Elucidating the genetic effect of vitamin D on mesenchymal stem cell differentiation in vitro

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Research Article

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Abstract

The active form of vitamin D (1, Alpha 25-Hydroxyvitamin D₃; [1α,25(OH)₂D₃]) is associated with multiple cellular processes, including bone formation. Severe vitamin D deficiency (as defined by serum 25-Hydroxyvitamin D <30nmol/L) typically results in growth retardation, rickets and osteomalacia. Conversely, 1α,25-(OH)₂D₃ treatment demonstrates anabolic effects on bone, which could be explained via its action on differentiating mesenchymal stem cells (MSCs). This study investigated the effect of 1α,25-(OH)₂D₃ on osteogenic and adipogenic differentiation of human MSCs (hMSC). We examined 12,000 human genes and expressed sequence tags on the array Human Genome U95A via Affymetrix DNA array. We confirmed genes with higher and lower expression by reverse transcription-polymerase chain reaction. We found that differentiating hMSC treated with 1α,25-(OH)₂D₃ exhibited a significantly higher expression of distinct osteogenic genes (CYP24A1, DSP, FBLN2, HYAL3, MNT1 and TBCD) and adipogenic genes (Fibulin 2 [FBLN2], DSP and G0S2) when compared to undifferentiating hMSCs. In addition, FBLN2 showed a significantly higher expression when treated with 1α,25-(OH)₂D₃ in both adipogenic and osteogenic conditions. Meanwhile, CYP24A1, which is associated with 1α,25-(OH)₂D₃ degradation, had reduced expression in adipogenic compared to osteogenic and MSCs growth media. In summary, our gene array analysis identified a direct effect of 1α,25-(OH)₂D₃ on a set of genes required for hMSCs differentiation, thus improving our understanding of the effect of the active form on vitamin D on bone metabolism.

Introduction

Bone loss begins in the third decade of life due to a progressive increase in bone resorption and a decline in bone formation. The processes underlying bone loss are multifactorial and are largely driven by reduced osteoblast survival, function, and number. This process is accelerated during aging and menopause, resulting in osteoporosis and increased fracture risk [1]. The key biological aspects that underlie the bone loss process with aging include changes to the mesenchymal stem cell (MSC) lineage. For example, in a healthy state, MSCs can differentiate into osteoblast (Ob) and adipocytes, with a balance achieved at a normal and steady rate [1]. However, an imbalance occurs with aging, resulting in an increased shift towards adipocyte formation [2, 3]. This imbalance can be further exacerbated in osteoporosis, and as a result, the bone marrow milieu is disrupted, which ultimately contributes to higher bone marrow adiposity and bone loss [3].

Interestingly, the presence of high bone marrow fat is considered an early predictor and diagnostic marker of bone loss and fragility [4, 5]. Specifically, elevated bone marrow adiposity is associated with increased loss of trabecular bone, reduced bone quality at the femoral neck, and loss of spine compressive strength in older adults [6]. Marrow adipocytes produce fatty acids, predominantly palmitic acid (PA), a 16-carbon saturated fatty acid toxic to O Obs and osteocytes, impairing their differentiation, function, and survival [7–11]. As such, the fundamental mechanisms underpinning the negative impacts of lipotoxicity on Obs include the induction of apoptosis and dysfunctional autophagy, which have been shown to result in reduced osteoblastogenesis and Ob mineralization [8, 9, 12].

Vitamin D, a pro-hormone produced via the skin when exposed to ultraviolet light, is commonly prescribed to preserve bone health due to its vital biological role in calcium absorption and regulation of bone turnover [13]. Vitamin D is metabolized via the liver and the kidney to its biologically active form, 1,25-dihydroxy vitamin D (1,25(OH)₂D), and then binds to its nuclear receptor, the vitamin D receptor (VDR). Much of the effect of 1α,25(OH)₂D on bone is through the intestinal absorption of calcium (Ca²⁺) and phosphorus which are necessary for mineralization of the bone matrix and prevention of bone-related diseases such as rickets and osteomalacia [14, 15].

In addition to this, the biological actions of 1α,25(OH)₂D directly affect the cellular features of aging bone. 1α,25(OH)₂D₃ stimulates bone anabolism through a direct effect on osteoblastogenesis and inhibition of adipogenesis [16–18]. In cultured human primary O Obs, 1α,25(OH)₂D₃ can enhance mineralization and the formation of bone nodules by increasing Ob survival, differentiation, and function [19, 20]. Although the effect of 1α,25(OH)₂D₃ on MSC differentiation and survival
has been assessed both in vitro and in vivo[17, 21], the characteristics of MSCs response to 1α,25(OH)2D3 – specifically their differentiation pathways – remain partially explored. Therefore, the current study investigated the genes associated with osteogenic and adipogenic differentiation in human MSCs (hMSCs) treated with 1α,25(OH)2D3.

**Materials And Methods**

**Cell culture**

hMSCs were obtained from Lonza (Victoria, Australia) and cultured and maintained in MSC growth medium (GM) (Lonza, Australia) according to the manufacturer's instructions. Cells at passage five were used in the present study.

**Osteogenic Differentiation of hMSCs In Vitro**

The induction of osteogenic differentiation of the hMSCs was done as described previously [22]. Briefly, hMSCs were plated at a density of 5 × 10^5 cells per well in 150-cm² dishes containing GM with 10% fetal calf serum (FCS) and incubated at 37°C. After the cells reached 70% confluence, the medium was replaced with either GM or osteoblastogenesis induction medium (OIM) (prepared with GM, 10% FCS, 0.2 mM dexamethasone, 10 mmol/l β-glycerol phosphates, and 50 µg/ml ascorbic acid), changed every 3 days for 21 days. 1,25(OH)2D3 was used at 10⁻⁸ M concentration based on previously identified osteoblastogenic and anti-apoptotic dose of 1α,25-(OH)2D3 in vitro [20]. Cells were then washed 4 times and collected for RNA analysis.

**Adipogenic Differentiation of hMSCs In Vitro**

For adipocyte differentiation, hMSCs were obtained and plated as previously described [17]. After passage 3, hMSCs were cultured in adipogenic induction medium (AIM) (prepared with DMEM, 4.5 g/L glucose, 1 µM dexamethasone, 0.2 mM indomethacin, 1.7 µM insulin, 0.5 mM 3-isobutyl-1-methylxanthine, 10% FCS, 0.05 U/ml penicillin, and 0.05 µg/ml streptomycin) and plated in 6-well plates at a density of 5 × 10^5 cells per well in 150-cm² dishes for 21 days. To promote the adipogenic phenotype, cells were then incubated for 3 days in adipogenesis maintenance medium (DMEM, 4.5 g/L glucose, 1.7 µM insulin, 10% FBS, 0.05 U/ml penicillin, and 0.05 µg/ml streptomycin) and then switched to induction media again. Cells were treated with 1,25(OH)2D3 as previously described. After 4 cycles of induction/maintenance (day 21), cells were then washed 4 times and collected for RNA analysis.

**cDNA Microarray Analysis of hMSCs**

Total RNA was extracted from hMSCs treated with either MSCGM, OIM or AIM on day 21 of differentiation using an Easy-Kit miniprep (Qiagen, Valencia, CA). Generation of cDNA, fluorescent labeling, hybridization to the gene chip, and data analysis was performed by the Genomics Laboratory at McGill University as previously described [23]. We examined 12,000 human genes and expressed sequences tags on the array Human Genome U95A (Affymetrix, Inc., Santa Clara, CA, http://www.affymetrix.com) and analyzed the results using the MicroDB Software. Genes with significant changes were then grouped depending on their known function. The biological function of each gene product was obtained from literature searches in medical databases (GenBank). This experiment was performed in triplicates, and significant changes in gene expression were determined by biological duplicates as previously described [23].

**Confirmatory RT-PCR.**

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was used to confirm the expression of vitamin D-dependent genes found by transcriptional profiling of hMSCs. To prepare cDNA, total RNA was first reverse transcribed using an oligo dT primer RNeasy Plus Mini Kit (QIAGEN, Australia). The RNA was kept on ice and immediately quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific, Life Technologies). qPCR was performed using the QIAGEN OneStep RT-PCR Kit following the manufacturer's instructions and specific primers (Supplementary Table 1). Amplifications were performed
in Bio-Rad CFX Connect upgrade to CFX96 Touch™ Real-Time PCR System under the following cycling conditions: 95°C 10 min, 40 cycles of 95°C 15 s, 60°C 15 s, 72°C 30 s.

**Statistics**

Data analysis was performed with SigmaStat (version 2.0) and IBM SPSS (version 25.0). The multiples of change (fold-change) in gene expression reported by the Affymetrix software (MAS 4.0) of triplicate experiments were used to determine whether a statistically significant change in expression had occurred. A 2-fold change was set as a threshold for significance. Means and 95% confidence intervals on the transformed data for each sequence were computed, and a gene was considered to have undergone a statistically significant change in expression if the 95% confidence intervals of the mean excluded zero as previously described [23]. All statistically significant changes in expression were filtered by two post hoc criteria. In the current study, we excluded any “absent” by the array reading software in any of the control samples, including the three conditions (MSCGM, OIM or AIM, for downregulated genes) or in any of the samples obtained from cells treated with 1,25(OH)\(_2\)D\(_3\) (for upregulated genes). In addition, sequences were excluded if the change in geometric mean expression was less than one-fold.

**Results**

1α,25-(OH)\(_2\)D\(_3\)-regulates genes associated with hMSCs function and survival

To evaluate the changes in gene expression of differentiating hMSC in response to 1α,25-(OH)\(_2\)D\(_3\), cells were cultured in MSCGM for 21 days. Compared to undifferentiating hMSCs, cells treated with 1α,25-(OH)\(_2\)D\(_3\) in GM exhibited a significantly higher expression (more than two-fold change significance) of distinct genes associated with proliferation and differentiation such as cytochrome P450 family 24 subfamily A member 1 (CYP24A1), the RUN and SH3 domain containing 2 (RUSC2) gene and the G0/G1 switch gene 2 (G0S2), which regulates quiescence in hematopoietic stem cells. Fibulin-2 calcium-binding (FBLN2) and desmoplakin (DSP) were significantly upregulated in hMSCs treated with 1α,25-(OH)\(_2\)D\(_3\) (Figure 1A). A notable change was an increased expression of bone morphogenetic protein 6 (BMP6), a key gene associated with motility, differentiation, and function of MSCs. In addition, 1α,25-(OH)\(_2\)D\(_3\) downregulated Bcl-2 associated X-protein (BAX), a pro-apoptotic member of the Bcl-2 gene family that plays a critical role in regulating intrinsic apoptosis. Furthermore, cells incubated in GM and treated with 1α,25-(OH)\(_2\)D\(_3\) also had downregulated expression of mitochondrial calcium uptake 1 (MICU1), twinfilin actin-binding protein 2 (TWF2), and hyaluronan synthase 1 (HAST).

Expression of osteogenic genes is altered in hMSCs treated with 1α,25-(OH)\(_2\)D\(_3\)

As shown in Figure 1A, 1α,25-(OH)\(_2\)D\(_3\) treatment modulated gene expression in hMSCs that were differentiated into Obs in OIM media. Significant changes were observed in gene expression (more than two-fold change significance) of distinct osteogenic genes associated with osteogenic differentiation and mineralization (CYP24A1 and DSP) as well as menigioma 1 (MN1) gene, an essential gene associated with motility, differentiation, and function of MSCs. Furthermore, tubulin folding cofactor D (TBCD) was significantly upregulated in response to 1α,25-(OH)2D\(_3\) treatment, a gene specifically associated with bone metabolism.

1α,25-(OH)\(_2\)D\(_3\) regulates adipogenic genes in differentiating hMSCs cultured in AIM media

hMSCs cultured in AIM media and treated with 1α,25-(OH)\(_2\)D\(_3\) exhibited a significantly higher expression (more than two-fold change significance) of distinct adipogenic genes that play a crucial role in regulating skeletal lipid content (DSP, FBLN2, MN1, G0S2 and CYP24A1) as shown in Figure 1B. 1α,25-(OH)\(_2\)D\(_3\) significantly downregulated lymphocyte antigen 6 family member E (LY6E) and retinol dehydrogenase 5 (RDH5), which both play a role in cell death and apoptosis.
1α,25-(OH)₂D₃ modulated the expression of specific genes associated with adipogenic versus osteogenic differentiation in hMSCs

In response to 1α,25-(OH)₂D₃ treatment, expression of CYP24A1, DSP and FBLN2 – genes associated with MSCs differentiation and function – was significantly upregulated in adipogenic conditions similarly to osteogenic and GM in Figure 2B. In addition, 1α,25-(OH)₂D₃ significantly downregulated RDH5 and LY6E in both adipogenic and osteogenic conditions compared to GM. Notably, patatin-like phospholipase domain-containing protein 2 (PNPLA2), also known as adipose triglyceride lipase, was significantly downregulated in AIM with 1α,25-(OH)₂D₃ but not to the same extent as the changes seen in the 1α,25-(OH)₂D₃ treated OIM cells. Conversely, the opposite effect was observed in osteogenic conditions in cells treated with 1α,25-(OH)₂D₃, in which FBLN2 expression was significantly decreased. Meanwhile, the expression of CYP24A1, a metabolic enzyme responsible for the degradation of 1α,25-(OH)₂D₃, was significantly lower in adipogenic conditions compared to osteogenic and GM.

Treatment of hMSC with 1α,25-(OH)₂D₃ affects specific molecular, cellular, and biological processes and pathways

hMSCs cultured with 1α,25-(OH)₂D₃ under osteogenic and adipogenic conditions showed specific genetic responses associated with specific osteogenic/adipogenic pathways (Figure 3).

Discussion

1α,25-(OH)₂D₃ induces favorable changes to the osteogenic lineage of hMSCs in vitro [24, 25]. Studies have demonstrated that vitamin D deficiency results in impaired bone formation and skeletal deformity in preclinical models in vivo [26] and human trials [27], highlighting the importance of maintaining optimal serum levels throughout the lifespan. In this study, we identified changes in gene expression with 1α,25-(OH)₂D₃ in conditions of osteogenic and adipogenic differentiation of hMSCs to better understand the effect of 1α,25-(OH)₂D₃ on bone metabolism and elucidate novel pathways with therapeutic potential.

1α,25-(OH)₂D₃ regulates hMSCs differentiation and skeletal homeostasis through autocrine/paracrine mechanisms [28]. It has previously been demonstrated that the differentiation of hMSCs into osteoblasts can be stimulated by 1α,25(OH)₂D in a tightly regulated manner [29]. The active form of vitamin D, 1α,25-(OH)₂D₃, is generated through two sequential hydroxylation steps with the second step mediated by the enzyme 25(OH)D₃-1α-hydroxylase, (CYP27B1, encoded by the gene CYP27B1) in the kidney [30]. Not surprisingly, given the crucial role in vitamin D metabolism, results from this study showed that CYP24A1 is highly expressed in both osteogenic and GM conditions when treated with 1α,25-(OH)₂D₃. Aside from metabolizing vitamin D into its bioactive form, CYP24A1 has also been shown to be associated with the proliferation and differentiation of hMSCs, which has been confirmed in knockdown and inhibitory cell studies [30]. Ablation of CYP24A1 inhibits the antiproliferative and pro-differentiation effects of 1α,25-(OH)₂D₃ on hMSCs [30], further confirming the importance of this gene in enabling the biological effects of vitamin D. Conversely, the enzyme responsible for vitamin D removal and breakdown, 25(OH)D-24-hydroxylase, (CYP24A1 gene encoded by CYP24A1), has also been implicated in bone metabolism [31]. Inactivating mutations of the CYP24A1 gene result in hypercalcemia and hypercalciuria secondary to diminished 1α,25-(OH)₂D degradation [32].

Another interesting finding from our study was the effect of 1α,25-(OH)₂D₃ to significantly upregulate the HYAL3 gene in all growth conditions, but to a lesser extent in both OIM and AIM than GM. Encoded by the HYAL3 gene, hyaluronidase-3 is an enzyme similar in structure to hyaluronidases. Hyaluronidases intracellularly degrade hyaluronan, one of the major glycosaminoglycans of the extracellular matrix (ECM). Hyaluronan is involved in cell proliferation, migration and differentiation and forms a major component of the ECM of cartilage, thus contributing to the structural and functional integrity of the bone [33, 34]. Whilst vitamin D increased the expression of HYAL3 in all conditions, the presence of
significantly higher fold increase found in the GM-treated group alone suggests that vitamin D may play an underappreciated role in regulating the extracellular matrix (ECM) via the MSCs. Further studies are needed to expand on the hypothesis that vitamin D could potentiate the changes in the ECM to preserve and/or improve the function and structure of various tissues.

Several genes are associated with osteogenic differentiation in hMSCs [35], and we found 1α,25-(OH)₂D₃ treatment to induce changes in expression in a few of them compared to untreated hMSCs. For example, significantly upregulated in GM and OIM conditions treated with vitamin D, the DSP gene is associated with bone and dentine osteogenic activity and odontogenic differentiation of bone marrow MSC [36]. Supporting this, a study has found that overexpression of DSP promoted mineralization of pluripotent MSC, and together with the expression of early odontogenic marker genes, implied that these cells differentiate into functional osteoblast cells [37]. Furthermore, 1α,25-(OH)₂D₃ upregulated multiple genes that are associated with MSC function and survival such as MN1, RUSC2 and BMP6 [38, 39].

Importantly, 1α,25-(OH)₂D₃ significantly downregulated PNPLA2 in osteogenic media, a gene that encodes for adipose triglyceride lipase. Mutations in the PNPLA2 gene is associated with neutral lipid storage disease [40]. Further to this, results from our study may suggest that vitamin D treatment could play a role that can reduce the harmful pathophysiological effects involved in lipotoxicity in bone. On the other hand, 1α,25-(OH)₂D₃ decreased multiple genes associated with cell death and apoptosis in all three conditions. Notably, 1α,25-(OH)₂D₃ downregulated phosphomevalonate kinase (PMVK), which encodes a cytoplasmic enzyme catalyzing the conversion of mevalonate 5-phosphate to mevalonate 5-diphosphate in the mevalonate pathway in many cell types, including MSC [41]. Similarly, BAX is a pro-apoptotic member of the Bcl-2 gene family that plays a critical role in regulating intrinsic apoptosis in bone [20] that was also downregulated by 1α,25-(OH)₂D₃.

The harmful effects of increased bone marrow adiposity on bone mass and strength are now well recognized. These effects are partially due to the toxic nature of saturated free fatty acids on Oabs [8, 9]. It has been shown previously that 1α,25-(OH)₂D₃ can protect osteoblasts from lipotoxicity and increase survival, differentiation potential and mineralization [19]. Our microarray data demonstrated that 1α,25-(OH)₂D₃ significantly influences multiple genes involved in regulating fat metabolism. In particular, we showed 1α,25-(OH)₂D₃ increased the expression of G0S2, a gene that plays a key role in the regulation of skeletal lipid content and oxidative metabolism [42].

In conclusion, gene microarrays of hMSCs undergoing differentiation can be used to identify candidates for new downstream targets for transcription and mechanistic factors that drive osteogenesis and adipogenesis. Here, we used this strategy to identify critical drivers of the osteogenesis and adipogenesis processes in the presence of 1α,25-(OH)₂D₃ treatment. The findings from this study improve our understanding of the relationship between 1α,25-(OH)₂D₃ and the specific pathways associated with optimizing bone health and preventing lipotoxicity.

Declarations

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Author’s Roles: Study design: AAS, RK and GD. Study conduct and experiments: AAS, DAD and GD. Data collection: AAS, DAD and GD. Data analysis: AAS, DAD, and GD. Data interpretation: AAS, DAD, AH, RK and GD. Drafting manuscript: AAS, DAD, and AH. Revising manuscript content: AAS, DAD, AH, RK and GD. Approving final version of manuscript: AAS, DAD, AH, RK and GD.

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**Supplementary Table**

**Supplementary Table 1. Specific PCR primers used to confirm 1,25-dihydroxyvitamin D-regulated gene expression in hMSCs**

| Gene Name | Forward primer | Reverse primer |
|-----------|---------------|----------------|
|           | Sequence, 5-3 | Sequence, 5-3 |
| Cytochrome P450 Family 24 Subfamily A Member 1 (CYP24A1) Hs.89663 | GCCCAGCCGGGAACTC | AAATACCACCATCTGAG |
| Fibulin 2 (FBLN2) Hs.2558 | CCGCAGCTCCCAACCACAAATAAGCT | TCGAAATAACACAAACCCTCGACGACGCC |
| G0/G1 Switch 2 (G0S2) | GGCCTGATGGAGACTGTGTG | CTTGCTTCTGGAGAGCCTGT |
| Retinol Dehydrogenase 5 (RDH5) AA587372 | CTTGGAGACCATGACATAGATGG | GGTAGATACAGTACCAGAGGTTC |
| Lymphocyte Antigen 6 Family Member E (LY6) Hs.77667 | GTGATATAGGGGTTGTAGGCCATA | GACAAAGTGAAAGCTGCAAAAAGTT |
| Late Cornified Envelope 2B (LCE2B) Hs.200478 | GAGCCGAAAGCTGAGCCATG | GTACCGAGTCGAAAGCGCAGG |

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Effect of 1α,25-(OH)₂D₃ treatment on gene expression in differentiating human mesenchymal stem cells (hMSC) cultured in osteoblastogenesis induction medium (OIM) or adipogenic induction medium (AIM) compared to general growth media (GM). (A) Heat map representation of the log2-fold change (FC) for the top 24 up and down-regulated genes of hMCS cultured in OIM and treated with vitamin D (OIM+1α,25-(OH)₂D₃) compared to GM+1α,25-(OH)₂D₃. (B) Heat map representation of the log2 fold change (FC) for the top 13 most up and down-regulated genes for the cells cultured in AIM treated with vitamin D (AIM+1α,25-(OH)₂D₃) compared to GM+1α,25-(OH)₂D₃. Experiments performed in triplicate and statistically significant (more than 2-fold change) comparison between hMSCs in GM+VEH and hMSCs in GM+1α,25-(OH)₂D₃; OIM+VEH and hMSCs in OIM+1α,25-(OH)₂D₃ and AIM+VEH and hMSC in AIM+1α,25-(OH)₂D₃ are presented.
Figure 2

Comparison between the effect of 1α,25-(OH)₂D₃ treatment on gene expression in differentiating mesenchymal stem cell (MSC) cultured in osteoblastogenesis induction medium (OIM) and adipogenic induction medium (AIM). (A) The heat map represents the log2 fold change (FC) for the top 32 most upregulated and downregulated genes for the OIM+1α,25-(OH)₂D₃ group compared to AIM+1,25OH. (B) Comparison between top-ranked up-and down-regulated genes from cells cultured in GM, AIM or OIM and treated with 1α,25-(OH)₂D₃. Experiments were performed in triplicate and were statistically significant (more than 2-fold change). Comparison between hMSCs in OIM+VEH and hMSCs in OIM+ 1α,25-(OH)₂D₃ and AIM+VEH and hMSCs in AIM+ 1α,25-(OH)₂D₃ are presented. (C) Confirmatory PCR for 1α,25-(OH)₂D₃ genes found by Affymetrix gene profile array. RNA was pooled from 3 independent experiments and subjected to RT-PCR using gene-specific primers (Supplementary Table 1) to confirm 1α,25-(OH)₂D₃ responsive gene expression and presented as quantification cycle (Cq).
Figure 3

Functional profiling of 1α,25-(OH)2D3 expressed genes. (A) hMSCs cultured in the three different conditions with 1α,25-(OH)2D3 associated with specific functional, metabolic, and biological pathways in gene profiling. (B) Biological pathway of the genes. (C) Biological processes of the genes. (D) Molecular function of the genes. (E) the cellular component associated with the genes.