

# Calcium Sulfate: Analysis of MG63 Osteoblast-like Cell Response by Means of a Microarray Technology

Francesco Carinci,<sup>1</sup> Adriano Piattelli,<sup>2</sup> Giordano Stabellini,<sup>3</sup> Annalisa Palmieri,<sup>4</sup> Luca Scapoli,<sup>4</sup> Gregorio Laino,<sup>5</sup> Sergio Caputi,<sup>2</sup> Furio Pezzetti<sup>6</sup>

<sup>1</sup> Maxillofacial Surgery, University of Ferrara, Corso Giovecca, 203, 44100 Ferrara, Italy

<sup>2</sup> Dental Clinic, University of Chieti, via Schiucchi 63, 66100 Chieti, Italy

<sup>3</sup> Institute of Histology, University of Milano, via Mangiagalli 31, 20133 Milano, Italy

<sup>4</sup> Department of Morphology and Embryology, University of Ferrara, via Fossato di Mortara 64/B, 44100 Ferrara, Italy

<sup>5</sup> Dental Clinic, Second University of Naples, via De Crecchio 8, 80138 Naples, Italy

<sup>6</sup> Institute of Histology, University of Bologna, and Centre of Molecular Genetics, CARISBO Foundation, via Belmeloro 8, 40126 Bologna, Italy

Received 9 September 2003; revised 4 March 2004; accepted 5 March 2004

Published online 22 September 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.b.30133

**Abstract:** Calcium sulfate (CaS) is an highly biocompatible material that has the characteristic of being one of the simplest as well as one of the synthetic bone-like graft with the longest clinical history, spanning more than 100 years. Solidified or crystallized CaS is very osteogenic *in vivo*. As the surface CaS dissolves in body fluid, the calcium ions form calcium phosphate that reprecipitates on the surface forming an osteoblast “friendly” environment. How this “friendly” environment alters osteoblast activity to promote bone formation is poorly understood. We therefore attempted to address this question by using microarray techniques to identify genes that are differently regulated in osteoblasts exposed to CaS. By using DNA microarrays containing 19,200 genes, we identified in osteoblast-like cells line (MG-63) cultured with CaS (SurgiPlaster, Classiimplant, Roma, Italy) several genes that expression was significantly upregulated. The differentially expressed genes cover a broad range of functional activities: (a) immunity, (b) lysosomal enzymes production, (c) cell cycle regulation, (d) and signaling transduction. It was also possible to detect some genes whose function is unknown. The data reported are, to our knowledge, the first genetic portrait of CaS effects. They can be relevant to better understand the molecular mechanism of bone regeneration and as a model for comparing other materials with similar clinical effects. © 2004 Wiley Periodicals, Inc. *J Biomed Mater Res Part B: Appl Biomater* 71B: 260–267, 2004

**Keywords:** calcium sulfate; DNA microarray; gene expression; gene profiling

## INTRODUCTION

Several graft materials have been proposed in implant dentistry. Autogenous bone is the golden standard but usually donor oral sites have limited amount of graft material. Consequently, surgeons harvest bone from extraoral sites with increased morbidity and the need of general anesthesia.<sup>1,2</sup> An

alloplastic material avoids the need of a second surgical field but it should be safe, resorbable, able to maintain space, and cheap.<sup>3,4</sup> Calcium sulfate (CaS) is highly biocompatible, and it is one of the synthetic graft with the longest clinical history (more than 100 years).<sup>5–12</sup> It has been utilized in periodontal disease, endodontic lesions, alveolar bone loss, and maxillary sinus augmentation.<sup>3,4,13–18</sup> CaS has been used as a membrane to facilitate healing and to prevent loss of other grafting materials.<sup>19</sup> When associated with other bone-like grafts it seems to have a favorable effect on osteogenesis.<sup>2,20</sup> CaS rapidly resorbs and leaves a calcium phosphate lattice, which promotes osteogenic activity.<sup>21,22</sup> Ricci et al.<sup>23</sup> demonstrated that CaS induces new bone formation in dogs after 2 weeks, and that it is almost completely resorbed after 1 months.

Correspondence to: A. Piattelli, via F. Sciucchi 63, 66100 Chieti, Italy (e-mail: apiattelli@unich.it)

Contract grant sponsor: University of Ferrara, Italy (to F.C.)

Contract grant sponsor: Fondazione CARIFE (to F.C.)

Contract grant sponsor: Guya-bioscience, Ferrara, Italy (to F.P.)

Contract grant sponsor: Fondazione CARISBO (to F.P.)

Because the mechanism by which CaS stimulates osteoblast activity to promote bone formation is poorly understood, we therefore attempted to address this question by using microarray techniques.

DNA microarray is a molecular technology that enables the analysis of gene expression in parallel on a very large number of genes, spanning a significant fraction of the human genome. Gene expression is performed by a process of (1) RNA extraction, (2) reverse transcription, and (3) labeling of cDNA. Reference (i.e., untreated cells) and investigated (i.e., cells cultured with CaS) cDNA are labeled with different dyes and then hybridized on slides containing cDNA fragments. Then the slides are scanned with a laser system, and two false color images are generated for each hybridization with cDNA from the investigated and reference cells. The overall result is the generation of a so-called genetic portrait.<sup>24–26</sup> It corresponds to up- or downregulated genes in the investigated cell system.

In the present study we define the genetic effect of CaS on cells by using an osteoblast-like cell line (MG63) and microarray slides containing 19,200 different oligonucleotides.

## MATERIALS AND METHODS

### Cell Culture

Osteoblast-like cells (MG63) were cultured in sterile Falcon wells (Becton Dickinson, Franklin Lakes, NJ) containing Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) (Sigma Chemical Co., St. Louis, MO) and antibiotics (Penicillin 100 U/mL and Streptomycin 100 µg/mL, Sigma Chemical Co.). Cultures were maintained in a 5% CO<sub>2</sub> humidified atmosphere at 37°C.

MG63 cells were collected and seeded at a density of  $1 \times 10^5$  cells/mL into 9 cm<sup>2</sup> (3 mL) wells by using 0.1% trypsin, 0.02% EDTA in Ca<sup>++</sup>- and Mg-free Eagle's buffer for cell release. One set of wells were added with CaS (Surgiplaster, Classimplant, Roma, Italy) at the concentration of 0.001 mg/mL. After 24 h, when cultures were subconfluent, cells were processed for RNA extraction. Another set of wells were added with 0.01 mg/mL of CaS, but this concentration was toxic, and after 24 h all cells died. We try three times to cultured MG63 at this CaS concentration, but the result was always negative.

### DNA Microarrays Screening and Analysis

The protocol was the same as a previous experiment.<sup>26</sup> RNA was extracted from the cells by using RNAzol. Ten micrograms of total RNA were used for each sample. cDNA was synthesized by using Superscript II (Life Technologies, Invitrogen, Milano, Italy) and amino-allyl dUTP (Sigma). Monoreactive Cy3 and Cy5 esters (Amersham Pharmacia, Little Chalfont, UK) were used for indirect cDNA labeling. RNA extracted from untreated cells was labeled with Cy3 and used as control against the Cy5-labeled treated (CaS) cDNA in the first experiment and then switched. Human 19.2 K

DNA microarrays were used (Ontario Cancer Institute, Toronto, Canada). For 19.2-K slides 100 µL of the sample and control cDNAs in DIG Easy hybridization solution (Roche, Basel, Switzerland) were used in a sandwich hybridization of the two slides constituting the 19.2. K set at 37°C overnight. Washing was performed three times for 10 min with 1× saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at 42°C, and three times for 5 min with 0.1× SSC at room temperature. Slides were dried by centrifugation for 2 min at 2000 rpm. The experiment was repeated twice and the dyes switched. A GenePix 4000a DNA microarrays scanner (Axon, Union City, CA) was used to scan the slides, and data were extracted with GenePix Pro. Genes with expression levels, after removing local background, of less than 1000 were not included in the analysis, because ratios are not reliable at that detection level.

## RESULTS

### DNA Microarrays

After scanning of the two slides containing the 19,200 human genes in duplicate, local background was calculated for each target location. A normalization factor was estimated from ratios of median. Normalization was performed, by adding the log<sub>2</sub> of the normalization factor to the log<sub>2</sub> of the ratio of medians. The log<sub>2</sub> ratios for all the targets on the array were then calibrated using the normalization factor, and log<sub>2</sub> ratios outside the 99.7% confidence interval (the median ± 3 times the SD = 0.52) were determined as significantly changed in the treated cells. Thus, genes are significantly modulated in expression when the absolute value of their log<sub>2</sub> expression level is higher than 1.56, or else there is a threefold difference in expression between treated cells and reference. GenePix Pro software was used to report genes above the threshold and with less than 10% difference in three different statistical evaluation of the intensity ratio, thus effectively enabling an automated quality control check of the hybridized spots. Furthermore, all the positively passed spots were finally visually inspected. SAM (significance analysis of microarray) program was then performed and SAM score was obtained (T-statistic value).<sup>24–26</sup>

The genes differentially expressed in cells treated with CaS are reported in Tables I, whereas in Figure 1 is reported the SAM plot.

## DISCUSSION

CaS is an highly biocompatible material.<sup>1–23</sup> Solidified or crystallized CaS is very osteogenic *in vivo*. As the surface CaS dissolves in body fluid, the calcium ions form calcium phosphate that reprecipitates on the surface forming an osteoblast “friendly” environment. How this “friendly” environment alters osteoblast activity to promote bone formation is poorly understood. We therefore attempted to address this

TABLE I. Upregulated Genes

CloneID	Name	Symbol	Chromosome	Score
128290	<i>Homo sapiens</i> -transcribed sequence with weak similarity to protein ref:NP_062553.1 (H.sapiens)	EST		203.180062
188230	ataxia telangiectasia mutated (includes complementation groups A, C, and D)	ATM	11q22–q23	189.4630148
182747	MAP/microtubule affinity-regulating kinase 1	MARK1	1q42.11	166.1802723
127592	transmembrane 9 superfamily member 1	TM9SF1	14q11.2	164.684725
130391	cathepsin S	CTSS	1q21	146.4742615
138400	<i>Homo sapiens</i> -transcribed sequences	EST		144.1357331
177612	RAB6A, member RAS oncogene family	RAB6A	11q13.3	141.6334113
200918	CD58 antigen, (lymphocyte function-associated antigen 3)	CD58	1p13	131.3135017
37996	Sapiens, clone IMAGE:3957507, mRNA	EST		130.5896109
5431259	glyceraldehyde-3-phosphate dehydrogenase	GAPD	12p13	129.3363762
112831	<i>Homo sapiens</i> -transcribed sequence with weak similarity to protein ref:NP_060312.1 (H.sapiens)	EST		127.3618882
146234	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	PPP2CA	5q23–q31	125.7410337
4746020	ubiquitin specific protease 3	USP3	15q22.3	123.1548725
141842	Sapiens cDNA FLJ35508 fis, clone SMINT2011958.	EST		123.1413986
156918	ATPase, H <sup>+</sup> transporting, lysosomal 38kDa, V0 subunit d isoform 1	ATP6V0D1	16q22	115.3472842
211361	<i>Homo sapiens</i> -transcribed sequences	EST		115.3340398
146479	Sapiens, clone IMAGE:3929520, mRNA	EST		112.7213263
503670	C3HC4-type zinc finger protein	LZK1	17q21.1	111.6576297
4711393	ferritin, light polypeptide	FTL	19q13.3–q13.4	110.959649
113150	lipidosin	BG1	15q23–q24	109.94988
3916591	U5 snRNP-specific 40-kDa protein (hPrp8-binding)	HPRP8BP	1p35.1	109.782933
149286	similar to RAN-binding protein 2-like 1 isoform 1; sperm membrane protein BS-63; RAN-binding protein 2-like 1	Na	2p11.2	108.6257557
135545	Sapiens, clone IMAGE:5288497, mRNA	EST		107.3679651
137669	interleukin 1 receptor accessory protein	IL1RAP	3q28	106.9939993
115376	<i>Homo sapiens</i> -transcribed sequence with weak similarity to protein ref:NP_060190.1 (H.sapiens)hypothetical protein FLJ20234 [Homo sapiens]	EST		103.9013874
5839943	disrupter of silencing 10	SAS10	4q13.3	103.6210568
206806	hyaluronoglucosaminidase 1	HYAL1	3p21.3–p21.2	97.96440077
146793	zinc-finger protein 317	ZNF317		97.37549663
182877	prickle-like 2 (Drosophila)	PRICKLE2	3p21.1	97.08282314
202332	phosphodiesterase 2A, cGMP-stimulated	PDE2A	11q13.3	97.0003003
150216	<i>Homo sapiens</i> -transcribed sequence with moderate similarity to protein ref:NP_060219.1 (H.sapiens)hypothetical protein FLJ20294 [Homo sapiens]	EST		96.32832313
206717	CHK1 checkpoint homolog (S. pombe)	CHEK1	11q24–q24	96.29710943
130974	<i>Homo sapiens</i> -transcribed sequences	EST		94.57304528
283078	similar to zinc-finger protein Zec	NA	19q13.43	94.56675564
157828	runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	RUNX1	21q22.3	93.08834526
173188	pregnancy-induced growth inhibitor	OKL38	16q23.3	90.53672503
147071	zinc-finger protein 294	ZNF294	21q22.11	89.7595522
295101	ferritin, light polypeptide	FTL	19q13.3–q13.4	89.24437416
485058	basic leucine zipper transcription factor ATF-like	BATF	14q24.3	89.15756258

TABLE I. (Continued)

CloneID	Name	Symbol	Chromosome	Score
158110	hypothetical protein MGC5395	MGC5395	11q12.2	88.42385136
261698	hypothetical protein FLJ13154	FLJ13154	16q13	87.60870133
304947	Sapiens cDNA FLJ39344 fis, clone OCBBF2019108.	EST		86.69067134
152226	spermidine/spermine N1-acetyltransferase	SAT	Xp22.1	85.80766474
172811	gem (nuclear organelle) associated protein 5	GEMIN5	5q33.2	85.00656504
154604	plasmalemma vesicle associated protein	PLVAP	19p13.2	82.45621216
134887	epithelial membrane protein 2	EMP2	16p13.2	78.54099109
179016	ribonuclease 6 precursor	RNASE6PL	6q27	78.02007156
3002315	zinc-finger protein 255	ZNF255	19q13.2	77.67155417
146689	pregnancy specific beta-1-glycoprotein 3	PSG3	19q13.2	75.80046847
301007	mitochondrial ribosomal protein L54	MRPL54	19p13.3	74.95007578
153209	hypothetical protein DKFZp434N1923	DKFZP434N 1923	8q24.3	74.48433194
156726	cadherin-like 24	CDH24	14q11.2	72.72093835
129683	hypothetical protein DKFZp434K1421	DKFZP434K 1421	17q11.2	72.39493684
204258	Sapiens mRNA full-length insert cDNA clone EUROIMAGE 200999.	EST		70.27008804
150451	<i>Homo sapiens</i> -transcribed sequences	EST		70.0586532
3907199	Wilms tumor 1-associated protein	WTAP	6q25-q27	69.92274729
207199	heat-shock 70 kDa protein 4	HSPA4	5q31.1-q31.2	69.79101752
5239635	ribosomal protein S7	RPS7	2p25	67.09322958
203557	hypothetical protein FLJ31842	FLJ31842	1p22.1	65.6732234
488208	quinoid dihydropteridine reductase	QDPR	4p15.31	65.10054965
491346	<i>Homo sapiens</i> -transcribed sequence with weak similarity to protein ref:NP_060312.1 (H.sapiens)hypothetical protein FLJ20489 [ <i>Homo sapiens</i> ]	EST		64.84846564
308924	hemoglobin, epsilon 1	HBE1	11p15.5	64.82562116
146832	cadherin 11, type 2, OB-cadherin (osteoblast)	CDH11	16q22.1	64.08069281
201514	<i>Homo sapiens</i> -transcribed sequences	EST		64.04506263
204541	asialoglycoprotein receptor 1	ASGR1	17p13.2	63.59669971
204285	<i>Homo sapiens</i> -transcribed sequence with moderate similarityto protein ref:NP_060312.1 (H.sapiens)hypothetical protein FLJ20489 [ <i>Homo sapiens</i> ]	EST		62.41511489
154567	hypothetical protein LOC155036	LOC155036	7q36.1	62.01624562
3847469	U5 snRNP-specific 40 kDa protein (hPrp8- binding)	HPRP8BP	1p35.1	59.06658543
202712	hypothetical protein FLJ33817	FLJ33817	17p13.3	58.38702567
47723	fibroblast growth factor receptor 1 (fms- related tyrosine kinase 2, Pfeiffer syndrome)	FGFR1	8p11.2-p11.1	58.30087504
46747	replication factor C (activator 1) 5, 36.5kDa	RFC5	12q24.2	58.28282281
4891768	calcium/calmodulin-dependent protein kinase kinase 1, alpha	CAMKK1	17p13.3	56.95053377
111834	<i>Homo sapiens</i> -transcribed sequences	EST		56.89418036
202492	<i>Homo sapiens</i> -transcribed sequence with weak similarityto protein ref:NP_060265.1 (H.sapiens)hypothetical protein FLJ20378 [ <i>Homo sapiens</i> ]	EST		56.82799984
137545	cortactin binding protein 2	CORTBP2		56.82035997
200611	hydroxymethylbilane synthase	HMBS	11q23.3	56.70250989
204244	cytochrome P450, family 39, subfamily A, polypeptide 1	CYP39A1	6p21.1-p11.2	56.56148438
417978	<i>Homo sapiens</i> -transcribed sequence with moderate similarityto protein ref:NP_054848.1 (H.sapiens)PRO0478 protein [ <i>Homo sapiens</i> ]	EST		56.40403369
194400	<i>Homo sapiens</i> -transcribed sequences	EST		56.35864512

TABLE I. (Continued)

CloneID	Name	Symbol	Chromosome	Score
239516	THO complex 2	THOC2	Xq25–q26.3	56.27577796
3930678	<i>Homo sapiens</i> -transcribed sequence with strong similarity to protein pir:S22655 (H.sapiens)S22655 translation elongation factor eEF-1 gamma chain—human	EST		55.85788085
46977	<i>Homo sapiens</i> -transcribed sequence with weak similarity to proteinref:NP_054848.1 (H.sapiens)PRO0478 protein [Homo sapiens]	EST		55.58310056
152746	Sapiens cDNA FLJ30137 fis, clone BRACE2000078.	EST		55.50820159
417759	TAF10 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 30kDa	TAF10	11p15.3	55.10232199
127657	<i>Homo sapiens</i> -transcribed sequences	EST		54.9398604
300990	<i>Homo sapiens</i> -transcribed sequences	EST		54.53667481
133274	solute carrier family 31 (copper transporters), member 1	SLC31A1	9q31–q32	54.37876775
295290	<i>Homo sapiens</i> -transcribed sequence with weak similarity to proteinref:NP_055301.1 (H.sapiens)neuronal thread protein [Homo sapiens]	EST		54.33303119
162406	Sapiens cDNA FLJ35653 fis, clone SPLEN2013690.	EST		54.32927727
150304	inositol polyphosphate-5-phosphatase, 145kDa	INPP5D	2q36–q37	54.27866481
147079	<i>Homo sapiens</i> -transcribed sequence with weak similarity to proteinref:NP_060265.1 (H.sapiens)hypothetical protein FLJ20378 [Homo sapiens]	EST		53.96268851
200247	Sapiens mRNA full-length insert cDNA clone EUROIMAGE 200247.	EST		53.84314649
5839199	immunoglobulin kappa constant	IGKC	2p12	53.32970192
179923	Sapiens cDNA FLJ38931 fis, clone NT2NE2013189.	EST		53.17099252
128023	ALL1 fused gene from 5q31	AF5Q31	5q31	53.14350374
44263	hypothetical protein FLJ20300	FLJ20300	9q31.1	52.76298657
4696228	tissue inhibitor of metalloproteinase 2	TIMP2	17q25	52.42261996
178524	<i>Homo sapiens</i> -transcribed sequences	EST		52.1763595
145946	zinc-finger protein 226	ZNF226	19q13.2	52.1396788
4450636	DKFZP566I1024 protein	DKFZP566I1024	7q11.1	51.94750674
154568	nuclear mitotic apparatus protein 1	NUMA1	11q13	51.85506409
5836188	hypothetical protein FLJ20312	FLJ20312	1p36.11	51.69272803
147932	<i>Homo sapiens</i> -transcribed sequence with weak similarity to protein ref:NP_060265.1 (H.sapiens) hypothetical protein FLJ20378 [Homo sapiens]	EST		51.50838243
126265	<i>Homo sapiens</i> -transcribed sequences	EST		51.25882648
343096	zinc-finger protein 136 (clone pHZ-20)	ZNF136	19p13.2–p13.12	49.97533595

question by using microarray techniques to identified genes that are differently regulated in osteoblasts exposed to CaS.

We cultured MG63 with two concentration of CaS: 0.001 and 0.01 mg/mL. The last was toxic, and after 24 h all cells died. It is not surprising because an *in vitro* system differs considerably from an *in vivo* system. Probably the highest concentration of CaS modifies the pH of the medium that has not great amount of phosphate.

Hybridization of cDNA (derived from MG63 cultured with 0.001 mg/mL of CaS) to cDNA microarrays allowed us to perform systemic analysis of expression profiles for thousands of genes simultaneously and to provide primary information on transcriptional changes related to CaS. We identified several genes whose expression was definitely upregulated.

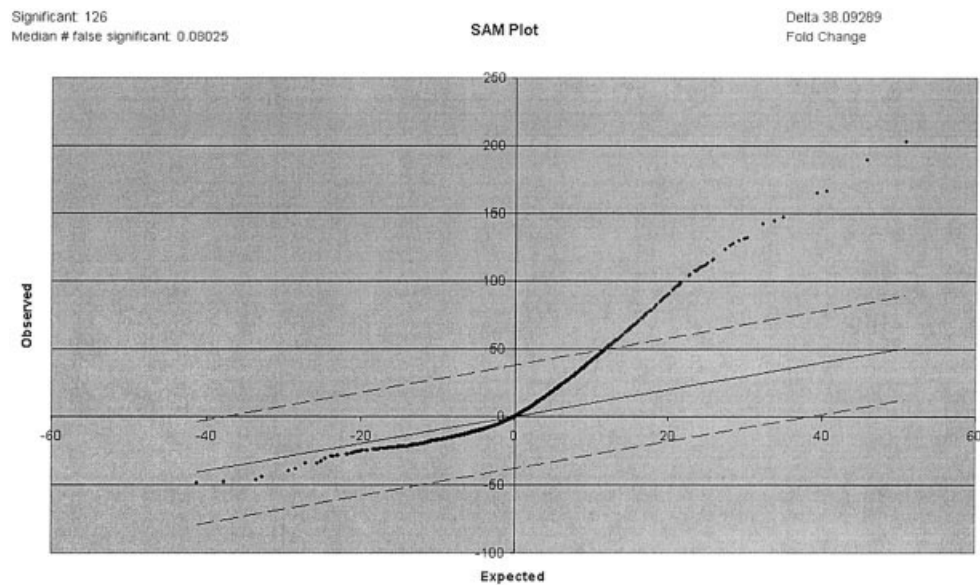
#### Upregulated Genes (Table I)

Among the upregulated genes some are involved in cell cycle regulation and signaling transduction. ATM belongs to the

PI3/PI4-kinase family. This kinase is an important cell cycle checkpoint. It functions as a regulator of a wide variety of downstream proteins, including tumor suppressor proteins p53 and BRCA1, checkpoint kinase CHK2, checkpoint proteins RAD17 and RAD9, and DNA repair protein NBS1. ATM and the closely related kinase ATR are thought to be master controllers of cell cycle checkpoint signaling pathways that are required for cell response to DNA damage and for genome stability. Mutations in this gene are associated with ataxia telangiectasia, an autosomal recessive disorder.<sup>27</sup>

Among the signaling transduction upregulated genes there is CAMKK1. It belongs to the Serine/Threonine protein kinase family, and to the Ca<sup>2+</sup>/calmodulin-dependent protein kinase subfamily. This protein plays a role in the calcium/calmodulin-dependent (CaM) kinase cascade.<sup>28</sup>

FGFR1 is a member of the fibroblast growth factor receptor family, where amino acid sequence is highly conserved between members and throughout evolution. FGFR family



**Figure 1.** SAM (significance analysis of microarray) plot of MG63 treated for 24 h with calcium sulfate at the concentration of 0.001 mg/mL.

members differ from one another in their ligand affinities and tissue distribution. A full-length representative protein consists of an extracellular region, composed of three immunoglobulin-like domains, a single hydrophobic membrane-spanning segment, and a cytoplasmic tyrosine kinase domain. The extracellular portion of the protein interacts with fibroblast growth factors, setting in motion a cascade of downstream signals, ultimately influencing mitogenesis and differentiation. This particular family member binds both acidic and basic fibroblast growth factors, and is involved in limb induction. Mutations in this gene can lead to Pfeiffer syndrome and Jackson-Weiss syndrome, syndromes characterized by anomalies of craniofacial and limb skeleton.<sup>29</sup>

CaS acts also on a gene-related immunity system. CD58 is an immunoglobulin superfamily receptor. It interacts with CD2 receptor and aids the activity of helper T cells.<sup>30</sup> Interleukin 1 induces synthesis of acute phase and proinflammatory proteins during infection, tissue damage, or stress, by forming a complex at the cell membrane with an Interleukin 1 receptor and an accessory protein. IL1RAP encodes an interleukin 1 receptor accessory protein,<sup>31</sup> and it is upregulated when MG63 cells are treated with CaS.

Several upregulated proteins are enzymes contained in lysosomes. CTSS, a member of the peptidase C1 family, is a lysosomal cysteine proteinase that may participate in the degradation of antigenic proteins to peptides for presentation on MHC class II molecules. The encoded protein can function as an elastase over a broad pH range in alveolar macrophages.<sup>32</sup> ATP6V0D1 encodes a component of vacuolar ATPase (V-ATPase), a multisubunit enzyme that mediates acidification of eukaryotic intracellular organelles. V-ATPase dependent organelle acidification is necessary for such intracellular processes as protein sorting, zymogen activation, and receptor-mediated endocytosis. V-ATPase is comprised of a cytosolic V1

domain and a transmembrane V0 domain.<sup>33</sup> HYAL1 encodes a lysosomal hyaluronidase. Hyaluronidases intracellularly degrade hyaluronan, one of the major glycosaminoglycans of the extracellular matrix. Hyaluronan is thought to be involved in cell proliferation, migration, and differentiation. This enzyme is active at an acidic pH, and is the major hyaluronidase in plasma. Mutations in this gene are associated with mucopolysaccharidosis type IX, or hyaluronidase deficiency.<sup>34</sup>

TIMP2 belongs to the TIMP gene family. The proteins encoded by this gene family are natural inhibitors of the matrix metalloproteinases, a group of peptidases involved in degradation of the extracellular matrix. Unlike the inducible expression of some other TIMP gene family members, the expression of this gene is largely constitutive.<sup>35</sup>

The genes discussed are only a limited number among those differentially expressed and reported in Table I. We briefly analyzed some of those with a better known function.

In conclusion, CaS is able to upregulate some functional activities of osteoblast-like cells: cell cycle regulation, signal transduction, immunity, and production of lysosomal enzymes. These last proteins act on components of immunity system and on turnover of the extracellular matrix. It is not clear, at the moment, the precise interaction among the discussed genes and, above all, which of them have the most relevant role in bone formation.

It is worth noting that MG63 are a cell line and not normal osteoblasts. Notwithstanding the advantages of using a cell line is related to the fact that the reproducibility of the data is higher because there is not the variability of the patient studied. Primary cell cultures provide a source of normal cells, but they also contain contaminating cells of different types and cells in variable differentiation states. Moreover, we have chosen to perform the experiment after 24 h to get information on the early stages of stimulation. It is our

knowledge, therefore, that more investigations with other osteoblast-like cell lines, primary cultures, and different time points are needed to get a global comprehension of the molecular events related to CaS action.

Finally, we believe that the reported data can be a model to compare different substances (i.e., BMPs) with similar effects.

## REFERENCES

- Guarnieri R, Bovi M. Maxillary sinus augmentation using pre-hardened calcium sulfate: A case report. *Int J Periodontics Restorative Dent* 2002;22:503–508.
- McNeill SR, Cobb CM, Rapley JW, Glaros AG, Spencer P. In vivo comparison of synthetic osseous graft materials. A preliminary study. *J Clin Periodontol* 1999;26:239–245.
- Pecora G, De Leonardis D, Ibrahim N, Bovi M, Cornelini R. The use of calcium sulfate in the surgical treatment of a “through and through” periradicular lesion. *Int Endod J* 2001;34:189–197.
- Yoshikawa G, Murashima Y, Wadachi R, Sawada N, Suda H. Guided bone regeneration (GBR) using membranes and calcium sulphate after apicectomy. A comparative histomorphometrical study. *Int Endod J* 2002;35:255–263.
- Alderman N. Sterile plaster of Paris as an implant in the infrabony environment: A preliminary study. *J Periodontol* 1969;40:11–13.
- Alexander DI, Manson NA, Mitchell MJ. Efficacy of calcium sulfate plus decompression bone in lumbar and lumbosacral spinal fusion: Preliminary results in 40 patients. *Can J Surg* 2001;44:262–266.
- Gitelis S, Piasecki P, Turner T, Haggard W, Charters J, Urban R. Use of a calcium sulfate-based bone graft substitute for benign bone lesions. *Orthopedics* 2001;24:162–166.
- Peltier LF. The use of plaster of Paris to fill large defects in bone. *Am J Surg* 1959;97:311.
- Pietrzak WS, Ronk R. Calcium sulfate bone void filler: A review and a look ahead. *J Craniofac Surg* 2000;11:327–333.
- Shaffer D, App G. The use of plaster of Paris for treating infrabony periodontal defects in humans. *J Periodontol* 1971;42:685–690.
- Sottosanti JS. Calcium sulfate aided bone regeneration: A case report. *Periodontal Clin Invest* 1995;17:10–15.
- Coetzee AS. Regeneration of bone in the presence of calcium sulfate. *Arch Otolaryngol* 1980;106:405–409.
- Bier SJ, Sinensky MC. The versatility of calcium sulfate: Resolving periodontal challenges *Compend Contin Educ Dent* 1999;20:655–661.
- Kim CK, Ki HY, Chai JK, Cho KS, Moon IS, Choi SH, Sottosanti JS, Wikesjo UM. Effect of calcium sulfate implant with calcium sulfate barrier on periodontal healing in 3-wall intrabony defects in dogs. *J Periodontol* 1998;69:982–988.
- Orsini M, Orsini G, Benlloch D, Aranda JJ, Lazaro P, Sanz M, De Luca M, Piattelli A. Comparison of calcium sulfate and autogenous bone graft to bioabsorbable membranes plus autogenous bone graft in the treatment of intrabony periodontal defects. A split-mouth study. *J Periodontol* 2001;72:296–302.
- Pecora G, Baek SH, Rethnam S, Kim S. Barrier membrane techniques in endodontic microsurgery. *Dent Clin North Am* 1997;41:585–602.
- Pecora GE, De Leonardis D, Della Rocca C, Cornelini R, Cortesini C. Short-term healing following the use of calcium sulfate as a grafting material for sinus augmentation. A clinical report. *Int J Oral Maxillofac Implants* 1998;13:866–873.
- Kelly CM, Wilkins RM, Gitelis G, Hartjen C, Watson JT, Kim PT. The use of a surgical grade calcium sulfate as a bone graft substitute. Results of a multicenter study. *Clin Orthop* 2001;382:42–50.
- Maragos P, Bissada NF, Wang R, Cole RP. Comparison of three methods using calcium sulfate as a graft barrier material for the treatment of Class II mandibular molar furcation defects. *Int J Periodontics Restorative Dent* 2002;22:493–501.
- Al Ruhaimi KA. Effects of adding calcium sulfate to grafting materials in early bone regeneration in osseous defects in rabbits. *Int J Oral Maxillofac Implants* 2000;15:859–864.
- Damien CJ, Ricci JL, Christel P, Alexander H, Patat JL. Formation of a calcium phosphate-rich layer on absorbable calcium carbonate bone graft substitutes. *Calcif Tissue Int* 1994;55:151–158.
- Tay BK, Patel VV, Bradford DS. Calcium sulfate and calcium phosphate-based bone substitutes. Mimicry of the mineral phase of bone. *Orthop Clin North Am* 1999;30:615–623.
- Ricci JL, Alexander H, Nadkarni P, Hawkins M, Turner J, Rosenblum S, Brezenoff L, De Leonardis D, Pecora G. Biological mechanisms of calcium sulfate replacement by bone. In Davies JE, editor. *Bone engineering*. Toronto, Canada: Em Squared Incorporated; 2000. p 332–344.
- Carinci F, Francioso F, Rubini C, Fioroni M, Tosi L, Pezzetti F, Venturoli L, Volinia S, Piattelli A. Genetic portrait of malignant granular cell odontogenic tumour. *Oral Oncol* 2003;9:69–77.
- Carinci F, Francioso F, Piattelli A, Rubini C, Fioroni M, Evangelisti R, Tosi L, Pezzetti F, Carinci P, Volinia S. Genetic expression profiling of six odontogenic tumors. *J Dent Res* 2003;82:551–557.
- Carinci F, Pezzetti F, Volinia S, Francioso F, Arcelli D, Farina E, Piattelli A. Zirconium oxide: Analysis of MG63 osteoblast-like cell response by means of a microarray technology. *Biomaterials* 2004;25:215–228.
- Shiloh Y. ATM and related protein kinases: Safeguarding genome integrity. *Nat Rev Cancer* 2003;3:155–168.
- Strausberg RL, Feingold EA, Grouse LH, Derge JG, Klausner RD, Collins FS, Wagner L, Shenmen CM, Schuler GD, Altschul SF, Zeeberg B, Buetow KH, Schaefer CF, Bhat NK, Hopkins RF, Jordan H, Moore T, Max SI, Wang J, Hsieh F, Diatchenko L, Marusina K, Farmer AA, Rubin GM, Hong L, Stapleton M, Soares MB, Bonaldo MF, Casavant TL, Scheetz TE, Brownstein MJ, Usdin TB, Toshiyuki S, Carinci P, Prange C, Raha SS, Loquellano NA, Peters GJ, Abramson RD, Mullahy SJ, Bosak SA, McEwan PJ, McKernan KJ, Malek JA, Gunaratne PH, Richards S, Worley KC, Hale S, Garcia AM, Gay LJ, Hulyk SW, Villalón DK, Muzny DM, Sodergren EJ, Lu X, Gibbs RA, Fahey J, Helton E, Kettman M, Madan A, Rodrigues S, Sanchez A, Whiting M, Madan A, Young AC, Shevchenko Y, Bouffard GG, Blakesley RW, Touchman JW, Green ED, Dickson MC, Rodriguez AC, Grimwood J, Schmutz J, Myers RM, Butterfield YS, Krzywinski MI, Skalska U, Smailus DE, Schnerch A, Schein JE, Jones SJ, Marra MA; Mammalian Gene Collection Program Team. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences *Proc Natl Acad Sci USA* 2002;99:16899–16903.
- Dode C, Levilliers J, Dupont JM, De Paeppe A, Le Du N, Soussi-Yanicostas N, Coimbra RS, Delmaghani S, Compain-Nouaille S, Baverel F, Pecheux C, Le Tessier D, Cruaud C, Delpech M, Speleman F, Vermeulen S, Amalfitano A, Bachelot Y, Bouchard P, Cabrol S, Carel JC, Delemarre-van de Waal H, Goulet-Salmon B, Kottler ML, Richard O, Sanchez-Franco F, Saura R, Young J, Petit C, Hardelin JP. Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. *Nat Genet* 2003;33:463–465.

30. Barber DF, Long EO. Coexpression of CD58 or CD48 with intercellular adhesion molecule 1 on target cells enhances adhesion of resting NK cells. *J Immunol* 2003;170:294–299.
31. Smith DE, Hanna R, Della Friend, Moore H, Chen H, Farese AM, MacVittie TJ, Virca GD, Sims JE. The soluble form of IL-1 receptor accessory protein enhances the ability of soluble type II IL-1 receptor to inhibit IL-1 action. *Immunity* 2003;18:87–96.
32. Bania J, Gatti E, Lelouard H, David A, Cappello F, Weber E, Camosseto V, Pierre P. Human cathepsin S, but not cathepsin L, degrades efficiently MHC class II-associated invariant chain in nonprofessional APCs. *Proc Natl Acad Sci USA* 2003;100:6664–6669.
33. Agarwal AK, White PC. Structure of the VPATPD gene encoding subunit D of the human vacuolar proton ATPase. *Biochem Biophys Res Commun* 2000;279:543–547.
34. Triggs-Raine B, Salo TJ, Zhang H, Wicklow BA, Natowicz MR. Mutations in HYAL1, a member of a tandemly distributed multigene family encoding disparate hyaluronidase activities, cause a newly described lysosomal disorder, mucopolysaccharidosis IX. *Proc Natl Acad Sci USA* 1999;96:6296–300.
35. Hammani K, Blakis A, Morsette D, Bowcock AM, Schmutte C, Henriet P, DeClerck YA. Structure and characterization of the human tissue inhibitor of metalloproteinases-2 gene. *J Biol Chem* 1996;271:25498–25505.