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ORIGINAL ARTICLE

Effects of Emdogain on osteoblast gene expression

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OBJECTIVE: Emdogain (EMD) is a protein extract purified from porcine enamel and has been introduced in clinical practice to obtain periodontal regeneration. EMD is composed mainly of amelogenins (90%), while the remaining 10% is composed of non-amelogenin enamel matrix proteins such as enamelins, tuftelin, amelin and ameloblastin. Enamel matrix proteins seem to be involved in root formation. EMD has been reported to promote proliferation, migration, adhesion and differentiation of cells associated with healing periodontal tissues *in vivo*.

DESIGN: How this protein acts on osteoblasts is poorly understood. We therefore attempted to address this question by using a microarray technique to identify genes that are differently regulated in osteoblasts exposed to enamel matrix proteins.

RESULTS: By using DNA microarrays containing 20 000 genes, we identified several upregulated and downregulated genes in the osteoblast-like cell line (MG-63) cultured with enamel matrix proteins (Emd). The differentially expressed genes cover a broad range of functional activities: (i) signaling transduction, (ii) transcription, (iii) translation, (iv) cell cycle regulation, proliferation and apoptosis, (v) immune system, (vi) vesicular transport and lysosome activity, and (vii) cytoskeleton, cell adhesion and extracellular matrix production.

CONCLUSIONS: The data reported are the first genome-wide scan of the effect of enamel matrix proteins on osteoblast-like cells. These results can contribute to our understanding of the molecular mechanisms of bone regeneration and as a model for comparing other materials with similar clinical effects.

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Keywords: DNA microarray; enamel matrix proteins; gene expression; gene profiling; MG63 cells; osteoblast gene expression

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Introduction

Emdogain (EMD) is a protein extract purified from porcine enamel and has been introduced in clinical practice to obtain periodontal regeneration (Parkar and Tonetti, 2004). EMD is mainly composed of amelogenins (90%) (Schwartz *et al*, 2000; Parkar and Tonetti, 2004; Shimizu *et al*, 2004): the remaining 10% is composed of non-amelogenin enamel matrix proteins such as enamelins, tuftelin, amelin and ameloblastin (Schwartz *et al*, 2000; Shimizu *et al*, 2004). Amelogenins are derived from a single gene by alternative splicing of the primary transcript and also by post-translational processing of the major peptide giving rise to a range of proteins and peptides (Parkar and Tonetti, 2004). Enamel matrix proteins seem to be involved in root formation (Baobaid *et al*, 2004) and amelogenin null mice showed a reduced expression of bone sialoprotein along the tooth root surface (cementoblasts) (Baobaid *et al*, 2004). EMD promotes the proliferation, migration, adhesion and differentiation of cells associated with healing periodontal tissues *in vivo* (Tokiyasu *et al*, 2000). *In vitro* studies show that periodontal ligament cells treated with EMD demonstrate an enhanced production of protein, cell proliferation and ability to promote mineral nodule formation (Tokiyasu *et al*, 2000). The mechanisms by which EMD promotes cell activity remain unknown (Tokiyasu *et al*, 2000).

Little is known about the effects of EMD on bone metabolism (Shimizu *et al*, 2004). Conflicting reports have been published in the literature. On the one hand, EMD has been found to alter genes associated with cementoblast and osteoblast maturation (Tokiyasu *et al*, 2000), to regulate cells in the osteoblastic lineage (Schwartz *et al*, 2000), to prolong cultured mouse primary osteoblast growth (Jiang *et al*, 2001), to influence bone metabolism through the activation of growth factors (Mizutani *et al*, 2003), to increase the number of osteoblasts (Mizutani *et al*, 2003), to enhance cell proliferation and viability of human osteoblasts on sandblasted and acid-etched surfaces (Schwartz *et al*, 2004), to increase the osteogenic capacity of bone marrow with an increase in cell numbers, increase in alkaline phosphatase activity and

increase in mineralized nodule formation (Keila *et al*, 2004). Moreover, EMD has been reported to stimulate the biosynthesis and regeneration of trabecular bone and the volume fraction of mineralized tissue appeared to be higher in EMD-applied bone than in controls (Sawae *et al*, 2002). EMD has also been found to be an effective matrix for enhancing new trabecular bone induction and resulting attachment of orthopedic prostheses to the recipient bone (Shimizu-Ishiura *et al*, 2002). On the other hand, Cornelini *et al* (2004), in a study in rabbit tibia, did not find differences in bone regeneration between sites treated with EMD and control sites. No beneficial effects on bone formation around titanium implants in rabbits were reported (Franke Stenport and Johansson, 2003) and in a study in the rat ramus, the use of EMD did not offer any added benefit in terms of new bone formation (Donos *et al*, 2005).

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

In the present study we tried to define the genetic effects of EMD on cells by using an osteoblast-like cell line (MG-63) and microarray slides containing 20 000 different oligonucleotides.

Materials and methods

Cell culture

The protocol was the same of previous experiments (Carinci *et al*, 2003, 2004a,b,c,d). Osteoblast-like cells (MG-63) were cultured in sterile Falcon wells (Becton Dickinson, Franklin Lakes, NJ, USA) containing Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) (Sigma Chemical Co., St Louis, MO, USA) and antibiotics (penicillin 100 U ml^{-1} and streptomycin 100 $\mu g\ ml^{-1}$; Sigma). Cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C.

MG-63 cells were collected and seeded at a density of 1×10^5 cells ml^{-1} into 9 cm^2 (3 ml) wells by using 0.1% trypsin, 0.02% ethylene diamine tetra-acetic acid in Ca++ and Mg-free Eagle's buffer for cell release. In one set of wells EMD (Emdogain; Biora AB, Malmo, Sweden) was added at the concentration of 250 $\mu g\ \mu l^{-1}$. After 24 h, when cultures were sub-confluent, cells were processed for RNA extraction.

DNA microarrays screening and analysis

Gene expression is performed by a process of (i) RNA extraction, (ii) reverse transcription and (iii) labeling of cDNA. Reference (i.e., untreated cells) and investigated (i.e., cells cultured with EMD) cDNA are labeled with different dyes and then hybridized on slides containing cDNA fragments. The slides are then 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 (Carinci *et al*, 2003, 2004a,b,c,d). It corresponds to upregulated or downregulated genes in the investigated cell system.

The protocol used in this study was in accordance with previous experiments (Carinci *et al*, 2003, 2004a,b,c,d). RNA was extracted from the cells by using RNAzol. Ten micrograms of total RNA was used for each sample. cDNA was synthesized by using Superscript II (Life Technologies, Invitrogen, Milan, Italy) and amino-allyl dUTP (Sigma). Mono-reactive 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 cDNA in the first experiment; in the second experiment the dyes were switched. Human 20 K DNA microarrays were used (MWG Biotech AG, Ebersberg, Germany). For 20 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 20 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 microarray scanner (Axon, Union City, CA, USA) 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, as ratios are not reliable at that detection level.

Results

DNA microarrays

After scanning of the two slides containing the 20 000 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 $\log_2$ of the normalization factor to the $\log_2$ of the ratio of medians. The $\log_2$ ratios for all the targets on the array were then calibrated using the normalization factor, and $\log_2$ ratios outside of 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 $\log_2$ 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 $<10\%$ difference in three different statistical evaluations 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. Significance analysis of microarray (SAM) program was then performed and SAM score was obtained (*t*-statistic value) (Carinci *et al*, 2003, 2004a,b,c,d).

The genes differentially expressed in cells treated with EMD are reported in Tables 1 and 2, whereas the SAM plot is reported in Figure 1.

Discussion

The treatment of different periodontal defects of various sizes with EMD stimulated the formation of a substantial periodontal regeneration with a high quantity of new cementum, Sharpey's fibers, periodontal ligament, and bone tissue (Cochran *et al*, 2003). A meta-analysis showed that EMD-treated sites presented a significant improvement of the clinical attachment level, and a pocket depth reduction when compared with flap surgery (Esposito *et al*, 2004). However, no evidence was found that more teeth could be saved and no differences were found in sites treated with EMD and guided tissue regeneration (Esposito *et al*, 2004). How EMD alters osteoblast activity to promote bone formation is poorly understood, and an evaluation of EMD in bone metabolism has been the focus of an intense research (Mizutani *et al*, 2003). We attempted to address this question by using a microarray technique to identify genes that were differently regulated in osteoblasts exposed to EMD.

Hybridization of cDNA (derived from M-G63 cultured with 250 μg μl^{-1} of EMD) to cDNA microarrays allowed us to perform a systemic analysis of the expression profiles for thousands of genes simultaneously and to provide primary information on transcriptional changes related to EMD. We identified several genes whose expression was definitely upregulated and downregulated.

Upregulated genes

Among the upregulated genes some are involved in signaling transduction (Table 1). Angiopoietins are members of the vascular endothelial growth factor (VEGF) family and the only known growth factors largely specific for vascular endothelium. Angiopoietin-1, angiopoietin-2, and angiopoietin-4 participate in the formation of blood vessels. ANGPTL2 protein is a secreted glycoprotein with homology to the angiopoietins and may exert a function on endothelial cells through autocrine or paracrine action (Davis *et al*, 1996; Horner *et al*, 2001; Arai *et al*, 2004) and mediates blood vessel maturation/stability. Angiogenesis is essential for bone growth and repair (Horner *et al*, 2001).

In the bone, Ang-1, Ang-2, and VEGF were detected in modeling and remodeling sitesHorner *et al* (2001). Ang-1 was detected in the majority of osteoblasts, osteoclasts, and in some marrow space cells.

These observations provided the first evidence for the expression of the angiopoietins in growing human bone *in vivo* (Horner *et al*, 2001). The distribution of Ang-1 and VEGF indicated that these factors may play a key role in the regulation of angiogenesis at sites of endochondral ossification, intramembranous ossification, and bone turnover in the growing human skeleton (Davis *et al*, 1996; Horner *et al*, 2001; Arai *et al*, 2004).

RAF1 is a mitogen-activated protein kinase (MAP) (MAP3K) which functions downstream of the Ras family of membrane-associated GTPases to which it binds directly (Alavi *et al*, 2003; O'Neill *et al*, 2004). Alavi *et al* (2003) concluded that RAF1 may be a pivotal regulator of endothelial cell survival during angiogenesis. O'Neill *et al* (2004) used proteomic analysis of RAF1 signaling complexes to show that RAF1 counteracts apoptosis by suppressing the activation of mammalian sterile 20-like kinase (MST2).

Once activated, Raf-1 can phosphorylate to activate the dual specificity protein kinases MEK1 and MEK2 which in turn phosphorylate to activate the serine/threonine-specific protein kinases ERK1 and ERK2. Activated ERKs are pleiotropic effectors of cell physiology and play an important role in the control of gene expression involved in cell division cycle, apoptosis, cell differentiation, and cell migration.

Transcription

FOXJ1 is a member of the forkhead gene family, which was originally identified in *Drosophila* and is composed of transcription factors (Lin *et al*, 2004). The forkhead family is composed of transcription factors with a conserved 100-amino acid DNA-binding motif. Lin *et al* (2004) concluded that FOXJ1 antagonizes proinflammatory transcriptional activities and potentially enforces lymphocyte quiescence.

TCEA2 is found in the nucleus, where it functions as an SII class transcription elongation factor. Elongation factors in this class are responsible for releasing RNA polymerase II ternary complexes from transcriptional arrest at template-encoded arresting sites. The encoded protein has been shown to interact with general transcription factor IIB, a basal transcription factor.

Translation

EEF1D encodes a subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome (van Damme *et al*, 1990; Sanders *et al*, 1991; von der Kammer *et al*, 1991; Pizzuti *et al*, 1993; Chambers *et al*, 2001).

The modification of proteins with ubiquitin is an important cellular mechanism for targeting abnormal or short-lived proteins for degradation. Ubiquitination involves at least three classes of enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin-protein ligases (E3s). UBE2J1 encodes a member of the E2 ubiquitin-conjugating enzyme family. This enzyme is located in the membrane of the endoplasmic reticulum (ER) and may contribute to quality control ER-associated degradation by the ubiquitin-proteasome system.

RNPS1 encodes a protein that is part of a postsplicing multiprotein complex involved in both mRNA nuclear export and mRNA surveillance. mRNA surveillance detects exported mRNAs with truncated open reading frames and initiates nonsense-mediated mRNA decay (NMD). When translation ends upstream from the last exon–exon junction, this triggers NMD to degrade mRNAs containing premature stop codons. This pro-

Table 1 Upregulated genes

GenBank	*Name*	*Symbol*	*Cytoband*	*Score (d)*
AK075144	Chloride intracellular channel 5	CLIC5	6p12.1–21.1	3,392213542
NM_021631	Apoptosis inhibitor	FKSG2	8p11.2	2,609932384
NM_002373	Microtubule-associated protein 1A	MAP1A	15q13-qter	2,545345232
AF134414	ABO blood group	ABO	9q34.1-q34.2	2,479912902
AY040829	Tumor protein p73	TP73	1p36.3	2,468325911
AF118124	Myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	1q21	2,462491919
AL138578	Adiponutrin	ADPN	22q13.31	2,392299523
AF020352	NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15 kDa (NADH-coenzyme Q reductase)	NDUFS5	1p34.2-p33	2,378955157
L39833	Potassium voltage-gated channel, shaker-related subfamily, β-member 1	KCNAB1	3q26.1	2,365748613
AF312864	Chromosome 1 open reading frame 21	C1orf21	1q25	2,342914164
BC005922	Islet cell autoantigen 1, 69 kDa	ICA1	7p22	2,34142417
M97260	ATPase, Ca++ transporting, plasma membrane 2	ATP2B2	3p25.3	2,338674601
L31860	Glycophorin A (includes MN blood group)	GYPA	4q28.2-q31.1	2,319310601
BC016392	Dedicator of cytokinesis 7	DOCK7	1p31.3	2,278347984
AF041245	Hypocretin (orexin) receptor 2	HCRTR2	6p11-q11	2,272894599
AF523361	CD34 antigen	CD34	1q32	2,272853764
AF180475	CCR4-NOT transcription complex, subunit 4	CNOT4	7q22-qter	2,271786411
NM_004934	Cadherin 18, type 2	CDH18	5p15.2-p15.1	2,244129109
NM_014776	G protein-coupled receptor kinase interactor 2	GIT2	12q24.1	2,229969374
NM_001851	Collagen, type IX, α-1	COL9A1	6q12-q14	2,229118952
NM_002209	Integrin, α-L	ITGAL	16p11.2	2,213735116
AK024550	Eukaryotic translation elongation factor 1 delta	EEF1D	8q24.3	2,180316528
AF174598	F-box protein 10	FBXO10	9p13.2	2,175119146
AY112721	UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase-like 4	GALNTL4	11p15.3	2,13873834
NM_006851	GLI pathogenesis-related 1 (glioma)	GLIPR1	12q21.1	2,122620245
BC021244	Centaurin, delta 2	CENTD2	11q13.4	2,121049624
D26445	Protein phosphatase 2, regulatory subunit B (B56), gamma isoform	PPP2R5C	14q32	2,120183348
BC032625	Somatostatin	SST	3q28	2,097646218
NM_002121	Major histocompatibility complex, class II, DP β-1	HLA-DPB1	6p21.3	2,094478177
AF125175	Angiopoietin-like 2	ANGPTL2	9q34	2,083719037
AK025438	Rho GTPase activating protein 12	ARHGAP12		2,078407147
AB013803	Contactin 5	CNTN5	11q21-q22.2	2,077731009
NM_014394	Growth hormone inducible transmembrane protein	GHITM	10q23.1	2,075804404
NM_005512	Glycoprotein A repetitions predominant	GARP	11q13.5-q14	2,075142082
AF448510	SET domain-containing protein 7	SET7	4q28	2,069508895
NM_006336	Chromosome 9 open reading frame 60	C9orf60	9q34.11	2,067491321
BC027710	Mitochondrial ribosomal protein L23	MRPL23	11p15.5-p15.4	2,051353053
NM_001454	Forkhead box J1	FOXJ1	17q22–17q25	2,042339077
BC011710	Hypoxia-inducible factor prolyl 4-hydroxylase	PH-4	3p21.31	2,031936972
AF036977	HLA complex group 4	HCG4	6p21.3	2,015811037
NM_004021	Dystrophin (muscular dystrophy, Duchenne and Becker types)	DMD	Xp21.2	1,99942993
AF202922	LRP16 protein	LRP16	11q11	1,994928832
AF151888	Chromosome 6 open reading frame 74	C6orf74	6q13-q24.3	1,984992376
NM_138928	Molybdenum cofactor synthesis 1	MOCS1	6p21.3	1,979164428
NM_003433	Zinc finger protein 132 (clone pHZ-12)	ZNF132	19q13.4	1,976214642
M15182	Glucuronidase, beta	GUSB	7q21.11	1,966941083
U03626	Arrestin 3, retinal (X-arrestin)	ARR3	Xcen-q21	1,965917487
BC005361	Proteasome (prosome, macropain) subunit, α-type, 4	PSMA4	15q25.1	1,964982999
AF047445	Killer cell lectin-like receptor subfamily A, member 1	KLRA1	12p13-p12	1,962875998
BC018896	Transcription elongation factor A (SII), 2	TCEA2	20q13.33	1,953438301
AB030733	Insulin-like growth factor 2, antisense	IGF2AS	11p15.5	1,94459361
AY129653	Potassium channel regulator	KCNRG	13q14.2	1,939623692
AF119386	Plasma glutamate carboxypeptidase	PGCP	8q22.2	1,935244212
AK000253	Development and differentiation enhancing factor-like 1	DDEFL1	1p36.12	1,930748181
AF078776	Tumor protein p53 binding protein, 1	TP53BP1	15q15-q21	1,929641353
AF062595	Adenylate kinase 5	AK5	1p31	1,929117604
AF225986	Sodium channel, voltage-gated, type III, alpha	SCN3A	2q24	1,922904869
L37368	RNA binding protein S1, serine-rich domain	RNPS1	16p13.3	1,91732159
NM_002880	V-raf-1 murine leukemia viral oncogene homolog 1	RAF1	3p25	1,911348033
AK001197	Fanconi anemia, complementation group L	FANCL	2p16.1	1,90794487
AF361351	Calcium channel, voltage-dependent, gamma subunit 5	CACNG5	17q24	1,903302094
M16985	Mannose-6-phosphate receptor (cation dependent)	M6PR	12p13	1,900997698
NM_080915	Deoxyguanosine kinase	DGUOK	2p13	1,898796175
AF152515	Protocadherin gamma subfamily C, 3	PCDHGC3	5q31	1,896632913
BC008405	Pregnancy specific β-1-glycoprotein 4	PSG4	19q13.2	1,89087705
AF007790	Deafness, autosomal dominant 5	DFNA5	7p15	1,877557582

Table 1 Continued

GenBank	*Name*	*Symbol*	*Cytoband*	*Score (d)*
NM_080874	Ankyrin repeat and SOCS box-containing 5	ASB5	4q34.2	1,875061915
AF124491	G protein-coupled receptor kinase interactor 2	GIT2	12q24.1	1,872336314
AF308819	Zinc finger protein (C2H2 type) 277	ZNF277	7q31.1	1,863966632
NM_003682	MAP-kinase activating death domain	MADD	11p11.2	1,859402841

Table 2 Downregulated genes

GenBank	*Name*	*Symbol*	*Cytoband*	*Score (d)*
AF116827	Component of oligomeric Golgi complex 6	COG6	13q14.11	−5,057184176
AK096886	Nebulin-related anchoring protein	NRAP	10q24-q26	−3,760992043
AK024451	EH domain binding protein 1-like 1	EHBP1L1	11q13.1	−3,463225589
NM_001818	Aldo-keto reductase family 1, member C4	AKR1C4	10p15-p14	−3,223059558
NM_000030	Alanine-glyoxylate aminotransferase	AGXT	2q36-q37	−3,038442872
AK023184	Kinesin family member 1B	KIF1B	1p36.2	−2,675957476
AF016371	Peptidyl prolyl isomerase H (cyclophilin H)	PPIH	1p34.1	−2,650351661
BC013744	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	CXCL6	4q21	−2,649038913
AF305835	CUB and zona pellucida-like domains 1	CUZD1	10q26.13	−2,629362434
AL357538	Smooth muscle cell-associated protein-1	SMAP-1	15q26.1	−2,603741722
L10393	Zinc finger protein, Y-linked	ZFY	Yp11.3	−2,593536919
NM_002969	Mitogen-activated protein kinase 12	MAPK12	22q13.33	−2,581022755
AB017005	Postmeiotic segregation increased 2-like 2	POM121	7q11.23	−2,561081269
NM_014715	Rho GTPase-activating protein	RICS	11q24-q25	−2,496076254
NM_014368	LIM homeobox 6	LHX6	9q33.2	−2,342453911
AK096459	Vitelliform macular dystrophy 2-like 3	VMD2L3	12q14.2-q15	−2,298340409
AF198488	Transcription factor CP2-like 1	TFCP2L1	2q14	−2,254579571
AK026765	Chromosome 6 open reading frame 59	C6orf59	6q26	−2,253986354
BC017887	Eukaryotic translation initiation factor 3, subunit 6	EIF3S6	8q22-q23	−2,209915163
XM_040910	Chromosome 14 open reading frame 73	C14orf73	14q32.32	−2,208248963
BC005261	Sarcolipin	SLN	11q22-q23	−2,204266979
NM_004702	Cyclin E2	CCNE2	8q22.1	−2,196302549
AF205888	Axin 2 (conductin, axil)	AXIN2	17q23-q24	−2,171933139
AF356518	Junctional adhesion molecule 3	JAM3	11q25	−2,168915413
M74096	Acyl-Coenzyme A dehydrogenase, long chain	ACADL	2q34-q35	−2,160570541
NM_002162	Intercellular adhesion molecule 3	ICAM3	19p13.3	−2,129369272
AJ308599	Keratin 6 irs	KRT6IRS	12q13.13	−2,073073141
BC009499	Syntaxin binding protein 6 (amisyn)	STXBP6	14q12	−2,039285736
AF213460	EPH receptor A3	EPHA3	3p11.2	−2,031400597
NM_014629	Rho guanine nucleotide exchange factor (GEF) 10	ARHGEF10	8p23	−2,030953219
AF075575	Dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	DYSF	2p13.3-p13.1	−2,021966877
AB080265	Cytochrome P450, family 2, subfamily J, polypeptide 2	CYP2J2	1p31.3-p31.2	−1,991883625
AF098668	Lysophospholipase II	LYPLA2	1p36.12-p35.1	−1,984116509
J03591	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	SLC25A5	Xq24-q26	−1,962016713
U71204	Ras-like without CAAX 2	RIT2	18q12.3	−1,946770826
AK023133	Myelin expression factor 2	MYEF2	15q21.1	−1,914893606
AL137762	Protein kinase substrate MK2S4	MK2S4	1q22-q24	−1,906708023

tein binds to the mRNA and remains bound after nuclear export, acting as a nucleocytoplasmic shuttling protein.

Cell cycle regulation, proliferation, and apoptosis

MCL1 belongs to the Bcl-2 family. Alternative splicing occurs at this locus and two transcript variants encoding distinct isoforms have been identified. The longer gene product (isoform 1) enhances cell survival by inhibiting apoptosis while the alternatively spliced shorter gene product (isoform 2) promotes apoptosis and is death-inducing (Kozopas *et al*, 1993; Craig *et al*, 1994; Bae *et al*, 2000; Opferman *et al*, 2003; Leu *et al*, 2004).

PPP2R5C belongs to the phosphatase 2A regulatory subunit B family. Protein phosphatase 2A is implicated in the negative control of cell growth and division (McCright and Virshup, 1995; McCright *et al*, 1996; Muneer *et al*, 2002).

AK5 encodes a member of the adenylate kinase family, which is involved in regulating the adenine nucleotide composition within a cell by catalyzing the reversible transfer of phosphate groups among adenine nucleotides (Van Rompay *et al*, 1999). This member is located in the cytosol.

In mammalian cells, the phosphorylation of purine deoxyribonucleosides is mediated predominantly by two deoxyribonucleoside kinases, cytosolic deoxycytidine

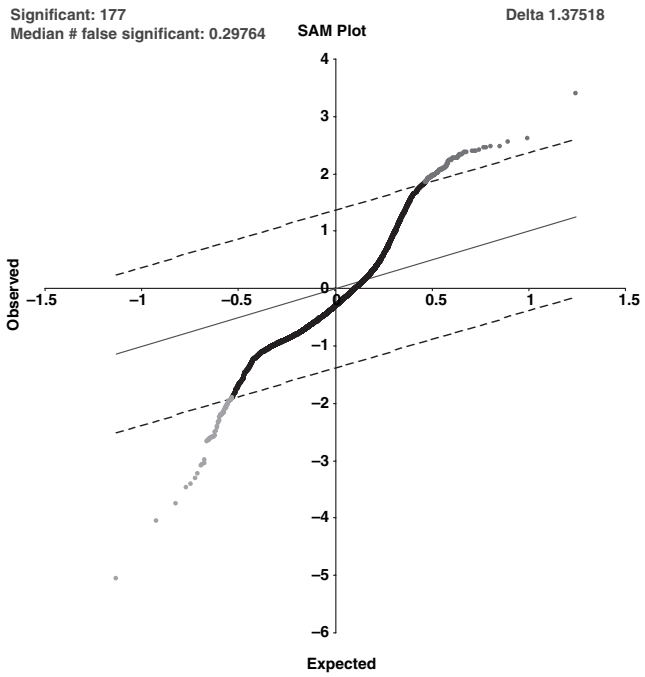

Figure 1 Significance analysis of microarray (SAM) plot of MG63 treated for 24 h with EMPs at the concentration of 250 μg μl^{-1}

kinase and mitochondrial deoxyguanosine kinase. DGUOK is responsible for phosphorylation of purine deoxyribonucleosides in the mitochondrial matrix (Johansson and Karlsson, 1996; Mandel *et al*, 2001; Salviati *et al*, 2002; Taanman *et al*, 2002).

Tumor necrosis factor-α (TNF-α) is a signaling molecule that interacts with one of two receptors on cells targeted for apoptosis (Aggarwal *et al*, 1985; Beattie *et al*, 2002; Allcock *et al*, 2004). The apoptotic signal is transduced inside these cells by cytoplasmic adaptor proteins.

Ota *et al* (2000) tested 192 sib pairs of adult Japanese women from 136 families for genetic linkage between osteoporosis and osteopenia phenotypes and allelic variants at the TNFA locus, using a dinucleotide repeat polymorphism located near the gene. Linkage with osteopenia was also significant in concordant affected pairs ($P = 0.017$). Analyses limited to the postmenopausal women in their cohort showed similar or even stronger linkage for both phenotypes.

MADD is a death domain-containing adaptor protein that interacts with the death domain of TNF-α receptor 1 to activate MAPK and propagate the apoptotic signal (Chow and Lee, 1996; Schievella *et al*, 1997; Chow *et al*, 1998).

Emdogain also acts on genes related to the immune system (Apte *et al*, 2001; All-Ericsson *et al*, 2002; Al-Jamal *et al*, 2003). ICA1 is a 69-kDa peptide autoantigen associated with insulin-dependent diabetes mellitus (Pietropaolo *et al*, 1993; Gaedigk *et al*, 1994, 1996; Winer *et al*, 2002). Alternate splicing within the coding region generates three transcript variants. All three splice variants encode the conserved T-cell epitope (in exon 2) recognized by autoreactive T cells in diabetic children.

Glycophorin A (GYPA) is a major sialoglycoprotein of the human erythrocyte membrane which bears the antigenic determinants for the MN blood group.

CD34 is a monomeric cell surface antigen with a molecular mass of approximately 110 kDa that is selectively expressed on human hematopoietic progenitor cells (Howell *et al*, 1991; He *et al*, 1992; Cheng *et al*, 1996).

Simmons *et al* (1992) isolated a cDNA clone for CD34. They stated that the gene is expressed by small vessel endothelial cells in addition to hematopoietic progenitor cells and is a sialomucin of as-yet-unknown function.

Hematopoietic stem cells (HSCs) give rise to all blood cells. CD34 is a sialomucin-like adhesion molecule that is expressed on a few percentage of primitive bone marrow cells. In human bone marrow, virtually all colony-forming unit activity resides in the population expressing human CD34.

ITGAL encodes the integrin α-L chain. Integrins are heterodimeric integral membrane proteins composed of an α-chain and a β-chain. This I-domain containing α-integrin combines with the β-2 chain (ITGB2) to form the integrin lymphocyte function-associated antigen-1 (LFA-1), which is expressed on all leukocytes. LFA-1 plays a central role in leukocyte intercellular adhesion through interactions with its ligands, intercellular adhesion molecules 1 through 3 (ICAMs 1–3), and also functions in lymphocyte costimulatory signaling.

Major histocompatibility complex belongs to the HLA class II β-chain paralogs (Richeldi *et al*, 1993; Fontenot *et al*, 2000; Rossman *et al*, 2003). This class II molecule is a heterodimer consisting of an α (DPA) and a β-chain (DPB), both anchored in the membrane. It plays a central role in the immune system by presenting peptides derived from extracellular proteins. Class II molecules are expressed in antigen presenting cells (APC: B lymphocytes, dendritic cells, macrophages).

Several upregulated proteins are enzymes contained in lysosomes and vesicular transport. CENTD2 associates with the Golgi, and mediates changes in the Golgi and the formation of filopodia (Nagase *et al*, 1998; Miura *et al*, 2002).

By screening brain cDNAs for the potential to encode proteins that are at least 50 kDa, Nagase *et al* (1998) identified a partial cDNA encoding CENTD2, which they called KIAA0782. The deduced KIAA0782 protein was predicted to be involved in cell signaling/communication. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis followed by enzyme-liked immunosorbent assay (ELISA) detected expression of KIAA0782 in all tissues tested, with highest levels in the ovary, followed by the lung, liver, and kidney.

M6PR is a receptor for mannose-6-phosphate groups on lysosomal enzymes (Dahms *et al*, 1987; Ludwig *et al*, 1992). The receptor forms a homodimer or homotetramer for intracellular targeting of lysosomal enzymes and export of newly synthesized lysosomal enzymes into cell secretions. The receptor is an integral membrane protein which localizes to the trans-Golgi reticulum, endosomes, and the plasma membrane. Receptors

specific for phosphomannosyl residues play a critical role in the segregation and targeting of lysosomal enzymes to lysosomes. As lysosomal enzymes share a common site of synthesis with both membrane and secretory proteins in the rough ER, they must be sorted from other proteins in order to be specifically delivered to lysosomes. This specific transport is accomplished by the generation of mannose 6-phosphate recognition markers on the lysosomal enzymes which then bind specifically to mannose 6-phosphate receptors located in the Golgi apparatus; the receptor–ligand complex is then translocated via vesicles to a prelysosomal compartment where the low pH stimulates dissociation.

Cytoskeleton, cell adhesion, and extracellular matrix components

MAP1A encodes a protein that belongs to the microtubule-associated protein family. The proteins of this family are thought to be involved in microtubule assembly (Hammarback *et al*, 1991; Fink *et al*, 1996; Ikeda *et al*, 2002).

CDH18 encodes a type II classical cadherin from the cadherin superfamily of integral membrane proteins that mediate calcium-dependent cell–cell adhesion (Shibata *et al*, 1997; Chalmers *et al*, 1999). Mature cadherin proteins are composed of a large N-terminal extracellular domain, a single membrane-spanning domain, and a small, highly conserved C-terminal cytoplasmic domain. Type II (atypical) cadherins are defined based on their lack of a HAV cell adhesion recognition sequence specific to type I cadherins. This particular cadherin is expressed specifically in the central nervous system and is putatively involved in synaptic adhesion, axon outgrowth, and guidance. Cadherins, a family of calcium-dependent cell–cell adhesion molecules, mediate neural cell–cell interactions and may play important roles in neural development. Cadherins are transmembrane proteins that associate with the cytoplasmic proteins α-catenin, *β*-catenin, plakoglobin, and p120, all of which regulate the functions of cadherins.

Northern blot analysis of human tissues detected 9.7-, 5.5-, and 3.9-kb CDH18 transcripts specifically in the central nervous system; CDH18 expression was also found in small-cell lung carcinoma cell lines, which have neuroectodermal cell phenotypes.

COL9A1 encodes one of the three α-chains of type IX collagen, the major collagen component of hyaline cartilage (Nakata *et al*, 1993; Fassler *et al*, 1994; Hagg *et al*, 1997; Czarny-Ratajczak *et al*, 2001; Loughlin *et al*, 2002). Type IX collagen is usually found in tissues containing type II collagen, a fibrillar collagen. Studies in knockout mice have shown that synthesis of the α-1 chain is essential for assembly of type IX collagen molecules, a heterotrimeric molecule. Type II collagen represents about 85% of the collagen of hyaline cartilage. In addition, there are several minor collagens. Its function is unknown. The triple helix of type IX collagen is composed of three genetically distinct polypeptide subunits – α-1(IX), α-2(IX), and α-3(IX). These are the products of genes whose exon structure is different from that of fibrillar collagens. Type IX collagen is also a proteoglycan. Chondroitin sulfate and dermatan sulfate chains are covalently linked to the α-2(IX) chain.

Czarny-Ratajczak *et al* (2001) identified a mutation in the COL9A1 gene in affected members of a family with multiple epiphyseal dysplasia.

Loughlin *et al* (2002) performed finer linkage mapping of a primary hip osteoarthritis susceptibility locus on chromosome 6 in affected sib pair families and defined a 11.4 cM female-specific interval flanked by markers D6S452 and 509-8B2, which map between 70.5 and 81.9 cM from the 6p telomere at 6p12.3-q13. As the COL9A1 gene maps within this interval, it was considered a logical candidate gene for osteoarthritis susceptibility.

Nakata *et al* (1993) generated transgenic mice expressing a truncated α-1(IX) chain, which was expected to interfere with stable triple helix formation and act as a trans-dominant mutation. Mice heterozygous for the transgene developed osteoarthritis in the articular cartilage of knee joints, while mice homozygous for the mutation developed mild chondrodysplasia as well. The phenotypic severity correlated well with the level of transgene expression.

Fassler *et al* (1994) found that mice with 'knockout' of the Col9a1 gene did not produce α-1(IX) mRNA or polypeptides and were born with no conspicuous skeletal abnormalities but postnatally developed early-onset osteoarthritis. Surprisingly, cartilage fibrils of all shapes and banding patterns found in normal newborn, adolescent, or adult mice were formed in transgenic animals, although they lacked collagen IX. Hagg *et al* (1997) concluded that collagen IX is not essential, and may be functionally redundant, for fibrillogenesis in cartilage *in vivo*. The protein is required, however, for long-term tissue stability, presumably by mediating interactions between fibrillar and extrafibrillar macromolecules.

CNTN5 is a member of the immunoglobulin superfamily. It is a glycosylphosphatidylinositol (GPI)-anchored neuronal membrane protein that functions as a cell adhesion molecule. It may play a role in the formation of axon connections in the developing nervous system (Kamei *et al*, 2000).

Downregulated genes

Among the downregulated genes some are involved in signaling transduction (Table 2). Activation of members of the MAPK family is a major mechanism for transduction of extracellular signals. Stress-activated protein kinases are one subclass of MAP kinases. MAPK12 functions as a signal transducer during differentiation of myoblasts to myotubes (Lechner *et al*, 1996; Li *et al*, 1996; Goedert *et al*, 1997).

Many downregulated genes are involved in transcription. ZFY encodes a zinc finger-containing protein that functions as a transcription factor (Muller and Schempp, 1989; Palmer *et al*, 1990; Dorit *et al*, 1995, 1996; Donnelly *et al*, 1996; Mahaffey *et al*, 1997). MSL3L1 encodes a nuclear protein and has similarity to drosophila male-specific lethal-3 gene (Prakash *et al*, 1999). The drosophila protein plays a critical role in a

dosage-compensation pathway, which equalizes X-linked gene expression in males and females. Thus this encoded protein is thought to play a similar function in chromatin remodeling and transcriptional regulation. LHX6 encodes a member of a large protein family that contains the LIM domain (Kimura *et al*, 1999; Lavdas *et al*, 1999; Gong *et al*, 2003). LHX6 functions as a transcriptional regulator and is involved in the control of differentiation and development of neural and lymphoid cells. The Axin-related protein, Axin2, presumably plays an important role in the regulation of the stability of β-catenin in the Wnt signaling pathway (Mai *et al*, 1999; Liu *et al*, 2000; Dong *et al*, 2001; Lammi *et al*, 2004). The deregulation of β-catenin is an important event in the genesis of a number of malignancies.

By Northern blot analysis, Dong *et al* (2001) detected ubiquitous expression of a 3.0-kb AXIN2 transcript, with highest levels in the thymus, prostate, testis, ovary and small intestine, lower levels in the colon, and barely detectable levels in blood leukocytes. In a Finnish family in which severe permanent tooth agenesis (oligodontia) and colorectal cancer segregated with dominant inheritance, Lammi *et al* (2004) identified an arg656-to-ter mutation in the AXIN2 gene (R656X). At least eight permanent teeth were missing in 11 members of the family, two of whom developed only three permanent teeth. Colorectal cancer or precancerous lesions of variable types were found in eight of the patients with oligodontia. In addition, Lammi *et al* (2004) identified a *de novo* frameshift mutation in the AXIN2 gene in an unrelated young patient with severe tooth agenesis. Both mutations were expected to activate Wnt signaling. The results provided the first evidence of the importance of Wnt signaling for the development of dentition in humans and suggested that an intricate control of Wnt signal activity is necessary for normal tooth development, as both inhibition and stimulation of Wnt signaling may lead to tooth agenesis. EPHA3 belongs to the ephrin receptor subfamily of the protein-tyrosine kinase family. EPH and EPH-related receptors have been implicated in mediating developmental events, particularly in the nervous system (Boyd *et al*, 1992; Wicks *et al*, 1992, 1994; Fox *et al*, 1995). The ephrin receptors are divided into two groups based on the similarity of their extracellular domain sequences and their affinities for binding ephrin-A and ephrin-B ligands. This gene encodes a protein that binds ephrin-A ligands.

Translation

The nuclear envelope creates distinct nuclear and cytoplasmic compartments in eukaryotic cells. It consists of two concentric membranes perforated by nuclear pores, large protein complexes that form aqueous channels to regulate the flow of macromolecules between the nucleus and the cytoplasm. These complexes are composed of at least 100 different polypeptide subunits, many of which belong to the nucleoporin family. POM121 is an integral membrane protein that localizes to the central spoke ring complex and participates in anchoring the nuclear pore complex to the nuclear envelope.

Cell cycle regulation, proliferation, and apoptosis

EZH2 encodes a member of the Polycomb-group (PcG) family. PcG family members form multimeric protein complexes, which are involved in maintaining the transcriptional repressive state of genes over successive cell generations (Chen *et al*, 1996; Cardoso *et al*, 1998, 2000; Dohner *et al*, 1998; Cao *et al*, 2002).

CCNE2 belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle (Lauper *et al*, 1998; Zariwala *et al*, 1998; Gudas *et al*, 1999). Cyclins function as regulators of CDK kinases. Cyclins bind to and activate cyclin-dependent kinases (CDKs) to form serine/threonine kinase holoenzyme complexes that regulate the eukaryotic cell cycle. Cyclins A, D, and E (CCNE1) are required for mammalian cells to traverse G1 and enter S phase. Cyclin E controls the initiation of DNA synthesis by activating CDK2, the KIP1 and CIP1 proteins bind and inhibit cyclin E-CDK2 complexes. Northern blot analysis revealed that the 2.8-kb cyclin E2 mRNA is expressed in several normal human tissues, with the highest levels in the testis, thymus, and brain.

ICAM3 is a member of the ICAM family. All ICAM proteins are type I transmembrane glycoproteins and bind to the leukocyte adhesion LFA-1 protein (Fawcett *et al*, 1992; Vazeux *et al*, 1992; Bossy *et al*, 1994). ICAM1 and ICAM2 are members of the immunoglobulin superfamily. The interaction of LFA-1 with ICAM provides essential accessory adhesion signals in many immune interactions, including those between T and B lymphocytes and cytotoxic T cells and their targets. Both ICAMs are expressed at low levels on resting vascular endothelium; ICAM1 is strongly upregulated by cytokine stimulation and plays a key role in the arrest of leukocytes in blood vessels at sites of inflammation and injury. Resting leukocytes were shown to express a third ligand, ICAM3, for LFA-1. ICAM3 is potentially the most important ligand for LFA-1 in the initiation of the immune response because the expression of ICAM-1 on resting leukocytes is low.

This protein is constitutively and abundantly expressed by all leukocytes and may be the most important ligand for LFA-1 in the initiation of the immune response. It functions not only as an adhesion molecule, but also as a potent signaling molecule.

Several downregulated proteins are enzymes contained in lysosomes or are proteins related to vesicular transport.

Multiprotein complexes are key determinants of Golgi apparatus structure and its capacity for intracellular transport and glycoprotein modification. Several complexes have been identified, including the Golgi transport complex (GTC), the low density lipoprotein receptor defect c-complementing protein complex, which is involved in glycosylation reactions (Coon *et al*, 1999), and the SEC34 complex (Suvorova *et al*, 2001; Ungar *et al*, 2002), which is involved in vesicular

transport. The SEC34 complex is involved in vesicular transport.

Northern blot analysis revealed abundant and ubiquitous expression of a 4.5-kb transcript, with the highest level in the pancreas and the testis and the lowest level in the lung.

These three complexes are identical and have been termed the conserved oligomeric Golgi (COG) complex, which includes COG6 (Hirosawa *et al*, 1999; Whyte and Munro, 2001). Multiprotein complexes are key determinants of Golgi apparatus structure and its capacity for intracellular transport and glycoprotein modification. Several complexes have been identified, including the GTC, the LDLC complex, which is involved in glycosylation reactions, and the SEC34 complex, which is involved in vesicular transport. These three complexes are identical and have been termed the conserved oligomeric Golgi (COG) complex, which includes COG6 (Ungar *et al*, 2002).

By screening for cDNAs with the potential to encode large proteins expressed in brain, Hirosawa *et al* (1999) identified a partial cDNA encoding COG6, which they called KIAA1134. RT-PCR analysis detected weak expression in the brain and the ovary, with little or no expression in other tissues. Within the brain, expression was highest in the amygdala and the cerebellum.

The product of the human AGXT protein is normally localized in the peroxisomes of the liver where it is involved in the detoxification of glyoxylate (Danpure *et al*, 1987).

Cytoskeleton, cell adhesion, and extracellular matrix components

DYSF belongs to the ferlin family and is a skeletal muscle protein found associated with the sarcolemma. It is involved in muscle contraction and plays a role in calcium-mediated membrane fusion events, suggesting that it may be involved in membrane regeneration and repair (Anderson *et al*, 1999; Aoki *et al*, 2001; Bansal *et al*, 2003). Anderson *et al* (1999) raised a monoclonal antibody to dysferlin and studied the expression of the protein. Immunolabeling with the antibody demonstrated a polypeptide of approximately 230 kDa on Western blots of skeletal muscle, and microscopy at both the light and electron microscopic levels localized dysferlin to the muscle fiber membrane. A specific loss of labeling of dysferlin was observed in patients with mutations in the DYSF gene. Furthermore, patients with two different frameshift mutations demonstrated very low levels of immunoreactive protein in a manner reminiscent of the dystrophin expressed in many Duchenne patients. Analysis of human fetal tissue showed that dysferlin was expressed at the earliest stages of development, at Carnegie stage 15 or 16 (embryonic age 5–6 weeks). Dysferlin is present, therefore, at a time when the limbs start to form regional differentiation. Anderson *et al* (1999) suggested that lack of dysferlin at this critical time may contribute to the pattern of muscle involvement that develops later, with the onset of a muscular dystrophy primarily affecting proximal or distal muscles.

RICS is a neuron-associated GTPase-activating protein that may regulate dendritic spine morphology and strength (Nagase *et al*, 1998; Nakamura *et al*, 2002; Moon *et al*, 2003; Okabe *et al*, 2003). By sequencing clones obtained from a size-fractionated brain cDNA library, Nagase *et al* (1998) cloned KIAA0712. RT-PCR ELISA detected expression in all tissues, with highest levels in the brain and the ovary, intermediate levels in the lung, kidney, pancreas, and testis, and lower levels in the heart, liver, skeletal muscle, and spleen.

Northern blot analysis detected high expression of 9.0-, 6.0-, and 3.2-kb transcripts in the brain and the testis, and lower expression in the pancreas and the colon. *In situ* hybridization of embryonic day 19.5 rats revealed abundant expression of Grit only in the central and peripheral nervous system and in parts of the gastrointestinal tract. Grit is a GTPase-activating protein of the Rho family and it regulates neurite extension.

Using selected armadillo repeats of mouse β-catenin as bait in a yeast two-hybrid screen, Okabe *et al* (2003) cloned mouse RICS from a 17-day mouse embryo cDNA library. Northern blot analysis detected a doublet of 9.8 and 10.0 kb expressed at high levels in the kidney, brain, testis, and heart, and at low levels in the skeletal muscle, liver, lung, and spleen.

Tight junctions represent one mode of cell–cell adhesion in epithelial or endothelial cell sheets, forming continuous seals around cells and serving as a physical barrier preventing solutes and water from passing freely through the paracellular space. JAM3 is an immunoglobulin superfamily gene member and is localized in the tight junctions between high endothelial cells (Arrate *et al*, 2001; Liang *et al*, 2002; Santoso *et al*, 2002; Gliki *et al*, 2004). JAM3 is a member of the junctional adhesion molecule (JAM) family.

By searching an EST database for sequences similar to JAM2, followed by amplification of a fetal brain cDNA library, Arrate *et al* (2001) isolated a cDNA encoding JAM3. Northern blot analysis revealed wide expression of an approximately 3.3-kb transcript, with highest levels in the placenta, brain, and kidney. Expression was also detected in cultured endothelial cells. Binding analysis by Arrate *et al* (2001) showed that unlike JAM2, JAM3 is unable to adhere to leukocyte cell lines and only forms weak homotypic interactions. However, JAM3 was found to interact strongly with JAM2.

K6IRS1 belongs to a family of type II keratins that are specifically expressed in the inner root sheath of hair follicles (Langbein *et al*, 2002, 2003).

The genes discussed in this study are only a limited number among those differentially expressed and reported in Tables 1 and 2. We briefly evaluated some of those with a better known function.

Previous reports have analyzed the genetic effect of EMD on cells (Schwartz *et al*, 2000; Tokiyasu *et al*, 2000; Brett *et al*, 2002; Inoue *et al*, 2004; Parkar and Tonetti, 2004) and the absence of several genes already associated with the effect of EMD is not surprising for several reasons. First, MG-63 are a cell line and not normal osteoblasts. Second, an *in vitro* system differs

from an *in vivo* system. In fact, a monolayer cell stratum differs significantly from bone tissue. The advantage of using a cell line and not a primary culture is related to the fact that the reproducibility of the data is higher because of the absence of variability in the patient. MG-63 cells have also been well characterized and they show many traits of the osteoblasts, including production of increased levels of alkaline phosphatase and osteonectin synthesis in response to 1,25-$(OH)_2D_3$ (Schwartz *et al*, 2000). Primary cell cultures not only provide a source of more normal, non-malignant cells, but they also contain a heterogeneous cell population, often containing contaminating cells of different types and cells in variable differentiation states. This variability in cell type could lead to a less precise demonstration of the effect of EMD on osteoblasts. Third, we have chosen to perform the experiment after 24 h in order to get information on the early effect of EMD. Fourth, some genes already associated with EMD effect were not present on the array used in this study. Moreover, because no cDNA microarray has a complete genome printed-up, more studies based on different system are needed for (i) a cross-validation of different results and (ii) a wider comprehension of molecular pathways involved in EMD cell stimulation.

In conclusion, EMD is able to modulate a broad range of osteoblast biologic processes: (i) signaling transduction, (ii) transcription, (iii) translation, (iv) cell cycle regulation, proliferation and apoptosis, (v) immune system, (vi) vesicular transport and lysosome activity, and (vii) cytoskeleton, cell adhesion and extracellular matrix production. Most probably the most important upregulated genes are those (angiopoietins, RAF1) involved in the regulation of endothelial cell survival during angiogenesis. It is well established that blood vessel formation precedes and plays a pivotal role in the bone formation processes. Also, the genes that antagonize proinflammatory transcriptional activities and potentially enforce lymphocyte quiescence (FOXJ1) are important in the overall effect of EMD and could help in explaining the slight, non-significant activation of the immune system reported in humans during the first year following EMD application (Nikolopoulos *et al*, 2002). Also of significance is the relationship of some of the upregulated genes with bone disorders such as osteoporosis and osteopenia and multiple epiphyseal dysplasia (TNF-α, COL9A1), and with the long-term stability of the fibrillar macromolecules of collagen (COL9A1). To date, the precise interaction between, and the role of, these genes is not clear. More investigations with other osteoblast-like cell lines, primary cultures, and different time points are needed for better understanding the molecular events related to the action of EMD. Experimental studies have shown a great potential for periodontal regeneration. In the periodontal ligament it is possible to find progenitor cells that can differentiate into periodontal ligament fibroblasts, osteoblasts, and cementoblasts. It is also not known whether osteoblasts and cementoblasts originate from a common precursor cell line, or whether distinct precursor cell lines exist. Thus, there is limited knowledge about how cell diversity evolves in the space between the developing root and the alveolar bone. Finally, we believe that the reported data can be a useful model for comparing the effects on different types of cells, e.g., osteoblasts and cementoblasts.

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