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# EFFECTS OF $1,25(OH)_2D_3$ AND VITAMIN D ANALOGS ON DEVELOPMENTAL CONTROL OF CELL GROWTH AND TISSUE-SPECIFIC GENE EXPRESSION DURING OSTEOBLAST DIFFERENTIATION

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**ABSTRACT**

Cultured normal diploid osteoblasts provide a model for identifying selective effects of vitamin D and $1,25(OH)_2D_3$ analogs on expression of cell growth and tissue-specific genes that are dependent on the differentiated state of the bone cell. Transcription of the bone-specific osteocalcin gene is responsive to the vitamin D receptor complex together with other cellular signaling factors.

Vitamin D influences proliferation and the phenotypic properties of a broad spectrum of normal and tumor cells (osteoblasts, adipocytes, keratinocytes, etc.) by modulating expression of cell growth and tissue-related genes (reviewed in [1-2]) . These observations provide a basis for postulating effects of vitamin D on cells and tissues whereby genes associated with proliferation and differentiation are selectively responsive to the hormone at specific developmental stages resulting in consequent effects on mechanisms that support progressive differentiation and maintenance of cell phenotypes.

The anabolic and catabolic effects of vitamin D on bone extracellular matrix and mineral metabolism have been longstanding and well documented. At the cellular and molecular levels, this may in part reflect differential effects of vitamin D on expression of cell growth and bone specific genes as a function of progressive development of the osteoblast phenotype. Understanding mechanisms by which the hormone can regulate gene expression at specific stages of osteoblast differentiation can therefore provide insight into developmental control.

## Cell Cycle and Tissue-Specific Genes Expressed During Osteoblast Differentiation are Developmentally Responsive to Vitamin D.

Insight into vitamin D mediated regulatory mechanisms operative during osteoblast differentiation is provided by the sequential expression of genes that supports progressive osteoblast phenotype development (Figure 1). Primary cultures of calvarial-derived rat osteoblasts develop a mineralized extracellular matrix with a bone tissue-like organization analogous to osteoblast differentiation in vivo [2-4]. Genes supporting cell cycle progression (e.g. histone), cell growth control (e.g. proto-oncogene encoded nuclear proteins) and

extracellular matrix biosynthesis (e.g. collagen, fibronectin) are expressed during the initial proliferation period. At the first key transition point, when genes associated with the proliferation process are down-regulated (Figure 2A), genes related to maturation and organization of the bone extracellular matrix become induced (e.g. alkaline phosphatase, matrix Gla protein (MGP)). Subsequently, at the second transition point, matrix mineralization signals upregulation of a unique set of genes (e.g. osteocalcin and osteopontin) together with genes involved with maintenance of structural and functional properties of the fully differentiated bone cell (e.g. collagenase)[5]. The transitions have been experimentally defined as restriction points during osteoblast differentiation to which developmental expression of genes can proceed but cannot pass without additional signaling mechanisms. These observations are the basis of cellular competency for progression toward development of a mature osteoblast phenotype and permit identification of vitamin D-mediated signaling pathways operative at the developmental transition points by which genes are selectively activated and/or suppressed.

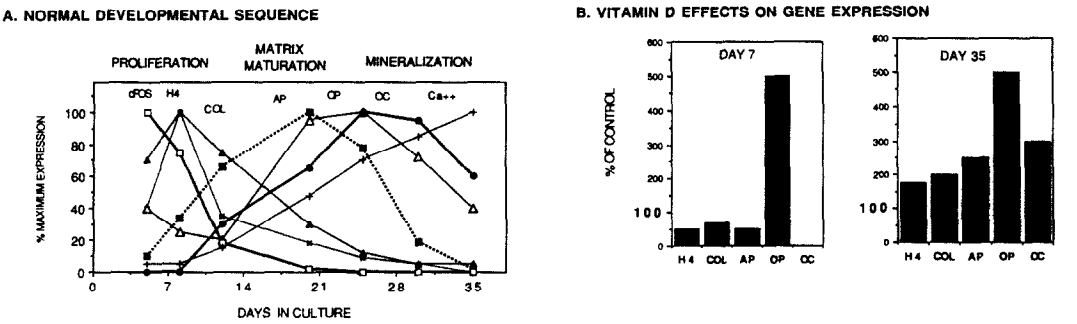


**Fig. 1A. Temporal expression of cell growth and bone cell-related genes during development of the osteoblast phenotype.** Cellular RNA was isolated at the times indicated from primary cultures of normal diploid calvarial-derived rat osteoblasts. Steady state levels of gene transcripts were quantitated by Northern blot analysis. Three periods of gene expression are indicated with expression of representative genes: proliferation--H4 histone, c-fos and Type collagen (COL); matrix maturation--alkaline phosphatase (AP); mineralization--osteopontin (OP) and osteocalcin (OC). Calcium (CA++) accumulation is indicated.

**Fig. 1B. Effects of vitamin D on gene expression during the proliferation and extracellular matrix mineralization periods of osteoblast differentiation.** Osteoblast cultures were treated with $1,25(OH)_2D_3$ ($10^{-7}$) for 48 hrs on day 7 or 35. Cellular levels of H4 histone (H4), Type I collagen (COL), alkaline phosphatase (AP), osteopontin (OP) and osteocalcin (OC) were quantitated by Northern blot analysis.

Windows of competency for responsiveness to vitamin D during the developmental stages of osteoblast differentiation are reflected at the tissue level by preferential effects on expression of a series of vitamin D responsive genes. Vitamin D treated osteoblasts during the proliferation period alters levels of gene expression to reflect a profile characteristic of post-proliferative, mature bone cells; while at a later stage can promote expression of genes that reinitiates osteoblast activity (Figure 1B). For example, in proliferating osteoblasts, vitamin D down-regulates cell growth genes and genes associated with

biosynthesis and organization of bone extracellular matrix (e.g. collagen, alkaline phosphatase) while upregulating genes associated with the mature osteoblast phenotype (e.g. osteopontin and MGP). In contrast, in mature osteoblasts within a mineralized extracellular matrix, vitamin D stimulates expression of genes transcribed during growth and extracellular matrix formation. Vitamin D treatment, initiated during the early proliferation period and maintained throughout the three weeks of culture, blocks the developmental sequence of gene expression and osteoblast differentiation (Figure 2). As a consequence, effects of vitamin D are highly dependent on the differentiated stage of the bone cell.

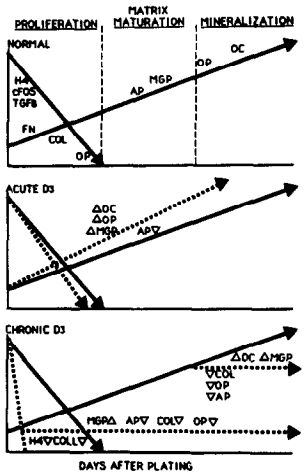


**Fig. 2. Model of the reciprocal relationship between proliferation and differentiation in normal diploid cells during the rat osteoblast developmental sequence (Top Panel) indicating effects of acute (Middle Panel) and chronic (Lower Panel) treatment with vitamin D.** The proliferation-differentiation relationships are schematically illustrated as arrows representing changes in expression of cell cycle and cell growth regulated genes (proliferation arrow) and genes associated with the maturation (differentiation arrow) of the osteoblast phenotype as the extracellular matrix develops and mineralizes in normal diploid cell cultures (Top Panel). The three periods of the osteoblast developmental sequence are designated with broken vertical lines (proliferation, extracellular matrix maturation and extracellular matrix mineralization). These broken lines indicate two experimentally established principal transition points in the developmental sequence exhibited by normal diploid osteoblasts during progressive acquisition of the bone cell phenotype. The Middle Panel schematically illustrates acceleration of the relationship between growth and differentiation by short term treatment with vitamin D (48 hrs). The hormone accelerates the onset of expression of cell growth and differentiation-related genes (broken arrows). The inhibitory effects of continuous vitamin D treatment initiated on day 5 or day 20 are expression of genes associated with development the osteoblast phenotype are indicated by broken arrows. Histone (H4), Type I collagen (COL), fibronectin (FN), alkaline phosphatase (AP), matrix gla protein (MGP), osteopontin (OP), osteocalcin (OC), c-fos and TGFB.

**Organization of the Bone Specific Osteocalcin Gene Promoter Supports the Integration of Physiological Regulatory Signals Mediating Developmental Competency for Vitamin D-Mediated Transcriptional Control.**

The bone specific osteocalcin gene is transcriptionally controlled and developmentally modified with respect to competency for responsiveness to Vitamin D within the context of a series of physiological regulatory signals during osteoblast differentiation. Vitamin D acts principally as an enhancer of osteocalcin gene transcription and the magnitude of enhancement is dependent on basal activity. This is reflected by the inability of vitamin D to induce transcription of osteocalcin in proliferating osteoblasts in the absence of basal expression, but to upregulate osteocalcin gene transcription only post-proliferatively in osteoblasts undergoing extracellular matrix mineralization when basal transcription is ongoing. Transcription is controlled by a modularly organized promoter with positive and negative

regulatory elements that interact in a sequence-specific manner with a diverse series of physiological mediators (Figure 3). Overlapping protein binding domains with sequence-specific recognition contribute to diversity of developmentally regulated responsiveness to vitamin D where protein/DNA together with protein/protein interactions influence transcription. An AP-1 site resides within the osteocalcin gene VDRE and overlaps one of the two steroid motifs characterizing this responsive element (Figure 3) [6-8]. Additionally, sequences which bind nuclear matrix protein complexes, NMP-1 and NMP-2, are found within this domain [9]. Osteopontin is the only other vitamin D regulated osteoblast-synthesized protein for which the VDRE has been identified [10] and contains two direct repeats, AGGTTCACGAGGTTCA.

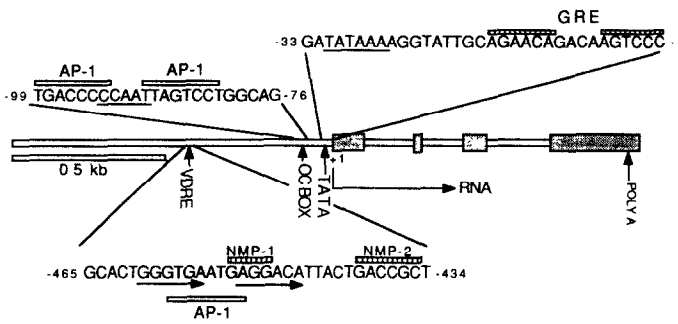


**Fig. 3. Structural organization of the rat osteocalcin gene indicating promoter sequences functioning as physiological regulatory elements.** Designated are the vitamin D responsive element (VDRE), the osteocalcin box containing a CCAAT motif as a central core and the TATA motif with a contiguous glucocorticoid-responsive element (GRE). Within the OC Box and VDRE elements are AP-1 sites which bind the oncogene-encoded Fos/Jun protein complex. Also indicated are two nuclear matrix protein binding sites designated NMP-1 and NMP-2. Both the OC Box and the NMP-2 site exhibit interactions with tissue-specific and gene specific transactivation factors. The intron and exon (shaded boxes) organization is illustrated.

A heterogenous series of tissue-specific and developmental stage-specific factors interact with primary DNA binding proteins contributing to regulatory specificity. Transcriptional control of the osteocalcin gene during differentiation also includes the biosynthesis and recruitment of transcription factors and the extent to which sequence-specific DNA binding proteins are phosphorylated. While complexity of an emerging repertoire of regulatory factors with potential for influencing activity is rapidly increasing, the flexibility for responsiveness to both transient and longterm transcriptional requirements is becoming apparent. It is evident that a molecular basis for selective developmental responsiveness to vitamin D by various genes may reside in subtleties of the vitamin D responsive element (VDRE) organization and the complement of receptor accessory factors available for participating in binding activity. Combinations or modifications of accessory factors with the vitamin D receptor may contribute to pleiotropic transcriptional control where either positive or negative activity is observed. For example, variations in the vitamin D receptor complex at the osteocalcin gene VDRE are found between non-expressing proliferative osteoblasts and differentiated cells or in the transformed ROS 17/2.8 line [11].

The overlap of the vitamin D receptor binding sequences by an AP-1 element which binds

the oncogene-encoded Fos and Jun proteins may relate to developmentally regulated competency for vitamin D mediated transcriptional enhancement. Experimental results that support the occupancy by Fos-Jun protein complexes at the VDRE as a potential molecular mechanism to account for the absence of osteocalcin gene expression in proliferating osteoblasts includes: 1) expression of c-fos and c-jun primarily during the proliferation period of the osteoblast developmental sequence; 2) AP-1 binding activity primarily in proliferating osteoblasts with a dramatic decrease post-proliferatively when osteocalcin gene transcription is initiated; 3) the absence of protein/DNA contacts at the residues within the AP-1 consensus sequences of the VDRE when the osteocalcin gene is actively transcribed; and 4) mutational analysis supporting mutually exclusive binding of the vitamin D receptor and the Fos-Jun heterodimeric complex at the VDRE (reviewed in [8]). Interestingly, the presence of two AP-1 sites overlapping the OC box which serves as the primary basal transcriptional regulatory element of the osteocalcin gene supports the concept of coordinate occupancy by Fos-Jun protein complexes at the VDRE and OC box as a basis for developmentally regulated, coordinate suppression of both basal and vitamin D-enhanced control of osteocalcin transcription.

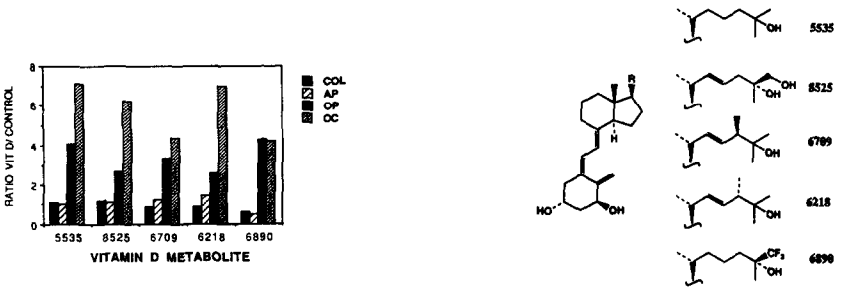


**Fig. 4. Effects of $1,25(OH)_2D_3$ (5535) and four vitamin D analogs (8525, 6709, 6218 and 6890) on development of the osteoblast phenotype.** Primary cultures of normal diploid calvarial-derived rat osteoblasts were treated with $1,25(OH)_2D_3$ or the four vitamin D analogs for 48 hrs beginning on day 16 in culture (post-proliferative extracellular matrix maturation period). Cellular levels of mRNA transcripts from the Type I collagen (COL), alkaline phosphatase (AP), osteopontin (OP) and osteocalcin (OC) genes were quantitated by Northern blot analysis. Structure of $1,25(OH)_2D_3$ and the four vitamin D analogs are shown.

**Conclusion: Vitamin D Analogs Can Selectively Influence Expression of Post-Proliferative Genes Supporting Development and Maintenance of the Mature Osteoblast Phenotype.**

The developmental sequence of gene expression supporting osteoblast growth and differentiation serves as a basis for evaluating the potential of vitamin D to therapeutically modulate the structural and metabolic status of bone. Here, one of the principal objectives is to maximize bone formation while minimizing metabolic calcium imbalances. This may be approached by the design and application of vitamin D analogs which selectively promote osteoblast growth and differentiation. Figure 4 indicates that analogs of vitamin D increase expression of collagen and alkaline phosphatase, while others decrease

expression of these genes. Osteocalcin and osteopontin, two genes associated with the mature differentiated state of the osteoblast, and may be related to bone turnover [5], are stimulated to different extents by the various vitamin D metabolites. Potential clinical applications for vitamin D analogs include, but are not restricted to, treatment of skeletal diseases such as osteoporosis and management of osteosarcomas.

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**References**

1. Lowe, K.E.; Maiyar, A.C.; Norman, A.W. *Crit. Rev. Eukaryotic Gene Exp.*, **1992**, 2, 65.

2. Lian, J.B.; Stein, G.S. *Nutrition and Gene Expression*, Hargrove; ed.; CRC Press: Boca Raton, **1992**; pp.391-429.

3. Bellows, C.G.; Aubin, J.E.; Heersche, H.N.M.; Antosz, M.E. *Calcif. Tissue Int.*, **1986**, 38, 143.

4. Owen, T.A.; Aronow, M.; Shalhoub, V.; Barone, L.M.; Wilming, L.; Tassinari, M.S.; Kennedy, M.B.; Pockwinse, S.; Lian, J.B.; Stein, G.S. *J. Cell. Physiol.*, **1990**, 143, 420.

5. Shalhoub, V; Conlon, D.M.; Tassinari, M.; Quinn, C.; Partridge, N.; Stein, G.S.; Lian, J.B. *J. Cell. Biochem.*, **1992**,425.

6. Demay, M.B.; Kiernan, M.S.; DeLuca, H.F.; Kronenberg, H.M. *Mol. Endocrin.*, **1992**, 557.

7. Terpening, C.M.; Haussler, C.A.; Jurutka, P.W.; Galligan, M.A.; Komm, B.S.; Haussler, M.R. *Mol. Endocrin.*, **1991**, 5, 373.

8. Owen, T.A.; Bortell, R.; Shalhoub, V.; Heinrichs, A.; Stein, J.L.; Stein, G.S.; Lian, J.B. *Proc. Natl. Acad. Sci. USA*, **1993**, in press.

9. Bidwell, J.P.; van Wijnen, A.J.; Fey, E.G.,; Dworetzky, S.; Penman, S.; Stein, J.L.; Lian, J.B.; Stein, G.S. *Proc. Natl. Acad. Sci. USA*, **1993**, in press.

10. Noda, M.; Vogel, R.L.; Craig, A.M.; Prahl, J.; DeLuca, H.F.; Denhardt, D.T. *Proc. Natl. Acad. Sci. USA*, **1990**, 87, 9995.

11. Bortell, R.; Owen, T.A.; Shalhoub, V.; Heinrichs, A.; Aronow, M.A.; Rochette-Egly, C.; Lutz, Y.; Stein, J.L.; Lian, J.B.; Stein, G.S. *Proc. Natl. Acad. Sci. USA*, **1993**, in press.