Published in final edited form as:

J Steroid Biochem Mol Biol. 2013 July ; 136: 156–159. doi:10.1016/j.jsbmb.2012.09.002.

Histone Deacetylation Mediates the Rejuvenation of Osteoblastogenesis by the Combination of 25(OH)D₃ and Parathyroid Hormone in MSCs from Elders

Shuanhu Zhou^{a,b}, Shuo Geng^{a,c}, and Julie Glowacki^{a,*}

Shuanhu Zhou: szhou@rics.bwh.harvard.edu; Shuo Geng: shuogeng.hmu@gmail.com; Julie Glowacki: jglowacki@rics.bwh.harvard.edu

^aDepartment of Orthopedic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

^bHarvard Stem Cell Institute, Harvard University, Cambridge, MA 02138, USA

^cDepartment of Orthopedic Surgery, The First Affiliated Hospital of Harbin Medical University, Harbin Medical University, Harbin, Heilongjiang Province 150001, China

Abstract

Vitamin D metabolites are important effectors of bone and mineral homeostasis. Human bone marrow stromal cells (hMSCs) are targets of 1 α ,25-dihydroxyvitamin D [1 α , 25(OH)₂D] action to promote their differentiation to osteoblasts. Osteoblastogenesis is also stimulated by 25-hydroxyvitamin D [25(OH)D], an effect that requires conversion to 1 α , 25(OH)₂D₃ by 25-hydroxyvitamin D₃ 1 α -hydroxylase (CYP27B1). These findings support an autocrine/paracrine role of vitamin D metabolism in osteoblastogenesis of hMSCs. In this study, we assessed whether and by what mechanisms osteoblastogenesis could be rejuvenated with hMSCs from elders. First, knockdown studies with VDR-siRNA showed that both the pro-differentiation and anti-proliferative effects of 1 α , 25(OH)₂D₃ required VDR. Second, 100 nM 25(OH)D₃ ($p < 0.01$ vs. control, ANOVA) and 100 nM PTH1-34 ($p < 0.05$) significantly stimulated alkaline phosphatase activity (a measure of osteoblastogenesis), with a synergistic effect when combined ($p < 0.001$). Scriptaid, an inhibitor of histone deacetylase, blocked the effect of 25(OH)D₃ and PTH on osteoblastogenesis. Scriptaid alone downregulated VDR in hMSCs. These data demonstrate that histone deacetylation is required for the synergistic effect of 25(OH)D₃ and PTH on osteoblastogenesis in hMSCs. Both VDR siRNA and Scriptaid downregulated VDR mRNA and inhibited osteoblastogenesis. Thus, epigenetic regulation of the VDR may be central to rejuvenating osteoblastogenesis in hMSCs from elders.

Keywords

Vitamin D; Vitamin D Receptor; Parathyroid Hormone; Epigenetic Regulation; Osteoblastogenesis; Human Marrow Stromal Cells

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*Corresponding author: Orthopedic Research, Brigham and Womens Hospital, 75 Francis Street, Boston, MA 02115 USA, Tel: 617-732-5397, Fax: 617-732-6937.

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1. Introduction

Vitamin D metabolites are important effectors of bone and mineral homeostasis. Classically, $1\alpha, 25(\text{OH})_2\text{D}$ mediates its actions through activation of the Vitamin D Receptor (VDR), a ligand-dependent transcription factor. Human bone marrow stromal cells (hMSCs) are a target of $1\alpha, 25(\text{OH})_2\text{D}$ action to promote their differentiation to osteoblasts [1]. Osteoblastogenesis is also stimulated by 25-hydroxyvitamin D [$25(\text{OH})\text{D}_3$] [2], an effect that requires conversion to $1\alpha, 25(\text{OH})_2\text{D}_3$ by 25-hydroxyvitamin D_3 1α -hydroxylase (CYP27B1) [3]. CYP27B1 in hMSCs is upregulated by 25OHD [2], IGF-I [2], and PTH [4] and is downregulated by $1\alpha, 25(\text{OH})_2\text{D}$ [2] and with age of the subject [4]. These findings support an autocrine/paracrine role of vitamin D metabolism in osteoblastogenesis of hMSCs. There are striking age-associated declines in baseline osteoblast differentiation of hMSCs [4–7], as well as in *in vitro* stimulation of osteoblastogenesis by $1\alpha, 25(\text{OH})_2\text{D}_3$ [8], $25(\text{OH})\text{D}_3$ [4], and PTH (1-34) [9]. The combination of $25(\text{OH})\text{D}_3$ and PTH-pretreatment, however, rejuvenated osteoblast differentiation in hMSCs from elders [4]. The studies herein test the hypotheses 1) that VDR is required for actions of $1\alpha, 25(\text{OH})_2\text{D}_3$ in hMSCs, 2) that simultaneous treatment with PTH and $1\alpha, 25(\text{OH})_2\text{D}_3$ stimulates osteoblastogenesis, and 3) that an epigenetic mechanism mediates the rejuvenation of osteoblastogenesis by a combination of $25(\text{OH})\text{D}_3$ and PTH.

2. Materials and methods

2.1 Human Marrow Stromal Cells (hMSCs)

Bone marrow samples were obtained with IRB approval as femoral tissue discarded during primary hip arthroplasty for osteoarthritis, with exclusion criteria and isolation methods as described previously [2]. The hMSCs were maintained in phenol red-free α -MEM, 10% fetal bovine serum-heat inactivated (FBS-HI), 100 u/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Invitrogen, Carlsbad, CA).

2.2 RNA interference with VDR-siRNA

Transient transfection of siRNA into hMSCs was performed by electroporation with the Human MSC Nucleofector Kit (Lonza/Amara Biosystems) with either VDR siRNA (Invitrogen) or non-silencing control siRNA (a non-homologous, scrambled sequence equivalent) (Santa Cruz Biotech., CA) according to the manufacturer's instructions and as described [3]. For proliferation assays, cells were transferred to 12-well plates in growth medium $\pm 10 \text{ nM}$ $1\alpha, 25(\text{OH})_2\text{D}_3$ for 3 days. Cells were re-suspended with 0.05% trypsin-EDTA (Invitrogen), and cell number was determined by hemacytometer. For differentiation assays, cells were cultured until confluent in 60-mm dishes and treated $\pm 10 \text{ nM}$ $1,25(\text{OH})_2\text{D}_3$ in osteoblastogenic medium (α -MEM with 1% FBS-HI, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 10^{-8} M dexamethasone, 5 mM β -glycerophosphate, 50 $\mu\text{g}/\text{ml}$ ascorbate-2-phosphate) for 3 days and assayed for ALP gene expression by RT-PCR [3].

2.3 Osteoblast differentiation

Upon confluence of passage 2 hMSCs, medium was changed to osteoblastogenic medium $\pm$ treatments. As indices of osteoblast differentiation, alkaline phosphatase (ALP) enzyme activity at day 7 was measured as described [6]. Previous studies showed that, with various stimuli, changes in ALP activity corresponded to changes in osteoblastic genes and mineralization [2–4, 9]. Scriptaid (10 μM) was added 45 minutes before the hormone treatments. Reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

2.4 Statistical analysis

Experiments were performed at least in triplicate. Group data are presented as Mean $\pm$ SEM, unless otherwise indicated. Quantitative data were analyzed with non-parametric tools; if data allowed, parametric tools were used, either t-test for two group or one-way ANOVA for multiple group comparisons.

3. Results

3.1 VDR is required for anti-proliferative and pro-osteoblastogenic effects of 1,25(OH)₂D₃ in adult human mesenchymal stem cells

Transfection with VDR-siRNA resulted in reduced VDR expression (Fig. 1A), with no effect on viability (Fig. 1B). In a short-term assay of proliferation of hMSCs that had been transfected with control siRNA (NC), the number of cells after exposure to 1 α , 25(OH)₂D₃ for 3 days was 65.8 $\pm$ 3.3% of cells not exposed to 1,25(OH)₂D₃ ($p < 0.0001$, Fig. 1C). In sharp contrast, the number of cells that had been transfected with VDR-siRNA was not affected by exposure to 1 α , 25(OH)₂D₃ (103.1 $\pm$ 1.0%, compared with those not exposed to 1 α , 25(OH)₂D₃) (Fig. 1C). Although cultures looked similar (Fig. 1B), there were fewer cells with VDR siRNA compared with control siRNA (Fig. 1C); this may be related to information that short interfering RNAs can sequence-dependently induce unexpected and divergent changes in the levels of untargeted proteins, such as p53 and p21, in mammalian cells [10]. As expected, in control hMSCs, 1 α , 25(OH)₂D₃ stimulated ALP gene expression (160% of untreated, Fig. 1D). In contrast, in hMSCs that had been transfected with VDR-siRNA, there was no stimulation of ALP by 1 α , 25(OH)₂D₃ (90% compared with those not exposed to 1 α , 25(OH)₂D₃).

3.2 CYP27B1/1 α -hydroxylase is required for 25(OH)D₃ to stimulate osteoblastogenesis in hMSCs

Human MSCs were cultured for 7 days in osteoblastogenic medium $\pm$ 25(OH)D₃ (10 nM) $\pm$ cytochrome P450 inhibitor ketoconazole (10 μ M). There was significant stimulation of osteoblastogenesis by 25(OH)D₃, but not in the presence of ketoconazole (Fig. 2A). The inhibition of alkaline phosphatase activity by ketoconazole alone may be due to its inhibition of other enzymes [11] or of hydroxylation of endogenous vitamin D in serum [12].

3.3 Histone deacetylase is required for 25(OH)D₃ to stimulate osteoblastogenesis in hMSCs

We tested the effect of Scriptaid, an inhibitor of histone deacetylase (HDAC), on constitutive VDR gene expression in hMSCs from a 61-year-old man. There was a dose-dependent down-regulation of the VDR by Scriptaid (Fig. 2B).

In an experiment on regulation of osteoblast differentiation, hMSCs were cultured for 7 days in osteoblastogenic medium $\pm$ 25(OH)D₃ (10 nM) $\pm$ Scriptaid. Stimulation of ALP activity by 25(OH)D₃ (158% relative to control, $p < 0.05$) was blocked by Scriptaid (Fig. 2C).

3.4 Histone deacetylation mediates the synergistic stimulation by PTH (1-34) and 25(OH)D₃ on osteoblastogenesis in hMSCs

To test the effect of simultaneous treatment of hMSCs with PTH and 25(OH)D₃ on osteoblastogenesis, we treated hMSCs from a 76-year-old man with PTH (1-34) (100 nM) and/or 25(OH)D₃ (100 nM) in osteoblastogenic medium for 7 days (Fig. 3A). There was 170% more ALP activity with PTH (1-34) ($p < 0.05$, compared with control) and 280% more with 25(OH)D₃ ($p < 0.01$). The simultaneous combination of 25(OH)D₃ and PTH (1-34)

resulted in 650% of control levels of ALP, with statistical significance compared with all the other groups ($p < 0.001$).

Blocking histone deacetylases with Scriptaid inhibited the synergistic stimulation of PTH and $25(\text{OH})\text{D}_3$ on osteoblastogenesis (Fig. 3B). These findings indicate that chromatin deacetylation is required for their synergistic effect on osteoblastogenesis in hMSCs.

Further studies on interactions between $25(\text{OH})\text{D}_3$ and PTH revealed that PTH (1-34) upregulated VDR gene expression (Fig. 3C) and that $25(\text{OH})\text{D}_3$ (10 nM) upregulated PTHR1 gene expression (Fig 3D). The effect of $25(\text{OH})\text{D}_3$ on PTHR1 mRNA appeared to be a direct effect that did not require CYP27B1; the cytochrome P450 inhibitor ketoconazole (10 μM) did not inhibit the up-regulation of PTHR1 by $25(\text{OH})\text{D}_3$. These data about interactions of $25(\text{OH})\text{D}_3$ and PTH with their mutual receptors add information about the mechanisms of their synergy in promoting osteoblastogenesis in hMSCs from elders.

4. Discussion

These studies indicate 1) that the steroid hormone nuclear VDR is required for the pro-differentiation and anti-proliferative actions of 1α , $25(\text{OH})_2\text{D}_3$ in hMSCs and 2) that histone deacetylation mediates the rejuvenation of osteoblastogenesis by a synergizing combination of $25(\text{OH})\text{D}_3$ and PTH. Studies with differentiated osteoblasts have shown that many [13] but not all actions of 1α , $25(\text{OH})_2\text{D}_3$ on them are mediated via genomic actions of the classical VDR [14]. Many rapid effects of 1α , $25(\text{OH})_2\text{D}_3$ and, in fact, $25(\text{OH})\text{D}_3$ occur via membrane-associated receptors [15].

We previously determined that pretreatment of hMSCs with PTH (1-34) upregulated CYP27B1 and rendered hMSCs from elders responsive to the pro-osteoblastogenic effects of $25(\text{OH})\text{D}_3$ [4]. We show herein that simultaneous treatment with PTH and $25(\text{OH})\text{D}_3$ had a synergistic effect on stimulating osteoblastogenesis and that histone deacetylation was required for that rejuvenation. The VDR may be central in this epigenetic regulation (Fig. 4), as suggested by Scriptaid's inhibition of VDR and inhibition of stimulation of osteoblastogenesis by $25(\text{OH})\text{D}_3$.

These studies add further support for the view that vitamin D metabolism in hMSCs serves an autocrine/paracrine role in osteoblast differentiation. The first lines of evidence showed that 1α -hydroxylation was required for the effects of $25(\text{OH})\text{D}_3$ [3] and that PTH promoted the biosynthesis of 1α , $25(\text{OH})_2\text{D}_3$ in hMSCs from elders [4]. This study shows that osteoblast differentiation with hMSCs from elders was stimulated by simultaneous treatment with $25(\text{OH})\text{D}_3$ and PTH, with each hormone stimulating the receptor for the other. The pro-osteoblastogenic effects of $25(\text{OH})\text{D}_3$ alone and when combined with PTH were prevented by Scriptaid, which also had a direct effect to down-regulate VDR gene expression.

There is other information about *in vivo* interactions between PTH and vitamin D metabolism. Samadfam *et al.* found greater increases in bone density by PTH in mice that had an intact 1α , $25(\text{OH})_2\text{D}$ -synthesizing system [16]. The concept that PTH and vitamin D interact to potentiate osteoblast differentiation is supported by an analysis of factors associated with heterogeneity in skeletal response to clinical PTH therapy for osteoporosis [17]. Of all variables examined, only the change in serum 1α , $25(\text{OH})_2\text{D}$ explained larger gains in bone density in response to PTH. More information about the molecular mechanisms of the osteoanabolic effects of PTH may help to reduce the variability seen in its clinical efficacy to increase bone density [18].

These studies demonstrate that histone deacetylation was required for the synergistic effect of $25(\text{OH})\text{D}_3$ and PTH on osteoblastogenesis in hMSCs and begin to unravel mechanisms of

skeletal aging. Epigenetic regulation of the VDR may be central to rejuvenating osteoblastogenesis.

Acknowledgments

These studies were supported by grants from the National Institutes of Health R01 AG025015 and R01 AG028114 (J.G.), the China Scholarship Council (S.G.), the American Federation for Aging Research grant A09052 (S.Z.), and the BWH-BRI Fund (S.Z.). Discarded marrow was obtained and studied with approval and annual review from the Partners Human Research Committee.

Abbreviations

1α	25(OH) $_2$ D $_3$, 1 α ,25-dihydroxyvitamin D $_3$
25(OH)D$_3$	25-hydroxyvitamin D $_3$
hMSCs	Human Marrow Stromal Cells
PTH	Parathyroid Hormone
VDR	Vitamin D Receptor

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Highlights

- The pro-differentiation and anti-proliferative effects of $1\alpha, 25(\text{OH})_2\text{D}$ in human marrow stromal cells (MSCs) required VDR.
- With age there are declines in osteoblast differentiation of human MSCs and in its stimulation by $25(\text{OH})\text{D}$.
- $25(\text{OH})\text{D}_3$ and PTH act synergistically to stimulate osteoblastogenesis in MSCs from elders.
- Histone deacetylation is required for the synergistic effect of $25(\text{OH})\text{D}_3$ and PTH.
- Epigenetic regulation of VDR may rejuvenate osteoblastogenesis in hMSCs of elders.

Fig. 1.

Requirement for VDR in the anti-proliferative and pro-osteoblastogenic effects of 1α , $25(\text{OH})_2\text{D}_3$ in hMSCs. A) Knockdown of VDR gene expression was achieved with 10 or 100 pmole of VDR-siRNA, compared with non-silencing control siRNA (NC). B) There was no visible difference in cellularity with the method. C) Proliferation of control hMSCs (NC) was inhibited with 1α , $25(\text{OH})_2\text{D}_3$ (10 nM, *** $p < 0.0001$), but there was no inhibitory effect of 1α , $25(\text{OH})_2\text{D}_3$ in cells transfected with 100 pmole of VDR-siRNA ($p = 0.62$). D) Gel electrophoretogram shows that stimulation of ALP gene expression by 1α , $25(\text{OH})_2\text{D}_3$ (10 nM, 3 days in osteoblastogenic medium) in control hMSCs (NC) was not present in cells transfected with VDR-siRNA (100 pmole).

Fig. 2.

Roles of 1α -hydroxylase and histone deacetylases in stimulation of osteoblastogenesis by $25(\text{OH})\text{D}_3$. A) After 7 days treatment, Ketoconazole ($10\text{ }\mu\text{M}$) blocked the stimulation of ALP activity by $25(\text{OH})\text{D}_3$ (10 nM) in hMSCs ($p<0.05$, $25(\text{OH})\text{D}_3$ vs. control; $p<0.001$, $25(\text{OH})\text{D}_3$ vs. $25(\text{OH})\text{D}_3$ & Ketoconazole; NS, not significant, Ketoconazole vs. $25(\text{OH})\text{D}_3$ & Ketoconazole; $n=4$, ANOVA). B) Electrophoretogram shows a dose-dependent decrease in VDR expression by Scriptaid in hMSCs from a 61-year-old man (24 hours). C) Stimulation of ALP by $25(\text{OH})\text{D}_3$ was blocked by Scriptaid ($10\text{ }\mu\text{M}$), a histone deacetylase inhibitor ($p<0.05$, $25(\text{OH})\text{D}_3$ vs. control; NS, Scriptaid vs. $25(\text{OH})\text{D}_3$ & Scriptaid; $n=4$, ANOVA).

Fig. 3.

Role of histone deacetylation in the synergistic effects of 25(OH)D₃ and PTH1-34 on osteoblastogenesis in hMSCs from elders. A) There was stimulation of Alkaline Phosphatase activity in hMSCs from a 76-year-old man by PTH (1-34) (100 nM, * $p < 0.05$) and by 25(OH)D₃ (100 nM, ** $p < 0.01$, and with greater stimulation by the simultaneous combination of PTH and 25(OH)D₃ than with either alone (** $p < 0.001$). B) Treatment with Scriptaid (10 μM) inhibited the stimulation of 25(OH)D₃ & PTH on osteoblastogenesis. Electrophoretograms show C) that PTH (1-34) (100 nM) upregulated VDR mRNA at 6–8 hours of treatment, and D) that 25(OH)D₃ (10 nM) upregulated PTHR1 mRNA and that ketoconazole (10 μM) did not blocked the upregulation of PTHR1 by 25(OH)D₃ in hMSCs from a 72-year-old woman (24 hours).

Fig. 4.

A summary scheme proposing the role of VDR in the interacting effects of PTH and 25(OH)D₃ on osteoblastogenesis in hMSCs.