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Vitamin D_3 metabolites induce osteogenic differentiation in human dental pulp and human dental follicle cells

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ABSTRACT

Vitamin D_3 metabolites regulate the bone metabolism and $1\alpha,25$-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) is known to play an important role in teeth mineralization. However, little is known about the potential of vitamin D as an osteogenic inducer in human dental pulp (hDPCs) and dental follicle cells (hDFCs) *in vitro*. Therefore, we investigated the effects of vitamin D_3 metabolites $1\alpha,25(OH)_2D_3$ and 25-hydroxyvitamin D_3 ($25OHD_3$) on proliferation and osteogenic differentiation of hDPCs and hDFCs *in vitro*. We also examined whether vitamin D_3 metabolic enzymes were regulated in hDFCs and hDPCs. Cell proliferation was decreased by both metabolites in hDPCs and hDFCs. Vitamin D_3 metabolites increased ALP activity and induced mineralization when osteogenic supplements (OS; L-ascorbic acid-2-phosphate + β-glycerophosphate) were added, though the expression of osteocalcin (OC) and osteopontin (OPN) were regulated without the addition of OS. CYP24 and CYP27B1 expressions were upregulated by vitamin D_3 metabolites and $25OHD_3$ was converted into $1\alpha,25(OH)_2D_3$ in the culture medium. These results confirm that $1\alpha,25(OH)_2D_3$ (10 and 100 nM) and $25OHD_3$ (500 nM) can be used as osteogenic inducers synergistically with osteogenic supplements for differentiation of hDPCs and hDFCs. Furthermore, our findings strengthen our knowledge about the role of hDPCs and hDFCs as vitamin D_3 target cells.

1. Introduction

Vitamin D_3 metabolites such as $1\alpha,25$-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) and 25-hydroxycholecalciferol ($25OHD_3$) are key regulatory factors of bone metabolism [1–3]. The circulating $25OHD_3$ metabolite is hydroxylated to $1\alpha,25(OH)_2D_3$ by $25OHD_3$-1α-hydroxylase (1α-hydroxylase, CYP27B1) in the kidneys and other vitamin D target organs [4]. The biological effects of vitamin D_3 can be mediated by the vitamin D receptor (VDR), a member of the superfamily of nuclear hormone receptors that functions as a transcription factor or the action of vitamin D_3 might be mediated non-genomically via a different receptors such as membrane-associated rapid response steroid receptor [5–7]. The actions of $1\alpha,25(OH)_2D_3$, as well as $25OHD_3$, are inactivated by 24-hydroxylase (CYP24) in the kidneys and in the other vitamin D_3 target tissue. CYP24 is a mitochondrial enzyme which catalyses the hydroxylation and thereby inactivates $1\alpha,25(OH)_2D_3$ while $25OHD_3$ is converted to $24,25(OH)_2D_3$ [8]. The expression of the CYP24 gene has been used as an indicator of transcriptional activity of vitamin D_3 metabolites [9].

Dental tissues derived cells are a source of multipotent mesenchymal stem cells that can be differentiated into osteogenic, chondrogenic, adipogenic and neurogenic cell types *in vitro*, as reported in other studies including one from our group [10,11]. These cells can be isolated from impacted human third molar teeth based on their anatomic locations and expression of stem cell markers. Human dental pulp cells (hDPCs) reside in the central cavity of the teeth and are a source of progenitor cells that can undergo differentiation towards odontoblastic, osteoblastic, neurogenic and adipocytic cell types *in vitro* [12]. It has been reported that stem cells derived from dental pulp are able to differentiate into osteoblastoids under high serum conditions and are a potential source of autologous bone produced *in vitro* [13,14]. Recent studies have identified dental follicle cells (hDFCs) which are isolated from the connective tissue surrounding the developing tooth germ before tooth eruption [15]. This tissue contains progenitor cells that give rise to the periodontium including cementum, periodontal ligament cells (PDL), and alveolar bone [16].

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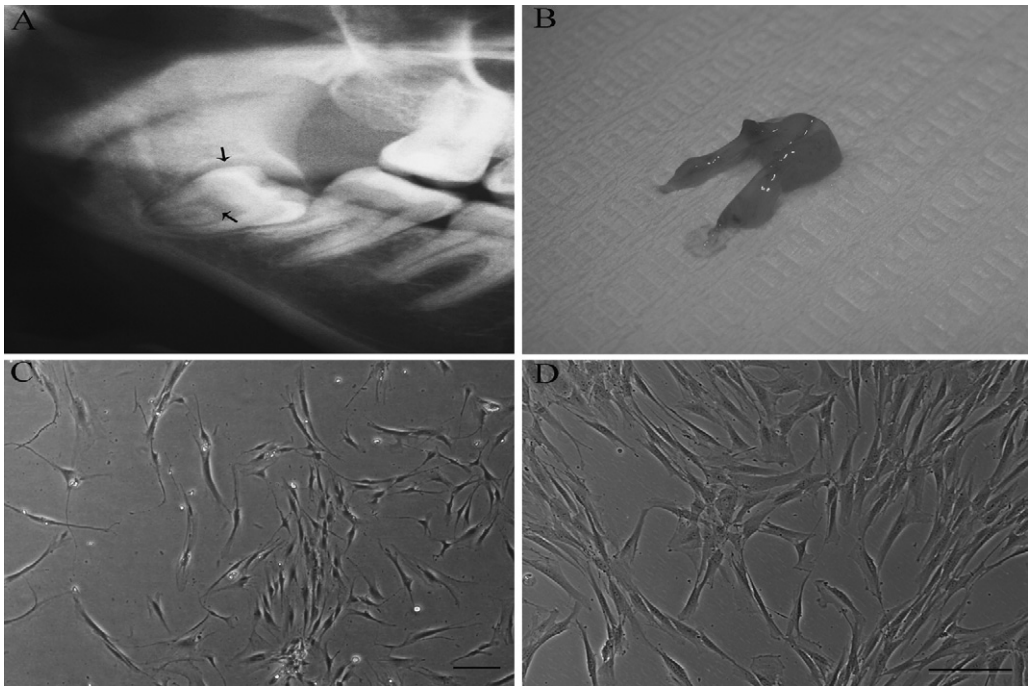

Fig. 1. Anatomical location and morphological appearances of human teeth derived cells. Radiographic image (A) showing anatomic location of dental follicle (arrow above) or dental pulp (arrow below) tissues and (B) human dental pulp tissue obtained from the pulp chamber and canals. Light microscopic appearance of (C) hDFCs (scale bar = 1 mm) and (D) hDPCs (scale bar = 100 μM).

Dexamethasone (Dex) a glucocorticosteroid, in combination with osteogenic supplements (OS; L-ascorbic acid-2-phosphate + β-glycerophosphate) is the most commonly used osteogenic inducer for human mesenchymal stem cells (MSCs) such as adipose tissue derived [17] and bone marrow derived stem cells *in vitro* [18]. Studies have shown that human dental follicle derived cells differentiate into osteoblasts like cells when induced by Dex [19,20]. Glucocorticosteroid, however have deleterious effects on bone *in vivo*, resulting in glucocorticoid-induced osteoporosis [21]. There are some studies where $1\alpha,25(OH)_2D_3$ in addition to OS has been used for osteogenic differentiation of MSCs [22–24]. However, the effects of vitamin D_3 metabolites to induce osteogenic differentiation in hDPCs and hDFCs is not yet clearly understood *in vitro*. Moreover, the role of $1\alpha,25(OH)_2D_3$ in tooth formation is well known from *in vivo* and clinical studies, deficiency of $1\alpha,25(OH)_2D_3$ results in hypocalcification of the dentin and enamel leading to unmineralized dental structure [25]. Excessive doses of $1\alpha,25(OH)_2D_3$ cause hypercementosis, formation of pulp stones and hypercalcification in dental tissues [26]. Additionally, $1\alpha,25(OH)_2D_3$ has been reported to promote the function of osteoclastogenesis in the periodontium [27]. Taken together, we hypothesized that cells derived from dental tissues when treated with vitamin D_3 metabolites might optimally induce osteogenic differentiation *in vitro*.

Therefore, this study was designed to understand the role of different concentrations of $25OHD_3$ and $1,25(OH)_2D_3$ on proliferation and osteogenic differentiation of hDPCs and hDFCs *in vitro*. In this series of investigations, we also tested whether vitamin D_3 metabolic enzymes in hDFCs and hDPCs were regulated *in vitro*.

2. Materials and methods

2.1. Cell isolation and culture

Human impacted third molars were obtained with informed consent from Finnish Student Health Services, Tampere, Finland. The collection of stem cells from tooth samples was approved by the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (R06009). Human dental pulp and dental follicle tissue explants were obtained from partially or completely impacted third molar teeth of 12 patients, patients aged 21–26 years (23 ± 2.5 years). The tooth samples were brought from the health centre to the laboratory in Phosphate buffered saline (PBS; BioWhittaker Lonza, Verviers, Belgium) containing 2% antibiotics/antimycotics (a/a; 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 mg/ml amphotericin B; Invitrogen, Paisley, Scotland, UK). The dental tissues were isolated in the laboratory under laminar hood; the teeth were cleaned with PBS before isolating cells. The human dental pulp and follicle tissue explants were derived from third molar teeth based on their anatomic location as shown in (Fig. 1A and B). Dental Pulp tissues were obtained by exposing the pulp chamber of the teeth and dental follicle from the tissues surrounding the mineralized tooth. Following the dental pulp and follicle tissue extraction, the tissue fragments were minced using sterilized scalpels. Tissues were then digested in collagenase type I 3 mg/ml (Invitrogen) and dispase 4 mg/ml (Invitrogen) for 1 h at 37 °C. Once digestion was completed the obtained cell suspension was passed through a 70 μm cell strainer (Falcon, BD Labware, Franklin lakes, NJ, USA) and cells were seeded in 6 well culture plates (Nunc, Roskilde, Denmark) in basic cell culture medium (BM) consisting of DMEM/F-12 1:1 (Invitrogen), 10% FBS (Invitrogen), L-glutamine (GlutaMAX I; Invitrogen), and 1% antibiotics/antimycotic (100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B; Invitrogen), and then incubated at 37 °C in 5% CO_2. After 14 days of culture, cells were detached using trypsin in PBS (Lonza) and then cells were expanded in 75 cm^2 culture flasks (Nunc, Roskilde, Denmark) containing BM. Cell culture plates and flasks were monitored daily for cell growth, with medium changes taking place three times per week. All assays were performed using cells between passage 3 and 4 and experiments were repeated using cells derived from 3 different donors each for hDPCs and hDFCs.

Table 1
Primers sequence for quantitative RT-PCR.

Name	5′-Sequence-3′	Product size	Accession number
RPLPO	Forward AATCTCCAGGGGCACCATT Reverse CGTTGGCTCCCACTTTGT	70	NM_001002
Osteocalcin	Forward AGCAAAGGTGCAGCCTTTGT Reverse GCGCCTGGGTCTCTTCACT	63	NM_000711
Osteopontin	Forward GCCGACCAAGGAAAACTCACT Reverse GGCACAGGTGATGCCTAGGA	71	J04765
CYP24	Forward GCCCAGCCGGGAACTC Reverse AAATACCACCATCTGAGGCGTATT	61	NM_000782
CYP27B1	Forward TTGGCAAGCGCAGCTGTAT Reverse TGTGTTAGGATCTGGGCCAAA	75	NM_000785
VDR	Forward CCTTCACCATGGACGACATG Reverse CGGCTTTGGTCACGTCACT	77	NM_000376

2.2. Cell proliferation assay

Cell proliferation assay was done to measure the viability and induction of cell proliferation by the action of vitamin D_3 metabolites alone, such as $1\alpha,25(OH)_2D_3$ (Sigma–Aldrich, MO, USA) and $25OHD_3$ (Sigma) on hDPCs and hDFCs. The cells were treated with different concentrations of $1\alpha,25(OH)_2D_3$ (1,25; 10 and 100 nM) and $25OHD_3$ (25; 100 and 500 nM) as follows. Human DPCs and hDFCs each were seeded at 10,000 cells/well in 24-well plates in BM with different concentrations of vitamin D_3 metabolites. The control samples were maintained in BM and blank values were also measured for non-specific binding. The culture media was changed after 3 and 4 days for each media concentration. The plates were incubated at 37 °C in a 5% CO_2 containing humidified atmosphere. Cell numbers and viability were quantified at 1, 7 and 14 days time points using the colorimetric reagent WST-1 (Takara Bio Inc., Otsu, Japan). The absorbance was measured directly with a plate reader Victor 1420 (Perkin Elmer life Sciences, Turku, Finland) using wavelength of 450 nm.

2.3. Alkaline phosphatase activity

To further investigate the combined effect of osteogenic supplements (OS; 50 μM L-ascorbic acid 2-phosphate (AA; Sigma) and 10 mM β-glycerophosphate (Sigma)) and vitamin D_3 metabolites on hDPCs and hDFCs, cells were plated in 24-well plates at a density of 10,000 cells/well, respectively, and incubated for 24 h in BM. Thereafter, cells were cultured in BM containing $1\alpha,25(OH)_2D_3$ (10 and 100 nM) or $25OHD_3$ (100 and 500 nM) with and without OS. As a control, cells were cultured in BM and for comparing the effects of different metabolites cells were cultured in BM containing dexamethasone (Dex) 10 nM + OS. Culture medium was replaced with fresh medium every 3 and 4 days. After 7 and 14 days of culture, cell proliferation and alkaline phosphatase (ALP) activity were analyzed with a commercially available *p*-nitrophenyl phosphate tablet set (Sigma, St. Louis, MO, USA) and cell proliferation kit (Premix WST-1 Cell Proliferation Assay System; Takara Bio Inc., Shiga, Japan), with modifications [28]. Cell proliferation (WST-1 absorbance) was analyzed according to the manufacturer's protocol. Briefly, WST-1 reagents was added to each well containing fresh medium (50 μl of WST-1/500 μl of medium in each well of 24-well plate), incubated for 60 min, the absorbance was measured at 450 nm using a microplate reader (Victor 1420, Finland). After WST-1 analysis, each well was washed twice with PBS and *p*-nitrophenyl phosphate solution was added (400 μl/well for 24-well plates). After 10 min of incubation at 37 °C, conversion of *p*-nitrophenyl phosphate into *p*-nitrophenol by cellular ALP was stopped with the equivalent amount of 3N NaOH and the absorbance of *p*-nitrophenol was measured at 450 nm using a microplate reader. Alkaline phosphatase-specific activity is expressed as *p*-nitrophenol absorbance (OD; 405 nm)/WST-1 absorbance (OD; 450 nm), which is designed to assess the ALP activity/no. of viable cells.

2.4. Mineralization assay (alizarin red staining)

The cell culture conditions used were similar as described for ALP activity. After 21 days of cell culture in 24-well plates, *in vitro* mineralization was analyzed by alizarin red staining. For alizarin red S staining, cells were fixed with ice-cold 70% ethanol for 60 min at −20 °C. Then, cells were washed twice with distilled water and stained with 40 mM Alizarin red S solution (Sigma) for 10 min at room temperature. The pH value of the solution was adjusted to 4.2 with 25% ammonium hydroxide prior to staining. After staining, excess dye was washed with distilled water and digital images of stained mineral deposits were taken.

2.5. Real-time quantitative PCR

Next, we analyzed the time course effect of the vitamin D_3 metabolites without OS at mRNA level. Human DPCs and hDFCs were seeded at a density of 10,000 cells/well in 6 well plate with different concentrations of $1\alpha,25(OH)_2D_3$ (10 and 100 nM) and $25OHD_3$ (100 and 500 nM) in BM. The control samples were maintained in the BM. The Total RNA was extracted at 24, 48, 72 h time points by Eurozol (Euroclone S.p.A, Pero, Italy). First-strand cDNA synthesis were performed by a High Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK). Real-time quantitative PCR (qRT-PCR) was conducted using osteocalcin (OC), osteopontin (OPN), and RPLP0 (human acidic ribosomal phosphoprotein), VDR (vitamin D receptor), CYP24 (24-hydroxylase) and $25OHD_3$-1α-hydroxylase (1α-hydroxylase, CYP27B1) primer sequences as shown in (Table 1). To exclude signals from contaminating DNA, the forward and reverse sequence of each primer was designed on different exons. The Power SYBR Green PCR Master Mix (Applied Biosystems) was used for Quantitative PCR reactions according to the manufacturer's instructions. The reactions were performed with AbiPrism 7300 Sequence Detection System (Applied Biosystems) at 95 °C 10 min, and then 45 cycles at 95 °C/15 s and 60 °C/60 s. The Ct values for OC, OPN, VDR and CYP24 were normalized to that of the housekeeping gene RPLP0, as described elsewhere [29].

2.6. 1,25-Dihydroxy vitamin D_3, enzyme immunoassay (EIA)

The 1,25-dihydroxy vitamin D_3 EIA kit (Immunodiagnostic Systems Ltd, Boldon, UK) was used to measure the conversion of $25OHD_3$ into $1\alpha,25(OH)_2D_3$ by 1α-hydroxylase in hDPCs and hDFCs. The cells were cultured in BM + $25OHD_3$ (500 nM) and BM + $25OHD_3$ (500 nM) + inhibitor (ketoconazole; 10 μM) for 24 h. The control samples were maintained in BM without the addition of vitamin D_3 metabolites and inhibitor. 10,000 cells/well were

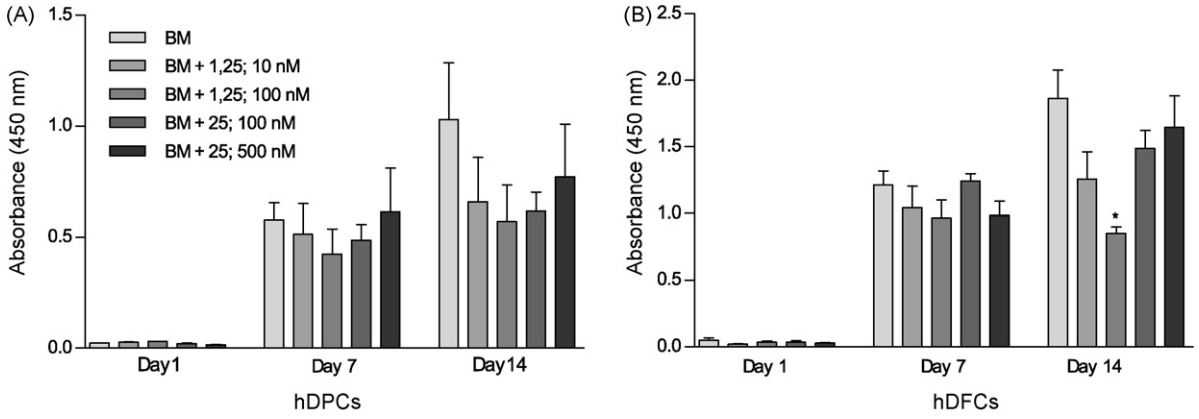

Fig. 2. Effect of $1\alpha,25(OH)_2D_3$ and $25OHD_3$ on cell proliferation. Cell numbers were analyzed in (A) hDPCs and (B) hDFCs at 1, 7 and 14 days time periods. Columns represent mean ± SEM ($n = 3$). Statistically significant difference when treated samples were compared to the control of each time point, $^*p < 0.05$.

seeded in 6 well plate and after overnight incubation at 37 °C in a 5% CO_2 humidified atmosphere different metabolites were added. After 24 h the culture media for all the samples were taken for immunoextraction following quantitation by enzyme immunoassay as per manufacturer's protocol. Samples were obtained from three different patient samples for both the tissues which were tested independently. Briefly, 100 μl of the delipidated samples and controls were added to the appropriately labeled immunocapsules in duplicates per sample. Primary antibody was added to the immunoextracts and incubated overnight at +4 °C. Next day, secondary antibody was added which was followed by the addition of enzyme conjugate and tetramethylbenzidine (TMB) substrate. An acidic stop solution was added to terminate the reaction which resulted in the color change from blue to yellow. The intensity of the yellow color is inversely proportional to the concentration of $1\alpha,25(OH)_2D_3$. The absorbance was measured within 30 min of the addition of the stop solution using a plate reader (Victor 1420) at a wavelength of 450 nm. The results were calculated by measuring the absorbance of the calibrators provided with the kit and creating a calibration curve by plotting the percent bind on the *y*-axis and the amount of $1\alpha,25(OH)_2D_3$ on the *x*-axis. The percent bind values for the samples were calculated and then interpolated amount of $1\alpha,25(OH)_2D_3$ using the calibration curve.

2.7. Statistical analysis

The statistical analyses of the results were performed with GraphPad Prism 5.01. The data is presented as mean ± standard error of the mean (SEM) for all quantitative assays and experiments were carried out in triplicate for cells derived from three donor samples. All statistical analyses were performed at the significance level $p < 0.05$. One-way analysis of variance (ANOVA) with Dunnett's post hoc test for multiple comparisons was used for the analysis.

3. Results

3.1. Cell morphology

Human DPCs and hDFCs were isolated by enzyme-digestion method. Both cell types exhibited initial triangular, stellate or spindle shape cell morphology after initial plating. The time required to form confluent cell cultures varied from 1 to 2 weeks for hDPCs and hDFCs. The hDFCs appeared spindle or stellate in shape as observed under phase contrast microscope (Fig. 1C). After first passage, the hDPCs appeared stellate in shape or some cultures formed patterns of net like structure as shown by phase contrast microscope (Fig. 1D).

3.2. Cell proliferation effect (days 1, 7 and 14)

Time course effects of different concentrations of $1\alpha,25(OH)_2D_3$ (10 and 100 nM) and $25OHD_3$ (100 and 500 nM) at days 1, 7 and 14 on proliferation of hDPCs and hDFCs were analyzed. The addition of vitamin D_3 metabolites, to hDPCs and hDFCs induced decrease in cell proliferation when compared to the cells treated without the metabolites, as shown in (Fig. 2A). While cell proliferation was significantly inhibited by $1\alpha,25(OH)_2D_3$ (100 nM) in hDFCs by day 14 (Fig. 2B).

3.3. Osteogenic differentiation

3.3.1. Alkaline phosphatase activity (days 7 and 14)

The effects of vitamin D_3 metabolites with or without OS on osteogenic differentiation of hDPCs and hDFCs were analyzed by measuring their ALP activities. Time course experiments showed significant increases in ALP activity of hDPCs on day 7 after treatment with OS + $1\alpha,25(OH)_2D_3$ (10 nM), OS + $1\alpha,25(OH)_2D_3$ (100 nM) and OS + $25OHD_3$ (500 nM) in comparison to cells treated without OS and the untreated control. Following day 14, ALP activity was significantly increased in cells treated with OS + $1\alpha,25(OH)_2D_3$ (100 nM), OS + $25OHD_3$ (500 nM) and OS + Dex (10 nM) in hDPCs, as shown in (Fig. 3A and B).

In addition, hDFCs treated with OS + $1\alpha,25(OH)_2D_3$ (100 nM), OS + $25OHD_3$ (500 nM) at day 7 significantly increased ALP activity. Following the 14 days time course, hDFCs expressed significant increase in ALP activity in cells treated with OS + $1\alpha,25(OH)_2D_3$ (10 nM), OS + $1\alpha,25(OH)_2D_3$ (100 nM); though cells treated without OS and OS + $25OHD_3$ (100 nM) and OS + Dex (10 nM) did not significantly increase the ALP activity of hDFCs (Fig. 3C and D). Human DPCs and hDFCs treated in combination with OS and vitamin D_3 metabolites showed significant ALP activity.

3.3.2. Mineralization

Treatment with vitamin D_3 metabolites in combination with OS promoted biomineralization of hDPCs and hDFCs as shown in (Fig. 4A and B). Human DFCs and hDPCs exposed to $1\alpha,25(OH)_2D_3$ (10 and 100 nM) and $25OHD_3$ (100 and 500 nM) without OS did not induce matrix mineralization (data not shown). There were differences in the intensities of alizarin red staining between cells derived from different donors.

3.3.3. The expression of bone markers at mRNA level (24, 48 and 72 h time points)

To observe the time course effect of vitamin D_3 metabolites on bone markers such as OPN and OC at mRNA level qRT-PCR was done. OC expression was up regulated by all the concentra-

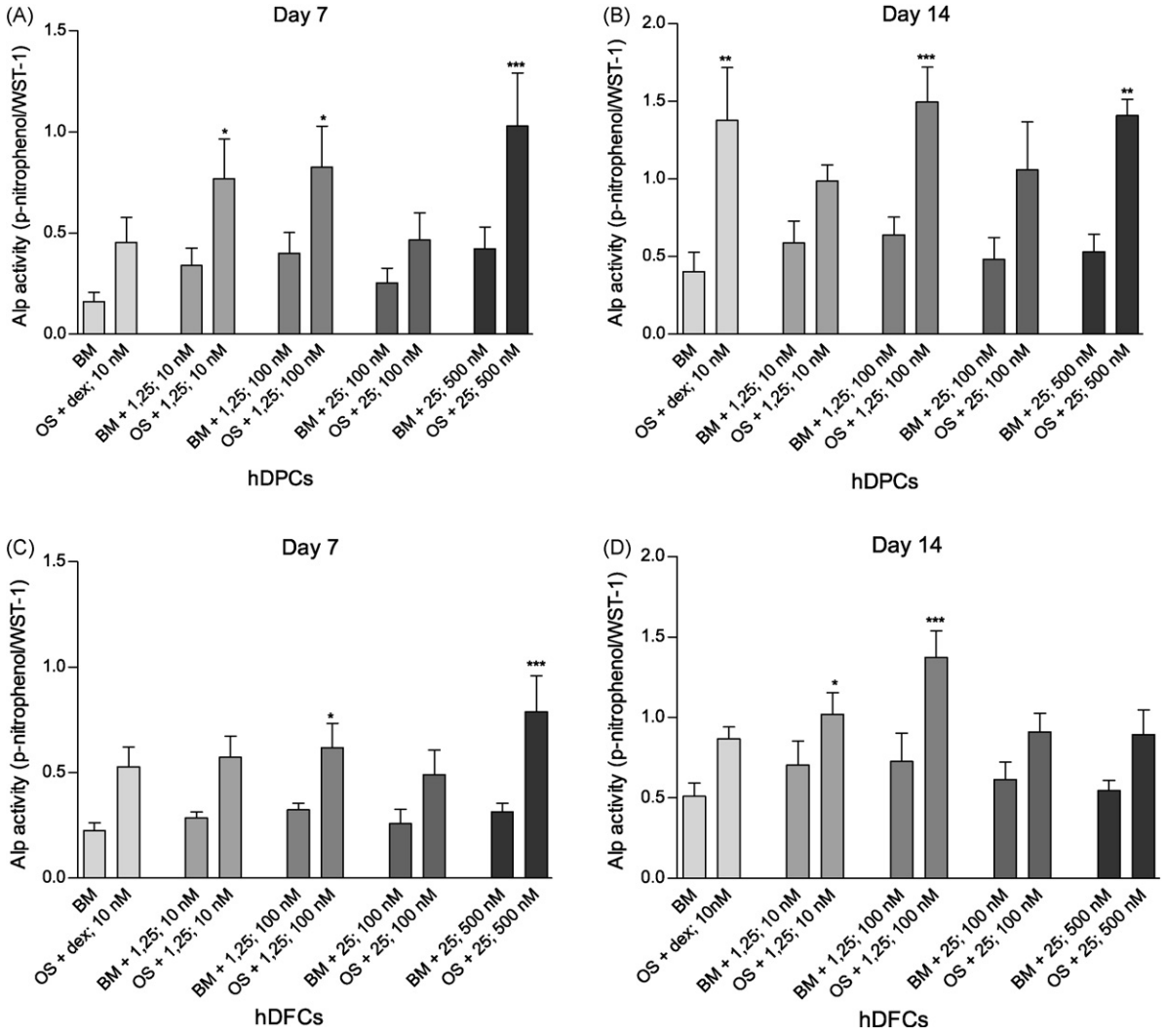

Fig. 3. ALP activity of cells cultured in $1\alpha,25(OH)_2D_3$ and $25OHD_3$ in the presence of osteogenic supplements (OS; L-ascorbic acid-2-phosphate + β-glycerophosphate) in comparison to cells cultured in Dex. ALP activity of hDPCs (A and B) and hDFCs (C and D) was assessed after 7 and 14 days of differentiation. Columns represent mean ± SEM ($n = 3$). Statistically significant difference when treated samples were compared to BM of days 7 and 14, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

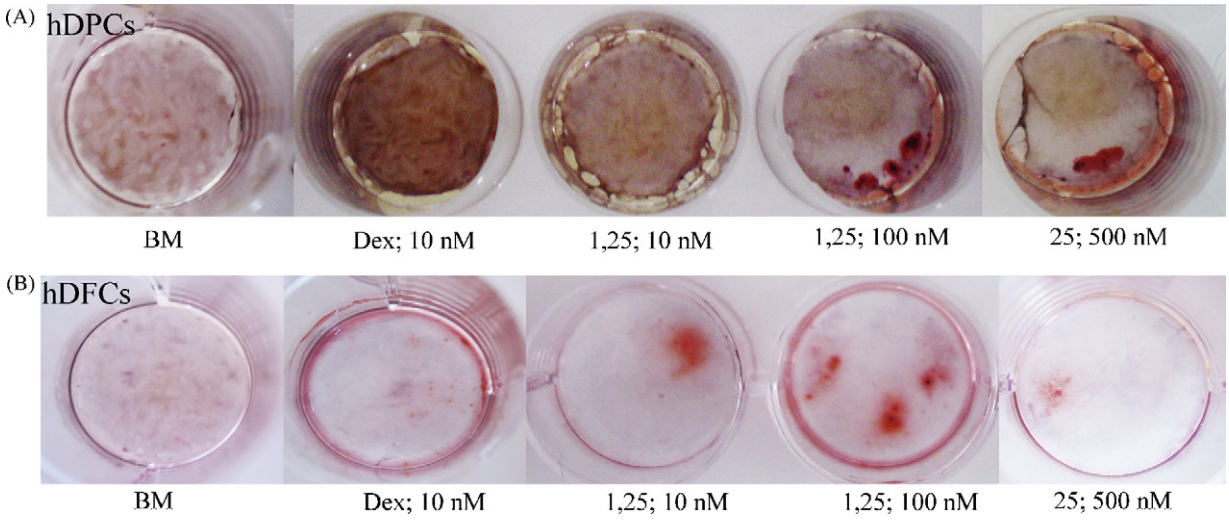

Fig. 4. Alizarin red staining of mineralized deposits after exposure to vitamin D_3 metabolites and Dex. Human DPCs (A) and hDFCs (B) were cultured in (1,25) 10 and 100 nM, (25) 500 nM and (Dex) 10 nM with osteogenic supplements (OS; L-ascorbic acid-2-phosphate + β-glycerophosphate) for 3 weeks. Data are representative of one cell culture ($n = 3$).

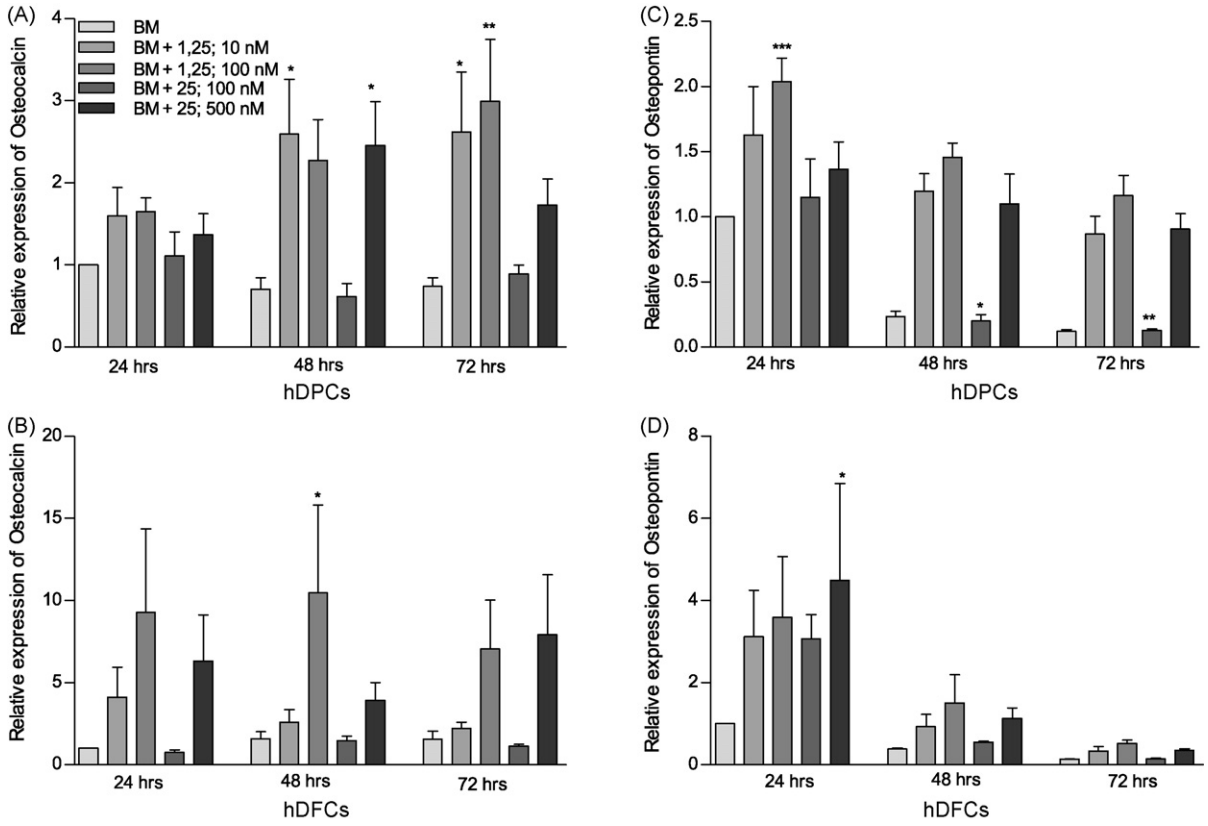

Fig. 5. Expression of bone markers were quantified by qRT-PCR. Relative mRNA expression of OC in hDPCs and hDFCs (A and B) and OPN in hDPCs and hDFCs (C and D) at 24, 48 and 72 h were analyzed. Data are normalized to housekeeping gene RPLP0. Results are reported as change in gene expression relative to untreated control (basic medium; BM) at 24 h time point. Columns represent mean ± SEM ($n = 3$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

tions of vitamin D_3 metabolites used except for $25OHD_3$ (100 nM) between 48 and 72 h time course in hDPCs (Fig. 5A), wherein, $1\alpha,25(OH)_2D_3$ (100 nM) alone significantly increased the expression of OC at 48 h time point in hDFCs (Fig. 5B). OPN expression was significantly upregulated by $1\alpha,25(OH)_2D_3$ (100 nM) in hDPCs and $25OHD_3$ (500 nM) in hDFCs at 24 h. Thereafter, downregulation in the expression was seen from 48 to 72 h in both hDPCs (Fig. 5C) and hDFCs (Fig. 5D).

3.4. Expression of VDR, CYP24 and $1\alpha,25(OH)_2D_3$ production

Vitamin D_3 regulating genes especially CYP24 expression was significantly upregulated by $1\alpha,25(OH)_2D_3$ (100 nM) and $25OHD_3$ (500 nM) but there was down regulation seen by $25OHD_3$ (100 nM) in hDPCs (Fig. 6A) and hDFCs (Fig. 6B). In addition, the expression of VDR was low at 24 h time point for both hDPCs and hDFCs. The VDR mRNA expression increased significantly following 48–72 h time point in the cells treated with $1\alpha,25(OH)_2D_3$ (10 and 100 nM) in both hDPCs (Fig. 6C) and hDFCs (Fig. 6D). The VDR mRNA expression was significantly upregulated by $25OHD_3$ (500 nM) concentration in hDPCs while expression was minimally regulated by $25OHD_3$ (100 nM) in both hDPCs and hDFCs. The activity of the CYP27B1 enzyme was confirmed by evaluating the conversion of $25OHD_3$ into $1\alpha,25(OH)_2D_3$ in hDPCs and hDFCs by enzyme immunoassay. $25OHD_3$ (500 nM) was converted into $1\alpha,25(OH)_2D_3$ at pM concentration by both hDPCs (Fig. 7A) and hDFCs (Fig. 7B). The conversion was significantly inhibited by cells cultured in the presence of the inhibitor. Subsequently, the mRNA expression of CYB27B1 was analyzed which was upregulated only by the higher concentration of $25OHD_3$ in both hDPCs (Fig. 7C) and hDFCs (Fig. 7D).

4. Discussion

Our study shows that osteoblast differentiation in hDPCs and hDFCs was stimulated by both $1\alpha,25(OH)_2D_3$ and $25OHD_3$. In addition, the current study provides evidence that $25OHD_3$ can be converted into $1\alpha,25(OH)_2D_3$ *in vitro* by hDPCs and hDFCs. Differentiation of MSCs such as those derived from adipose tissue, bone marrow tissue or from dental tissue into osteoblast like cells is induced *in vitro* by treating the cells with Dex, ascorbic acid and β-glycerophosphate as reported in many studies [19,30]. Interestingly, it has been reported that $1\alpha,25(OH)_2D_3$ may be superior to dexamethasone as an agent that induces osteogenic differentiation in human adipose derived cells [23,24,31]. Considering the previous reports it was reasonable to confirm the effects of vitamin D_3 metabolites such as $1\alpha,25(OH)_2D_3$ (10 and 100 nM) and $25OHD_3$ (100 and 500 nM) with or without the addition of osteogenic supplements (OS; L-ascorbic acid 2-phosphate and β-glycerophosphate) on proliferation and osteogenic differentiation of hDPCs and hDFCs. Osteogenic differentiation of MSCs *in vitro* is normally characterized by early expression of ALP activity, extracellular matrix mineralization and expression of osteoblasts associated genes.

Vitamin D_3 is also reported to function locally by binding with the VDR to inhibit proliferation of certain cell types such as osteoblasts and osteoclasts [32]. Our study revealed that, the addition of vitamin D_3 metabolites to hDPCs and hDFCs did not increase cell proliferation. Moreover, $1\alpha,25(OH)_2D_3$ (100 nM) inhibited cell proliferation in hDFCs. Our results are consistent with a recent study conducted on hMSCs, wherein, $1\alpha,25(OH)_2D_3$ inhibited cell proliferation of hMSCs [22]. These results also suggest that when

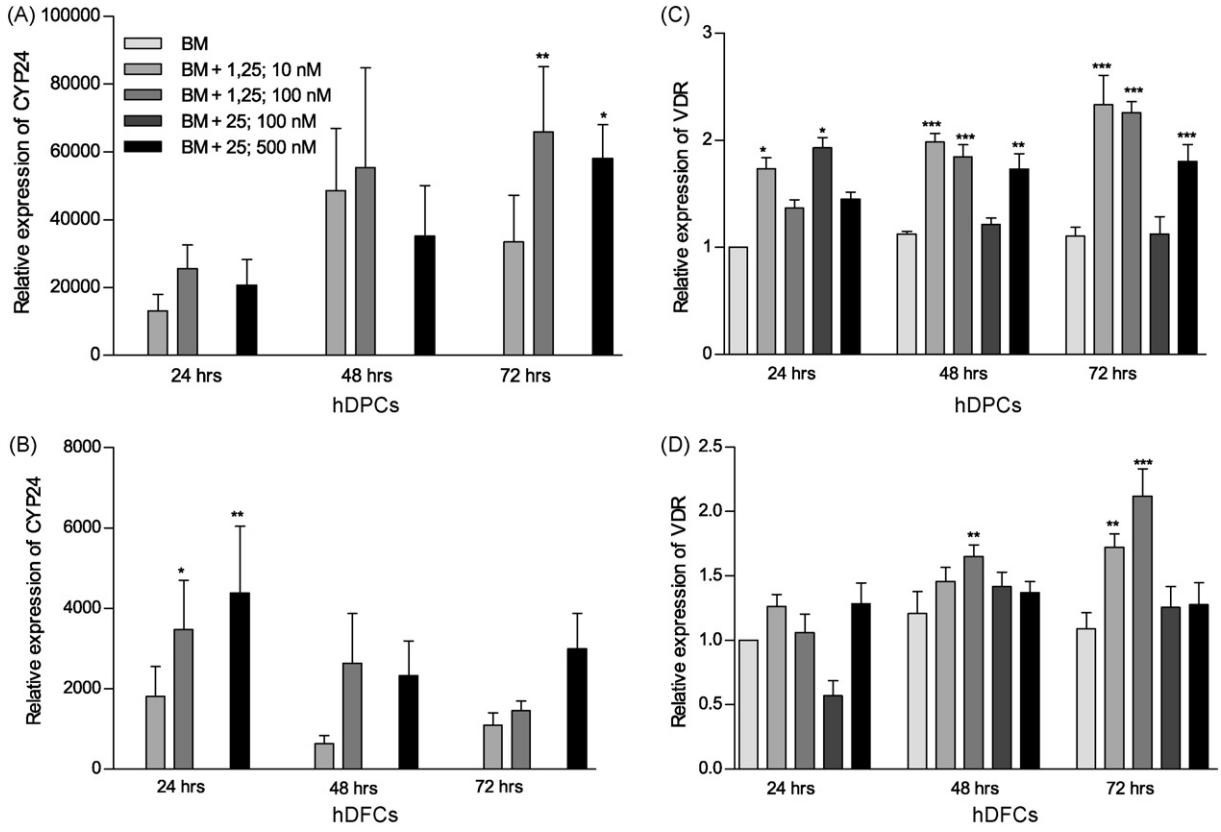

Fig. 6. Expression of vitamin D_3 regulating genes were quantified by qRT-PCR. Relative mRNA expression of CYP24 in hDPCs and hDFCs (A and B) and VDR in hDPCs and hDFCs (C and D) at 24, 48 and 72 h were analyzed. Data are normalized to housekeeping gene RPLP0. Results are reported as change in gene expression relative to untreated control (basic medium; BM) at 24 h time point. Columns represent mean ± SEM ($n = 3$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

cells are differentiating osteogenically, cell proliferation will be inevitably suppressed.

So far studies towards the direct effects of vitamin D_3 on osteogenic differentiation of enzymatically isolated hDPCs and hDFCs have been limited. To confirm osteogenic capacity of the hDPCs and hDFCs, ALP expression was studied, which is membrane bound enzyme and is an early marker of osteogenic differentiation [33]. It was reported that the addition of $1\alpha,25(OH)_2D_3$ metabolite to OS + Dex significantly increased Alp activity of hDPCs [34]. Here, we show that $1\alpha,25(OH)_2D_3$ (10 and 100 nM) and Dex in addition with OS increased the ALP activity in hDPCs. Additionally, for the first time we have shown that $25OHD_3$ (500 nM) also increased the ALP activity in hDPCs and hDFCs. In correlation with the ALP expression, the initiation of osteogenesis resulted in progression of mineralized matrix formation when cells were cultured in $1\alpha,25(OH)_2D_3$ (100 nM) and $25OHD_3$ (500 nM) in hDPCs. Furthermore, we observed difference in response to the treatments between patient samples in forming calcified nodules *in vitro*. The most likely reason for this could be associated with donor variability in response to various treatments. Whereas for hDFCs, previous reports suggest that, Dex + OS stimulated ALP activity and mineralization [19,35] when cells were cultured for 4 weeks [20]. However, we could not observe increase in ALP activity when cells were cultured with Dex for 2 weeks and weak response to mineralization was seen when hDFCs were cultured for 3 weeks, contrary to what is known. This discrepancy in our results can be explained by referring to the long term cultures made to observe increase in ALP activity and mineralization in the reported literature and variability between patient samples. Moreover, effect of the vitamin D_3 metabolites on ALP activity and mineralization of hDFCs has not been yet elucidated. Here we report that, hDFCs formed mineralized matrix when treated with vitamin D_3 metabolites in the presence of OS. Interestingly, vitamin D_3 metabolites had better effect on mineralization than Dex (10 nM) in combination with OS in hDPCs and hDFCs. These results highlight that vitamin D_3 could be used as an alternative to Dex for *in vitro* mineralization considering the potential catabolic effects of Dex when used *in vivo* [21].

The studies on osteoblastic cells *in vitro* have shown that $1,25(OH)_2D_3$ increases alkaline phosphatase activity [36] and stimulates the expression of some of the non-collagenous proteins of bone such as OC, which is a small protein found in abundance in bone, cementum, and dentin [36,37]. $1,25(OH)_2D_3$ has been observed to play an essential role in synthesizing OC in hDPCs and bone cells *in vitro* [37,38]. The correlation between decreased OC production and hypocalcified dentin formation due to vitamin D deficiency has also been reported in hDPCs [37]. Our results verify that OC expression was upregulated by $1\alpha,25(OH)_2D_3$ in hDFCs and hDPCs. The expression of other non-collagenous protein such as OPN, which promotes bone resorption and stimulation of bone deposition [39], is also regulated by $1,25(OH)_2D_3$ in osteoblast cells [40]. It has been shown in clonal rat dental pulp cells that OPN produced by pulp cells by action of $1\alpha,25(OH)_2D_3$ causes mineralization to form reparative dentin and pulp stones [41]. Our data shows that $1\alpha,25(OH)_2D_3$ (100 nM) upregulated expression of OPN in hDPCs at 24 h and following 72 h time course showed gradual down regulation. Moreover, the induction of OPN expression in hDFCs by $25OHD_3$ has not been reported previously.

From *in vivo* observation it is concluded that $1\alpha,25(OH)_2D_3$ is important in mineralization of dental tissues, as shown by the presence of VDR in human dental tissues such as ameloblasts and odontoblasts in mouse model [7,42]. We report here that addition of $1\alpha,25(OH)_2D_3$ (10 and 100 nM) upregulated VDR expression in hDPCs and hDFCs. Moreover, $25OHD_3$ has also been

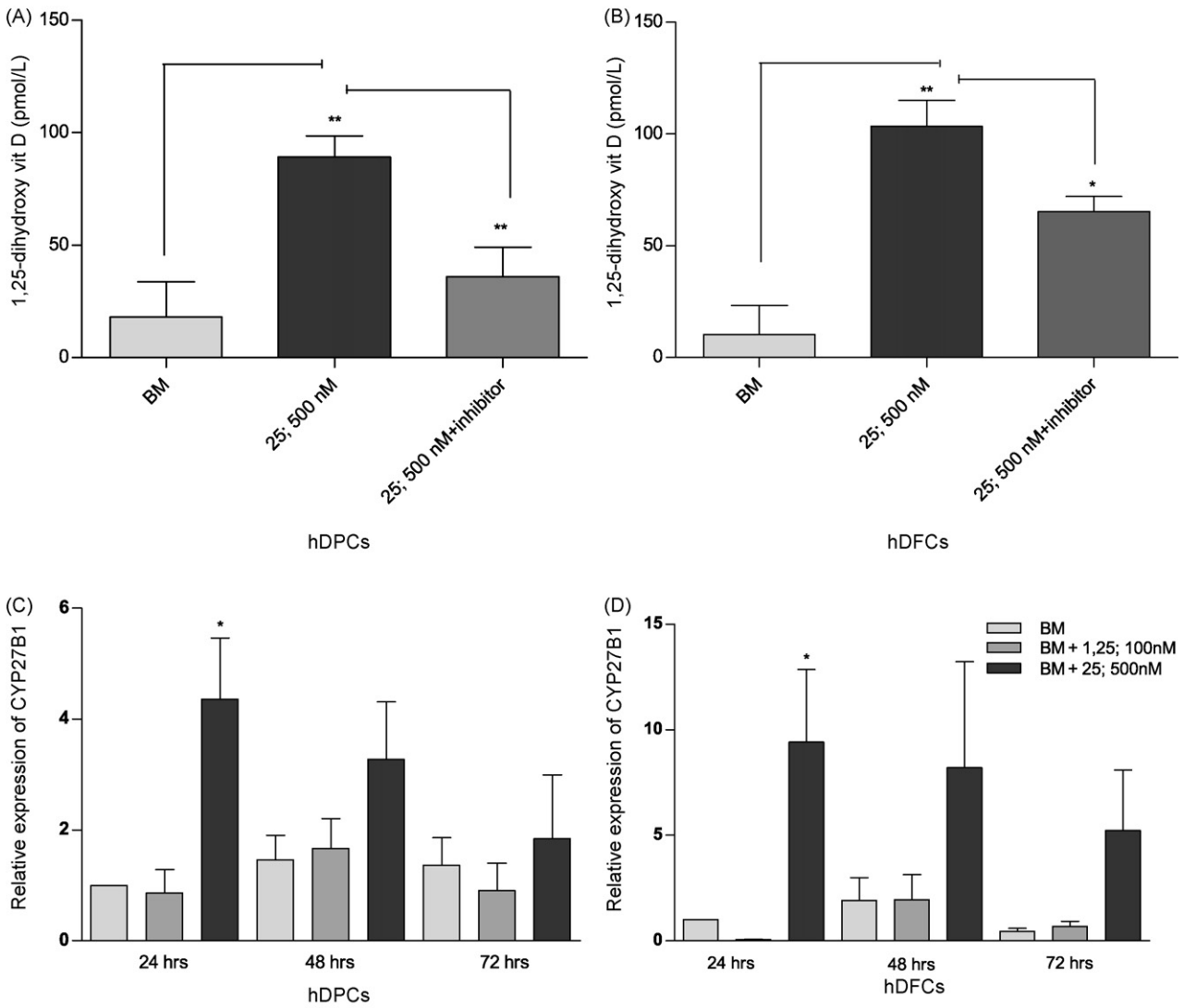

Fig. 7. Enzymatic activity and expression of CYP27B1 in hDFCs and hDPCs. Enzyme immunoassay showed the conversion of 25; 500 nM into $1\alpha,25(OH)_2D_3$ in (A) hDFCs and (B) hDPCs. The conversion was evaluated as $1\alpha,25(OH)_2D_3$ (pM) concentration. The results were considered significant when BM was compared to 25; 500 nM treated sample and 25; 500 nM treated sample was compared with 25; 500 + inhibitor. Relative mRNA expression of CYP27B1 in hDPCs and hDFCs (C and D) was analyzed. Results are reported as change in gene expression relative to untreated control (basic medium; BM) at 24 h time point. Columns represent mean ± SEM ($n = 3$); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

reported to show biological activity through VDR, but the response is 500–1000 fold lower than $1\alpha,25(OH)_2D_3$ [43]. In this study, higher concentration of $25OHD_3$ upregulated VDR expression in hDPCs. Furthermore, the metabolic conversion of $25OHD_3$ into $1\alpha,25(OH)_2D_3$ by hDPCs and hDFCs suggested that both of these cell types express functional CYP27B1 enzyme. Further evidence of the effect of vitamin D_3 metabolites on hDPCs and hDFCs, can be described by the upregulation of CYP24, which metabolizes vitamin D_3 and thereby inactivates the conversion $25OHD_3$ into $1\alpha,25(OH)_2D_3$ [44]. The present study shows that higher concentrations of $1\alpha,25(OH)_2D_3$ (100 nM) and $25OHD_3$ (500 nM) upregulated CYP24 gene expression in hDPCs and hDFCs. These results confirm the regulation of synthesis and inactivation of $1\alpha,25(OH)_2D_3$ in hDPCs and hDFCs is similar to as shown in cells derived from bone marrow and bone cells [45,46].

A recent study reported that vitamin D_3 deficiency was treated with $25OHD_3$ which resulted in effective bone turnover in haemodialysis patients [3]. It is worthwhile to note that our studies were performed in cultures treated with $25OHD_3$ (500 nM), which resulted in osteogenic differentiation *in vitro*. Considering the report and our results we can conclude that $25OHD_3$ metabolite could be considered as a potential clinical osteogenic inducer for bone tissue engineering. This research also shows the potential of hDPCs and hDFCs as an alternative to other MSCs, obtained from extracted human third molars (i.e. wisdom teeth) with no tissue site morbidity, often discarded tissue that may be valuable source of cells for future research.

In summary, vitamin D_3 metabolites regulated the expression of vitamin D_3 regulating and bone marker genes at mRNA level in the hDPC and hDFC cultures, while increase in ALP enzyme activity was mediated by the presence of L-ascorbic acid-2-phosphate or β-glycerophosphate. This research also indicates the essentiality of the synergists to achieve osteoblastic differentiation in addition to vitamin D_3 and thereby assisting mineralization *in vitro*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2010.08.001.

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