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Vitamin D Metabolism in Human Bone Marrow Stromal (Mesenchymal Stem) Cells

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Abstract

There are many human extra-renal tissues and cells that biosynthesize 1α,25-dihydroxyvitamin D (1α,25(OH)$_2$D) by the action of CYP27B1/1α-hydroxylase. Human marrow stromal cells (hMSCs), also known as mesenchymal stem cells, were isolated from marrow discarded from well-characterized, consented subjects during common orthopedic procedures. Human MSCs can give rise to osteoblasts, chondrocytes, adipocytes, and other lineages. Their *in vitro* differentiation to osteoblasts is stimulated by 1α,25(OH)$_2$D, and recent evidence indicates that they have the capacity to metabolize vitamin D in a regulated manner. Human MSCs express the vitamin D receptor, 25-hydroxylases, 1α-hydroxylase, and 24-hydroxylase; stimulation of *in vitro* osteoblastogenesis by 25(OH)D depends on the activity of CYP27B1/1α-hydroxylase. Finding that hMSCs are a both a producer and target of 1α,25(OH)$_2$D suggests a potential autocrine/paracrine role of vitamin D metabolism in osteoblast differentiation. Expression and enzyme activity of CYP27B1/1α-hydroxylase are upregulated by substrate 25(OH)D and Parathyroid Hormone (PTH) and are downregulated by 1α,25(OH)$_2$D. With subject age, there is a decrease in basal osteoblast potential and in stimulation of osteoblastogenesis by 1α,25(OH)$_2$D, 25(OH)D, and PTH. *In vitro* treatment with a combination of 25(OH)D and PTH rejuvenated osteoblastogenesis with hMSCs from elders; this was attributable to increases in CYP27B1/1α-hydroxylase and in receptor for each hormone by the reciprocal factor. Other clinical variables beside age, i.e. low serum 25(OH)D or low estimated glomerular filtration rate, are correlated with reduced osteoblastogenesis. These studies suggest that osteoblastogenesis may not be optimal unless there is sufficient serum 25(OH)D substrate for hMSCs to synthesize and respond to local 1α,25(OH)$_2$D.

Keywords

Osteoblast differentiation; CYP27B1; Aging; 25-hydroxyvitamin D

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Conflict of Interest
The authors declare that there is no conflict of interest associated with this manuscript.

Contributions
SG, SZ, ZB, and JG wrote and revised components of this manuscript. SG prepared Table 1. JG prepared Table 2. JG, SZ, and SG prepared and revised the Figures.

1. Introduction

Human bone marrow stromal cells (hMSCs) are also known as human mesenchymal stem cells or marrow-derived skeletal stem cells. Human MSCs are multipotential progenitor cells capable of differentiation into osteoblasts, chondrocytes, adipocytes, other connective tissue cells [1–3], and possibly other cell types such as neuronal cells [4] or hepatocytes [5]. The active metabolite of vitamin D, 1α,25-dihydroxyvitamin D (1α,25$(OH)_2D_3$) or calcitriol, is an important regulator of mineral and bone metabolism. Calcitriol regulates proliferation, differentiation, and function of many cell types, both normal and malignant [6]. Human MSCs are a target of calcitriol action to promote their differentiation to osteoblasts [7]. Osteoblastogenesis is also stimulated by 25-hydroxyvitamin D_3 (25$(OH)D_3$) [8], an effect that requires conversion to 1α,25$(OH)_2D_3$ by 1α-hydroxylase (CYP27B1) [9]. This review summarizes the latest information concerning the significance of vitamin D metabolism in hMSCs.

2. Impact of method to isolate hMSCs

Stem cells or progenitors are defined by the ability to proliferate and to differentiate; for hematopoietic cells, those properties are monitored by colony assays in semi-solid media. For MSCs, proliferative capacity is recognized by the development of monolayer colonies of plastic-adherent cells from a single cell; these are termed Colony-Forming Unit-Fibroblasts (CFU-F). MSCs are defined functionally by adherence to plastic surfaces and by the potential to give rise to osteoblasts, chondrocytes, and adipocytes [1–3]. *In vitro* differentiation is demonstrated by specific lineage markers that arise when the cells are cultured in lineage-specific media and supplements for osteoblasts [8–13], adipocytes [14–16], or chondrocytes [16]. Because there are changes in cell behaviors associated with prolonged culture, such as culture stress or *in vitro* senescence, it is important to use hMSCs from different patients at the same passage for each experiment [17]. In addition, many *in vitro* behaviors and baseline characteristics of hMSCs depend on clinical features of the subjects from whom the cells were isolated, including age [10–13,17], gender [17], vitamin D status [8,18], and kidney status [18]. Human MSCs can be obtained from marrow aspirates from volunteers, from cadaveric bones, or from tissues discarded during orthopedic surgery, for example, for joint replacement due to debilitating hip or shoulder osteoarthrosis. It is also important to be aware of the method by which the hMSCs were prepared because of potential impact of accompanying cell types and their products that are present within the preparation.

Recent studies demonstrate that various tissues and organs are sources of multipotential progenitors, including marrow, adipose tissue, and dental tissues, but different MSC populations can exhibit significant differences in their proliferation, differentiation, and molecular phenotype [19]. The major methods for isolation hMSCs from bone marrow are outlined here to highlight differences in the composition of the sample. Bone marrow contains hematopoietic stem cells and the adherent fraction that gives rise to hMSCs. When whole marrow is transferred to tissue culture dishes, the hematopoietic fraction does not adhere to plastic, whereas the skeletal progenitor cells are adherent. The adherent layer also includes mature cells like macrophages, macrophage colonies, endothelial cells, and epithelioid cells. When the hematopoietic cells are left in the dish and an adherent stromal layer is allowed to develop, the former will adhere to the attached cells and will persist, depending on the factors produced by the stroma. The interactions between those fractions of cells depend on cell-to-cell contacts and are referred to as “juxtacrine” stimulation between cell surface ligands on one cell type and cell-surface receptors on the other; the interactions can be reciprocal [20]. When whole marrow is cultured at high initial seeding density, the adherent layer forms rapidly and produces factors that support continuous

hematopoiesis *in vitro* [21]. If the non-adherent hematopoietic cells are thoroughly removed a day or two after seeding, the adherent cells expand and gives rise to proliferative MSCs, thus diluting the non-adherent and non-proliferating cells. The early principles for identification and characterization of MSCs were established by Friedenstein [22] and others [23] for animal species that have small amounts of available marrow. The general procedure for studying mouse MSCs includes mature and progenitor cells and small aggregates of cells. Many studies with human cells follow a similar protocol. It is important to appreciate the methods used in order to properly interpret results and to compare studies.

2.1 Human MSCs from whole marrow cultures

There are several ways to establish whole marrow cultures for hMSC studies. In D'Ippolito's study, for example, bone marrow was obtained from trabecular bone chips by gentle rocking with medium [24]. Adherent cells from this preparation include fibroblastic spindle-shape cells, monocytes, macrophages, endothelial cells, and multinucleated osteoclasts.

Other studies use hMSCs prepared from single-cell-colonies and from pooled colonies [2]. Marrow aspirates and surgical fragments of trabecular bone and marrow were scraped gently and repeatedly to release a suspension of marrow cells. The hMSCs from single-colonies varied widely in differentiation capacities; some strains developed extensive bone and hematopoietic tissue *in vitro*, some strains formed little bone, and others formed only fibrous tissue. Clonal stromal cell lines immortalized by transfection with SV40 for studies of regulation of stromal support of hematopoiesis [25] have been used for comparison of properties with normal hMSCs [16,26]. Although immortalized MSCs and cell lines can be useful because of the availability of large numbers of cells, they may not represent the biology of normal cells.

2.2 Human MSCs from density centrifugation protocols

This method is designed to enrich for progenitor cells by centrifugation with a viscous medium that separates cells based upon their density. We developed a standardized protocol that isolates hMSCs from bone and marrow discarded during orthopedic surgery for hip and shoulder osteoarthrosis (Figure 1), with a yield of low-density mononuclear cells between 40 to 800 million per subject [27]. This fraction contains progenitors for both hematopoietic and mesenchymal cells, but the non-adherent hematopoietic progenitor cells can be removed to allow the adherent hMSCs to proliferate. Chang *et al.* compared the ability of two products, Ficoll and Percoll, to isolate MSCs from human bone marrow [28]. They demonstrated that the Ficoll methodology was superior in isolating cells with osteoblast lineage potential. A Ficoll-containing device was commercialized and shown to increase yield of hMSCs from marrow aspirate, compared with a manual procedure [29].

Currently, there are several commercial sources of hMSCs. The companies state that they use Ficoll or Histopaque protocols for their products.

2.3 Human MSCs from sorting with various antibodies

Fluorescence Activated Cell-Sorting (FACS) methods can be used to isolate a population of cells enriched for a phenotype on the basis of cell-surface epitopes. The sorting machine physically separates a mixture of cells into different containers, one cell at a time, based upon cell size and amount of attached fluorescent antibody. STRO-1 antibody is one of the first antibodies shown to enrich hMSCs, when it was found to bind with high affinity to an uncharacterized cell surface epitope expressed by hMSCs and erythrocytic cells [30]. In a direct comparison of protocols, we found similar biological properties in cell populations isolated with STRO-1 sorting and with the simpler Ficoll method [13]. Enrichment of

hMSCs by FACS can be achieved by combining antibodies [31–33]. Alternatively, immunomagnetic isolation methods can be used for smaller samples [34].

Although sorting techniques are helpful in separating MSCs from hematopoietic cells, the methods are approximations dependent on the specificity and binding affinity of antibodies and on compromises between threshold settings and recovery yields. Wagner et al. have warned that cell surface markers were not useful to discriminate between human MSCs from different sources and fibroblasts that have no differentiation potential [35]. A panel of 22 surface markers did not discern any differences in hMSCs from marrow, adipose tissue, and cord blood, but neither could it distinguish those hMSCs from dermal fibroblasts.

3. Vitamin D metabolism

Cholecalciferol (vitamin D_3) is synthesized from 7-dehydrocholesterol in the skin by exposure to ultraviolet light from the sun. Alternatively, vitamin D, in the form of ergocalciferol (vitamin D_2) from plants or vitamin D_3 (from animals), can be obtained from supplements or dietary sources. Biological activation of vitamin D, a two-step process, starts with carbon-25-hydroxylation to calcidiol (25-hydroxyvitamin D, 25(OH)D) primarily by the cytochrome enzymes CYP2R1 and CYP27A1, and subsequent carbon-1α-hydroxylation by CYP27B1/1α-hydroxylase [36]. The CYP24A1/24-hydroxylase regulates and inactivates 1α,25$(OH)_2$D or 25(OH)D in kidney, skin, and bone cells [37–39].

Cells that contain functional CYP27B1/1α-hydroxylase, such as kidney cells, can convert calcidiol (25(OH)D) to 1α,25$(OH)_2$D. Emerging data suggest that besides kidney cells, many other cells including bone cells have the ability to generate 1α,25$(OH)_2$D (Table 1). There are differences that have been reported for extra-renal biosynthesis of 1α,25$(OH)_2$D. For example, the regulation of 1α,25$(OH)_2D_3$ production in keratinocytes is more sensitive to inhibition by exogenous 1,25$(OH)_2$D than is the renal production of 1α,25$(OH)_2$D [40,41]. Thus, at normal circulating levels of free 1α,25$(OH)_2$D, production of that metabolite by epidermal cells may be more inhibited than is its production by renal tubules. Biosynthesis of 1α,25$(OH)_2D_3$ also occurs by cells in the immune system [42], such as human monocyte-derived dendritic cells [43], myelomonocytic cell line [44], cultured alveolar macrophages [45], and in human prostate and other cancer cells [46].

4. Effects of vitamin D on osteoblasts and hMSCs

Cells with the vitamin D receptor (VDR) can be targets of vitamin D action, depending on the receptor's affinity for the metabolites. 1,25$(OH)_2D_3$, is the most active metabolite, with high affinity for VDR. *In vivo*, 1,25$(OH)_2D_3$ acts to maintain normocalcemia by regulating intestinal calcium absorption and PTH activity. In addition to its role in calcium homeostasis, 1α,25$(OH)_2D_3$ affects cell proliferation, differentiation, and function [6]. The differentiation of hMSCs to osteoblasts is enhanced by 1α,25$(OH)_2D_3$ [7]. Our finding that both 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ stimulated osteoblastogenesis in hMSCs and, in some cases, to equal extents [8,9] suggests a potential autocrine/paracrine role of vitamin D metabolism in osteoblast differentiation. Similar ideas have been proposed for 25$(OH)D_3$ metabolism in regulating bone matrix formation by differentiated human osteoblasts [47].

The presence of CYP27B1 in extra-renal tissues especially in bone raises several important questions: (1) What are the effects of vitamin D on bone cells? (2) What is the amount of local 1,25$(OH)_2$D synthesis in bone? (3) Do hMSCs have all the vitamin D enzymes? (4) Is extra-renal CYP27B1 in hMSCs regulated in a manner like kidney cells? (5) Does age affect the relative expression of vitamin D enzymes? (6) What is the significance of marrow synthesis of 1α,25$(OH)_2$D?

5. Vitamin D metabolism in bone and in marrow

Human bone tissue includes osteoblasts, osteoclasts, and osteocytes; marrow contains hMSCs, adipocytes, and hematopoietic lineage cells. In 1981, Howard *et al.* reported that human osteoblasts activate and inactivate $25(OH)D_3$ [48], subsequently confirmed by others as being dependent on 1α-hydroxylase/CYP27B1 [39,48,49]. *In vitro*, $1\alpha,25(OH)_2D$ stimulates bone formation and matrix mineralization but also stimulates bone resorption under different circumstances. Because cells of the monocyte/macrophage lineage are known to express CYP27B1 and convert $25(OH)D_3$ into $1\alpha,25(OH)_2D$ [44], it was of interest whether differentiated osteoclasts would also do so. Kogawa *et al.* determined that osteoclasts that were derived *in vitro* from human peripheral blood monocytes (PBMCs) produced $1\alpha,25(OH)_2D$ from added $25(OH)D_3$ [50,51]. In addition, $25(OH)D_3$ significantly reduced bone resorption in other osteoclast models [50].

In addition to bone cells *per se*, Li *et al.* found that 1α-hydroxylase was expressed in adipose tissue, with measurable 1α-hydroxylase enzymatic activity in adipocytes (1.16 ± 0.07 pmol/mg protein/h) [52] comparable to that in other cell lines, including prostate (0.07 – 3.08) [46], vascular endothelial cells (0.32) [53], human bone marrow stromal cells (1.50 – 4.41) [12], and in human renal tissue (0.60) [54]. Thus, the combined presence of CYP27B1 and VDR in various skeletal cells and their progenitors indicate possible autocrine/paracrine roles for $25(OH)D_3$ to regulate bone cell growth, differentiation, and skeletal homeostasis.

The observation that both $1\alpha,25(OH)_2D_3$ and $25(OH)D_3$ stimulated osteoblastogenesis in the majority of hMSCs samples led to the discovery that hMSCs expressed the VDR and vitamin D hydroxylases, CYP27B1, CYP27A1, CYP24A1 [8], as well as CYP2R1 (unpublished). It is notable that the *in vitro* hydroxylation of $25(OH)D_3$ to $1\alpha,25(OH)_2D$ and the stimulation of osteoblastogenesis by $25(OH)D_3$ were blocked by ketoconazole, a cytochrome P450 inhibitor [8], and by knock-down of CYP27B1 with gene silencing technology [9]. These lines of evidence indicate that 1α-hydroxylation of $25(OH)D_3$ to $1\alpha,25(OH)_2D$ by CYP27B1 is necessary for the biological effects of $25(OH)D_3$. In the kidney, CYP24A1 converts vitamin D metabolites to water-soluble forms for excretion [37]. In hMSCs, basal expression of CYP24A1 is usually low and is upregulated by 1 nM $1\alpha,25(OH)_2D_3$ and by 1 μM $25(OH)D_3$ [9]. *In vitro* studies [8] showed that CYP27B1 in hMSCs is regulated by substrate feed-forward stimulation and product feedback inhibition (Figure 2). There was dose-dependent upregulation of CYP27B1 by $25(OH)D_3$ and its downregulation by $1\alpha,25(OH)_2D_3$. Further, high doses of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ upregulated CYP24A1 in hMSCs. Another important way that regulation of CYP27B1 in hMSCs is similar to that in renal cells is in upregulation by PTH [12]. Thus, hMSCs can regulate the concentration of $1\alpha,25(OH)_2D$ by both the rates of its production and inactivation [8,9].

6. Effects of age on osteoblastogenesis and on vitamin D metabolism in hMSCs

A decline in the numbers of or differentiation potential of stem cell populations in adult organs can contribute to human aging and age-related disease such as arthrosis, tendinosis, and osteoporosis [55]. There are many properties of hMSCs that are dramatically affected by the age of the subject (Table 2). The basal differentiation of hMSCs to osteoblasts declines with age, as shown by our group [10–13] and by others [24]. In addition, we showed that there are age-related intrinsic changes in hMSCs associated with decreased proliferation and differentiation potential [13]. There is also an age-related decline in stimulation of osteoblastogenesis by $1\alpha,25(OH)_2D_3$ [18].

Previously, we found that in two-thirds of hMSCs from elders osteoblastogenesis was stimulated by both $25OHD_3$ and $1\alpha,25(OH)_2D_3$ [8]. Indeed, in hMSCs, there was an age-related decline in expression and activity of CYP27B1, in biosynthesis of $1\alpha,25(OH)_2D$, and in stimulation of osteoblastogenesis by $25(OH)D_3$ [12]. Expression of CYP27B1 in MSCs from subjects older than 55 years of age was 56% of that in MSCs from subjects younger than 50 years of age. In studies with rats, Ishida M *et al.* showed conversion of $25OHD_3$ to $1\alpha,25(OH)_2D$ in the kidney was decreased with age of the animal [56].

7. Rejuvenation of osteoblastogenesis and vitamin D metabolism in hMSCs from elders

Findings from our lines of research on PTH effects on hMSCs and on vitamin D metabolism in hMSC converged in an endeavor to rejuvenate osteoblast differentiation in hMSCs from elders. PTH is an important stimulus of CYP27B1 transcription and activity in the kidney [57] and likewise dose-dependently upregulated CYP27B1 expression and biosynthesis of $1\alpha,25(OH)_2D$ in hMSCs [57,58]. To date, other than hMSCs, there is no evidence that PTH upregulates CYP27B1 in extra-renal cells or tissue. In contrast to the bone-resorptive effects from chronically elevated PTH, it is clear that intermittent administration of low doses of PTH is osteoanabolic [59]. PTH is also known to prevent osteoblast apoptosis [60]. PTH stimulates osteoblast differentiation of hMSCs, but the responsiveness to low doses declines with the age of the subject [11]. Several model systems show that the actions of PTH to stimulate bone formation are mediated by skeletal insulin-like growth factor-I (IGF-I) [61,62]. In hMSCs, PTH induced IGF-I and IGF-signaling (Figure 3); moreover, experiments with small molecule signaling inhibitors revealed that PTH induction of CYP27B1 was mediated directly through CREB and indirectly by IGF-I signaling [12]. Not only does IGF-I stimulate osteoblast differentiation in hMSCs, it stimulates biosynthesis of $1\alpha,25(OH)_2D$ in synergy with $25OHD_3$ [9]. Finding that with age there is a decline in PTH/PTHrP receptor (PTHR1) expression and consequent decline in PTH signaling of CREB and β-catenin helps to explain why PTH stimulation of osteoblastogenesis decreases with age of the subject from whom the hMSCs were obtained (Table 2). As proof-of-principle, we showed that dexamethasone upregulated PTHR1 and restored the effects of PTH on hMSCs [11]. More recent evidence that $25(OH)D_3$ upregulates PTHR1 [63] indicates further beneficial interactions between PTH and vitamin D metabolism in hMSCs.

In sum, hMSCs from elders are resistant to stimulation of osteoblastogenesis by $1\alpha,25(OH)_2D_3$ [18], by $25(OH)D_3$ [12], and by PTH [11] (Table 2). That PTH upregulated CYP27B1 in a dose-dependent manner even in hMSCs from elders [12] suggested possibile synergy between PTH and $25OHD_3$. In the first series of studies, 12-hour pre-treatment of hMSCs from elders with PTH(1-34) resulted in biosynthesis of $1\alpha,25(OH)_2D$ equivalent to that in hMSCS from young subjects [12]. The increases in biosynthesis and CYP27B1 expression were mediated through CREB and IGF-I pathways. Accordingly, 12-hour pre-treatment with PTH1-34 provided hMSCs from elders with responsiveness to the pro-osteoblastogenic effects of $25(OH)D_3$, with increased osteoblast differentiation. In the second series of studies, hMSCs were treated simultaneously and continuously with PTH and $25(OH)D_3$ [63]. Osteoblast differentiation was significantly stimulated 170% by PTH1-34 (100 nM) and 280% by $25(OH)D_3$, but by 650% with simultaneous combination of PTH1-34 and $25(OH)D_3$. Not only was the synergy due to upregulation of CYP27B1, but in addition, PTH upregulated the VDR, and $25OHD_3$ upregulated PTHR1. Further, the synergistic effects on osteoblast differentiation were blocked in the presence of a small molecule inhibitor of histone deacetylase, Scriptaid$^{(R)}$ [63]. Thus, epigenetic regulation may be central to rejuvenating osteoblastogenesis in hMSCs from elders.

8. Clinical implications

Loss of bone mass associated with human skeletal aging can be explained in part by the age-related decline in *in vitro* osteoblast differentiation [10–13,24] and by the age-related increase in *in vitro* osteoclast differentiation [64] with bone cell progenitors from bone marrow. Finding vitamin D-hydroxylases and regulated activity in hMSCs provides support for the hypothesis of an autocrine/paracrine role of vitamin D metabolism in human osteoblast differentiation. Analysis of a cohort of subjects whose hMSCs were used for osteoblast differentiation experiments showed that several clinical attributes were significantly associated with *in vitro* behavior. Stimulation of osteoblastogenesis by α, $25(OH)_2D_3$ was reduced in hMSCs from subjects with advanced age, with low serum 25(OH)D levels, and with low estimated glomerular filtration rate (eGFR) [18]. Those observations suggest that it is clinically important to correct vitamin D-deficiency, especially in elders, in order to enhance bone cell differentiation and bone formation. There is an ongoing controversy about the optimal level of serum 25(OH)D to ensure skeletal health [65–67] and circulating 25(OH)D may be very important to support non-renal production of $1\alpha,25(OH)_2D$. It is also controversial whether to monitor/correct serum 25(OH)D in patients with chronic kidney disease (CKD) [68]. We recently reported that all three metabolites, D_3, $25(OH)D_3$, and $1\alpha,25(OH)_2D_3$, stimulated *in vitro* osteoblastogenesis with hMSCs from a subject who had been undergoing hemodialysis for 2+ years as well as an age/gender-matched control subject [69]. We propose that osteoblastic bone formation in CKD patients may not be optimal unless there is sufficient serum 25(OH)D substrate for MSCs to synthesize and respond to local $1\alpha,25(OH)_2D$.

The synergistic and reciprocal interactions of $25(OH)D_3$ and PTH we found in hMSCs may be of relevance to anabolic therapy for osteoporosis. A synthetic form of PTH, teriparatide, has been approved by the US FDA for osteoporosis because of its action to stimulate bone formation [59]. There is some information about the importance of vitamin D status in teriparatide therapy. Samadfam *et al.* showed that intermittently administered PTH increased bone density in 1α-hydroxylase$^{-/-}$ mice, but that there was a greater effect in mice with an active $1\alpha,25(OH)_2D$-synthesizing system [70]. They concluded that PTH and endogenous vitamin D may interact to optimize osteoblast differentiation. This concept is also supported by an analysis of factors associated with heterogeneity in skeletal response to full-length PTH therapy for osteoporosis [71]. Of all the variables tested, only an increase in serum 1α, $25(OH)_2D$ explained larger gains in bone density in response to PTH. There are data from a PTH trial (Fracture Prevention Trial, PFT) that there was no difference in teriparatide anti-fracture efficacy and bone markers between subjects that were vitamin D- sufficient or insufficient [72], but that trial excluded subjects with serum 25(OH)D <10 ng/mL or with elevated PTH levels that is a secondary response to low 25(OH)D levels. In other words, they compared groups with mean serum 25(OH)D of 24 vs. 38 ng/mL, i.e. “Insufficiency or Sufficiency”. A subsequent analysis of that trial and the Male Osteoporosis Trial, notably all with baseline mean serum 25(OH)D levels of 30–32, i.e. sufficiency, indicated that PTH significantly increased $1\alpha,25(OH)_2D$ levels and lowered serum 25(OH)D [73]. The authors concluded that conversion of 25(OH)D to $1\alpha,25(OH)_2D$ may contribute to the biological effects of teriparatide and that the PTH-induced reduction in serum 25(OH)D may be of clinical importance and should be monitored and corrected, as needed.

There are a number of clinical trials showing efficacy of PTH in elders with osteoporosis, but none directly compared young and old subjects. One analysis [74], for example, compared groups ⟨/⟩75-years with a mean age of 66.5 vs. 78.3 years. A recent meta-regression analysis of 15 randomized, placebo-controlled trials showed that PTH-induced increments in spine bone density were reduced with increasing age [75]. This suggest that a

different clinical regimen of PTH & vitamin D may be needed to optimize their synergy to stimulate bone formation in elders, especially those with osteoporosis or healing disorders.

9. Conclusions

There has been considerable progress in our understanding of vitamin D metabolism and its biological activities. The discoveries of CYP27B1 in a wide variety of extra-renal tissues provide plausible mechanisms for local function of 1α,25$(OH)_2$D, especially in the bone microenvironment. This may explain the clinical consequences of vitamin D-deficiency in the elderly and the marked age-related increase in risk for hip fracture. We summarized features of hMSCs, compared different methods to isolate them, and described the effects of vitamin D on them, and regulation of vitamin D metabolism in them. The striking effects of age on osteoblast differentiation and on responsiveness to 1α,25$(OH)_2$D, 25(OH)D, and PTH suggest potential approaches for rejuvenation. Vitamin D deficiency is common in elders and is associated with impaired calcium absorption and secondary hyperparathyroidism which, in turn, stimulates bone resorption and bone loss. Impaired renal production of 1α,25$(OH)_2$D is seen in CKD, which also increases with age. Discarded orthopedic tissues are precious resources that can provide new information about bone cell differentiation when obtained with consent from well-characterized subjects. Using hMSCs from elders and from subjects with vitamin D-deficiency or with poor renal status allows for new insights into pathophysiological mechanisms of aging and skeletal disorders.

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ABBREVIATIONS

1α	25$(OH)_2$D, 1α,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
ALP	alkaline phosphatase activity
CFU-F	colony-forming unit-fibroblasts
eGFR	estimated glomerular filtration rate
FACS	fluorescence activated cell sorting
FBS-HI	heat-inactivated fetal bovine serum
hMSCs	human marrow stromal cells
IGF-I	insulin-like growth factor-I
PBMCs	peripheral blood monocytes
PTH	parathyroid hormone
PTHR1	PTH/PTHrP receptor
VDR	vitamin D receptor

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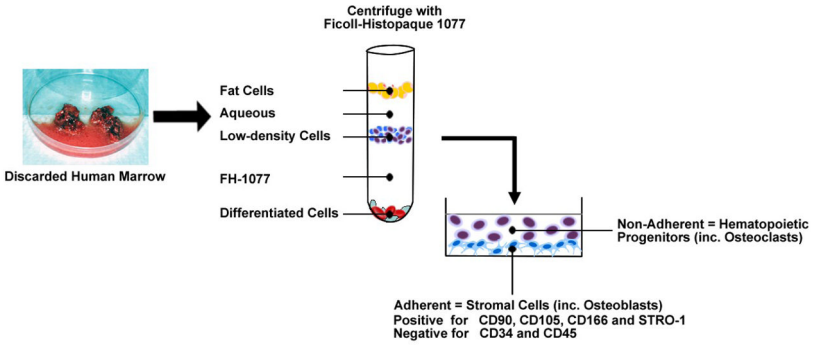

Figure 1. Ficoll isolation method for hMSCs

Discarded human bone and marrow tissues are minced in PBS with EDTA to release cells. After passage through a sieve to remove bone particles, cells are collected by centrifugation and resuspension in PBS. Ficoll-Histopaque 1077 (FH-1077, a medium with density of 1.077 g/mL) is carefully added beneath the aqueous cell suspension. Centrifugation separates marrow cells into three major fractions. The fat cells float at the top, and the mature, differentiated erythrocytes and leukocytes sediment at the bottom. Low-density undifferentiated cells accumulate at the interface between the aqueous and FH-1077 layers. This fraction contains progenitors for both hematopoietic and mesenchymal cells. They are collected, washed, and seeded into tissue culture dishes. After 2–3 days incubation, the non-adherent hematopoietic lineage cells, which includes osteoclast progenitors, are removed, allowing the adherent stromal cells to expand. The enriched adherent cells are called hMSCs, which are positive for CD90, CD105, CD166, and STRO-1, and negative for CD34 and CD45.

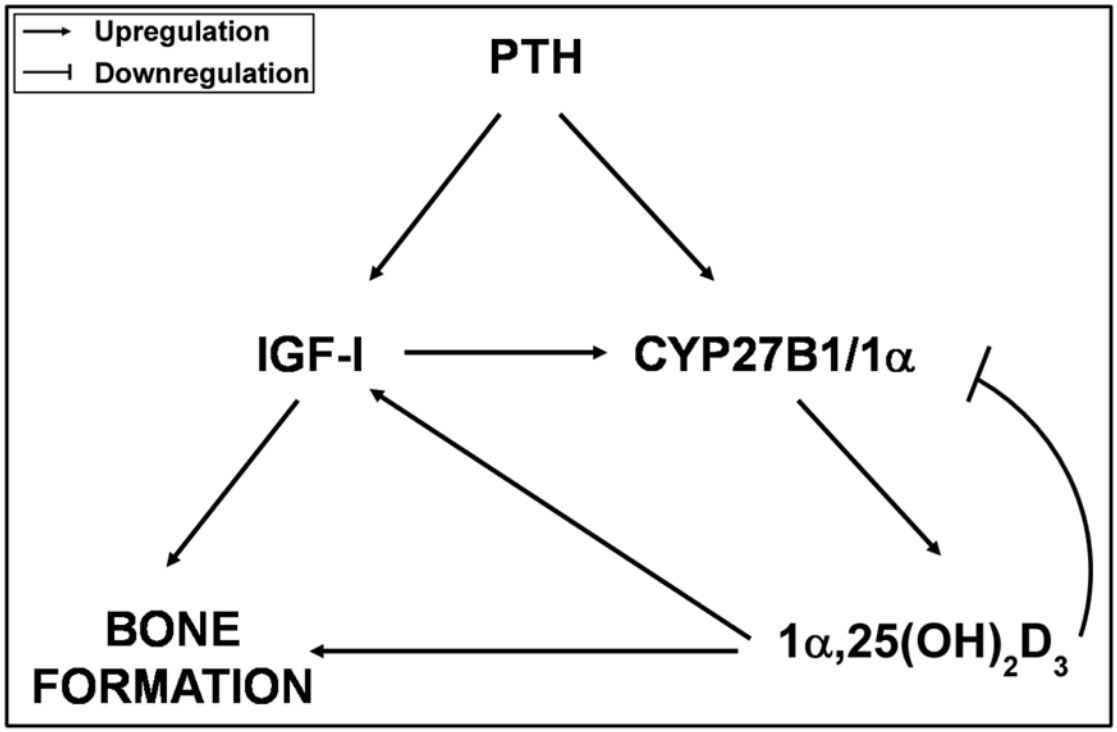

Figure 2. Vitamin D metabolism, regulation, and action in hMSCs

Vitamin D_3 (cholecalciferol) is hydroxylated at carbon-25 by CYP27A1 to $25(OH)D_3$ (calcidiol), which downregulates CYP27A1 and upregulates CYP27B1 and, at higher concentrations, upregulates CYP24A1. In a dose-dependent manner, $25(OH)D_3$ is hydroxylated at carbon-1α by CYP27B1 to $1\alpha,25(OH)_2D_3$ (calcitriol). Higher concentrations of $1\alpha,25(OH)_2D_3$ downregulate CYP27B1 and upregulate CYP24A1. Both $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ upregulate IGF-I which mediates their stimulation of osteoblast differentiation in hMSCs. These findings indicate an autocrine/paracrine role for vitamin D metabolism in human osteoblastogenesis in hMSCs.

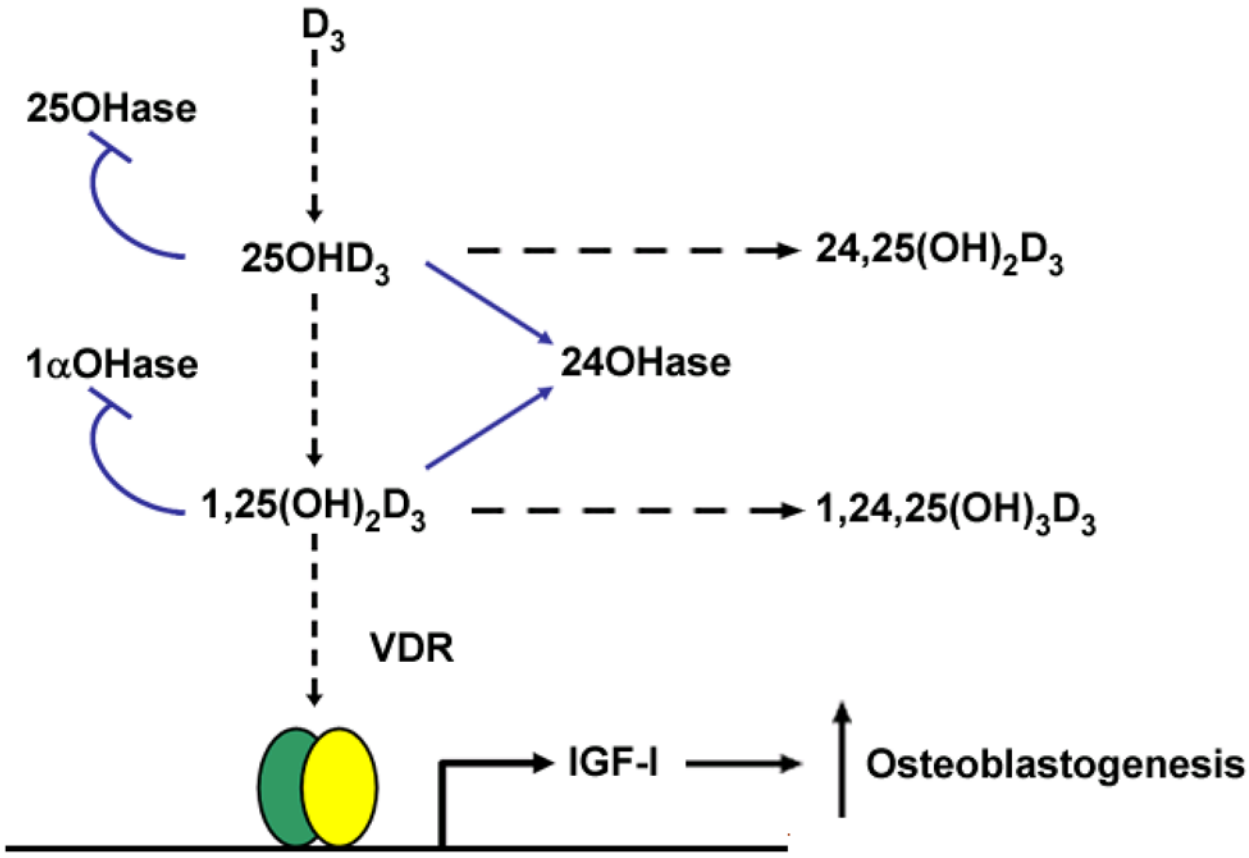

Figure 3. Summary of the interactions among PTH, IGF-I, and 1,25(OH)$_2$D$_3$ on CYP27B1/1α-hydroxylase expression and bone formation in hMSCs

PTH upregulates IGF-I [12] which stimulates bone formation in hMSCs [8]. PTH upregulates CYP27B1 expression and stimulates 1α,25(OH)$_2$D$_3$ production [12], and at higher concentration, 1α,25(OH)$_2$D$_3$ downregulates CYP27B1 [8]. In addition, IGF-I upregulates CYP27B1 expression and stimulates 1α,25(OH)$_2$D$_3$ biosynthesis [8]. 1α,25(OH)$_2$D$_3$ induces IGF-I and bone formation in hMSCs [8].

Table 1

Expression of CYP27B1 in hMSCs, Kidney, and Other Tissues/Cells

Tissue	Cell type	RNA	Protein	Activity	References
Bone Marrow	hMSCs	yes	WB	1	[8,9,12]
Kidney	Human kidney	yes	yes	2	[76]
Bone	Bone biopsy	yes	N.D.	N.D.	[39]
	Human primary ostoblast	yes	N.D.	1	[48]
	Osteoclast RAW 264.7 cells	yes	IF	1	[39,50,51]
Human osteoblast-like cells	MG-63	yes	N.D.	1	[39]
	SAOS-2	yes	N.D.	1	[47]
	G-292	yes	N.D.	1	[47]
	HOS	yes	IF	1	[49]
	SV-HFO	yes	WB	1	[39]
Human keratinocytes	Primary human keratinocytes HPK1A cell line	yes	ICC	3	[41,77]
Prostate	Primary normal prostatic epithelial cells	yes	WB	2	[46]
	Cancer-derived prostatic epithelial cells	yes	WB	2	[46]
	Benign prostatic hyperplasia	yes	N.D.	2	[46]
Human prostate cancer cell lines	LNcaP	yes	WB	2	[46]
	PC-3	yes	WB	2	[46]
Colon	Human normal and malignant Colon mucosa and colon tumors	yes	WB IF IHC	N.D.	[42]
Immune system Blood cells	Peripheral blood Mononuclear cells Dendritic cells Macrophages	yes	ICC	2	[42–45]
Human colon cell line	Caco-2	yes	N.D.	3	[78]
	BBe	yes	N.D.	3	[78]
Adipocyte	3T3-L1	yes	N.D.	2	[52]
Others	human umbilical vein endothelial cells	yes	WB IHC	2	[53]
	Brain (cerebellum/purkinje cells)	yes	IHC	N.D.	[42]
	Epidermis (statum basalis)	yes	IHC	N.D.	[42]
	Parathyroid glands	yes	IHC	N.D.	[79]

Tissue	Cell type	RNA	Protein	Activity	References
	Pancreatic islets	yes	IHC WB ICC	N.D.	[42]
	Mammary, breast (cancer or normal)	yes	IHC	N.D.	[80]
	Ovary	yes	IHC	2	[81]
	Human trophoblast cells	yes	N.D.	N.D.	[82]
	Human Vascular Smooth Muscle Cells	yes	N.D.	4	[83]
	Lymph node Spleen cells	yes	IHC	2	[42,84]
	Human small cell lung cancer cell line NCI H82	N.D.	N.D.	3	[85]

RNA: yes means by *in situ* hybridization, RT-PCR, or real-time RT-PCR;

Protein: detected by Western immonblot (WB), Immunohistochemistry (IHC) or Immuno Fluorescence (IF);

Activity: 1. by radioimmunoassay or enzyme-linked immunosorbent assay kits from IDS; 2. by thin-layer chromatography; 3. by high-performance liquid chromatography; 4. by radioimmunoassay kit from DiaSorin.

N.D.: Not detected.

Table 2

Effects of Age on Human Marrow Stromal (Mesenchymal Stem) Cells

Decrease with Age	Increase with Age
Proliferation potential Osteoblast differentiation Stimulation of osteoblastogenesis by 1α,25$(OH)_2D_3$, 25$(OH)D_3$, and PTH Constitutive expression of CYP27B1 and biosynthesis of 1α,25$(OH)_2D_3$ Constitutive expression PTHR1 PTH signaling of CREB and β-catenin Constitutive expression of anti- osteoclastogenic Osteoprotegerin	Cells positive for Senescence Associated-β-galactosidase Apoptotic cells Constitutive expression of *P53, P21, BAX* Support of osteoclastogensis by increased constitutive expression of pro-osteoclastogenic RANKL, IL-6, IL- 11