased expression of P21 and P27 were also observed in monocytes leukaemia, which preceded the appearance of dephosphorylated pRb [28]. The findings from the cited studies above, suggest that VD3 treatment in the OP9 stromal cells and in E14 cells lines up-regulates the gene expressions of P21 and P27. Similar to in other cell lines, cyclin-dependent kinase inhibitor protein (CDKs-CDIs) through p21 and p27 might influence cell-phase arrest by VD3 in proliferating E14 and OP9 cells.

## VI. CONCLUSION

These results with E14 and OP9 cells indicate that adequate VD3 concentration enhances cellular differentiation and inhibits proliferation. The results also suggest that if E14 and OP9 cells were co-cultured and VD3-treated, there would be further-enhanced differentiation of ESCs into blood cells.

## ACKNOWLEDGMENT

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## REFERENCES

- [1] M. J. Evans and M. H. Kaufman, "Establishment in culture of pluripotential cells from mouse embryos," *Nature*, vol. 292, pp. 154-156, 1981.
- [2] S.-I. Nishikawa, L. M. Jakt, and T. Era, "Embryonic stem-cell culture as a tool for developmental cell biology," *Nature Reviews Molecular Cell Biology*, vol. 8, pp. 502-507, 2007.
- [3] M. A. Vodyanik, J. A. Bork, J. A. Thomson, and I. I. Slukvin, "Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential," *Blood*, vol. 105, pp. 617-626, 2005.
- [4] H. F. DeLuca, "Overview of general physiologic features and functions of vitamin D," *The American journal of clinical nutrition*, vol. 80, pp. 1689S-1696S, 2004.
- [5] N. I. zurNieden, F. D. Price, L. A. Davis, R. E. Everitt, and D. E. Rancourt, "Gene profiling on mixed embryonic stem cell populations reveals a biphasic role for  $\beta$ -catenin in osteogenic differentiation," *Molecular Endocrinology*, vol. 21, pp. 674-685, 2007.
- [6] P. H. Anderson, B. May, and H. A. Morris, "Vitamin D metabolism: new concepts and clinical implications," *The Clinical Biochemist Reviews*, vol. 24, p. 13, 2003.

- [7] A. Grande, M. Montanari, E. Tagliafico, R. Manfredini, T. Z. Marani, M. Siena, et al., "Physiological levels of  $1\alpha, 25$  dihydroxyvitamin D3 induce the monocytic commitment of CD34+ hematopoietic progenitors," *Journal of leukocyte biology*, vol. 71, pp. 641-651, 2002.
- [8] H. F. Luderer and M. B. Demay, "The vitamin D receptor, the skin and stem cells," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 121, pp. 314-316, 2010.
- [9] T. C. Chen, G. G. Schwartz, K. L. Burnstein, B. L. Lokeshwar, and M. F. Holick, "The in vitro evaluation of 25-hydroxyvitamin D3 and 19-nor- $1\alpha, 25$ -dihydroxyvitamin D2 as therapeutic agents for prostate cancer," *Clinical Cancer Research*, vol. 6, pp. 901-908, 2000.
- [10] J. O'Kelly, J. Hisatake, Y. Hisatake, J. Bishop, A. Norman, and H. P. Koeffler, "Normal myelopoiesis but abnormal T lymphocyte responses in vitamin D receptor knockout mice," *Journal of Clinical Investigation*, vol. 109, pp. 1091-1099, 2002.
- [11] S. Yetgin and S. Yalçin, "The effect of vitamin D3 on CD34 progenitor cells in vitamin D deficiency rickets," *TURKISH JOURNAL OF PEDIATRICS*, vol. 46, pp. 164-166, 2004.
- [12] H. Okuno, K. N. Kishimoto, M. Hatori, and E. Itoi, " $1\alpha, 25$ -dihydroxyvitamin D3 enhances fast-myosin heavy chain expression in differentiated C2C12 myoblasts," *Cell Biology International*, vol. 36, pp. 441-447, 2012.
- [13] C. M. Bunce, G. Brown, and M. Hewison, "Vitamin D and hematopoiesis," *Trends in Endocrinology & Metabolism*, vol. 8, pp. 245-251, 1997.
- [14] L. Sproat, B. Bolwell, L. Rybicki, R. Dean, R. Sobecks, B. Pohlman, et al., "Vitamin D level after allogeneic hematopoietic stem cell transplant," *Biology of Blood and Marrow Transplantation*, vol. 17, pp. 1079-1083, 2011.
- [15] G. R. MacGregor, B. P. Zambrowicz, and P. Soriano, "Tissue non-specific alkaline phosphatase is expressed in both embryonic and extraembryonic lineages during mouse embryogenesis but is not required for migration of primordial germ cells," *Development*, vol. 121, pp. 1487-1496, 1995.
- [16] M. D. O'Connor, M. D. Kardel, I. Iosfina, D. Youssef, M. Lu, M. M. Li, et al., "Alkaline Phosphatase-Positive Colony Formation Is a Sensitive, Specific, and Quantitative Indicator of Undifferentiated Human Embryonic Stem Cells," *Stem cells*, vol. 26, pp. 1109-1116, 2008.
- [17] P. A. Hershsberger, R. A. Modzelewski, Z. R. Shurin, R. M. Rueger, D. L. Trump, and C. S. Johnson, " $1, 25$ -dihydroxycholecalciferol ( $1, 25$ -D3) inhibits the growth of squamous cell carcinoma and down-modulates p21Waf1/Cip1 in vitro and in vivo," *Cancer research*, vol. 59, pp. 2644-2649, 1999.
- [18] R. H. Getzenberg, B. W. Light, P. E. Lapco, B. R. Konety, A. K. Nangia, J. S. Acierno, et al., "Vitamin D inhibition of prostate adenocarcinoma growth and metastasis in the Dunning rat prostate model system," *Urology*, vol. 50, p. 999, 1997.
- [19] X. Zhang, F. Jiang, P. Li, C. Li, Q. Ma, S. V. Nicosia, et al., "Growth suppression of ovarian cancer xenografts in nude mice by vitamin D analogue EB1089," *Clinical Cancer Research*, vol. 11, pp. 323-328, 2005.
- [20] K. W. Colston, S. K. Chander, A. G. MacKay, and R. C. Coombes, "Effects of synthetic vitamin d analogues on breast cancer cell proliferation in vivo and in vitro," *Biochemical pharmacology*, vol. 44, pp. 693-702, 1992.
- [21] K. Nakagawa, A. Kawaura, S. Kato, E. Takeda, and T. Okano, " $1\alpha, 25$ -Dihydroxyvitamin D3 is a preventive factor in the metastasis of lung cancer," *Carcinogenesis*, vol. 26, pp. 429-440, 2005.
- [22] M. Simboli-Campbell, C. J. Narvaez, M. Tenniswood, and J. Welsh, " $1, 25$ -Dihydroxyvitamin D<sub>3</sub> induces morphological and biochemical markers of apoptosis in MCF-7 breast cancer cells," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 58, pp. 367-376, 1996.
- [23] D. Mantell, P. Owens, N. Bundred, E. Mawer, and A. Canfield, " $1\alpha, 25$ -dihydroxyvitamin D3 inhibits angiogenesis in vitro and in vivo," *Circulation research*, vol. 87, pp. 214-220, 2000.
- [24] J. Wade Harper, G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge, "The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases," *Cell*, vol. 75, pp. 805-816, 1993.
- [25] H. Toyoshima and T. Hunter, "p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21," *Cell*, vol. 78, pp. 67-74, 1994.
- [26] G. Hager, M. Formanek, C. Gedlicka, D. Thurnher, B. Knerer, and J. Kornfehl, " $1, 25$  (OH)<sub>2</sub> vitamin D3 induces elevated expression of the cell cycle-regulating genes P21 and P27 in squamous carcinoma cell

- lines of the head and neck," *Actaoto-laryngologica*, vol. 121, pp. 103-109, 2001.
- [27] L. Verlinden, A. Verstuyl, R. Convents, S. Marcelis, M. Van Camp, and R. Bouillon, "Action of  $1, 25 (OH)_2 D_3$  on the cell cycle genes, cyclin D1, p21 and p27 in MCF-7 cells," *Molecular and cellular endocrinology*, vol. 142, pp. 57-65, 1998.
- [28] K. T. Kanatani Y., Okabe-Kado J., Yamamoto-Yamaguchi Y., Naokazu N., Motoyoshi K. & Honma Y. , "Role of CD14 expression in the differentiation-apoptosis switch in human monocytic leukemia cells treated with  $1\alpha, 25$ -dihydroxyvitamin D3 or dexamethasone in the presence of transforming growth factor beta1," *Cell Growth Differ.*, vol. 10, pp. 705-12., Oct 1999.