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Microarray Analysis of 1,25(OH)$_2$D$_3$ Regulated Gene Expression in Human Primary Osteoblasts

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ABSTRACT

Though extensive studies have been conducted, questions regarding the molecular effectors and pathways underlying the regulatory role of 1,25(OH)$_2$D$_3$ in human osteoblasts other than cell differentiation and matrix protein production remain unanswered. This study aims to identify genes and pathways that are modulated by 1,25(OH)$_2$D$_3$ treatment in human osteoblasts. Primary osteoblast cultures obtained from human bone tissue samples were treated with 1,25(OH)$_2$D$_3$ (10^{-7} M) for 24 h and their transcritptomes were profiled by microarray analysis using the Affymetrix GeneChip®. Statistical analysis was conducted to identify genes whose expression is significantly modulated following 1,25(OH)$_2$D$_3$ treatment. One hundred and fifty-eight genes were found to be differentially expressed. Of these, 136 were upregulated, indicating clear transcriptional activation by 1,25(OH)$_2$D$_3$. Biostatistical evaluation of microarray data by Ingenuity Pathways Analysis (IPA) revealed a relevant modulation of genes involved in vitamin D metabolism (CYP24), immune functions (CD14), neurotransmitter transporters (SLC1A1, SLC22A3), and coagulation [thrombomodulin (THBD), tissue plasminogen activator (PLAT), endothelial protein C receptor (PROCR), thrombin receptor (F2R)]. We identified a restricted number of highly regulated genes and confirmed their differential expression by real-time quantitative PCR (RT qPCR). The present genome-wide microarray analysis on 1,25(OH)$_2$D$_3$-treated human osteoblasts reveals an interplay of critical regulatory and metabolic pathways and supports the hypothesis that 1,25(OH)$_2$D$_3$ can modulate the coagulation process through osteoblasts, activates osteoclastogenesis through inflammation signaling, modulates the effects of monoamines by affecting their reuptake. J. Cell. Biochem. 113: 640–649, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: 1,25(OH)$_2$D$_3$; HUMAN PRIMARY OSTEOBLASTS; TRANSCRIPTIONAL PROFILING; MICROARRAY

Vitamin D requires two metabolic conversions, 25-hydroxylation in the liver and 1α-hydroxylation in the kidney, before its hormonal form, 1,25(OH)$_2$D, can bind to the vitamin D receptor (VDR) to modulate gene transcription and regulate mineral ion homeostasis [Bouillon et al., 2008]. It is estimated that VDR activation may regulate directly and/or indirectly the expression of a very large number of genes (0.5–5% of the total human genome i.e., 100–1250 genes) [Carlberg 2003; Bouillon et al., 2008]. Variations in the experimental design (e.g., cell culture conditions, concentration of 1,25(OH)$_2$D, and time points after stimulation with 1,25(OH)$_2$D) might account for this wide range [Bouillon et al., 2008].

Consistent with the detection of VDR expression in osteoblasts [Haussler et al., 1995], several effects have been described when primary osteoblast cultures or established osteoblastic cell lines are treated with 1,25(OH)$_2$D in vitro. As previously reviewed [St-Arnaud 2008], 1,25(OH)$_2$D effects are strongly dependent on the species examined. In fact 1,25(OH)$_2$D transcriptionally represses the mouse osteocalcin gene [Ducy and Karsenty, 1995] whereas it activates the human one. In particular, the pioneering study of Owen et al. [1991] showed that 1,25(OH)$_2$D$_3$ can both positively and negatively regulate the expression of murine osteoblast phenotypic markers as a function of duration of hormone treatment and basal levels of gene expression, which is a reflection of bone matrix competency and the differentiated state of the osteoblast. In addition, in vitro analyses using both rat clonal osteosarcoma cell lines and primary osteoblasts have demonstrated a key role for 1,25(OH)$_2$D in the regulation of genes encoding bone matrix proteins, including type I collagen [Lichtler et al., 1989] and osteocalcin [Demay et al., 1990], the two most abundant matrix proteins. 1,25-Dihydroxyvitamin D

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induces the expression of receptor activator for nuclear factor κB ligand [Tsukii et al., 1998], an osteoblast-secreted molecule that is the key regulator of osteoclast differentiation.

More recent studies in mouse models after targeted manipulation of VDR expression in osteoblasts have further assessed the biological role of $1,25(OH)_2D$ by demonstrating that the VDR acts directly to inhibit the differentiation and function of osteoblasts [Tanaka and Seino, 2004; Yamamoto et al., 2004; Sooy et al., 2005]. Others demonstrated an increase in both trabecular and cortical bone by expressing a VDR transgene in mature osteoblasts using the osteocalcin gene promoter, suggesting an anabolic function for VDR-mediated signaling in osteoblasts [Gardiner et al., 2000; Misof et al., 2003; Baldock et al., 2006].

The complexity of the gene transcription scenario under $1,25(OH)_2D$ exposure is further expanded by the observation that $1,25(OH)_2D$ promotes the osteogenic differentiation of human bone marrow stromal cells, reflected by an increase in alkaline phosphatase activity and osteocalcin production [Liu et al., 1999]. These data are particularly intriguing, given the observation that the key regulator of osteoblast differentiation, Runx2, is transcriptionally repressed by 1,25-dihydroxyvitamin D [Drissi et al., 2002].

As reported above, the $1,25(OH)_2D$ pathway has been exhaustively studied in osteoblasts, but has not yielded all aspects of its functional spectrum. Questions regarding the molecular effectors and pathways underlying the regulatory role of $1,25(OH)_2D$ in human osteoblast biology aside from cell differentiation and matrix protein production remain unanswered. Microarray experiments provide a useful way to address this matter. Microarray analysis provides a wealth of gene expression data and is appropriate to the comparison of cellular samples subjected to specific physiologic or pharmacologic modifications [Young, 2000]. Given that $1,25(OH)_2D$ signals through a nuclear receptor that directly regulates gene transcription, its signaling is ideally suited for microarray analysis [White, 2004]. Microarray use may reveal the regulation of individual genes that might not be identified otherwise, and may identify clusters of genes undergoing concurrent regulation [Griffin et al., 2004].

Along this line of thought, gene expression induced by $1,25(OH)_2D_3$ was analyzed in primary human osteoblast culture. This well established human osteoblast-like cell model (hOB) allowed us to conduct a functional genomic study directed at examining the effect of $1,25(OH)_2D$ on the osteoblast phenotype in the absence of systemic or paracrine factors that could modulate or compensate the activation of the VDR signaling pathway in vivo. By applying Affymetrix® HG-U133 Plus 2.0 (Affymetrix, Santa Clara, CA) human microarrays to compare the gene expression profiles of hOB after stimulation with $1,25(OH)_2D_3$ versus their relative control samples, this study might clarify molecular effectors and pathways of the complex endocrine system that regulates osteoblast differentiation, maturation, and activities.

MATERIALS AND METHODS

HUMAN PRIMARY OSTEOBLAST-LIKE CELL (hOB) CULTURE

Bone cells were established in culture by a modification of the Geron Robey and Termine procedure [1985]. Bone samples were obtained from waste material derived from the intertrochanteric region of the proximal femur of female donors during arthroplasty procedures for degenerative diseases or traumatic fractures of the femoral neck requiring osteotomy procedures. None of the patients (60–85 years old) submitted to surgery had any malignant or metabolic bone diseases other than senile osteoporosis and osteoarthritis. All patients with a known history of medication influencing bone metabolism were excluded. The study was approved by the San Raffaele Scientific Institute Ethical Committee (Prot. AX-ORT-BMU-1-2004) and the patients gave written consent prior to participation.

Briefly, the trabecular bone was cut into small pieces ($2 \times 2 \times 2$ mm) and washed thoroughly with commercial standardized Joklik's modified MEM serum free medium (Sigma, St. Louis, MO) to remove nonadherent marrow cells. The bone pieces were incubated with the same medium containing 0.5 mg/ml collagenase (type IV, Sigma, St. Louis, MO) at 37°C for 30 min, with rotation. The collagenase digestion was stopped by adding Iscove's modified Dulbecco's medium (IMDM, Lonza Group Ltd, Basel, Switzerland) containing 10% fetal bovine serum (FBS, Euroclone, Milano, Italy). The bone pieces (8–10 from each patient) were then placed in 25-cm^2 flasks and cultured in IMDM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 50 U/ml mycostatin (Sigma, St. Louis, MO). Culture medium was changed every 2–3 days. Cells began to migrate within 1–2 weeks and reached confluence after 1 month. The cell population was tested for osteocalcin production after $1,25(OH)_2D_3$ to ensure that the cells were endowed with osteoblast characteristics. All cells were used at the first passage to reduce the possibility of phenotype changes. For mRNA analyses, after 48 h starving, confluent hOB were incubated with 10^{-7} M $1,25(OH)_2D_3$ for 24 h.

MICROARRAY ANALYSIS

Total RNA was extracted using TRIzol® reagent (Invitrogen Life Technology, Inc., Paisley, UK) and purified using the RNeasy Mini Kit (Qiagen SpA, Milan, Italy). RNA integrity was assessed using the Agilent® 2100 bioanalyzer and RNA Nano LabChip® Kit (Agilent Technologies Italia SpA, Cernusco s/N, Italy). The samples have been profiled on the Affymetrix® HG-U133 Plus 2.0, using 5 μg of total RNA and the new Affymetrix® protocol for cRNA synthesis (GeneChip® Expression 3′ Amplification One-Cycle Target Labeling kit, Affymetrix®). The hybridization was performed using the reagents included in the GeneChip® Hybridization Wash and Stain Kit (Affymetrix). Quality assessment was performed at each process level (bone samples, cell culture, RNA extraction, cRNA symthesis, microarray hybridization, and data analysis).

DATA ANALYSIS

The analysis has been performed on 22 hOB cell samples, each available in control and treated condition. Data handling was primarily done using the Bioconductor Affy Package [Ihaka and Gentleman, 1996]. The gene expression intensity levels calculation was conducted using the function for GC Robust Multi-array Average (GCRMA). The intensities were Lowess normalized in the R environment for statistical computing.

Differential expression analysis has been conducted for the purpose of identifying class-specific genes on filtered grade A probe

sets (Affymetrix® annotations release 22) using linear models for microarray data (LIMMA). Microarray analysis was conducted comparing each $1,25(OH)_2D_3$-treated hOB sample with its untreated control of the cell preparation derived from the same donor. Bayesian statistical analysis was carried out using LIMMA to identify statistically significant differentially expressed genes. A threshold was applied such that only genes differentially expressed by $\geq$ twofold (up or down) were selected.

Data were then analyzed for functional annotations through the use of IPA (Ingenuity® Systems, www.ingenuity.com). Genes from the dataset that were attributed a significant *P*-value by LIMMA and were associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base, were considered for the analysis. Fisher's exact test was used to calculate the probability (*P*-value) that each biological function and/or disease assigned to that data set is due to chance alone.

IPA was also used to calculate networks of differentially expressed genes or gene products, represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. Therefore, this information adds to the relative relation between modulated genes and their products.

Real Time quantitative PCR: Validation of microarray data for a selected number of genes was performed by real-time quantitative PCR (RT qPCR) on an ABI Prism® 7700 Sequence Detection System® (AppliedBiosystems, Weiterstadt, Germany), using TaqMan® MGB Probes (AppliedBiosystems) according to the manufacturer's instructions. mRNA levels were quantified using the comparative threshold-cycle (Ct) method [Pfaffl, 2001]. First, the amount of target mRNA in each sample was normalized to the amount of the housekeeper mRNA (18s), designated as a calibrator, to give ΔCt ($Ct_{target} - Ct_{18s}$). Second, the amounts of target mRNA in the samples were expressed using the formula: Amount of target mRNA $= 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ (sample 1) $- \Delta Ct$ (untreated sample), assuming that the efficiencies of the PCR reaction were close to 1. Three replicates were performed for each experimental point and experiments were repeated with the cells obtained from the different donors. Differences between control and treated samples were assessed by means of Wilcoxon rank signed test for nonparametric data.

RESULTS

MICROARRAY ANALYSIS OF HUMAN PRIMARY OSTEOBLASTS AFTER STIMULATION WITH $1,25(OH)_2D_3$

Before proceeding to process hOB samples for the microarray experiments, we analyzed the mRNA levels for bone gamma-carboxyglutamate protein (BGLAP) in each hOB preparation before and after treatment with $1,25(OH)_2D_3$ as a positive control for the real capability of the cells to respond to $1,25(OH)_2D_3$ treatment. As expected, BGLAP mRNA was significantly ($P < 0.000$) upregulated in all treated samples.

Using the criteria described in the Materials and Methods section, we were able to identify 158 differentially expressed transcripts after treatment with $1,25(OH)_2D_3$, indicating that gene expression in hOB was significantly affected by this stimulus. Among the identified genes, 22 transcripts were downregulated and 136 transcripts were upregulated. The most relevant upregulated and downregulated genes are summarized in Table I.

Furthermore, we identified the biological functions and/or diseases that were most significant to the data set: Of 158 genes modulated, 100 were associated with functional annotations in the Ingenuity Pathways Knowledge Base. The four top biological functions (ranked by significance; *P*-value) affected by $1,25(OH)_2D_3$ in hOB are named as follows: "Cellular Movement", "Inflammatory Disease", "Hematological System Development and Function", and "Immune Response", and are all related to immunological functions.

Of the 67 genes that were associated with the four top biological function annotations, 19 are also annotated in Ingenuity with biological functions related to bone and bone cell biology, while another 19 genes are associated with arthritis or connective tissue disorders (Table II).

CYP24A1, which appears at the top of the differentially expressed genes being upregulated more than 100-fold after $1,25(OH)_2D_3$ treatment, belongs to the metabolic pathways of vitamin D and is known to be regulated by a feedback mechanism. CYP26B1, which appears as slightly modulated (fold change: 1.21; Limma *P*-value: 3.52E-02), belongs to the retinoic acid pathway and is responsible for the inactivation of all-trans retinoic acid; it is also known to be involved in limb development and chondrocyte maturation.

The remaining genes (APBB1IP, AVIL, BHLHB3, CDCP1, COL12A1, CRIP1, DOK5, FAIM2, FAM20C, GRK5, KCNK3, LPL, MAN1C1, MN1, NID2, NINJ1, OSR1, OSR2, PDP2, PREB, RASSF5, RGMA, RHOU, ROM1, SERPINB1, SERPINB9, SGCD, SLC1A1, SLC5A3, TM4SF1, TMEM158) have sparse biological functions.

The top canonical metabolic and signaling pathways affected by $1,25(OH)_2D_3$ treatment and identified by IPA analysis in our data set are listed in Table III. We have found genes belonging to VDR/RXR signaling to be strongly upregulated. Interestingly, we have also found several genes belonging to the coagulation system to be significantly regulated.

IPA also calculates networks of differentially expressed genes. In this case, differentially expressed genes are overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. The most relevant gene networks in this data set are represented with their relevant biological functions (Fig. 1A–C). It is notable that genes with relevant roles in bone physiology and related pathways appear in all networks. There is also clearly a strong interplay between genes with roles in immune functions and in bone metabolism.

VERIFICATION OF CANDIDATE GENE EXPRESSION

For a method-independent validation of the expression patterns extracted from the microarray data, the abundance of selected transcripts was analyzed on each individual hOB sample in control and $1,25(OH)_2D_3$-treated conditions using semi-quantitative RT-qPCR. We have analyzed cell samples prepared from a total of 18 donors.

We have selected the following genes for this RT-qPCR validation: Known osteoblast markers (BGLAP, SPP1, ALPL) as

TABLE I. Genes Exhibiting the Highest-Fold Upregulation or Downregulation

Gene symbol	Gene title	Cellular process or molecular function—IPA	Fold change (over control)	*P*-value (Limma)
Top 15 upregulated genes				
CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1	Metabolism of Vit D	121.0	6.92E-23
BGLAP	Bone gamma-carboxyglutamate (gla) protein (osteocalcin)	Differentiation of bone cells	51.6	2.11E-18
CD14	CD14 molecule	Inflammation	19.6	5.62E-20
CLMN	Calmin (calponin-like, transmembrane)	Actin binding	14.9	4.91E-26
THBD	Thrombomodulin	Inflammation	13.4	1.11E-13
CILP	Cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	Negative regulation of insulin-like growth factor receptor signaling pathway	10.2	3.04E-10
LRRN3	Leucine rich repeat neuronal 3	Protein binding	8.1	1.66E-09
SHE	Src homology 2 domain containing E	Protein binding	7.7	2.11E-18
EFTUD1	Elongation factor Tu GTP binding domain containing 1	Translation	7.4	1.43E-21
TREM1	Triggering receptor expressed on myeloid cells 1	Inflammation	6.9	5.44E-10
EXPH5	exophilin 5	Intracellular protein transport	5.9	6.52E-07
OSR1	Odd-skipped related 1 (Drosophila)	Development of mesoderm	5.6	6.43E-10
BMP6	bone morphogenetic protein 6	Differentiation of bone cells	5.6	1.68E-10
KCNK3	Potassium channel, subfamily K, member 3	Ion transport	5.0	2.29E-06
TMEM37	Transmembrane protein 37	Ion transport	5.0	1.33E-05
Top 15 downregulated genes				
SCG2	Secretogranin II (chromogranin C)	Skeletal system development and function	0.3	1.42E-03
IER3	Immediate early response 3	Immune and limphatic system development	0.3	2.23E-04
CD24	CD24 molecule	Immune and limphatic system development	0.4	4.14E-03
CORO2B	Coronin, actin binding protein, 2B	Actin binding	0.4	1.83E-02
PDGFC	Platelet derived growth factor C	Skeletal system development and function	0.4	8.72E-06
B3GALT2	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	Protein amino acid glycosylation	0.4	1.78E-03
LIF	Leukemia inhibitory factor (cholinergic differentiation factor)	Immune and limphatic system development	0.4	2.16E-04
FGF1	Fibroblast growth factor 1 (acidic)	Immune and limphatic system development	0.4	3.28E-02
COL11A1	Collagen, type XI, alpha 1	Skeletal system development and function	0.4	4.74E-02
FILIP1L	Filamin A interacting protein 1-like	ND	0.4	5.20E-06
SHROOM2	Shroom family member 2	Ocular albinism type 1	0.4	2.63E-03
LFNG	LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	Skeletal system development and function	0.4	4.82E-02
ARMC9	Armadillo repeat containing 9	ND	0.5	1.28E-03
F2R	Coagulation factor II (thrombin) receptor	Immune and limphatic system development	0.5	7.25E-06
TBC1D12	TBC1 domain family, member 12	GTPase activator	0.5	1.04E-02

TABLE II. List of Genes Belonging to Biological Functions Most Affected by $1,25(OH)_2D_3$ and Their IPA Annotations for Biological Functions and/or Diseases Relevant to Bone Biology

Biological functions and/or diseases—IPA	Gene symbol
Developmental process of bone	BMP4, CSF1, FGF1, GADD45B, GHR, HIP1, LIF, PDGFC, SPP1, TGFB2, VEGFA
Differentiation of bone cell lines	BGLAP, BMP4, BMP6, CSF1, CXCL12, RGS2, SPP1;
Quantity of osteoclasts	CSF1, F2R, LIF, SPP1, VEGFA
Polarity of osteoclasts	ITGA6
Destruction of bone	SLP1
Deposition of calcium	TGM2
Arthritis	CALD1, CD24, CD97, CXCL12, CXCL14, DPP4, DUSP1, G0S2, GADD45B, IL7R, MAPK13, NFKBIA, PDGFRL, PLAT, SPP1, TNFRSF21, TNFSF13B, ZFP36
Connective tissue disorders	BCL2, CALD1, CD24, CD97, CXCL12, CXCL14, DPP4, DUSP1, G0S2, GADD45B, IL7R, MAPK13, NFKBIA, PDGFRL, PLAT, SIRPA, SPP1, TIMP3, TNFRSF21, TNFSF13B, ZFP36
Pathways of vitamin D	CYP24A1
Retinoic acid pathway	CYP26B1

they represent key positive controls; genes belonging to the coagulation pathway [thrombomodulin (THBD), tissue plasminogen activator (PLAT), endothelial protein C receptor (PROCR), thrombin receptor (F2R)]; two transporters (SLC22A3, SLC1A1) and CD14, for their unexpected and intriguing involvement in the studied biological process; and two genes, ALPL and TLR4, that are not in the list of the differentially expressed genes as negative controls.

Table IV summarizes results obtained with the two techniques for the selected candidate genes. The results, expressed as mean (±SEM) fold change values over control, obtained for osteoblast marker genes by RT-qPCR are shown in Figure 2A. Similarly, results from the other gene groups are plotted in Figure 2B–D. Results obtained with the two technological approaches are in full concordance.

DISCUSSION

Our genome-wide expression analysis provides the first insight into the global transcriptional activity that underlie the effects of $1,25(OH)_2D_3$ on human primary osteoblasts. This analysis has highlighted previously unknown significant effects of $1,25(OH)_2D_3$ on molecular effectors and pathways related to the coagulation system, the immune system, and to neurotransmitters transport molecules (albeit to a minor extent) in hOBs. The observation that two known osteoblast markers, osteocalcin (BGALP) and

TABLE III. Canonical Metabolic and Signaling Pathways Most Affected by $1{,}25(OH)_2D_3$ Treatment

Pathway	−Log (*P*-value)	Downregulated	Upregulated	Molecules
VDR/RXR Activation	5,94E00	0/80 (0%)	8/80 (10%)	CD14, TGFB2, SEMA3B, BGLAP, SPP1, SERPINB1, CYP24A1, THBD
Hepatic fibrosis/hepatic stellate cell activation	2,83E00	1/131 (1%)	5/131 (4%)	CD14, TGFB2, FGF1, VEGFA, BCL2, CSF1
Death receptor signaling	2,58E00	0/61 (0%)	4/61 (7%)	HSPB7, NFKBIA, TNFRSF21, BCL2
Coagulation system	2,26E00	1/35 (3%)	2/35 (6%)	F2R, PLAT, THBD
Axonal guidance signaling	2,11E00	1/387 (0%)	8/387 (2%)	SEMA6D, VEGFA, SEMA3B, CXCL12, PDGFC, RASSF5, BMP6, BMP4, SEMA3C
Glucocorticoid receptor signaling	2,06E00	0/265 (0%)	7/265 (3%)	SLPI, TGFB2, NFKBIA, DUSP1, BGLAP, BCL2, MAPK13
Toll-like receptor signaling	1,96E00	0/51 (0%)	3/51 (6%)	CD14, NFKBIA, MAPK13
IL-6 signaling	1,94E00	0/91 (0%)	4/91 (4%)	CD14, HSPB7, NFKBIA, MAPK13
p38 MAPK signaling	1,84E00	0/95 (0%)	4/95 (4%)	TGFB2, HSPB7, DUSP1, MAPK13
IL-10 signaling	1,68E00	0/68 (0%)	3/68 (4%)	CD14, NFKBIA, MAPK13

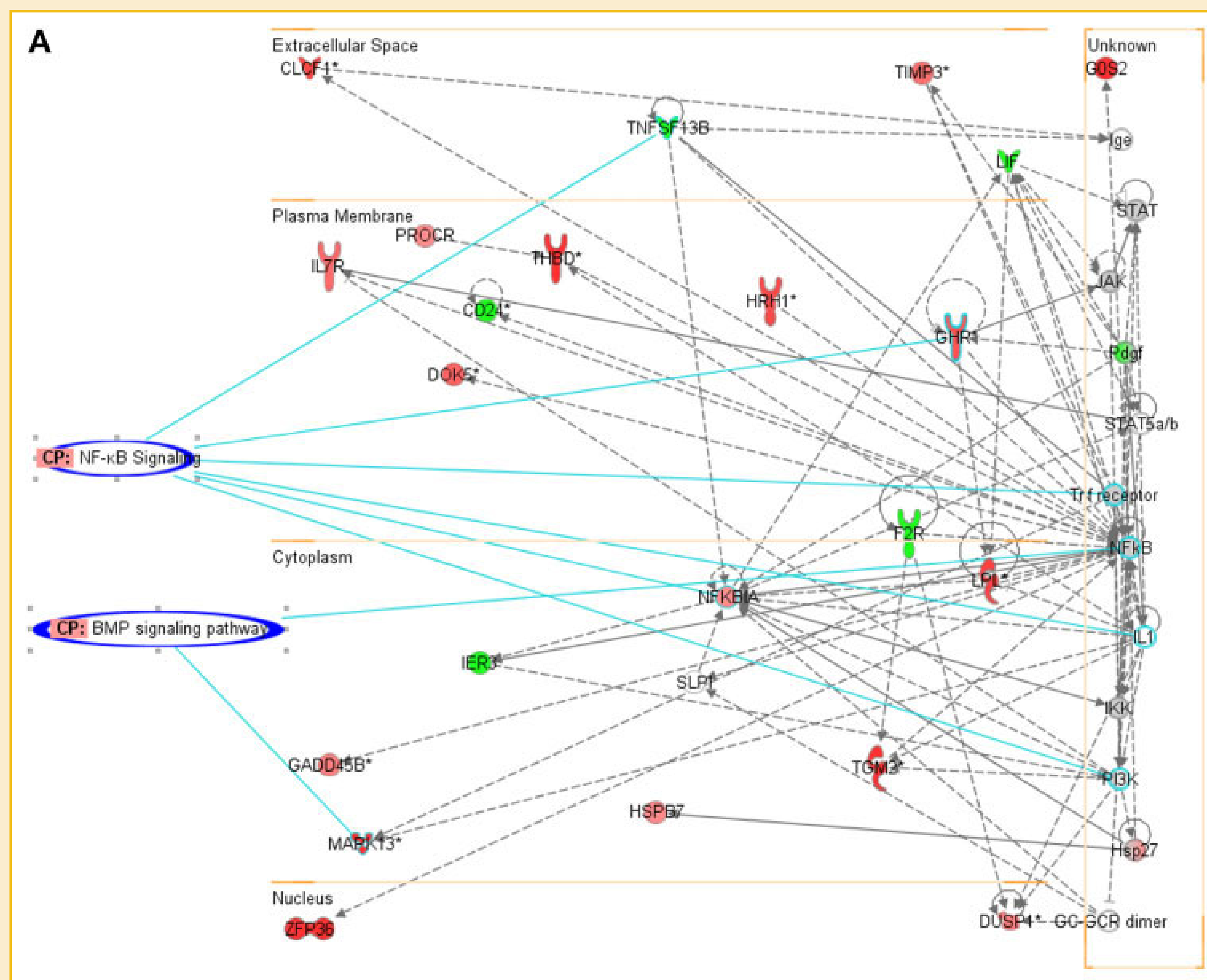

Fig. 1. Graphic representation of the most relevant gene networks identified by Ingenuity Pathway Analysis (IPA) in this data set with their relevant biological functions. Genes or gene products are represented as nodes, and the direct (solid lines) and indirect (dashed lines) biological relationship between two nodes are represented as an edge. A: Network 1. Upregulated (CLCF1, TIMP3, GOS2, IL7R, PROCR, THBD, HRH1, GHR, DOK5, NFKBIA, LPL, GADD45B, TGM2, MAPK13, HSPB7, Hsp27, ZFP36, DUSP1), downregulated (TNFSF13B, LIF, CD24, Pdgf, F2R, IER3) and not significantly regulated genes of the network are represented in the diagram with their reciprocal interactions and according to their cellular localization. Genes belonging to NF-κB and BMP signaling, two known canonical pathways relevant to bone biology, are highlighted. B: Network 2. Upregulated (SERPINE2, PLAT, BMP4, BMP6, BGLAP, SPP1, BIMP, RGMA, Tgfβ, TREM1, SLC1A1, CD97, BCL2, CYP24A1, NPC1, GUCY1B3, SERPINB1, NBL1, RGS2), downregulated (CORO2B) and not significantly regulated genes of the network are represented in the diagram with their reciprocal interactions and according to their cellular localization. Bone biology relevant genes are highlighted. C: Network 3. Upregulated (ANGPTL4, BGLAP, ITGA6, PDPN, ROM1, CDCP1, THBD, PROCR, MAPK13, PDE7A, TGM2, DH2, LMO2), downregulated (PDGFC, B3GALT2, BHLHB3, FILIP1L) and not significantly regulated genes of the network are represented in the diagram with their reciprocal interactions and according to their cellular localization. Bone biology relevant genes are highlighted. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

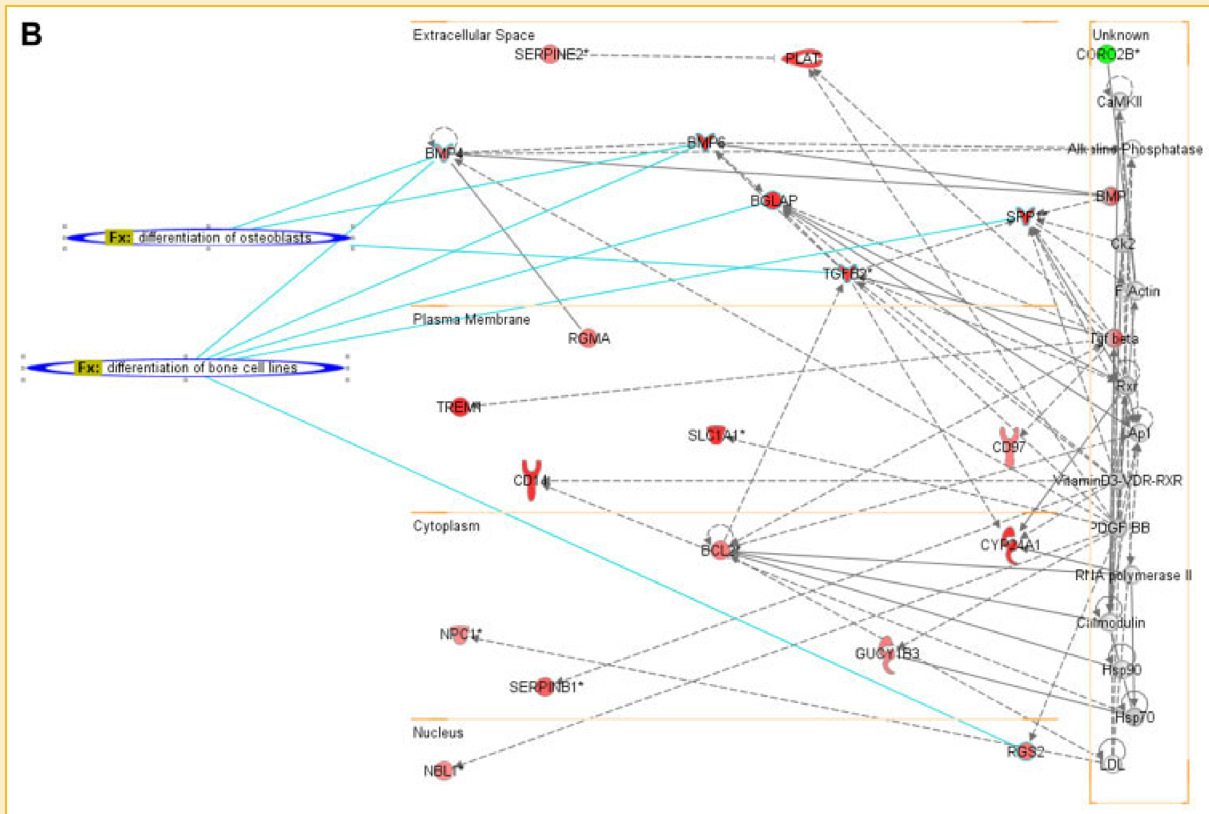

Fig. 1. (*Continued*)

osteopontin (SPP1), which are known to be under the control of VDR [Paredes et al., 2004] appear, as expected, in the list of top genes modulated by $1{,}25(OH)_2D_3$, confirms that the experimental conditions were adequate. These data were also confirmed by RT-qPCR, although osteocalcin seems to be less dramatically upregulated. The finding that another marker of osteoblast differentiation, alkaline phosphatase (ALPL), does not appear in the list of differentially expressed genes identified by microarray analysis is not to be ascribed to a bias in the Affymetrix technology, as it was confirmed by RT-qPCR. It is therefore likely that the ALPL gene is not strongly induced after 24 h treatment or that its expression is not modulated by $1{,}25(OH)_2D_3$ in human osteoblasts, at variance with what happens in osteosarcoma cells [Majeska and Rodan, 1982].

$1{,}25(OH)_2D_3$ DEGRADATION PATHWAY

In the present study, $1{,}25(OH)_2D_3$ upregulates the expression of the cytochrome P450 superfamily 24 gene (CYP24A1). CYP24A1 is a member of the cytochrome p450 enzyme family that is primarily responsible for catabolizing the active form of vitamin D ($1{,}25(OH)_2D_3$) to inactive calcitroic acid. Its increase in human osteoblast confirms a recent report [Ruiz-Gaspà et al., 2010] and suggests that the level and biological activity of $1{,}25(OH)_2D_3$ in osteoblast is not only related to the local rate of its synthesis by CYP27B1 [Atkins et al., 2007] but also to the rate of its degradation by CYP24A1. By maintaining locally a precise balance between the rates of vitamin D synthesis and degradation, the skeleton acts as an intracrine organ for vitamin D metabolism and constitutes autocrine and paracrine loops of vitamin D metabolism [see Anderson and Atkins, 2008 for review]. Understanding the role of locally produced and catabolized $1{,}25(OH)_2D_3$ in bone and its role in bone cell metabolism would impact on the existing strategies for optimal skeletal health.

GENES BELONGING TO THE COAGULATION SYSTEM

We have identified at least four genes involved in blood coagulation that are significantly regulated by $1{,}25(OH)_2D_3$ treatment in hOB: THBD, PLAT, and PROCR are upregulated, while F2R is downregulated. It is known that effectors playing a relevant role in coagulation also influence bone metabolism. This is the case for vitamin K, which, being responsible for protein γ-carboxylation, is fundamental for the maturation of coagulation factors (prothrombin (factor II); factors VII, IX, and X; protein C; protein S; and protein Z) and bone matrix noncollagenous proteins with subsequent metabolic effects on bone [Rubinacci, 2009]. In fact, long-term use of anticoagulants based on vitamin K antagonism has negative effects on bone mass and quality [Pearson, 2007]. Similar to vitamin K, which controls coagulation and calcification at the same time [Krueger et al., 2009], our results suggest that vitamin D might exert analogous functions. By considering that smooth muscle cells undergo to a transdifferentiation process acquiring an osteoblast phenotype [Mizobuchi et al., 2009] it is likely that vitamin D might modulate the functional state of the vascular wall and blood clots. An even deeper connection and crosstalk between the pathways

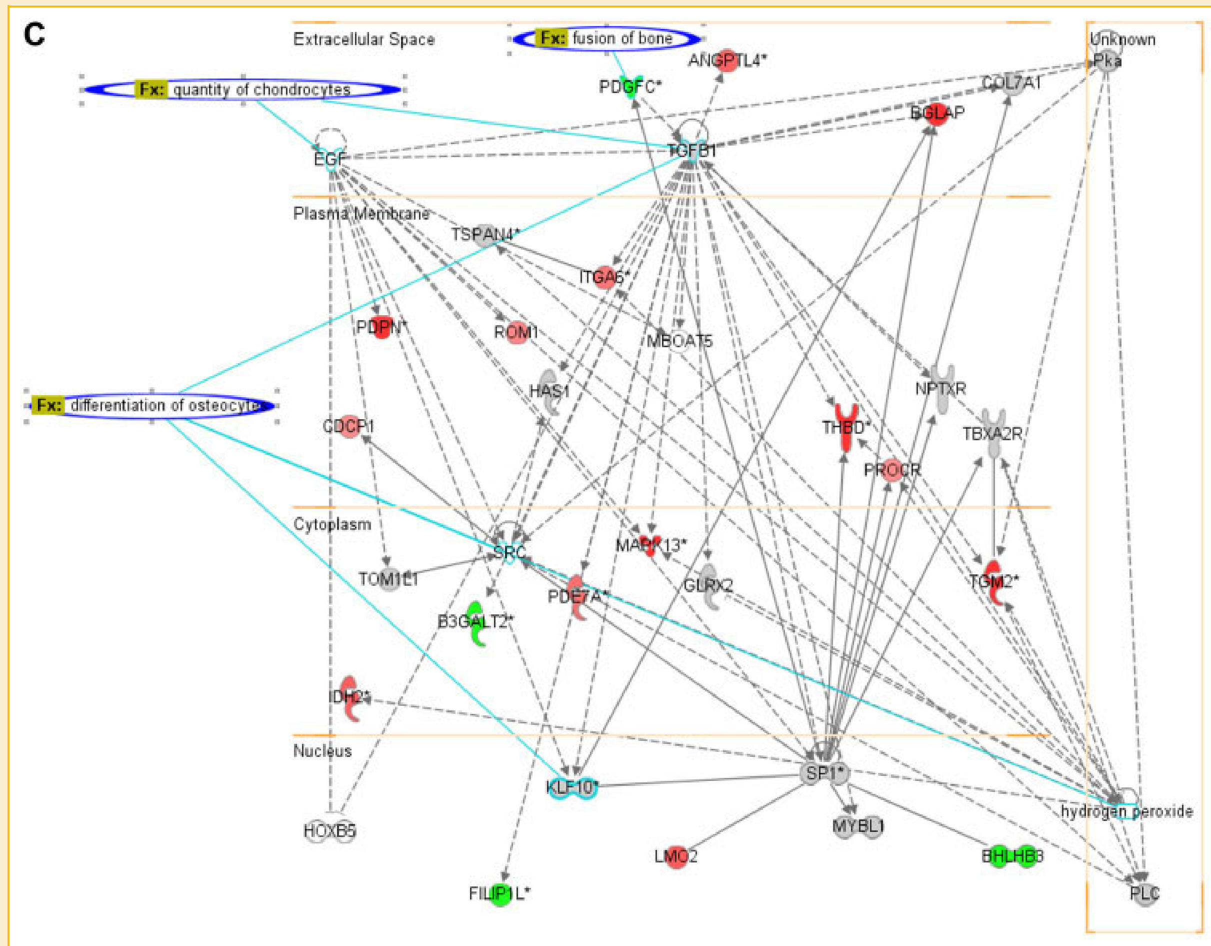

Fig. 1. (*Continued*)

regulating blood clots, vasogenesis, and bone metabolism through vitamin K and vitamin D might be likely.

IMMUNE FUNCTIONS

We found that a significant number of genes with immune functions are regulated in hOB after treatment with $1,25(OH)_2D_3$. Unexpectedly, we have found CD14, a fundamental constituent of the LPS receptor [Akira 2003; Yeh and Chen, 2003] with the coreceptor TLR4, at the top of the list of upregulated genes. LPS, a major constituent of gram-negative bacteria, is proposed to be a potent stimulator of bone resorption in inflammatory diseases [Nair et al., 1996]. Among upregulated genes, CD14 is just beneath CYP24A1, which belongs to the metabolic vitamin D pathway, and BGLAP, a major noncollagenous protein whose production by

TABLE IV. Comparison of Results Obtained With Affymetrix and RT-qPCR Techniques for the Selected Candidate Genes

		Affymetrix		RT-qPCR	
		Fold change	*P*-value (Limma)	Fold change	*P*-value (*t*-test)
Osteoblast markers	BGLAP	51.6	2.11E-23	2.62	1.58E-05
	SPP1	4.3	1.54E-05	2.34	1.55E-04
	ALPL	nd	nd	0.81	8.45E-02
Coagulation pathway	THBD	13.4	1.11E-13	11.68	1.69E-04
	PLAT	3.2	1.47E-04	1.81	2.43E-05
	PROCR	2.0	3.14E-02	2.44	1.28E-02
	F2R	0.46	7.25E-06	0.40	3.77E-04
Membrane transporters	SLC22A3	3.2	9.59E-03	2.61	8.11E-05
	SLC1A1	4.2	7.92E-05	2.89	1.04E-04
Innate immune system	CD14	19.6	5.62E-20	79.48	1.66E-04
	TLR4	nd	nd	1.22	3.06E-01

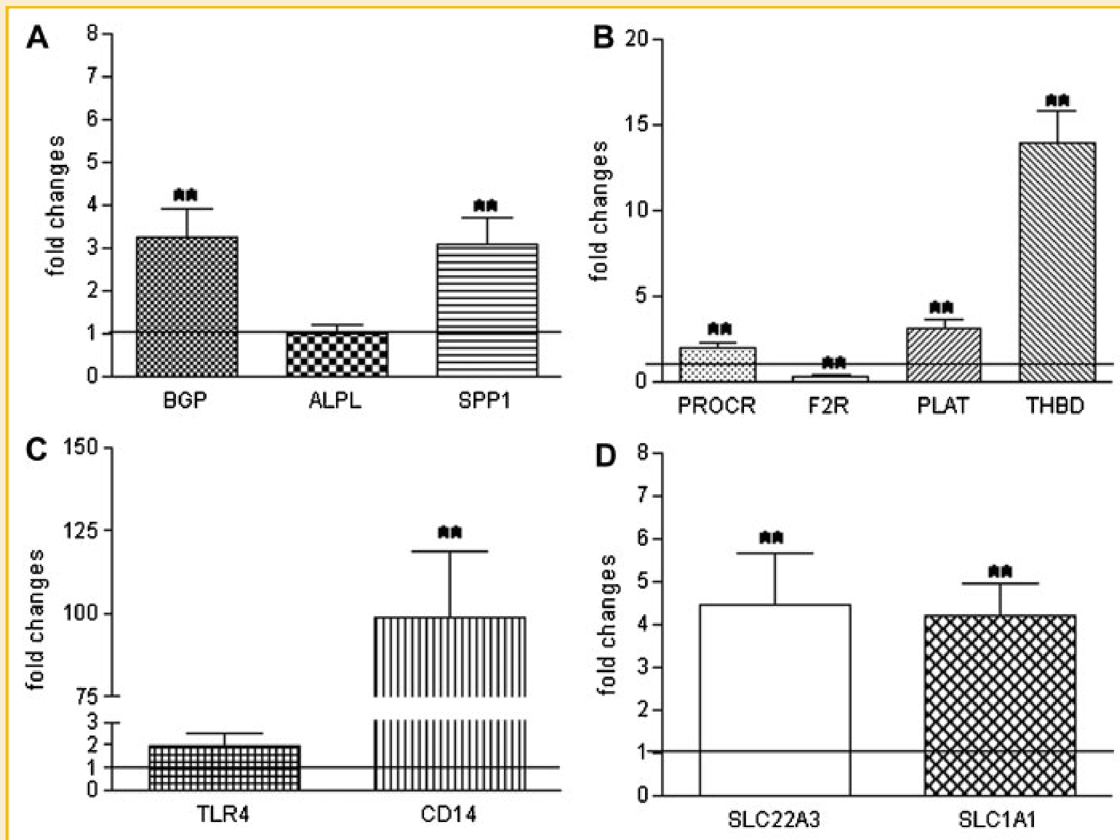

Fig. 2. Mean values ± SEM of fold change over control for mRNA levels determined by RT-qPCR before and after $1,25(OH)_2D_3$ (10^{-7} M) treatment in human osteoblast-like cells deriving from 18 different donors. A: BGLAP and SPP1 are significantly upregulated in treated samples compared to untreated samples (Wilcoxon rank test $P < 0.00005$ and $P < 0.0005$, respectively), while ALPL shows no significant trend. B: THBD, PLAT, and PROCR are significantly upregulated, while F2R expression is significantly downregulated (n = 18; $P < 0.0005$, $P < 0.00005$, $P < 0.05$, $P < 0.0005$, respectively). C: CD14 is significantly ($P < 0.0005$) upregulated, while TLR4 does not show any significant trend. D: SLC1A1 and SLC22A3 are significantly upregulated (n = 18; $P < 0.0001$).

osteoblasts is directly controlled by vitamin D through its nuclear receptor.

The role of CD14 in bone is at present unknown. CD14 or TLR4 knockout mice have increased bone mineral content, density, and size, as well as decreased body fat. Nevertheless, the cellular and biomolecular mechanisms underlying the associations between the specific phenotype and the observed mutations are unknown [Johnson et al., 2004]. Currently, the expression and the function of this LPS receptor in bone has been widely studied and recognized in osteoclasts, while it is less well understood in bone forming cells. Its expression has been identified in cementoblasts, specialized bone-forming cells of the tooth follicle, in the MC3T3-E1 osteoblast cell line, and in murine calvaria cells, were it is upregulated after induction of cell differentiation by ascorbic acid [Roman-Roman et al., 2003; Nemoto et al., 2008].

In vitro experiments suggest that LPS, through its receptor, induces osteoblast mediated osteoclast differentiation and activation [Kikuchi et al., 2001]. It has also been shown that the osteoblast is responsible for mouse strain-dependent osteoclastogenesis in response to LPS [Choi et al., 2007].

All these data suggest that LPS could activate osteoclastogenesis via osteoblasts, indicating that CD14 signaling might constitute an additional pathway by which $1,25(OH)_2D_3$ modulates osteoclastogenesis. This is in line with the observations that: (a) Bone and immune cells have overlapping regulatory mechanisms since many of the soluble mediators of immune cells, including cytokines, chemokines, and growth factors, are recognized to regulate the activities of osteoblasts and osteoclasts [Walsh et al., 2006; Takayanagi, 2007] and that (b) the active form of vitamin D upregulates CD14 expression though Sp-1 [Moeenrezakhanlou et al., 2008] in cells of the immune system, influencing their proliferation, differentiation, and immunogenic properties.

Recent in vivo exploration in humans has shown that during the early response to LPS injection (healthy men receiving once 2 ng/kg LPS *Escherichia Coli* endotoxin), there is a boost in osteopontin levels and a modification of bone biomarkers, indicating a decrease in the lytic activity of osteoclasts, accompanied by an increase in the activity of immature osteoblasts. [Grimm et al., 2010].

NEUROTRANSMITTER TRANSPORTERS

This study confirms that the glutamate transporter SLC1A1 is expressed in human primary osteoblasts and that its expression is induced by treatment with $1,25(OH)_2D_3$. This is in agreement with

the previous observations that a number of glutamate transporters are expressed in rat osteoblasts (SLC1A1, SLC1A2, and SLC1A3) and that active glutamate uptake occurs in these cells in vitro [Takarada et al., 2004]. There is now a wealth of evidence supporting a role for glutamate signaling in bone. Osteoblasts can release glutamate in a regulated manner and express functional glutamate receptors that influence their differentiation and osteogenic activity [For review see Skerry 2008; Takarada and Yoneda, 2008]. Furthermore, it has been reported that vitamin D modulates glutamate concentration in bone via the regulation of the enzyme glutamine synthetase [Olkku and Mahonen, 2008]. Therefore, it can be speculated that an intrinsic synaptic-like glutamatergic signaling network under the modulation of vitamin D exists in bone and is essential for in vitro osteoblast differentiation and survival [Genever and Skerry, 2001].

In the present study, $1,25(OH)_2D_3$ also upregulates the expression of SLC22A3, another transporter gene that encodes for the extraneuronal monoamine transporter OCT3. This protein transports a variety of neurotransmitters, including 1-methyl-4-phenylpyridinium ion (MPP+), and serotonin, as well as cationic neurotoxins. Neurotransmitter regulation of bone metabolism is a subject of increasing interest and investigation particularly regarding the emerging role of serotonin in the skeletal metabolism. Serotonin exerts an inhibitory effect on bone formation through a specific serotonin receptor (Htr1b) expressed on osteoblasts. Remarkably, patients taking chronically synthetic serotonin reuptake inhibitors, a class of drugs increasing extracellular serotonin concentration throughout the body, can have reduced bone mass [Richards et al., 2007]. Therefore, by affecting the efficiency of the clearing systems of monoamines in bone, $1,25(OH)_2D_3$ might subsequently modulate their effects on bone metabolism.

CONCLUSIONS

Although larger studies are required to define the clinically relevant gene expression patterns, the present genome-wide microarray analysis in osteoblasts revealed a wide range of critical regulatory and metabolic pathways influenced by $1,25(OH)_2D_3$ that supports the hypothesis that vitamin D modulates the coagulation process through osteoblasts, activates osteoclastogenesis through inflammation signaling, modulates the effects of monoamines by affecting their reuptake and utilizes bone as an intracrine organ for its metabolism and function.

REFERENCES

Akira S. 2003. Toll-like receptor signaling. J Biol Chem 278:38105–38108.

Anderson PH, Atkins GJ. 2008. The skeleton as an intracrine organ for vitamin D metabolism. Mol Aspects Med 29:397–406.

Atkins GJ, Anderson PH, Findlay DM, Welldon KJ, Vincent C, Zannettino AC, O'Loughlin PD, Morris HA. 2007. Metabolism of vitamin D3 in human osteoblasts: Evidence for autocrine and paracrine activities of 1 alpha,25-dihydroxyvitamin D3. Bone 40:1517–1528.

Baldock PA, Thomas GP, Hodge JM, Baker SU, Dressel U, O'Loughlin PD, Nicholson GC, Briffa KH, Eisman JA, Gardiner EM. 2006. Vitamin D action and regulation of bone remodeling: Suppression of osteoclastogenesis by the mature osteoblast. J Bone Miner Res 21:1618–1626.

Bouillon R, Carmeliet G, Verlinden L, van Etten E, Verstuyf A, Luderer HF, Lieben L, Mathieu C, Demay M. 2008. Vitamin d and human health: Lessons from vitamin D receptor null mice. Endocr Rev 29:726–776.

Carlberg C. 2003. Current understanding of the function of the nuclear vitamin D receptor in response to its natural and synthetic ligands. Recent Results Cancer Res 164:29–42.

Choi HG, Kim JM, Kim BJ, Yoo YJ, Cha JH. 2007. Mouse strain-dependent osteoclastogenesis in response to lipopolysaccharide. J Microbiol 45:566–571.

Demay MB, Gerardi JM, DeLuca HF, Kronenberg HM. 1990. DNA sequences in the rat osteocalcin gene that bind the 1,25-dihydroxyvitamin D_3 receptor and confer responsiveness to 1,25-dihydroxyvitamin D3. Proc Natl Acad Sci USA 87:369–373.

Drissi H, Pouliot A, Koolloos C, Stein JL, Lian JB, Stein GS, van Wijnen AJ. 2002. $1,25\text{-}(OH)_2$-vitamin D_3 suppresses the bone-related Runx2/Cbfa1 gene promoter. Exp Cell Res 274:323–333.

Ducy P, Karsenty G. 1995. Two distinct osteoblast-specific cis-acting elementscontrol expression of a mouse osteocalcin gene. Mol Cell Biol 15:1858–1869.

Gardiner EM, Baldock PA, Thomas GP, Sims NA, Henderson NK, Hollis B, White CP, Sunn KL, Morrison NA, Walsh WR, Eisman JA. 2000. Increased formation and decreased resorption of bone in mice with elevated vitamin D receptor in mature cells of the osteoblastic lineage. FASEB J 14:1908–1916.

Genever PG, Skerry TM. 2001. Regulation of spontaneous glutamate release activity in osteoblastic cells and its role in differentiation and survival: Evidence for intrinsic glutamatergic signaling in bone. FASEB J 15:1586–1588.

Griffin MD, Xing N, Kumar R. 2004. Gene expression profiles in dendritic cells conditioned by 1alpha,25-dihydroxyvitamin D3 analog. J Steroid Biochem Mol Biol 89–90:443–448.

Grimm G, Vila G, Bieglmayer C, Riedl M, Luger A, Clodi M. 2010. Changes in osteopontin and in biomarkers of bone turnover during human endotoxemia. Bone 47:388–391.

Haussler MR, Jurutka PW, Hsieh JC, Thompson PD, Selznick SH, Haussler CA, Whitfield GK. 1995. New understanding of the molecular mechanism of receptor-mediated actions of the vitamin D hormone. Bone 17:S33–S38.

Ihaka R, Gentleman R. 1996. R: A language for data analysis and graphics. J Comput Graph Stat 5:299–314.

Johnson GB, Riggs BL, Platt JL. 2004. A genetic basis for the "Adonis" phenotype of low adiposity and strong bones. FASEB J 18:1282–1284.

Kikuchi T, Matsuguchi T, Tsuboi N, Mitani A, Tanaka S, Matsuoka M, Yamamoto G, Hishikawa T, Noguchi T, Yoshikai Y. 2001. Gene expression of osteoclast differentiation factor is induced by lipopolysaccharide in mouse osteoblasts via Toll-like receptors. J Immunol 166:3574–3579.

Krueger T, Westenfeld R, Schurgers L, Brandenburg V. 2009. Coagulation meets calcification: The vitamin K system. Int J Artif Organs 32:67–74.

Lichtler A, Stover ML, Angilly J, Kream B, Rowe DW. 1989. Isolation and characterization of the rat alpha 1(I) collagen promoter. Regulation by 1,25-dihydroxyvitamin D. J Biol Chem 264:3072–3077.

Liu P, Oyajobi BO, Russell RG, Scutt A. 1999. Regulation of osteogenic differentiation of human bone marrow stromal cells: Interaction between transforming growth factor-beta and 1,25(OH)(2) vitamin D(3) In vitro. Calcif Tissue Int 65:173–180.

Majeska RJ, Rodan GA. 1982. The effect of 1,25(OH)2D3 on alkaline phosphatase in osteoblastic osteosarcoma cells. J Biol Chem 257:3362–3365.

Misof BM, Roschger P, Tesch W, Baldock PA, Valenta A, Messmer P, Eisman JA, Boskey AL, Gardiner EM, Fratzl P, Klaushofer K. 2003. Targeted overexpression of vitamin D receptor in osteoblasts increases calcium concentration without affecting structural properties of bone mineral crystals. Calcif Tissue Int 73:251–257.

Mizobuchi M, Ogata H, Koiwa F, Kinugasa E, Akizawa T. 2009. Vitamin D and vascular calcification in chronic kidney disease. Bone 45:S26–S29.

Moeenrezakhanlou A, Nandan D, Reiner NE. 2008. Identification of a Calcitriol-Regulated Sp-1 Site in the Promoter of Human CD14 using a Combined Western Blotting Electrophoresis Mobility Shift Assay (WEMSA). Biol Proced Online 10:29–35.

Nair SP, Meghji S, Wilson M, Reddi K, White P, Henderson B. 1996. Bacterially induced bone destruction: Mechanisms and misconceptions. Infect Immun 64:2371–2380.

Nemoto E, Honda T, Kanaya S, Takada H, Shimauchi H. 2008. Expression of functional Toll-like receptors and nucleotide-binding oligomerization domain proteins in murine cementoblasts and their upregulation during cell differentiation. J Periodontal Res 43:585–593.

Olkku A, Mahonen A. 2008. Wnt and steroid pathways control glutamate signalling by regulating glutamine synthetase activity in osteoblastic cells. Bone 43:483–493.

Owen TA, Aronow MS, Barone LM, Bettencourt B, Stein GS, Lian JB. 1991. Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: Dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in normal rat osteoblast cultures. Endocrinology 128: 1496–1504.

Paredes R, Arriagada G, Cruzat F, Villagra A, Olate J, Zaidi K, van Wijnen A, Lian JB, Stein GS, Stein JL, Montecino M. 2004. Bone-specific transcription factor Runx2 interacts with the 1alpha,25-dihydroxyvitamin D3 receptor to up-regulate rat osteocalcin gene expression in osteoblastic cells. Mol Cell Biol 24:8847–8861.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.

Pearson DA. 2007. Bone health and osteoporosis: The role of vitamin K and potential antagonism by anticoagulants. Nutr Clin Pract 22:517–544.

Richards JB, Papaioannou A, Adachi JD, Joseph L, Whitson HE, Prior JC, Goltzman D. 2007. Canadian Multicentre Osteoporosis Study Research Group. Effect of selective serotonin reuptake inhibitors on the risk of fracture. Arch Intern Med 167:188–194.

Robey PG, Termine JD. 1985. Human bone cells in vitro. Calcif Tissue Int 37:453–460.

Roman-Roman S, Garcia T, Jackson A, Theilhaber J, Rawadi G, Connolly T, Spinella-Jaegle S, Kawai S, Courtois B, Bushnell S, Auberval M, Call K, Baron R. 2003. Identification of genes regulated during osteoblastic differentiation by genome-wide expression analysis of mouse calvaria primary osteoblasts in vitro. Bone 32:474–482.

Rubinacci A. 2009. Expanding the functional spectrum of vitamin K in bone. Focus on: "Vitamin K promotes mineralization, osteoblast to osteocyte transition, and an anti-catabolic phenotype by {gamma}-carboxylation-dependent and -independent mechanisms". Am J Physiol Cell Physiol 297: C1336–C1338.

Ruiz-Gaspà S, Guañabens N, Enjuanes A, Peris P, Martinez-Ferrer A, de Osaba, MJ, Gonzalez B, Alvarez L, Monegal A, Combalia A, Parés A. 2010. Lithocholic acid downregulates vitamin D effects in human osteoblasts. Eur J Clin Invest 40:25–34.

Skerry TM. 2008. The role of glutamate in the regulation of bone mass and architecture. J Musculoskelet Neuronal Interact 8:166–173.

Sooy K, Sabbagh Y, Demay MB. 2005. Osteoblasts lacking the vitamin D receptor display enhanced osteogenic potential in vitro. J Cell Biochem 94:81–87.

St-Arnaud R., 2008. The direct role of vitamin D on bone homeostasis. Arch Biochem Biophys 473:225–230.

Takarada T, Hinoi E, Fujimori S, Tsuchihashi Y, Ueshima T, Taniura H, Yoneda Y. 2004. Accumulation of [3H] glutamate in cultured rat calvarial osteoblasts. Biochem Pharmacol 68:177–184.

Takarada T, Yoneda Y. 2008. Pharmacological topics of bone metabolism: Glutamate as a signal mediator in bone. J Pharmacol Sci 106:536–541.

Takayanagi H. 2007. Osteoimmunology: Shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol 7:292–304.

Tanaka H, Seino Y. 2004. Direct action of 1,25-dihydroxyvitamin D on bone: VDRKO bone shows excessive bone formation in normal mineral condition. J Steroid Biochem Mol Biol 89–90:343–345.

Tsukii K, Shima N, Mochizuki S, Yamaguchi K, Kinosaki M, Yano K, Shibata O, Udagawa N, Yasuda H, Suda T, Higashio K. 1998. Osteoclast differentiation factor mediates an essential signal for bone resorption induced by 1 alpha, 25-dihydroxyvitamin D3, prostaglandin E2, or parathyroid hormone in the microenvironment of bone. Biochem Biophys Res Commun 246:337–341.

Walsh MC, Kim N, Kadono Y, Rho J, Lee SY, Lorenzo J, Choi Y. 2006. Osteoimmunology: Interplay between the immune system and bone metabolism. Annu Rev Immunol 24:33–63.

White JH. 2004. Profiling 1,25-dihydroxyvitamin D3-regulated gene expression by microarray analysis. J Steroid Biochem Mol Biol 89–90: 239–244.

Yamamoto Y, Yoshizawa T, Fukuda T, Kawano H, Nakamura T, Yamada T, Karsenty G, Kato A. 2004. A genetic evidence of direct VDR function in osteoblasts. Generation and analysis of osteoblast-specific VDR KO mice. J Bone Miner Res 19:S26.

Yeh WC, Chen NJ. 2003. Immunology: Another toll road. Nature 424:736–737.

Young RA. 2000 Biomedical discovery with DNA arrays. Cell 102:9–15.