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Original Article

ENHANCING HUMAN OSTEOBLAST TO OSTEOCYTE-LIKE CELL DIFFERENTIATION USING 25-HYDROXYVITAMIN D3 AND ALL-TRANS RETINOIC ACID COMBINATION

A. Munir[1], A.E. Agger[1], A. Samara[1], J.E. Reseland[1,§] and L.B. Solberg[2,*,§]

[1]Department of Biomaterials, Institute of Clinical Dentistry, University of Oslo, 0317 Oslo, Norway
[2]Division of Orthopaedic Surgery, Oslo University Hospital, N-0424 Oslo, Norway
[§]These authors contributed equally.

Abstract

Background: The individual effects of the 25-hydroxyvitamin D3 (25(OH)D3) and all-trans retinoic acid (ATRA) on osteogenesis have been studied in two-dimensional (2D) *in vitro* models, but the combined effect of these vitamins remains largely unexplored. Therefore, to address this knowledge gap we evaluated the effect of different concentrations of 25(OH)D3 and ATRA, individually and in combination, on differentiation of primary human osteoblasts (hOBs) over a 21-day period. **Methods**: The growth of hOBs, alkaline phosphatase (*ALP*) activity (cell bound and secreted), and the expression of bone-related markers were comprehensively evaluated at both gene expression level using real-time polymerase chain reaction (RT-PCR) and the proteins secretion through multiplex immunoassays. **Results**: Both 25(OH)D3 and ATRA enhanced hOBs differentiation. However, the combination of 10^{-6} M 25(OH)D3 and 10^{-5} M ATRA demonstrated the most pronounced effect, characterized by reduction in cell growth and enhancement in differentiation. This was further validated by elevated relative expression of osteoblast and osteocyte markers at both mRNA level (collagen type I alpha 1 (*COL1A1*), podoplanin (*E11*), and dentin matrix acidic phosphoprotein 1 (*DMP1*), and at protein level (osteopontin (OPN), osteocalcin (*OCN*), sclerostin (*SOST*), and fibroblast growth factor 23 (*FGF23*)). Additionally, 25(OH)D3 and ATRA were found to regulate the expression of key receptors involved in osteoblasts differentiation, including retinoic acid receptor alpha (*RARα*), retinoid X receptor beta (*RXRβ*), and vitamin D receptor (*VDR*). **Conclusions**: This study demonstrates that a combination of 10^{-6} M 25(OH)D3 and 10^{-5} M ATRA effectively enhanced the differentiation of hOBs to osteocyte-like cells *in vitro*.

Keywords: Osteocytes, osteoblasts, atra, 25(OH)D3, *in vitro*.

***Address for correspondence**: L.B. Solberg, Division of Orthopaedic Surgery, Oslo University Hospital, N-0424 Oslo, Norway. Email: l.b.solberg@gmail.com.

Introduction

It is well-established that hepatic hydroxylation converts vitamin D to 25-hydroxyvitamin D3 (25(OH)D3), which is then metabolized in the kidney to its biologically active form 1,25-dihydroxyvitamin D3 (1,25$(OH)_2$D3) [1]. *In vitro* studies demonstrate that 1,25$(OH)_2$D3 decreases osteoblast proliferation, enhances the expression of osteoblast differentiation markers like osteocalcin [2], increases alkaline phosphatase (*ALP*) activity and promotes mineralization [3–5]. At each stage, 1,25$(OH)_2$D3, orchestrates its action through the vitamin D receptor (*VDR*) [6]. Upon binding of 1,25$(OH)_2$D3 to *VDR*, a heterodimer with retinoid X receptor (*RXR*) is formed, which initiates downstream targeted gene regulation [7].

25(OH)D3 has been shown to influence primary osteoblast function independently [2,8], but the underlying mechanisms are less well documented. 25(OH)D3 has been found to reduce the proliferation of osteoblasts and elevate the expression of bone markers in human mesenchymal cells [9], primary human osteoblasts (hOBs) [2,10], and a murine pre-osteoblast cell line [11]. Additionally, Howard *et al*. [12] reported that human bone cells can metabolize 25(OH)D3 to 1,25$(OH)_2$D3 and 24R,25$(OH)_2$D3. Similarly, recent findings by van der Meijden *et al*. [2] indicate that 25(OH)D3 could be metabolized to its active metabolites (1,25$(OH)_2$D3 and 24R,25$(OH)_2$D3) by osteoblasts, and directly bind to *VDR* receptors.

All-trans retinoic acid (ATRA) is the most abundant physiologically active metabolite of vitamin A, influencing various cellular functions like proliferation, differentiation, and apoptosis [13]. However, its role in osteogenic differentiation remains controversial and is not fully understood. Some studies have reported that ATRA induces osteogenic differentiation [14] while others have reported opposite results [15]. This diversity in the action of ATRA could be attributed to different concentrations of vitamin A. Existing studies present conflicting evidence of ATRA action, with ATRA at low-concentration inhibiting osteoblastic proliferation and differentiation [15–17] and down-regulating osteogenic markers (*ALP* and osteocalcin (*OCN*)) [15,16,18], whereas ATRA at higher concentrations can reduce the cell number and induce osteogenic differentiation markers (*OCN* and osteopontin (OPN)) [14]. Furthermore, ATRA's dual role in both promoting differentiation of osteoblast to osteocyte-like cells by upregulation of osteocyte specific markers (dentin matrix acidic phosphoprotein 1 (*DMP1*) and sclerostin (*SOST*)) [19,20], and as an inhibitor of mineralization [13] accentuates its complexity.

Several studies have shown individual effects of both vitamin A and vitamin D on the differentiation of osteoblasts [2–4,8,9,14,17], but there is not much information on the combined effect of these two vitamins. To the best of our knowledge, only a handful of studies have focused on the dual effect of these two vitamins. Retinoic acid (RA), calcitriol, and ascorbic acid (AA) were shown to induce osteoblast differentiation [21], whereas other studies reported antagonistic effects of vitamin A on vitamin D [22–28].

Previously, researchers have aimed to induce osteogenesis in two-dimensional (2D) and three-dimensional (3D) models in which cells like osteoblasts, mesenchymal cells, and osteoblast-like cell lines were routinely exposed to soluble factors and biomaterials to promote differentiation [29]. However, a robust and reproducible *in vitro* model to study the differentiation of hOBs to osteocyte-like cells, in the presence of essential bioactive molecules, remains lacking. Developing such a model is essential for advancing our understanding of bone remodeling.

We have previously shown that a combination of 25(OH)D3 and ATRA can induce the differentiation of hOBs to osteocyte-like cells in a 3D rotary culture setting [30]. Both osteoblasts and osteocytes play key roles in maintaining bone homeostasis and metabolism, making reliable models crucial to investigate these processes to offer insights into the molecular mechanisms driving this transition. Therefore, in this study we aimed to compare the effect of various dosages of vitamins, alone and in combination, on hOBs growth and differentiation for up to 21 days, along with the identification of the expression of transcription factors (*RAR*, *RXR* and *VDR*).

Materials and Methods

Cell Culture

Commercially available hOBs were obtained from Lonza (Walkerville, MD, USA). The cells were cultured in osteoblast basal medium (OBM; CC-3208; Lonza, Walkerville, MD, USA) supplemented with osteoblast growth medium (CC-4193; Lonza, Walkerville, MD, USA) containing 10 % fetal bovine serum (FBS), 0.1 % gentamicin sulphate/amphotericin B (GA) and 0.1 % ascorbic acid (AA). The cells were routinely cultured in a humidified incubator (37 °C, and 5 % CO_2) and supplemented with fresh medium every third day. As per internal standard operating procedure (SOP), cell lysates and media are routinely outsourced for mycoplasma testing (https://eurofinsgenomics.eu/) and was negative.

Cells were cultured in OBM with or without the following treatments: osteogenic differentiation medium (OM) containing 200 nM hydrocortisone (HC, SLBW3343) and 10 mM β-glycerophosphate (βGP, SLCD3944) (both from Sigma-Aldrich, St Louis, MO, USA), OM supplemented with all-trans-retinoic-acid (ATRA) (R26255; SLCG5473, Sigma-Aldrich, St Louis, MO, USA) at concentrations ranging from 0.5 to 4 $\times$ 10^{-5} M; OM supplemented with 25(OH)D3 at concentrations of 10^{-8} M and 10^{-6} M (Calcifediol CRS; European Pharacopoeia Reference Standard, EDQM, Strasbourg, France), either alone or in combination. Osteoblasts in basal medium (GM) and in osteogenic differentiation medium (OM) were used as controls. Cell culture medium, with and without stimulants, was replaced twice a week. Both cells and cell culture medium were harvested at the respective time points indicated in the specific procedures below.

hOBs Growth

hOBs seeded at the density of 5 $\times$ 10^3 cells/well in a 96-well plate were incubated at 37 °C, and 5 % CO_2 overnight to facilitate adherence and normal growth, and then cells were supplemented with fresh medium with or without respective treatment. Cells were harvested after 1, 3, 5, and 7 days of incubation, and the growth was assessed using AlamarBlue Cell Viability Reagent (DAL1100; 2346207; Invitrogen; Thermo Fisher Scientific, IL, USA). Briefly, 4 hours prior to harvest, the cell medium was replaced with freshly prepared AlamarBlue (1:10 ratio) in medium with or without respective treatments. An aliquot of 100 μl of supernatant was transferred to a 96-well plate, and fluorescence was measured at 530 nm excitation and 590 nm emission wavelength in a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Alkaline Phosphatase Enzyme Activity Assay

Both cell bound and secreted *ALP* activity were measured. Briefly, hOBs were seeded at a density of 5 × 10^4 cells/well in 12-well plates and incubated at 37 °C, and 5 % CO_2. Confluent cells were supplemented with fresh medium with or without the respective treatments. Cell medium was collected and stored at –20 °C after 3, 7, 14, and 21 days, and cells on day 7 and 14 were washed with phosphate buffered saline (PBS), fixed in 4 % paraformaldehyde (PFA), washed, and kept in PBS at 4 °C before further use.

Cells were washed with washing buffer (0.05 % Tween 20 in PBS) and incubated in BCIP/NBT solution (SIGMAFAST™ BCIPR/NBT tablet; Sigma-Aldrich) for 10 minutes in the dark, at room temperature. *ALP* stained areas were imaged using a Leica DM RBE microscope (Leica, Wetzlar, Germany) with a digital camera. Image J software (ImageJ 1.54f, NIH, Bethesda, MD, USA; https://imagej.nih.gov/ij/) was used to quantify the images.

Cell culture media were concentrated ten-fold using VWR centrifugal filters (FG7257, modified PES 3K; VWR, Darmstadt, Germany) with a 3 KDa cut-off. 25 μl of concentrated medium from each sample was incubated with 100 μl pNPP in the dark at room temperature for 30 minutes on a shaker. The reaction was stopped by adding 50 μl of 3M NaOH to each well. The absorbance of pNPP from *ALP* enzymatic activity was measured immediately at 405 nm using an ELISA plate reader (ELX800; BioTek, Winooski, VT, USA). A standard curve based on the calf intestinal *ALP* (5C04L75850, CIAP Promega, Madison, WI, USA) was used to quantify the *ALP* enzymatic activity readouts.

Quantification of Bone Related Proteins Secreted in the Cell Culture Medium

hOBs were seeded at a density of 5 × 10^4 cells/well in 12-well plates and incubated at 37 °C, and 5 % CO_2 until they reached confluence. The cells were then supplemented with fresh medium with or without respective treatment. Cell medium was collected and stored at –20 °C after 3, 7, 14, and 21 days, and concentrated ten-fold using VWR centrifugal filters (modified PES 3K; VWR, Germany) with a 3 kDa cut-off. Following the manufacturer's protocols the protein expression levels of the bone markers; osteopontin (OPN), osteocalcin (*OCN*), sclerostin (*SOST*), fibroblast growth factor (*FGF23*), and leptin (LEP), were determined using Milliplex Human Bone Panel (EMD Millipore, Billerica, MA, USA). Multianalyte profiling was performed using the Luminex 200 system (Luminex Corporation, Austin, TX, USA) employing xMAP technology. Acquired fluorescence data were analysed using the Luminex xPONENT 3.1 software. Concentrations of bone markers in the samples were calculated based on a logistic 5P weighted standard curve.

Gene Expression of Bone Markers

The mRNA expression of selected osteoblast and osteocyte markers (listed in Table 1) and the receptors involved in downstream pathways (Retinoic acid receptor alpha (*RAR*α), retinoic acid receptor beta (*RAR*β), retinoic acid receptor gamma (*RAR*γ), retinoid X receptor alpha (*RXR*α), retinoid X receptor beta (*RXR*β), retinoid X receptor gamma (RXRγ) and *VDR*) were evaluated by quantitative polymerase chain reaction (qPCR). hOB cells were seeded at a density of 5 × 10^4 cells/well in 12-well plates. Upon reaching confluency, cells were supplemented with fresh medium with or without respective treatment. On days 7 and 21, mRNA was isolated using Dynabeads™ mRNA DIRECT™ Purification Kit (01226117, Thermo Fisher Scientific, Carlsbad, CA, USA) following the manufacturer's protocol. Briefly, cells were lysed using lysis buffer, and mRNA was isolated using magnetic beads and suspended in 10 mM tris-hydrochloride (tris-HCL). The levels of extracted mRNA were quantified using a nano-drop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE, USA, with software version 3.3.1.) and stored at –80°C before further use.

cDNA was synthesized from isolated mRNA using Revert Aid First Strand cDNA Synthesis Kit (K1622, Thermo Fisher Scientific; Waltham, MA, USA) according to the manufacturer's instructions. Real-time polymerase chain reaction (RT-PCR) was carried out using IQ SYBR Green Supermix (Bio Rad; Laboratories, Hercules, CA, USA) in a total volume of 5 μl in CFX96 Touch Real-Time PCR Detection System (Bio Rad Laboratories, Hercules, CA, USA). Relative mRNA levels of genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and presented as $2^{-\Delta\Delta Ct}$. The sequences of primers used, and their respective annealing temperatures are listed in Table 1.

Statistical Analysis

Statistical analysis was performed using SigmaPlot software (version 14.0, Systat Software, San Jose, CA, USA). Data from cell growth, *ALP* activity, Luminex analysis, and qPCR ($\Delta\Delta Ct$ values) were compared to the untreated control using unpaired Student's *t*-test or Whitney Rank Sum test depending on their normal distribution. A probability of $\leq$0.05 was considered significant.

Results

Effect of 25(OH)D3 and ATRA on hOBs Growth and Differentiation

Based on the effect of various concentrations of ATRA tested on hOBs **(Supplementary Fig. S1A)**, 10^{-5} M ATRA (10^{-5} ATRA) was chosen for the subsequent analysis, as it reduced the hOBs growth. During the course of 7 days, administration of the combination of 10^{-8} M 25(OH)D3 (10^{-8} 25(OH)D3) and 10^{-5} ATRA induced a slight enhancement of the hOBs growth (5 % and 13 %

Table 1. Primer sequences.

Protein	Probe name		Sequence	Annealing temp. (°C)
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	F	CTCTGCTCCTCCTGTTCGAC	60
		R	ACGACCAAATCCGTTGACTC	
Runt-related transcription factor 2	*RUNX2*	F	GCGCTAGATGACACCCTCTC	60
		R	CGGGGTGACCTTATGAGAAA	
Collagen type I alpha 1	*COL1A1*	F	CCAAATCCG-ATGTTTCTGCT	60
		R	CATCTCCCCTTCGTTTTTGA	
Alkaline phosphatase	*ALPL*	F	AGACGCGCCTGGTAGTTGT	60
		R	GACAAGAAGCCCTTCACTGC	
Osteoprotegerin	*OPG*	F	GTGTCTTGGTCGCCATTTTT	60
		R	TGGGAGCAGAAGACATTGAA	
Osteocalcin	*BGLAP*	F	GCTTCACCCTCGAAATGGTA	60
		R	GCAAGTAGCGCCAATCTAGG	
Podoplanin	*E11*	F	GCTGTGTGGCCTCAAGATGA	60
		R	TCCCAGGCCAGGAGTCTTTT	
Dentin Matrix Acidic Phosphoprotein 1	*DMP1*	F	AAGCAGACAGCGAATCCAGT	60
		R	CTGCTGAGCTGCTGTGAGAC	
Sclerostin	*SOST*	F	CTGGTACACACAGCCTTCCG	60
		R	TTGTTGTTCTCCAGCTCCGG	
Fibroblast growth factor 23	*FGF23*	F	TGGAGGAATTGGTTGAGGGC	60
		R	TTTGCCAAAACAGCTCTCGC	
Matrix extracellular phosphoglycoprotein	*MEPE*	F	TCCCAAGCCCAGAAAAGTCC	60
		R	ACCGCTGCCTTCAAAATCAC	
Phosphate regulating endopeptidase homolog X-linked	*PHEX*	F	TGGAGGAATTGGTTGAGGGC	60
		R	TTTGCCAAAACAGCTCTCGC	
Retinoic acid receptor alpha	*RARα*	F	CAGAGCAGCAGTTCTGAAGAGATA	70
		R	GACACGTGTACACCATGTTCTTCT	
Retinoic acid receptor beta	*RARβ*	F	GTCACCGAGATAAGAACTGTGTTA	60
		R	ACTCAGCTGTCATTTCATAGCTCTC	
Retinoic acid receptor gamma	*RARγ*	F	CTGTATCATCAACAAGGTGACCA	60
		R	TGGTGATGAGCTCTTCTAACTGAG	
Retinoid X receptor alpha	*RXRα*	F	CTCTTTATGGATCTGTCATCCTCTC	60
		R	TGCTTCGTGTAAGCAAGTACATAAG	
Retinoid X receptor beta	*RXRβ*	F	TCTTAGTCAACCTGGGAAAGTACAG	60
		R	CCAGAGTCTCTTTTTACACTTCACC	
Retinoid X receptor gamma	RXRγ	F	GATCTAGAGGCAGATTCCTGACTAA	60
		R	CATGTTTACTCGTCAGTTCATGTTC	
Vitamin D receptor	*VDR*	F	AGGAGGCCTTGAAGGACAGT	60
		R	CTGGCAGAAGTCGGAGTAGG	

at day 3 and 5, respectively), in comparison to the combination of 10^{-6} M 25(OH)D3 (10^{-6} 25(OH)D3) and 10^{-5} ATRA which reduced the growth (8 % and 24 % at day 3 and 5, respectively), however, the effects of the combined treatments failed to be statistically significantly different from control (Fig. 1). It was also observed that administration of 10^{-8} 25(OH)D3 alone increased, while 10^{-6} 25(OH)D3 alone reduced hOBs growth over the period of 7 days **(Supplementary Fig. S2A).**

Effect of 25(OH)D3 and ATRA on the Expression of ALP

All treatment groups exhibited an increase in cell bound *ALP* activity on days 7 and 14 compared to their respective controls (Fig. 2**A**). 10^{-6} 25(OH)D3 + 10^{-5} ATRA enhanced the cell bound *ALP* activity (78 % and 340 % on day 7 and day 14, respectively) compared to controls (Fig. 2**B**). The *ALP* activity in the culture medium was, however, not influenced by the vitamins at days 7–21 (Fig. 2**C**; **Supplementary Fig. S2D**). These findings suggest that the 10^{-5} M ATRA and 10^{-6} M 25(OH)D3 combined treatment significantly increased *ALP* activity within the cells (Fig. 2**A,B**), indicating enhanced osteoblast differentiation.

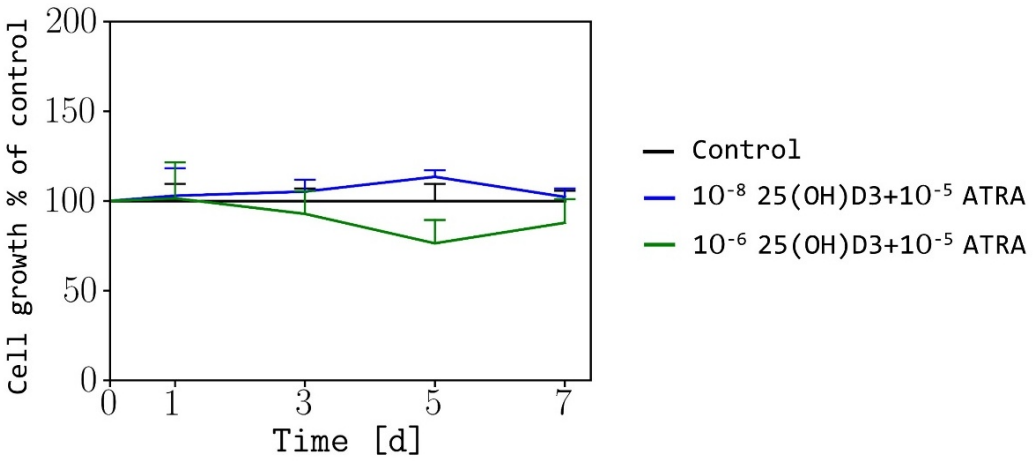

Fig. 1. Effect of combination of 25(OH)D3 and ATRA on growth of primary human osteoblasts (hOBs) at day 1, 3, 5, and 7. 10^{-5} M ATRA and 10^{-8} M 25(OH)D3 slightly enhanced hOBs growth, while the combination with 10^{-6} M 25(OH)D3 reduced growth over 7 days. However, these differences were not statistically significant. The data was calculated as % of control and presented as mean ± SD, n = 3. 25(OH)D3, 25-hydroxyvitamin D3; ATRA, all-trans retinoic acid.

The effects of the individually administrated vitamines on *ALP* activity (both as cell bound and in culture medium) are shown in **Supplementary Fig. S1B–D and Fig. S2B–D**.

Effects of 25(OH)D3 and ATRA on Bone Markers Secretion in the Cell Medium

The combination of 10^{-6} 25(OH)D3 and 10^{-5} ATRA resulted in elevated levels of secreted OPN (Fig. 3**A**) and *FGF23* (Fig. 3**D**) in the cell culture medium over 21 days. The levels of *OCN* (Fig. 3**B**) were significantly higher in the 10^{-6} 25(OH)D3 + 10^{-5} ATRA group at all time points compared to the 10^{-8} 25(OH)D3 + 10^{-5} ATRA group and control.

The combination of 10^{-6} 25(OH)D3 + 10^{-5} ATRA also increased the levels of sclerostin (Fig. 3**C**) from days 7 to 21, whereas the levels of leptin were almost abolished in the 10^{-6} 25(OH)D3 + 10^{-5} ATRA group compared to the 10^{-8} 25(OH)D3 + 10^{-5} ATRA group and control (Fig. 3**E**).

A similar but less pronounced effect on the levels of OPN, *OCN*, sclerostin, *FGF23* and leptin were observed by 10^{-6} 25(OH)D3 and 10^{-8} 25(OH)D3 **(Supplementary Fig. S3)**, mirroring the trend of 10^{-6} 25(OH)D3 + 10^{-5} ATRA and 10^{-8} 25(OH)D3 + 10^{-5} ATRA, respectively. Notably, the levels of osteoblast (OPN and *OCN*) and osteocyte (*SOST* and *FGF23*) markers remained low after treatment with 10^{-5} ATRA alone **(Supplementary Fig. S3)**. Effect of various concentrations of ATRA, individually, on expression of osteoblast and osteocyte markers is shown in **Supplementary Fig. S1E-I**.

These results suggest that the combination of 10^{-6} 25(OH)D3 and 10^{-5} ATRA has a pronounced effect on promoting osteogenic activity, as evidenced by elevated levels of key bone markers like OPN, *OCN*, sclerostin, and *FGF23* secreted in the cell media.

Effect of 25(OH)D3 and ATRA on the MRNA Expression of Bone Markers

After 21 days of incubation, the combination of 10^{-6} 25(OH)D3 + 10^{-5} ATRA induced a 1.9 fold ($p < 0.001$) increase in mRNA expression of runt-related transcription factor 2 (*RUNX2*) (Fig. 4**A**), a 9.6 fold ($p < 0.001$) increase in Collagen type I alpha 1 (*COL1A1*) (Fig. 4**B**), a 2.6 fold ($p < 0.001$) increase in osteoprotegerin (*OPG*) (Fig. 4**D**), a 3.7 fold ($p < 0.05$) increase in podoplanin (*E11*) (Fig. 4**F**), and 1.4 fold ($p < 0.05$) increase in phosphate regulating endopeptidase homolog X-linked (*PHEX*) (Fig. 4**K**) compared to untreated controls. Whereas 10^{-6} 25(OH)D3 + 10^{-5} ATRA increased mRNA expression of *ALP* (Fig. 4**C**) only at day 7.

The mRNA expression of *BGLAP* (Fig. 4**E**), *DMP1* (Fig. 4**G**), *SOST* (Fig. 4**H**), *FGF23* (Fig. 4**I**), and matrix extracellular phosphoglycoprotein (*MEPE*) (Fig. 4**J**) were not significantly different at either time point tested, compared to the controls.

10^{-8} 25(OH)D3 + 10^{-5} ATRA significantly increased mRNA expression of *RUNX2* (Fig. 4**A**) ($p < 0.01$), *COL1A1* (Fig. 4**B**) ($p < 0.05$), *OPG* (Fig. 4**D**) ($p < 0.01$), and significantly decreased *DMP1* mRNA expression (Fig. 4**G**) ($p < 0.01$) after 21 days of hOBs culture. The effects of the individually administrated vitamins are shown in **Supplementary Fig. S4**.

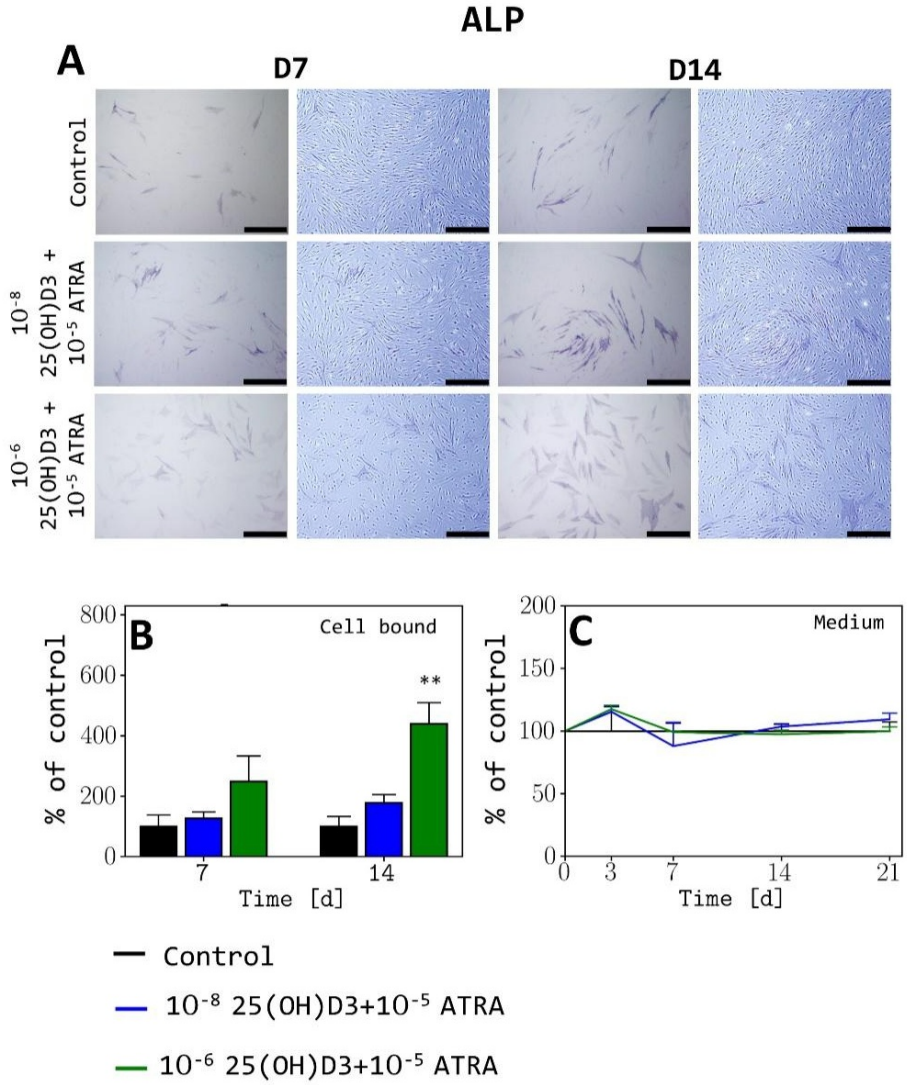

Fig. 2. Effect of combination of 25(OH)D3 and ATRA on *ALP* activity. (**A**) The cell bound *ALP* activity in hOBs at day 7 and day 14. (**B**) The quantitative expression of cell bound *ALP* activity in (**A**). (**C**) The *ALP* in medium at day 3, 7, 14, and 21. Scale bar = 500 μm. The data were calculated as % of control and presented as mean ± SD, n = 3. Significance $^{**}p \leq 0.01$. *ALP*, alkaline phosphatase.

Effect of 25(OH)D3 and ATRA on the Expression of ATRA and 25(OH)D3 receptors

The combination of 10^{-6} 25(OH)D3 + 10^{-5} ATRA significantly increased the mRNA expression of *RARα* (Fig. 5**A**), *RXRβ* (Fig. 5**E**), and *VDR* (Fig. 5**F**) by 2.2 fold ($p < 0.001$), 1.7 fold ($p < 0.01$) and 6.9 fold ($p < 0.01$), respectively, when compared to control after 21 days. *RARβ* (Fig. 5**B**), *RARγ* (Fig. 5**C**) mRNA expression remained lower, and *RXRα* (Fig. 5**D**) mRNA expression remained unchanged in 10^{-6} 25(OH)D3 + 10^{-5} ATRA group compared to the control after 21 days. RXRγ was not detectable in any of the groups (data not shown). The effects of the individually administrated vitamins are shown in **Supplementary Fig. S5**.

The results suggest that the combination of 25(OH)D3 and ATRA selectively enhances the expression of *RARα*, *RXRβ*, and *VDR* receptors, likely because they have a functional role in osteoblast differentiation in this setting.

Discussion

Building on our previous research that demonstrated the ability of a combination of 25(OH)D3 and ATRA to induce the differentiation of primary human osteoblasts into osteocyte-like cells within a 3D rotary culture environment, these 2D culture experiments confirmed that stimulating of human osteoblast (hOBs) with a combination of 10^{-6} 25(OH)D3 and 10^{-5} ATRA induces differentiation into osteocyte-like cells in a time-dependent manner. The novel findings are substantiated by a significant re-

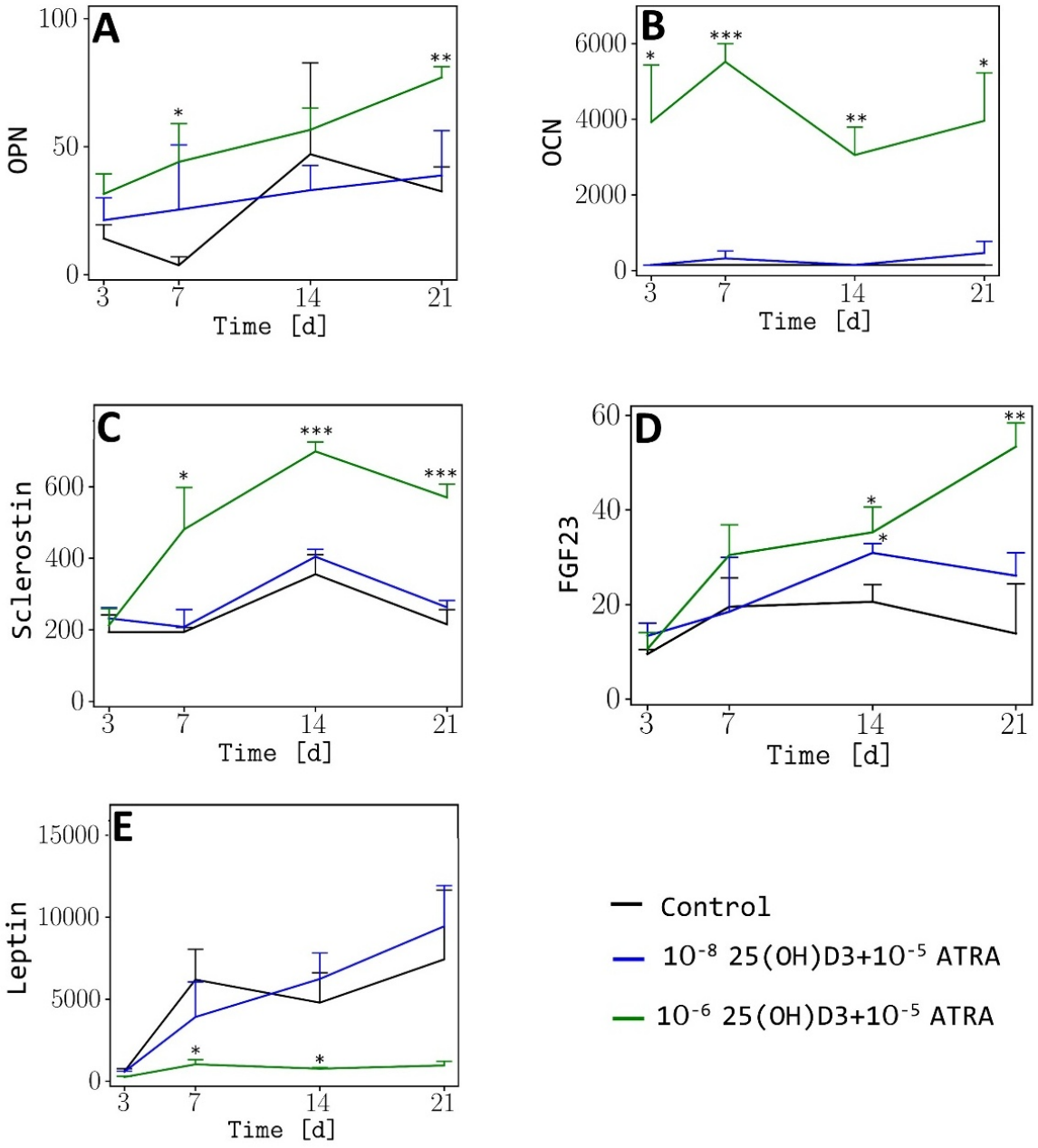

Fig. 3. Protein levels of osteoblast and osteocyte marker in cell culture medium. Graphs for (**A**) OPN, (**B**) *OCN*, (**C**) sclerostin, (**D**) *FGF23*, and (**E**) leptin show the concentration (pg/mL) of secreted proteins in the medium. Values are presented as the mean ± SD, n = 3. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$. OPN, osteopontin; *OCN*, osteocalcin; *FGF23*, fibroblast growth factor 23.

duction of cell growth, as well as an increase in the secretion and expression of osteoblast markers (alkaline phosphatase) *ALPL*, collagen type I (*COL1*), OPN, and *OCN*), and osteocyte-specific markers such as *E11*, *DMP1*, sclerostin, and *FGF23*.

The study addressed the literature gap regarding the combined effect of 25(OH)D3 and ATRA, and the molecular mechanisms guiding *in vitro* differentiation in human osteoblasts. 25(OH)D3 is a stable metabolite with a high cellular uptake [10–12], and has also been demonstrated to metabolize 25(OH)D3 into 1,25$(OH)_2$D3 in osteoblasts [10,12,31]. Studies further demonstrated that 25(OH)D3 can induce osteogenic differentiation at various concentrations such as 10^{-7}–10^{-5} M [2,10,32,33]. Serum levels of 25(OH)D3 above 5×10^{-7} M were documented to be safe but had no further effect on bone mineralization [34,35].

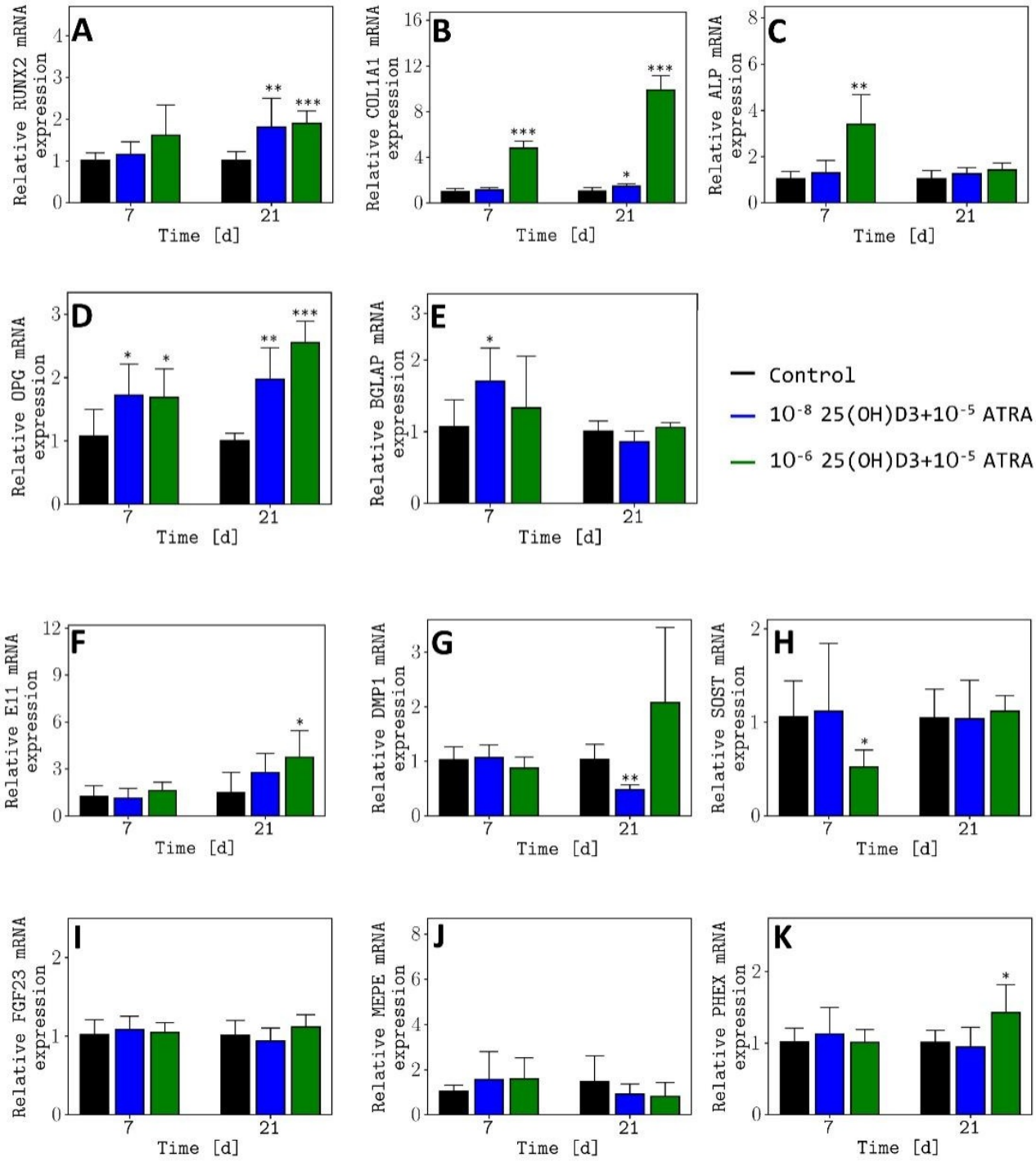

Fig. 4. Relative expression of osteoblast markers. *RUNX2* (A), *COL1A1* (B), *ALP* (C), *OPG* (D) and *BGLAP* (E) and osteocyte markers: *E11* (F), *DMP1* (G), *SOST* (H), *FGF23* (I), *MEPE* (J) and *PHEX* (K) in hOBs cultured at 7 and 21 days with a combination of 25(OH)D3 and ATRA. mRNA expression was normalized to *GAPDH* and is presented as relative to control. Values are presented as the mean ± SD, n = 3. Significance $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$. *COL1A1*, collagen type I alpha 1; *E11*, podoplanin; *DMP1*, dentin matrix acidic phosphoprotein 1; *SOST*, sclerostin; *PHEX*, phosphate regulating endopeptidase homolog X-linked; *RUNX2*, runt-related transcription factor 2; *MEPE*, matrix extracellular phosphoglycoprotein; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

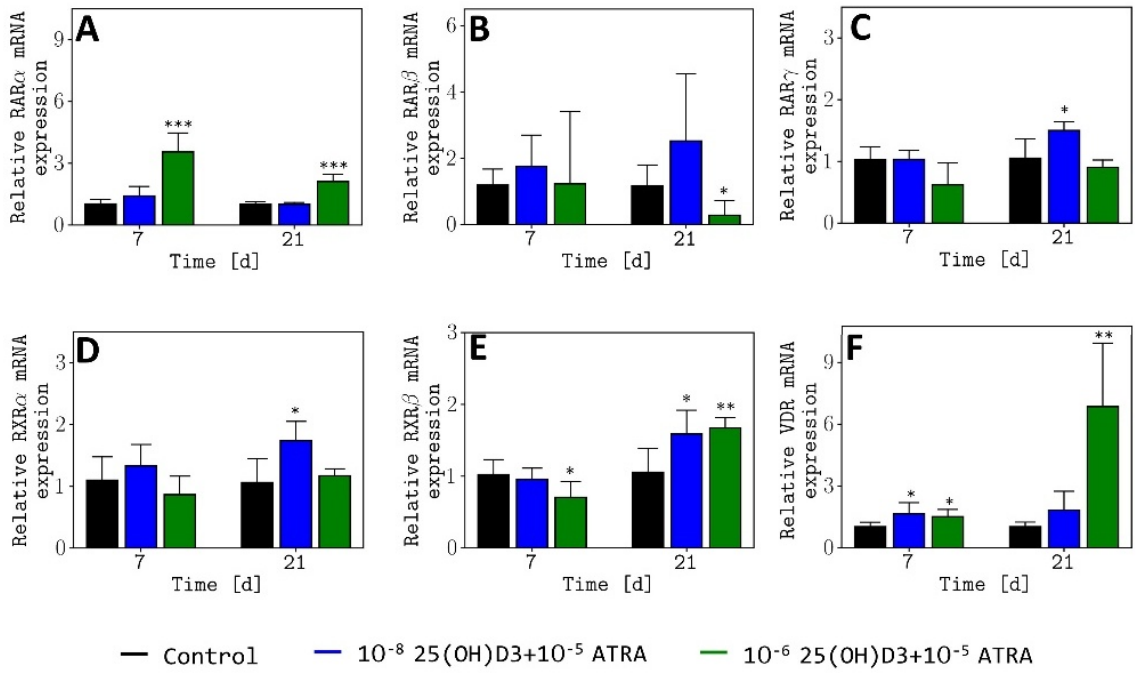

Fig. 5. Relative mRNA expression of *RARα* (A), *RARβ* (B), *RARγ* (C), *RXRα* (D), *RXRβ* (E), and *VDR* (F) receptors in hOBs cultured at 7 and 21 days with a combination of 25(OH)D3 and ATRA. mRNA expression was normalized to *GAPDH* and is presented as relative to control. Values are presented as the mean ± SD, n = 3. Significance $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$. *RARβ*, retinoic acid receptor beta; *RXRα*, retinoid X receptor alpha; *VDR*, vitamin D receptor; *RXRβ*, retinoid X receptor beta; *RARγ*, retinoic acid receptor gamma; *RARα*, retinoic acid receptor alpha.

We aimed to investigate the molecular mechanism of higher concentration of 25(OH)D3 alone and in combination with ATRA on primary human osteoblast.

High serum levels of vitamin A have been found to reduce bone mass and increase the risk of fracture [36,37], however, vitamin A is also found to induce differentiation of osteoblast to osteocytes [38]. Several studies in various species have shown that vitamin A antagonizes vitamin D action when vitamins are administrated in combination [22–28]. Although, an *in vitro* study showed reduced proliferation and a possible synergistic effect on the differentiation of the combination of 1,25(OH)$_2$D3 and ATRA [39]. However, the combined effect of 25(OH)D3 and ATRA has been less extensively studied, and the molecular mechanism in hOBs remains elusive.

We showed that 10^{-6} 25(OH)D3 + 10^{-5} ATRA reduced the growth of hOBs, which is in agreement with Choong *et al*. [21] who reported a reduced proliferation of rat osteoblasts upon treatment with 1,25(OH)$_2$D3 and RA. An anti-proliferative effect of ATRA alone has also been reported in murine osteoblasts [16], MC3T3 cells [40], and rat calvarial osteoblasts [41]. Similarly, 25(OH)D3 has also been reported to reduce the differentiation of rat calvarial osteoblasts [8], hOBs [2], and human marrow stromal cells (hMSCs) [42].

ALP was assessed as a marker of bone formation, and the results showed that the combined treatment significantly increased *ALP* activity within the cells, indicating enhanced osteoblast differentiation. Enhanced mRNA levels and cell bound *ALP* activities induced by 10^{-6} 25(OH)D3 + 10^{-5} ATRA have been verified by the combination of 1,25(OH)$_2$D3 and RA [21], as well as by the individual vitamin treatments [2,9,14,41].

Additionally, we quantified the secretion of selected bone markers in the cell culture media. Our results suggest that the combination of 10^{-6} 25(OH)D3 and 10^{-5} ATRA have a pronounced effect on promoting osteogenic activity, as evident by elevated levels of bone markers. Enhanced *OCN* secretion was observed in hOBs treated with 10^{-6} 25(OH)D3, both alone or in combination with 10^{-5} ATRA, whereas treatments with ATRA alone had opposing effects in our study, although the individual treatments with ATRA had opposite effects in our study. The observed effects of the individual vitamins were in agreement with Bosetti *et al*. [43] who reported an increase in *OCN* in human osteoblast by vitamin D, and Mattinzoli *et al*. [38], who reported decreased *OCN* in MC3T3 cells by RA.

We also observed that 10^{-6} 25(OH)D3 + 10^{-5} ATRA significantly increased collagen type 1 and OPN expression, however, to our knowledge there are no other stud-

ies investigating these factors with the combined vitamin treatment. Earlier studies have reported increased collagen 1 expression in both human bone cells [3] and in MC3T3 cells [4,44] by 1,25(OH)$_2$D3, and decreased collagen 1 expression in MC3T3 and primary mouse osteoblasts [38] by ATRA, which are in line with our findings. In addition, we also found an increase in OPN expression by 25(OH)D3 alone, which is in accordance with van der Meijden *et al*. [2] demonstrating an increased OPN expression in hOBs by 25(OH)D3. Contrary to our finding, an increase in OPN expression has been previously reported in rat MSCs rat bone marrow-derived mesenchymal stem cells (rBMSCs) [14] and calvarial osteoblasts [41] by ATRA and RA, respectively. This discrepancy on the effect of vitamin A on OPN expression may reflect a species-specific response.

Upregulation of both the early and the late osteocyte markers; *E11*, *DMP1*, and sclerostin by the combination of 10^{-6} 25(OH)D3 + 10^{-5} ATRA was observed; an expression pattern previously demonstrated in 3D culture model of hOBs [30]. The high expression levels of *E11* and *DMP1* at day 21 indicated differentiation into osteocyte-like cells, as *E11* is selectively detected in the pre-osteocytes, where it plays an important role in the development of osteocyte dendrites [45] while *DMP1* plays an important role in the transition of pre-osteocyte into mature osteocytes [46]. Similarly, the observed high levels of sclerostin and *FGF23* further manifested the presence of osteocyte-like cells [47,48]. Increased expression of osteocyte markers by hOBs in the presence of 10^{-6} 25(OH)D3 and ATRA individually, are in agreement with other studies showing that vitamin D and vitamin A alone increased *DMP1* [17,20,49], sclerostin [38,50–52] and *FGF23* [33,38] expression in different cells. Contrary to that, 4×10^{-7} M RA had reportedly decreased *SOST* expression in MC3T3-E1 [13]. This could have been due to the diversity of action of vitamin A metabolites at different concentrations.

The effects of vitamin D and RA are mediated by specific nuclear receptors (*RARs*, *RXRs* and *VDR*, respectively) [2,53–55]. Lou *et al*. [32] demonstrated that 25(OH)D3 can stabilize the *VDR*-ligand binding domain in the same way as its active metabolite 1,25 (OH)$_2$D3. Earlier study on the combined effect of vitamin D and vitamin A on the expression of receptors showed that ATRA regulates *VDR* expression in osteosarcoma cell line [39]. Our results are in agreement with the finding described above, as we observed that 10^{-6} 25 (OH)D3 and 10^{-5} ATRA both individually and in combination increased *VDR* receptor expression. The possible mechanism would involve ATRA-induced upregulation of *VDR* expression, enhancing the response to 25(OH)D3 and facilitating osteoblast maturation. In addition, ATRA binding to *RAR*, and forming heterodimers with *RXR* [56] may in turn regulate the gene transcription machinery involved in osteoblast differentiation [39]. As *RXR* can also form heterodimers with *VDR* [57], this cross-regulation could further induce the transcription of osteogenic genes. Thus, ATRA and 25(OH)D3 could synergistically promote osteoblast differentiation *in vitro*, by coordinating nuclear receptor activity.

The study has several limitations, one being that we have not assessed the effect of the combination of vitamins on receptors involved at an early time point, which may have led to a better understanding of the initial effect of combined vitamin treatment on primary human osteoblasts. Additionally, The BCIP/NBT method used to visualize alkaline phosphatase activity, has limitations when it comes to quantitative analysis due to variable staining intensity. Future studies could alternatively utilize Gomori's stain, to provide clearer differentiation between tissue components, and detection of enzymatic activity in tissue sections, particularly in histochemical studies. Furthermore, short tandem repeat (STR) analysis could have been used to further verify the authenticity of hOBs. However, we didn't include hOBs STR analysis in this study, as the hOBs were used at lower passage following purchase and were maintained in isolated incubators to ensure the authenticity. It would also have been beneficial to use more than one osteoblast donor to strengthen the generalizability, however, the use of human primary osteoblasts may better reflect the *in vivo* situation and the clinical effects. Furthermore, investigation such as mechanical testing could have been included to further confirm if differentiated cells behave like osteocytes or mature osteoblast. Additionally, our results show high mRNA expression of *ALP* and collagen at day 21, which are early osteoblast markers. We proposed that cell population by day 21 may be a mixture of osteoblast and osteocytes but a detailed profiling of early, mid, and late osteoblast markers could be beneficial and should be considered in future studies.

Our findings, however, are significant for several reasons. Osteocytes play a vital role in maintaining bone homeostasis and regulating bone metabolism. By demonstrating that the combination of 25(OH)D3 and ATRA can induce the differentiation of hOBs into osteocyte-like cells, the results promote the field of bone development enhancing our understanding of remodeling processes. Moreover, the insights gained into the mechanisms driving the transition from osteoblasts to osteocytes are essential for translational research focusing on how bone responds to stimuli. Research focused on remodeling strategies that target osteocyte formation is also clinically crucial for the design of new therapies for bone-related diseases, such as osteoporosis and other metabolic bone disorders, ultimately aimed at improving bone strength and ailing fractures. Thus, these findings pave the way for future investigations into the underlying molecular mechanisms governing osteoblast and osteocyte differentiation, as well as the potential synergistic effects of other compounds on bone health.

Conclusions

In summary, our results demonstrate that the combination of vitamins notably surged the population of osteocyte-like cells, persistently expressing *E11*, *DMP1*, *SOST*, and *FGF23*. Hence, our study unveils a compelling strategy to enhance osteoblast to osteocyte-like cell differentiation using 10^{-6} 25(OH)D3 and 10^{-5} ATRA in combination and can give the possibility not only to evaluate osteoblast to osteocyte differentiation at different stages but also evaluate the effect of other agents on bone cells.

List of Abbreviations

AA, ascorbic acid; *ALP*, alkaline phosphatase; ATRA, all-trans retinoic acid; *COL1A1*, collagen type I alpha 1; *DMP1*, dentin matrix acidic phosphoprotein 1; *E11*, podoplanin; FBS, fetal bovine serum; *FGF23*, fibroblast growth factor 23; GA, gentamicin sulphate/amphotericin B; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; HC, hydrocortisone; hOBs, human osteoblasts; LEP, leptin; *MEPE*, matrix extracellular phosphoglycoprotein; OBM, osteoblast basal medium; *OCN*, osteocalcin; OM, osteogenic differentiation medium; *OPG*, osteoprotegerin; OPN, osteopontin; PBS, phosphate buffered saline; PFA, paraformaldehyde; *PHEX*, phosphate regulating endopeptidase homolog X-linked; RA, retinoic acid; *RARα*, retinoic acid receptor alpha; *RUNX2*, runt-related transcription factor 2; *RXRβ*, retinoid X receptor beta; *SOST*, sclerostin; STR, short tandem repeat; *VDR*, vitamin D receptor; 25(OH)D3, 25-hydroxyvitamin D3; $1,25(OH)_2D3$, 1,25-dihydroxyvitamin D3; RT-PCR, real-time polymerase chain reaction; βGP, β-glycerophosphate; GM, osteoblasts in basal medium; *RARβ*, retinoic acid receptor beta; *RARγ*, retinoic acid receptor gamma; *RXR*, retinoid X receptor; *RXRα*, retinoid X receptor alpha; RXRγ, retinoid X receptor gamma; *COL1*, collagen type I; hMSCs, human marrow stromal cells; 2D, two-dimensional; 3D, three-dimensional; qPCR, quantitative polymerase chain reaction.

Availability of Data and Materials

Data available upon reasonable request to the corresponding author.

Author Contributions

AM conceived and designed the study, performed the experiments, data collection, data analysis/interpretation, and drafted the original manuscript. AEA assisted with luminex experiments and revised the manuscript. AS supervised gene expression experiment and revised the manuscript. JER conceived and designed the study, supervised the study, data interpretation, drafted the manuscript, and acquired funding. LBS conceived and designed the study, supervised the study, data interpretation, drafted the manuscript, and acquired funding. All authors have read and agreed to the final version of the manuscript. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Ethics Approval and Consent to Participate

This study did not require ethical approval.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.22203/eCM.v051a09.

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