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Gene profile in periodontal ligament cells and clones with enamel matrix proteins derivative

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Abstract

Aim: Evaluate enamel matrix proteins derivative effect on gene expression profiles in cultured human periodontal ligament cell population and its clones.

Material and Methods: Human periodontal ligament (PDL) cells were explanted. Cell cloning was performed and clones classified into fibroblastic (FB) and mineralized tissue forming (MTF) according to their capacity to express alkaline phosphatase and form mineralized tissue. All cell cultures were grown for 7 days, with and without enamel proteins added to the medium. Following RNA extraction, expression profiling was performed by hybridization with a DNA micro-array. Selected genes differed from the control at a significant level smaller than $p < 0.01$.

Results: Enamel proteins induced major qualitative changes in mRNA expression in all PDL cell populations, differently affecting the entire PDL cell population and its clones. In the entire PDL cell population, enamel proteins significantly enhanced PDL cell function, with a general effect on enhanced cell functional metabolism.

Conclusions: Enamel proteins enhanced gene expression responsible for protein and mineralized tissue synthesis in the entire PDL population. In the MTF clones, nucleic acid metabolism, protein metabolism and signal transduction related genes were up-regulated, while in the FB clones, up-regulated genes were related to cell adhesion, nucleic acid metabolism and signal transduction.

Key words: enamel proteins; genes; human; in vitro; periodontal ligament

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The periodontal ligament (PDL) cell population is heterogeneous, consisting of two main lineages: fibroblastic (FB) and mineralized tissue forming (MTF) (McCulloch & Melcher 1983, Liu et al. 1997). Cell kinetic studies suggest that part of these progenitor cells terminally differentiate into cementoblasts or osteoblasts (Roberts & Chase 1981, Hou & Yaeger 1993).

Enamel-related proteins that nucleate and regulate the growth of hydroxypatite crystals to form the mineralized enamel covering the tooth crown (Zeichner-David 2001) are also secreted by Hertwig's epithelial root sheath during tooth development and initiate the differentiation of cementoblasts and induce acellular cementum formation (Slavkin et al. 1989, McCulloch 1993, Sculean et al. 1999).

Heijl et al. (1997) suggest that enamel matrix proteins derivative (EMD) has the ability to promote true periodontal regeneration in humans by inducing new acellular cementum, PDL and bone formation. The active EMD product consists of hydrophobic enamel matrix proteins (Deutsch et al. 1991, Hammarström 1997), with amelogenin as the major protein, which is extracted from porcine developing embryonic enamel (Gestrelius et al. 1997a); however, amelogenin does not have significant effects on PDL cell proliferation or migration by itself, therefore, clinical effects of EMD require a combined interaction of several enamel matrix components (Chong et al. 2006). Models in vitro might explain its molecular and cellular mechanism action (Brett et al. 2002, Parker & Tonetti 2004). EMD-enhanced proliferation of odontoblasts and osteoblasts (Jiang et al. 2006), PDL cell proliferation, increased total protein

Conflict of interest and source of funding statement

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synthesis and promoted mineral nodule formation by PDL cells (Gestrelius et al. 1997a, b) favoured mesenchymal cell growth, while inhibiting epithelium growth (Hammarström et al. 1996). Exposure to EMD increased intracellular cAMP signalling and production of transforming growth factor-β1, interleukin 6 (IL-6), and platelet-derived growth factor-β (Lyngstadaas et al. 2001), while attenuating the release of TNF-α and IL-8 (Myhre et al. 2006)

Little information is available regarding EMD mechanism of action and its effect on the induction and expression of extracellular matrix proteins, growth factors and morphogenes, by the FB and MTF lineage in the PDL. The aim of this study was to characterize the effect of EMD after 7 days of culture through high-quality gene expression profiles derived from treated cultured human PDL cells and its derived clones.

Material and Methods

The explantation technique was used to obtain primary cultures of human PDL cells (Brunette et al. 1976) from healthy PDL of premolars of two patients who had undergone tooth extraction for orthodontic reasons. The patients signed an informed consent before providing the samples, according to the Helsinki Declaration. Explants for PLC cultures were obtained from the central part of the root.

Cultures were grown in medium containing αMEM supplemented with 12% foetal calf serum (FCS), (Biological Industries, Beth Haemek, Israel) and antibiotics. Cultures were maintained at 37°C, 100% humidity and 5% CO_2

Confluent cells surrounding the different explants were harvested with 0.25% trypsin–versene (Biological Industries, Beth Haemek, Israel) and transferred to 96- and 24-well plates (Nunc, Naperville, IL, USA).

Cell cloning

The limiting dilution technique was used to clone some of the harvested cell populations. Basically, 0.3 cells/well were cultured in 96-well microtitre plates in the same culture medium. Colonies that developed from a single cell were grown until confluent. Subsequently, each clone was harvested with 0.25% trypsin–versene, passaged to a 24-well plate, cultured until confluent and used for the following assays.

Alkaline phosphatase (AP) expression

Cells were plated in 16 mm wells (2000 cells/well) and cultured in αMEM supplemented with 12% FCS and antibiotics for 48 h. Cultures were washed, stained with Naphtol AS-MX and Fast Violet B salt (Sigma, St. Louis, MO, USA) for 30 min. and fixed in 70% ethanol for 10 min. The percentage of AP positive cells was determined among 300 cells.

Mineralized tissue formation

Cells obtained from the same cell suspensions used for AP expression were plated in 96-well microtitre plates (5000 cells/well; four wells for each cell type) and cultured in medium supplemented with 50 μg/ml Vitamin C (Merck, West Point, PA, USA), 10^{-8} M Dexamethasone and 10 mM β-glycerophosphate (Sigma, St. Louis, MO, USA). After 28 days, cultures were stained in a saturated solution of alizarin red S (BDH Chemicals Ltd, Poole, UK) (Noff et al. 1989). The amount of mineralized-like tissue formed by each culture was estimated (Pitaru et al. 1993).

Classification of progenitor clones

The different progenitor clones were classified according to their capacity to form mineralized tissue and express AP in culture. Clones with a high AP expression, i.e., 66–100% positive cells, and an ability to form mineralized tissue in culture, were named MTF clones. Clones with a low AP expression, i.e., 0–33% positive cells, and with no ability to form mineralized tissue in culture, were named FB clones.

Immortalization

The immortalization technique, which enables large cell populations to maintain their original phenotype, uses the recombinant retroviral vector LXSN-16E6E7 from Papilloma virus (provided by Prof. Sampath Narayanan, Seattle, WA, USA).

As confirmed in a preliminary study (data not shown), the immortalization process of clones as described did not change the phenotype of non-immortalized clones.

Immortalized MTF and FB clones, and non-immortalized entire PDL cell populations, were cultured with or without EMD (Emdogain®, Straumann Biologics, Basel, Switzerland) at 100 μg/ml, a concentration previously used by others (Tokiyasu et al. 2000, Van der Pauw et al. 2000, 2002, Hoang et al. 2002). EMD in the form of lyophilized proteins diluted in 0.1% acetic acid solution was added every other day for 7 days.

RNA extraction

Total cellular RNA was extracted from cells using a Tri Reagent isolation kit (MRC Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. RNA samples (20 μg) were separated on 0.9% agarose/formaldehyde gels (Amresco, Bolon, OH, USA) to determine the quality of the RNA. RNA extraction was carried out on cells cultured in a flask with an average cell density of 1,000,000 cells/flask.

RNA quantity and purity were measured with a spectrophotometer.

Array processing sample preparation and hybridization

Micro-array experiments were performed using the Affymetrix GeneChip Human Genome Focus Array (Affymetrix, Santa Clara, CA, USA), representing over 8500 verified sequence probes from the NCBI RefSeq database

www.affymetrix.com/products/arrays/specific/focus.affx

All experiments were carried out as described at

http://www.affymetrix.com/support/technical/datasheets/human_datasheet.pdf

Control and experiment RNA pools were obtained and independently assayed twice. Only genes that were expressed in both pools were analyzed.

Briefly, each total cellular RNA sample was used to generate cDNA and subsequent biotinylated target cRNA that was processed by an Affymetrix GeneChip Instrument System (Affymetrix, Santa Clara).

Data analysis

Treated and untreated samples were compared. Only genes with a significant change of expression ($p \leqslant 0.01$) and a

change of 1.5-fold or more and a presence by both repeats were selected, as either up-regulated or down-regulated.

Genes were classified into functional groups using the GO annotation tool and DAVID Database (NIH, Bethesda, MD, USA). The latter was used for Annotation, Visualization and Integrated Discovery. http://apps1.niaid.nih.gov/David/upload.asp.

Availability of micro-array data

For further discussion on experimental design and other details of the methods, see online address (http://eng.sheba.co.il/genomics).

Results

Results were evaluated for the effect of EMD on the entire population of PDL cells and MTF and FB clones separately and for differences in genetic expression between the two different clones when exposed to EMD. Please refer to the Appendices A–C for the complete list of genes.

Entire PDL cell population

Differences were detected in the expression of 202 genes cultured in the presence of EMD: up-regulated = 68, down-regulated = 134.

Up-regulated genes: functional groups

Up-regulation was shown in 13 functional genetic groups based on the functional activity of the corresponding protein (Fig. 1). Certain individual genes were markedly up-regulated among the different functional groups, such as bone morphogenetic protein 2; prostaglandin-endoperoxide synthase 2; laminin, β1; transforming growth factor, β receptor I; tumour necrosis factor receptor superfamily, member 21; and fibroblast growth factor 7. Among the down-regulated genes that played a role in epithelial and/or endothelial cell proliferation, synthesis of certain proteins and specific cell functions were of special interest, such as metallothionein $\times$ 1, 1H, 2A; interferon, α-inducible protein; endothelial differentiation-related factor 1; epithelial membrane protein 3; vascular endothelial growth factor B; and tumour necrosis factor (ligand) superfamily, member 15.

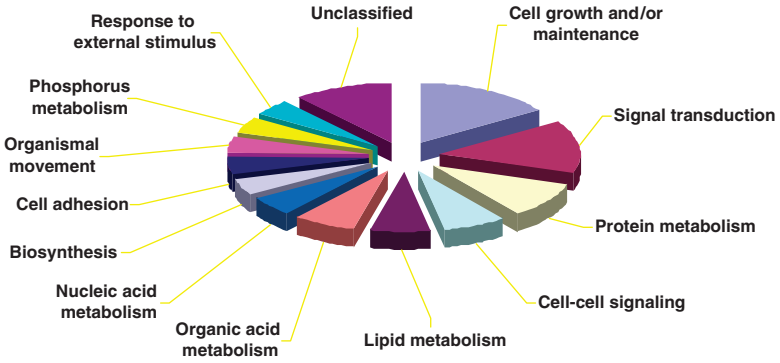

Fig. 1. Division of functional gene groups up-regulated in periodontal ligament cells in response to enamel matrix proteins derivative.

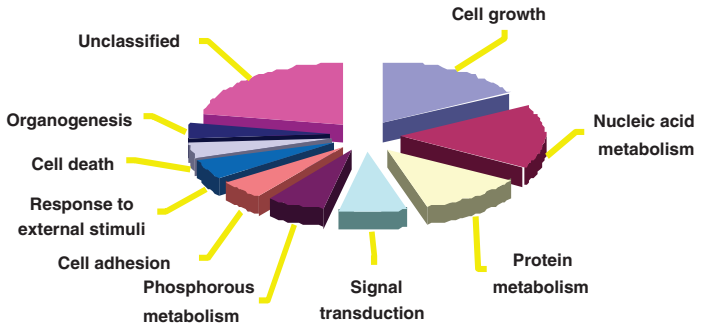

Fig. 2. Division of functional gene groups up-regulated in the mineralized tissue-forming clones in response to enamel matrix proteins derivative.

Down-regulated genes: functional groups

Down-regulation was shown in 16 functional genetic groups based on the functional activity of the corresponding protein. Most of these genes have a role in cell growth and/or maintenance, nucleobase, nucleoside, nucleotide and nucleic acid metabolism, protein metabolism, or response to external stimulus.

FB and MTF clones

Within the MTF clones, differences were detected in the expression of 78 genes after cultured in the presence of EMP: 54 increased and 24 decreased. In the FB clones, expression changed in 27 genes: up-regulated = 14, down-regulated = 13 with the addition of EMD compared with control clones.

MTF clones

Functional groups

Within MTF clones, up-regulation was shown in 10 functional genetic groups based on the functional activity of the corresponding protein (Fig. 2) and down-regulation in six based mostly on the functional activity of cell growth and/or maintenance, nucleobase, nucleoside, nucleotide and nucleic acid metabolism and response to external stimulus of the corresponding protein. Of interest among the individual genes in the MTF clones that were markedly up-regulated were IL 7 receptor, solute carrier family 16, kinesin family member 5B, myosin, light polypeptide kinase, and tumor necrosis factor receptor superfamily, member 10 day. The most significantly down-regulated genes were zinc finger protein, ATPase, Ca^{2+} transporting, ubiquitous and cathepsin C.

FB clones

Functional groups

Up-regulation was found in four functional genetic groups (Fig. 3) and down-regulation in four: immune response, response to external stimuli, signal transduction, unclassified. Of interest among the individual genes in the FB clones that were markedly up-regulated were syntrophin, β1; sialic acid-binding

Ig-like lectin 7; and IL-6 signal transducer. The most significantly down-regulated gene was chemokine (C-X-C motif) ligand 11.

Although some functional groups were both up- and down-regulated, each group contained several different genes with nearly every gene having a unique response to the presence of EMD in the culture medium.

Discussion

This study showed that the addition of EMD to an entire PDL cell culture and to both cell clone types elicited changes in the mRNA levels of certain genes related to various cell functions. All three culture cell types differently responded to EMD.

Entire PDL cell population

Up-regulation in gene expression, responsible for protein and mineralized tissue syntheses, was observed when EMD was added to the culture medium. Changes in genetic expression were marked as genes responsible for molecular functions and response to external stimulus were also significantly enhanced.

Integrins are involved in the interaction of PDL and gingival fibroblasts with EMD (Van der Pauw et al. 2002). In this study, EMD increased cell function as previously shown by proliferation, growth and metabolism of PDL cells (Gestrelius et al. 1997a, Sculean et al. 1999), thus favouring mesenchymal cell growth (Gestrelius et al. 1997b).

In this study, IL-6 signal transducer, prostaglandin-endoperoxide synthase 2 and EGF-like-domain, multiple 6, were up-regulated in the presence of EMD. Changes previously reported in the osteogenic activity of PDL cells (Brett et al. 2002, Ohyama et al. 2002, Takayanagi et al. 2006) when treated with EMD were represented in the genetic profile of this study. EMD contains transforming growth factor (TGF)-β and BMP-like growth factors that contribute to the induction of biomineralization during periodontal regeneration (Suzuki et al. 2005). Certain genes involved in mineralized tissue formation were up-regulated, such as Hyaluronan synthase 2, bone morphogenetic protein 2 and cartilage-linking protein 1. The results support the hypothesis that EMD enhances the expression of certain osteogenic markers and protein synthesis in bone stromal and committed cells (Schwartz et al. 2000, Haase & Bartold 2001, Keila et al. 2004, Carinci et al. 2006, Pischon et al. 2006, Reseland et al. 2006). As previously reported, fibroblasts (Grayson et al. 2006) and PDL cells release significantly higher levels of TGF β in the presence of EMD, suggesting an increase in the cementogenic capacity of these cells (Van der Pauw et al. 2000). In this study, enhanced collagen synthesis (Romanos et al. 1991) and collagen matrix remodelling (Grayson et al. 2006) is suggested, in the presence of EMD, due to the enhanced expression of collagen, type XI, α1, cartilage-linking protein 1, fibroblast growth factor 7 (keratinocyte growth factor) and TGF, β receptor I.

MTF and FB clones

EMD affected each clone differently, which was expected due to the different characteristics of each clone. Clearly, EMD enhanced cell metabolism and replication, mostly in MTF cell clones, while general cell response was up-regulated in FB cell clones. These changes were marked in the MTF where genes responsible for molecular functions, such as carrier activity, cation transporter activity, and ATPase activity, were significantly up-regulated. In the FB cell clones, changes in gene expression were mainly marked in certain biological processes, such as organismal physiological process, immune response, general response to stimulus response to biotic stimulus, and response to external stimulus. These results did not support the hypothesis that EMD would enhance the expression of osteogenic markers in the MTF clones, and, especially in the FB clones. No increase in these genes was found in both types of clones. The bioactive effects of EMD on mineralized tissue formation partially depend on the local environment (Sawae et al. 2002). The possibility that EMD effect is especially on non-committed cell populations (Ashkenazi & Shaked 2006) cannot be ruled out, therefore, in the MTF and FB clones, where cells are already differentiated, EMD might elicit minimal changes in their metabolism.

To the best of our knowledge, this is the first study that examines the effect of EMD on gene expression when added to a PDL cell clone in vitro after 7 days of culture. The effect of EMD on PDL cells have been genetically evaluated (Brett et al. 2002, Parker & Tonetti 2004). After 48 h of incubation, protein and DNA syntheses were significantly elevated in the presence of EMD (Van der Pauw et al. 2000, Brett et al. 2002). Recently, enhanced expression of genes encoding growth and repair-promoting molecules was reported in 4-day cultures with EMD (Parker & Tonetti 2004). The present findings also showed that major qualitative changes in mRNA expression in PDL cells were induced by EMD, which is in agreement with others (Brett et al. 2002, Parker & Tonetti 2004). The presented technique highlights a range of multiple gene activities that help clarify the fundamental molecular events of EMD effect on the entire PDL cell population and on its different cell lines.

Independent assays of the expression of specific genes, for example by Western and Northern blotting or by real time-PCR, are required to corroborate these results before definitive biological conclusions can be drawn.

EMD significantly influenced PDL cell function, by up- or down-regulating several gene expressions with a general effect on enhanced cell functional metabolism. EMD affected the entire PDL cells and each of its clones differently. EMD enhanced the gene expression responsible for protein and mineralized

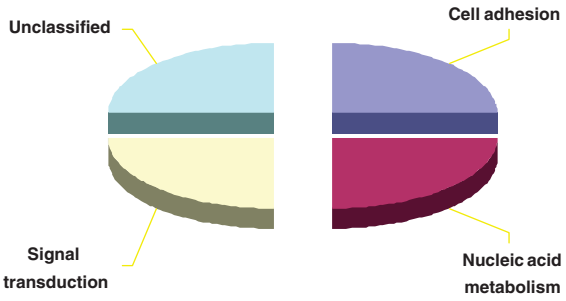

Fig. 3. Division of functional gene groups up-regulated in the fibroblastic clones in response to enamel matrix proteins derivative.

tissue syntheses in the entire PDL cell population, cell metabolism and replication in MTF cell clones, and general cell response in FB clones.

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Clinical Relevance

Scientific rationale for the study: PDL cell population is heterogeneous, consisting of two main lineages: fibroblastic and MTF. The effect of enamel proteins derivative was evaluated through high-quality gene expression profiling of cultured human PDL cells and its derived clones.

Principal findings: Enamel proteins differently affected the entire PDL cell populations and its clones. The general PDL cell cultures showed up-regulation in gene expression responsible for cell functional metabolism and for protein and mineralized tissue synthesis.

Practical implications: Results may explain the molecular mechanism of the events induced by the application of enamel proteins on periodontal tissues.

Appendix A

Table A1. List of genes up-regulated and down-regulated in the MTF clones in the presence of EMD, ordered according to LR (log ratio)

AffyID	GenBank	Gene symbol	Title	LR*
Fifty-four genes up-regulated in MTF clone				
205798_at	NM_002185	IL7R	Interleukin 7 receptor	4.6
207057_at	NM_004731	SLC16A7	Solute carrier family 16 (monocarboxylic acid transporters), member 7	3.8
205178_s_at	NM_006910	RBBP6	Retinoblastoma binding protein 6	3.3
213229_at	BF590131	DICER1	Dicer1, Dcr-1 homolog (*Drosophila*)	3.1
219528_s_at	NM_022898	BCL11B	B-cell CLL/lymphoma 11B (zinc finger protein)	3.1
214577_at	BG164365	MAP1B	Microtubule-associated protein 1B	2.9
205321_at	NM_001415	EIF2S3	Eukaryotic translation initiation factor 2, subunit 3 γ, 52 kDa	2.8
203590_at	NM_006141	DNCLI2	Dynein, cytoplasmic, light intermediate polypeptide 2	2.5
219024_at	NM_021622	PLEKHA1	Pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1	2.3
208662_s_at	AW510696	TTC3	Tetratricopeptide repeat domain 3	2.2
201991_s_at	BF223224	KIF5B	Kinesin family member 5B	2.1
209897_s_at	AF055585	SLIT2	Slit homolog 2 (Drosophila)	2
205168_at	NM_006182	DDR2	Discoidin domain receptor family, member 2	2
202102_s_at	NM_014299	BRD4	Bromodomain containing 4	1.9
220202_s_at	NM_018835	MNAB	Membrane-associated nucleic acid binding protein	1.8
204881_s_at	NM_003358	UGCG	UDP-glucose ceramide glucosyltransferase	1.8
207415_at	NM_007366	PLA2R1	Phospholipase A2 receptor 1, 180 kDa	1.7
212852_s_at	AL538601	SSA2	Sjogren syndrome antigen A2 (60 kDa, ribonucleoprotein autoantigen SS-A/Ro)	1.7
221009_s_at	NM_016109	ANGPTL4	Angiopoietin-like 4	1.6
205315_s_at	NM_006750	SNTB2	Syntrophin, β 2 (dystrophin-associated protein A1, 59 kDa, basic component 2)	1.6
201737_s_at	NM_005885	TEB4	Similar to *S. cerevisiae* SSM4	1.5
212318_at	NM_012470	TRN-SR	Transportin-SR	1.5
203825_at	NM_007371	BRD3	Bromodomain containing 3	1.5
217979_at	NM_014399	NET-6	Transmembrane 4 superfamily member tetraspan NET-6	1.5
204516_at	BG390306	SCA7	Spinocerebellar ataxia 7 (olivopontocerebellar atrophy with retinal degeneration)	1.4
202555_s_at	NM_005965	MYLK	Myosin, light polypeptide kinase	1.4
218237_s_at	NM_030674	SLC38A1	Solute carrier family 38, member 1	1.4
201730_s_at	BF110993	TPR	Translocated promoter region (to activated MET oncogene)	1.3
204771_s_at	AI632304	TTF1	Transcription termination factor, RNA polymerase I	1.3
204031_s_at	NM_005016	PCBP2	Poly(rC) binding protein 2	1.3

Table A1. (Contd.)

AffyID	GenBank	Gene symbol	Title	LR*
201810_s_at	AL562152	SH3BP5	SH3-domain binding protein 5 (BTK-associated)	1.3
204630_s_at	NM_004871	GOSR1	Golgi SNAP receptor complex member 1	1.3
203497_at	NM_004774	PPARBP	PPAR binding protein	1.3
211317_s_at	AF041461	CFLAR	CASP8 and FADD-like apoptosis regulator	1.3
203247_s_at	BC003566	ZNF24	Zinc finger protein 24 (KOX 17)	1.2
213348_at	N33167	CDKN1C	Cyclin-dependent Kinase inhibitor 1C (p57, Kip2)	1.2
205523_at	U30872	CRTL1	Centromere protein F, 350/400 ka (mitosin)	1.2
217802_s_at	NM_022731	NUCKS	Similar to rat nuclear ubiquitous casein kinase 2	1.2
206374_at	NM_004420	DUSP8	Dual specificity phosphatase 8	1.2
219571_s_at	NM_016265	GIOT-3	GIOT-3 for gonadotropin inducible transcription repressor-3	1.1
202274_at	NM_001615	ACTG2	Actin, γ 2, smooth muscle, enteric	1.1
205097_at	AI025519	SLC26A2	Solute carrier family 26 (sulphate transporter), member 2	1.1
204348_s_at	NM_013410	AK3	Adenylate kinase 3	1.1
220794_at	NM_022469	FLJ21195	Hypothetical protein FLJ21195 similar to protein related to DAC and cerberus	1.1
221829_s_at	AI307759	KPNB2	Karyopherin (importin) β 2	1.1
210654_at	AF021233	TNFRSF10D	Tumour necrosis factor receptor superfamily, member 10day, decoy with truncated death domain	1
204211_x_at	NM_002759	PRKR	Protein kinase, interferon-inducible double stranded RNA dependent	1
208022_s_at	NM_003671	CDC14B		1
203665_at	NM_002133	HMOX1	Heme oxygenase (decycling) 1	1
34478_at	X59740	RAB11B	Zinc finger protein, X-linked	1
212952_at	BE251303	CALR	Calreticulin	0.8
219935_at	NM_007038	ADAMTS5	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 5 (aggrecanase-2)	0.7
205063_at	NM_003616	SIP1	Survival of motor neuron protein interacting protein 1	0.7
39248_at	N74607	AQP3	Aquaporin 3	0.6
Twenty-four genes down-regulated in MTF clone				
208964_s_at	AL512760	FADS1	Fatty acid desaturase 1	− 0.5
203186_s_at	NM_002961	S100A4	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	− 0.5
215716_s_at	L14561	ATP2B1		− 0.7
219087_at	NM_017680	ASPN	Asporin (LRR class 1)	− 0.8
200832_s_at	AB032261	SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	− 0.8
219161_s_at	NM_016951	CKLF1	Chemokine-like factor	− 0.8
204170_s_at	NM_001827	CKS2	CDC28 protein kinase regulatory subunit 2	− 0.9
209146_at	AV704962	SC4MOL	Sterol-C4-methyl oxidase-like	− 1
206026_s_at	NM_007115	TNFAIP6	Tumour necrosis factor, α-induced protein 6	− 1
205081_at	NM_001311	CRIP1	Cysteine-rich protein 1 (intestinal)	− 1
209172_s_at	U20165	CENPF	Bone morphogenetic protein receptor, type II (serine/threonine kinase)	− 1.1
203646_at	NM_004109	FDX1	Ferredoxin 1	− 1.1
201656_at	NM_000210	ITGA6	Integrin, α 6	− 1.1
216237_s_at	AA807529	MCM5	MCM5 minichromosome maintenance deficient 5, cell division cycle 46 (*S. cerevisiae*)	− 1.2
221521_s_at	BC003186	LOC51659	HSPC037 protein	− 1.2
205034_at	NM_004702	CCNE2	Cyclin E2	− 1.3
205191_at	NM_006915	RP2	Retinitis pigmentosa 2 (X-linked recessive)	− 1.6
213590_at	AA705628	SLC16A5	Solute carrier family 16 (monocarboxylic acid transporters), member 5	− 1.6
201625_s_at	NM_005542	INSIG1	Insulin induced gene 1	− 1.9
201487_at	NM_001814	CTSC	Cathepsin C	− 3.1
207522_s_at	NM_005173	ATP2A3	ATPase, Ca^{2+} transporting, ubiquitous	− 3.5
205739_x_at	NM_016220	ZFD25	Zinc finger protein (ZFD25)	− 4.3

LR*, log ratio compared with control, the increased or decreased change in gene expression induced by EMD.
MTF, mineralized tissue formating.

Appendix B

Table B1. List of genes up-regulated in the general PDL cell population in the presence of EMD, ordered according to LR (log ratio)

AffyID	GenBank	Gene symbol	Title	LR*
Sixty-eight genes up-regulated in PDL				
202241_at	NM_025195	C8FW	Phosphoprotein regulated by mitogenic pathways	2.9
207891_s_at	NM_017518	TREX2	Three prime repair exonuclease 2	2.6
205034_at	NM_004702	CCNE2	Cyclin E2	2
205345_at	NM_000465	BARD1	BRCA1 associated RING domain 1	1.9

Table B1. (Contd.)

AffyID	GenBank	Gene symbol	Title	LR*
202068_s_at	NM_000527	LDLR	Low density lipoprotein receptor (familial hypercholesterolemia)	1.8
200832_s_at	AB032261	SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	1.7
203549_s_at	NM_000237	LPL	Lipoprotein lipase	1.7
204748_at	NM_000963	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1.6
207304_at	NM_003425	ZNF45	Zinc finger protein 45 (a Kruppel-associated box (KRAB) domain polypeptide)	1.6
210247_at	AW139618	SYN2	Synapsin II	1.5
204224_s_at	NM_000161	GCH1	GTP cyclohydrolase 1 (dopa-responsive dystonia)	1.4
207275_s_at	NM_001995	FACL1	Fatty-acid-Coenzyme A ligase, long-chain 1	1.4
205371_s_at	M27093	DBT	Dihydrolipoamide branched chain Transacylase (E2 component of branched chain keto acid dehydrogenase complex; maple syrup urine disease)	1.3
218311_at	NM_003618	MAP4K3	Mitogen-activated protein kinase kinase kinase kinase 3	1.3
201963_at	NM_021122	FACL2	Fatty-acid-Coenzyme A ligase, long-chain 2	1.3
208964_s_at	AL512760	FADS1	Fatty acid desaturase 1	1.2
40665_at	M83772	FMO3	Flavin containing monooxygenase 3	1.2
201505_at	NM_002291	LAMB1	Laminin, β 1	1.2
203680_at	NM_002736	PRKAR2B	Protein kinase, cAMP-dependent, regulatory, type II, β	1.2
206943_at	NM_004612	TGFBR1	Transforming growth factor, β receptor I (activin A receptor type II-like kinase, 53 kDa)	1.2
206432_at	NM_005328	HAS2	Hyaluronan synthase 2	1.2
206002_at	NM_005756	GPR64	G protein-coupled receptor 64	1.2
214443_at	NM_006505	PVR	Poliovirus receptor	1.2
219935_at	NM_007038	ADAMTS5	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 5 (aggrecanase-2)	1.2
203593_at	NM_012120	CD2AP	CD2-associated protein	1.2
219454_at	NM_015507	EGFL6	EGF-like-domain, multiple 6	1.2
220343_at	NM_018945	PDE7B	Phosphodiesterase 7B	1.2
208608_s_at	NM_021021	SNTB1	Syntrophin, β 1 (dystrophin-associated protein A1, 59 kDa, basic component 1)	1.2
204299_at	NM_021993	FUSIP1		1.2
221218_s_at	NM_022445	TPK1	Thiamin Pyrophosphokinase 1	1.2
209920_at	Time to run: 104 wallclock secs (100.44 usr+0.07 sys = 100.51 CPU)	BMPR2		1.2
217176_s_at	U86453	ZFX	Phosphoinositide-3-kinase, catalytic, δ polypeptide	1.2
209146_at	AV704962	SC4MOL	Sterol-C4-methyl oxidase-like	1.1
210567_s_at	BC001441	SKP2	S-phase kinase-associated protein 2 (p45)	1.1
214581_x_at	BE568134	TNFRSF21	Tumour necrosis factor receptor superfamily, member 21	1.1
205782_at	NM_002009	FGF7	Fibroblast growth factor 7 (keratinocyte growth factor)	1.1
208070_s_at	NM_002912	REV3L	REV3-like, catalytic subunit of DNA polymerase ζ (yeast)	1.1
206953_s_at	NM_012302	LPHH1	Latrophilin 1	1.1
205543_at	NM_014278	APG-1	Heat shock protein (hsp110 family)	1.1
220386_s_at	NM_019063	EML4	Echinoderm microtubule associated protein like 4	1.1
205289_at	AA583044	BMP2	Bone morphogenetic protein 2	1
204595_s_at	AI300520	STC1	Stanniocalcin 1	1
203525_s_at	AI375486	–	Adenomatosis polyposis coli	1
204022_at	AI668780	WWP2	Nedd-4-like ubiquitin-protein ligase	1
212774_at	AJ223321	ZNF238		1
212298_at	BE620457	NRP1	Neuropilin 1	1
36499_at	D87469	CELSR2	Cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)	1
37892_at	J04177	COL11A1	Collagen, type XI, α 1	1
215716_s_at	L14561	ATP2B1		1
204864_s_at	NM_002184	IL6ST	Interleukin 6 signal Transducer (gp130, oncostatin M receptor)	1
207038_at	NM_004694	SLC16A6	Solute carrier family 16 (monocarboxylic acid transporters), member 6	1
208396_s_at	NM_005019	PDE1A	Phosphodiesterase 1A, calmodulin-dependent	1
205701_at	NM_006390	RANBP8	Importin 8	1
219229_at	NM_013272	SLC21A11	Solute carrier family 21 (organic anion transporter), member 11	1
217894_at	NM_016121	NY-REN-45	NY-REN-45 antigen	1
221107_at	NM_017581	CHRNA9	Cholinergic receptor, nicotinic, α polypeptide 9	1
219087_at	NM_017680	ASPN	Asporin (LRR class 1)	1
220770_s_at	NM_022090	LOC63920	Transposon-derived Buster3 transposase-like	1
202422_s_at	NM_022977	FACL4	Fatty-acid-Coenzyme A ligase, long-chain 4	1
203879_at	U43328	PIK3CD	Cartilage linking protein 1	1

LR*, log ratio compared with control, the increased or decreased change in gene expression induced by EMD.
PDL, periodontal ligament.

Appendix C

Table C1. List of genes down-regulated in the general PDL cell population in the presence of EMD, ordered according to LR (log ratio)

AffyID	GenBank	Gene symbol	Title	LR*
One hundred and thirty four genes down-regulated in PDL				
202345_s_at	NM_001444	RPLP2	Fatty acid binding protein 5 (psoriasis-associated)	– 3.6
208581_x_at	NM_005952	MT1X	Metallothionein 1 ×	– 3.6
205081_at	NM_001311	CRIP1	Cysteine-rich protein 1 (intestinal)	– 3.2
206461_x_at	NM_005951	MT1H	Metallothionein 1H	– 2.7
204949_at	NM_002162	ICAM3	Intercellular adhesion molecule 3	– 2.2
202659_at	NM_002801	PSMB10	Proteasome (prosome, macropain) subunit, β type, 10	– 2.2
204170_s_at	NM_001827	CKS2	CDC28 protein kinase regulatory subunit 2	– 1.7
203186_s_at	NM_002961	S100A4	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	– 1.6
214290_s_at	AA451996	H2AFO	Histone 2, H2aa	– 1.5
202690_s_at	BC001721	SNRPD1	Small nuclear Ribonucleoprotein D1 polypeptide 16 kDa	– 1.5
217931_at	BC004423	TNRC5	Trinucleotide repeat containing 5	– 1.5
201441_at	NM_001863	COX6B	Cytochrome *c* oxidase subunit Vib	– 1.5
203571_s_at	NM_006829	APM2	Adipose specific 2	– 1.5
200085_s_at	NM_007108	TCEB2	Transcription elongation factor B (SIII), polypeptide 2 (18 kDa, elongin B)	– 1.5
220540_at	NM_022358	KCNK15	Potassium channel, subfamily K, member 15	– 1.5
213757_at	AA393940	EIF5A	Eukaryotic translation initiation factor 5A	– 1.4
209806_at	BC000893	H2BFT	Histone 1, H2bk	– 1.4
212952_at	BE251303	CALR	Calreticulin	– 1.4
203371_s_at	NM_002491	NDUFB3	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 3, 12 kDa	– 1.4
203454_s_at	NM_004045	ATOX1	ATX1 antioxidant protein 1 homolog (yeast)	– 1.4
202839_s_at	NM_004146	NDUFB7	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 7, 18 kDa	– 1.4
218381_s_at	NM_007279	U2AF65	U2 small nuclear ribonucleoprotein auxiliary factor (65 kD)	– 1.4
218213_s_at	NM_014206	C11orf10	Chromosome 11 open reading frame 10	– 1.4
221269_s_at	NM_031286	SH3BGRL3	SH3 domain binding glutamic acid-rich protein like 3	– 1.4
39248_at	N74607	AQP3	Aquaporin 3	– 1.3
203478_at	NM_002494	NDUFC1	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1, 6 kDa	– 1.3
200872_at	NM_002966	S100A10	S100 calcium binding protein A10 (annexin II ligand, calpactin I, light polypeptide (p11))	– 1.3
205792_at	NM_003881	WISP2	WNT1 inducible signalling pathway protein 2	– 1.3
204070_at	NM_004585	RARRES3	Retinoic acid receptor responder (tazarotene induced) 3	– 1.3
200826_at	NM_004597	SNRPD2	Small nuclear ribonucleoprotein D2 polypeptide 16.5 kDa	– 1.3
212185_x_at	NM_005953	MT2A	Metallothionein 2A	– 1.3
201568_at	NM_014402	QP-C	Low molecular mass ubiquinone-binding protein (9.5 kD)	– 1.3
219762_s_at	NM_015414	RPL36	Ribosomal protein L36	– 1.3
218011_at	NM_024292	UBL5	Ubiquitin-like 5	– 1.3
213606_s_at	AI571798	ARHGDIA	Rho GDP dissociation inhibitor (GDI) α	– 1.2
212716_s_at	AW083133	M9	Muscle specific gene	– 1.2
209911_x_at	BC002842	H2BFB	Histone 1, H2bd	– 1.2
209224_s_at	BC003674	NDUFA2	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 2, 8 kDa	– 1.2
209492_x_at	BC003679	ATP5I	ATP synthase, H+transporting, mitochondrial F0 complex, subunit e	– 1.2
200093_s_at	N32864	HINT1	Histidine triad nucleotide binding protein 1	– 1.2
217753_s_at	NM_001029	RPS26	ribosomal protein S26	– 1.2
204570_at	NM_001864	COX7A1	Cytochrome *c* oxidase subunit VIIa polypeptide 1 (muscle)	– 1.2
201106_at	NM_002085	GPX4	Glutathione peroxidase 4 (phospholipid hydroperoxidase)	– 1.2
203683_s_at	NM_003377	VEGFB	Vascular endothelial growth factor B	– 1.2
200925_at	NM_004373	COX6A1	Cytochrome *c* oxidase subunit VIa polypeptide 1	– 1.2
201403_s_at	NM_004528	MGST3	Microsomal glutathione S-transferase 3	– 1.2
202298_at	NM_004541	NDUFA1	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 1, 7.5 kDa	– 1.2
206790_s_at	NM_004545	NDUFB1	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 1, 7 kDa	– 1.2
203880_at	NM_005694	COX17	COX17 homolog, cytochrome *c* oxidase assembly protein (yeast)	– 1.2
202598_at	NM_005979	S100A13	S100 calcium binding protein A13	– 1.2
202927_at	NM_006221	PIN1	Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1	– 1.2
212647_at	NM_006270	RRAS	Related RAS viral (r-ras) oncogene homolog	– 1.2
218435_at	NM_013238	MCJ	DNAJ domain-containing	– 1.2
209058_at	AB002282	EDF1	Endothelial differentiation-related Factor 1	– 1.1
210532_s_at	AF116639	C14orf2		– 1.1
213735_s_at	AI557312	COX5B	Cytochrome *c* oxidase subunit Vb	– 1.1
213357_at	AV701318	–	Homo sapiens, Similar to CG14037 gene product, clone IMAGE:3640720, mRNA, partial cds	– 1.1
210605_s_at	BC003610	MFGE8	Milk fat globule-EGF factor 8 protein	– 1.1
213409_s_at	BF593727	RHEB2	Ras homolog enriched in brain 2	– 1.1
40489_at	D31840	DRPLA	Dentatorubral-pallidoluysian atrophy (atrophin-1)	– 1.1
201360_at	NM_000099	CST3	Cystatin C (amyloid angiopathy and cerebral haemorrhage)	– 1.1

Table C1. (Contd.)

AffyID	GenBank	Gene symbol	Title	LR*
203729_at	NM_001425	EMP3	Epithelial membrane protein 3	– 1.1
205644_s_at	NM_003096	SNRPG	Small nuclear ribonucleoprotein polypeptide G	– 1.1
218495_at	NM_004182	UXT	Ubiquitously-expressed transcript	– 1.1
203391_at	NM_004470	FKBP2	FK506 binding protein 2, 13 kDa	– 1.1
218563_at	NM_004542	NDUFA3	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 3, 9 kDa	– 1.1
218200_s_at	NM_004546	NDUFB2	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 2, 8 kDa	– 1.1
218226_s_at	NM_004547	NDUFB4	NADH dehydrogenase (ubiquinone) 1 β subcomplex, 4, 15 kDa	– 1.1
205483_s_at	NM_005101	G1P2	Interferon, α-inducible protein (clone IFI-15 K)	– 1.1
217756_x_at	NM_005770	SERF2	Small EDRK-rich factor 2	– 1.1
201058_s_at	NM_006097	MYL9	Myosin, light polypeptide 9, regulatory	– 1.1
202090_s_at	NM_006830	UQCR	Ubiquinol-cytochrome *c* reductase (6.4 kD) subunit	– 1.1
218357_s_at	NM_012459	TIMM8B	Translocase of inner mitochondrial membrane 8 homolog B (yeast)	– 1.1
202209_at	NM_014463	LSM3	LSM3 homolog, U6 small nuclear RNA Associated (*S. cerevisiae*)	– 1.1
204175_at	NM_015871	LOC51042	Zinc finger protein	– 1.1
218007_s_at	NM_015920	RPS27L	Ribosomal protein S27-like	– 1.1
217755_at	NM_016185	HN1	Haematological and neurological expressed 1	– 1.1
217733_s_at	NM_021103	TMSB10	thymosin, β 10	– 1.1
213892_s_at	AA927724	APRT	Adenine phosphoribosyltransferase	– 1
210908_s_at	AB055804	PFDN5	Prefoldin 5	– 1
210125_s_at	AF044773	BCRP1	Barrier to autointegration factor 1	– 1
202627_s_at	AL574210	SERPINE1	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	– 1
208904_s_at	BC000354	RPS28	Ribosomal protein S28	– 1
209477_at	BC000738	EMD	Emerin (Emery-Dreifuss muscular dystrophy)	– 1
200075_s_at	BC006249	GUK1	Guanylate kinase 1	– 1
214224_s_at	BE674061	PIN4	Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting, 4 (parvulin)	– 1
208680_at	L19184	PRDX1	Peroxiredoxin 1	– 1
201201_at	NM_000100	CSTB	Cystatin B (stefin B)	– 1
200824_at	NM_000852	GSTP1	Glutathione S-transferase pi	– 1
200823_x_at	NM_000992	RPL29	Ribosomal protein L29	– 1
207585_s_at	NM_001001	RPL36AL	Ribosomal protein L36a-like	– 1
201258_at	NM_001020	RPS16	Ribosomal protein S16	– 1
202592_at	NM_001487	GCN5L1	GCN5 general control of amino-acid synthesis 5-like 1 (yeast)	– 1
201841_s_at	NM_001540	HSPB1	Heat shock 27 kDa protein 1	– 1
205824_at	NM_001541	HSPB2	Heat shock 27 kDa Protein 2	– 1
204868_at	NM_001545	ICT1	Immature colon carcinoma transcript 1	– 1
202325_s_at	NM_001685	ATP5J	ATP synthase, H+transporting, mitochondrial F0 complex, subunit F6	– 1
201597_at	NM_001865	COX7A2	Cytochrome *c* oxidase subunit VIIa polypeptide 2 (liver)	– 1
202110_at	NM_001866	COX7B	Cytochrome *c* oxidase subunit VIIb	– 1
203725_at	NM_001924	GADD45A	Growth arrest and DNA-damage-inducible, α	– 1
203816_at	NM_001929	DGUOK	Deoxyguanosine kinase	– 1
201348_at	NM_002084	GPX3	Glutathione peroxidase 3 (plasma)	– 1
205133_s_at	NM_002157	HSPE1	Heat shock 10 kDa protein 1 (chaperonin 10)	– 1
202001_s_at	NM_002490	NDUFA6	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 6, 14 kDa	– 1
201400_at	NM_002795	PSMB3	Proteasome (prosome, macropain) subunit, β type, 3	– 1
203021_at	NM_003064	SLPI	Secretory leukocyte protease inhibitor (antileukoproteinase)	– 1
200703_at	NM_003746	DNCL1	Dynein, cytoplasmic, light polypeptide 1	– 1
203663_s_at	NM_004255	COX5A	Cytochrome *c* oxidase subunit Va	– 1
218101_s_at	NM_004549	NDUFC2	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2, 14.5 kDa	– 1
201757_at	NM_004552	NDUFS5	NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15 kDa (NADH-coenzyme Q reductase)	– 1
203667_at	NM_004607	TBCA	Tubulin-specific chaperone a	– 1
221085_at	NM_005118	TNFSF15	Tumour necrosis factor (ligand) superfamily, member 15	– 1
204971_at	NM_005213	CSTA	Cystatin A (stefin A)	– 1
200660_at	NM_005620	S100A11	S100 calcium binding protein A11 (calgizzarin)	– 1
202233_s_at	NM_006004	UQCRH	Ubiquinol-cytochrome *c* reductase hinge protein	– 1
201004_at	NM_006280	SSR4	Signal sequence receptor, delta (translocon-associated protein delta)	– 1
200055_at	NM_006284	TAF10	TAF10 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 30 kDa	– 1
202276_at	NM_006304	DSS1	Deleted in split-hand/split-foot 1 region	– 1
200701_at	NM_006432	NPC2	Niemann–Pick disease, type C2	– 1
200002_at	NM_007209	RPL35	Ribosomal protein L35	– 1
218117_at	NM_014248	RBX1	Ring-box 1	– 1
202857_at	NM_014255	TMEM4	Transmembrane protein 4	– 1
203534_at	NM_014462	LSM1	LSM1 homolog, U6 small nuclear RNA associated (*S. cerevisiae*)	– 1
205341_at	NM_014601	EHD2	EH-domain containing 2	– 1
217728_at	NM_014624	S100A6	S100 calcium binding protein A6 (calcyclin)	– 1

Table C1. (Contd.)

AffyID	GenBank	Gene symbol	Title	LR*
219161_s_at	NM_016951	CKLF1	Chemokine-like factor	– 1
208579_x_at	NM_017445	H2BFT		– 1
201812_s_at	NM_019059	TOM7	Homolog of Tom7 (*S. cerevisiae*)	– 1
208540_x_at	NM_021039	S100A11P	S100 calcium binding protein A11 pseudogene	– 1
217802_s_at	NM_022731	NUCKS	Similar to rat nuclear ubiquitous casein kinase 2	– 1

LR*, log ratio compared with control, the increased or decreased change in gene expression induced by EMD.
PDL, periodontal ligament.

Appendix D

Table D1. List of genes up-regulated and down-regulated in the FB clones in the presence of EMD, ordered according to LR (log ratio)

AffyID	GenBank	Gene symbol	Title	LR*
Thirteen genes up-regulated in FB clone				
205063_at	NM_003616	SIP1	Survival of motor neuron protein interacting protein 1	3.5
214590_s_at	AL545760	UBE2D1	Ubiquitin-conjugating enzyme E2D 1 (UBC4/5 homolog, yeast)	1.8
205713_s_at	NM_000095	COMP	Cartilage oligomeric matrix protein (pseudoachondroplasia, epiphyseal dysplasia 1, multiple)	1.2
203603_s_at	NM_014795	ZFHX1B	Zinc finger homeobox 1b	1.2
208608_s_at	NM_021021	SNTB1	Syntrophin, β 1 (dystrophin-associated protein A1, 59 kDa, basic component 1)	1.1
203945_at	NM_001172	ARG2	Arginase, type II	1
207379_at	NM_005711	EDIL3	EGF-like repeats and discoidin I-like domains 3	1
216537_s_at	AJ130713	SIGLEC7	Sialic acid binding Ig-like lectin 7	1
204864_s_at	NM_002184	IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	0.9
221009_s_at	NM_016109	ANGPTL4	Angiopoietin-like 4	0.7
204595_s_at	AI300520	STC1	Stanniocalcin 1	0.6
208070_s_at	NM_002912	REV3L	REV3-like, catalytic subunit of DNA polymerase ζ (yeast)	0.5
218311_at	NM_003618	MAP4K3	Mitogen-activated protein kinase kinase kinase kinase 3	0.5
Twelve genes down-regulated in FB clone				
206002_at	NM_005756	GPR64	G protein-coupled receptor 64	– 0.6
205483_s_at	NM_005101	G1P2	Interferon, α-inducible protein (clone IFI-15 K)	– 0.9
201721_s_at	NM_006762	LAPTM5	Lysosomal-associated multispanning membrane protein-5	– 1
204994_at	NM_002463	MX2	Myxovirus (influenza virus) resistance 2 (mouse)	– 1
204439_at	NM_006820	C1orf29	Chromosome 1 open reading frame 29	– 1
202086_at	NM_002462	MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	– 1.1
216860_s_at	AF028333	GDF11	Growth differentiation factor 11	– 1.1
203153_at	NM_001548	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	– 1.1
206704_at	NM_000084	CLCN5	Chloride channel 5 (nephrolithiasis 2, X-linked, Dent disease)	– 1.3
207557_s_at	NM_001035	RYR2	Ryanodine receptor 2 (cardiac)	– 1.3
209619_at	K01144	CD74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	– 1.8
210163_at	AF030514	CXCL11	Chemokine (C-X-C motif) ligand 11	– 3.8

LR*, log ratio compared with control, the increased or decreased change in gene expression induced by EMD.
FB, fibroblastic.