Archives of Oral Biology (2005) **50**, 481–496

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Archives of Oral Biology

www.intl.elsevierhealth.com/journals/arob

Effect of Vitamin C on pre-osteoblast gene expression

Francesco Carinci[a], Furio Pezzetti[b], Anna Maria Spina[c], Annalisa Palmieri[b,d], Gregorio Laino[c], Alfredo De Rosa[c], Ernesto Farina[c], Fausto Illiano[c], Giordano Stabellini[e], Vittoria Perrotti[f], Adriano Piattelli[f,*]

[a]*University of Ferrara, Italy*
[b]*Institute of Histology, University of Bologna, Italy*
[c]*Second University of Naples, Italy*
[d]*Centre of Molecular Genetics, CARISBO Foundation, Italy*
[e]*University of Milano, Italy*
[f]*Department of Oral Pathology, Dental Clinic, University of Chieti, Via dei vestini 31, 66013 Chieti, Italy*

Accepted 14 October 2004

KEYWORDS
Ascorbic acid;
DNA micro-array;
Gene expression;
Gene profiling;
Pre-osteoblast;
Stem cell

Summary Ascorbic acid (AA), also known as Vitamin C, is a cofactor required for the function of several hydroxylases. It is not synthesised in humans and has to be provided by diet. Its absence is responsible for scurvy, a condition related to the defective synthesis of collagen by the reduced function of prolylhydroxylase. AA is also a risk factor for periodontal disease.

Recently, it has been shown that AA induces embryonic stem cells to differentiate into osteoblasts. The mechanism by which AA sustains pre-osteoblast proliferation and commitment is mediated through the synthesis of collagen type I, interaction with alpha2- and beta1-integrin, activation of the mitogen-activated protein kinase pathway, and phosphorylation of osteoblast-specific transcription factors. However, the multifunctional role of AA is not fully elucidated.

MC3T3-E1 mouse calvaria-derived cell line is a well-defined in vitro model of pre-osteoblast differentiation, and AA is essential for the proliferation and differentiation of MC3T3-E1.

By using DNA micro-arrays containing 15,000 genes, we identified several genes in MC3T3-E1 cultured with AA for 24 h whose expression was significantly up or down-regulated. The differentially expressed genes covered a broad range of functional activities: (1) cell growth; (2) metabolism; (3) morphogenesis; (4) cell death; (5) cell communication. The data reported are, to our knowledge, the first genetic portrait of early stage stimulation of pre-osteoblasts by AA, and may be relevant to better

* Corresponding author. Present address: Via F. Sciucchi 63, 66100 Chieti, Italy. Tel.: +39 0871 358421/3554073/4083; fax: +39 0871 3554076.
E-mail address: apiattelli@unich.it (A. Piattelli).

0003-9969/$ – see front matter
doi:10.1016/j.archoralbio.2004.11.006

understand the molecular mechanism of pre-osteoblast proliferation and commitment. Elucidation of the molecular mechanism has important clinical implications because it may facilitate the correct use of AA to accelerate bone regeneration.

Introduction

Ascorbic acid (AA), also known as Vitamin C, is a cofactor required for the function of several hydroxylases. It is not synthesised in humans and has to be provided by diet. Its absence is responsible for scurvy, a condition related to a defective synthesis of collagen by the reduced function of prolylhydroxylase and production of collagen polypeptides lacking hydroxyproline, that causes incorrect assembling of stable triple-helical collagen molecules.

Studies of scorbutic gingivitis and the effects of AA on extracellular matrix (ECM) and immunological and inflammatory responses provide a rationale for hypothesising that AA is a risk factor for periodontal disease. Gingival bleeding increases significantly after AA depletion and returns to baseline values after AA repletion.[1] Moreover, a relationship exists between dietary intake of AA and periodontal disease in smokers.[2]

Recently, it has been shown that AA, together with beta-glycerophosphate (beta-GP) and 1alpha,25-OH Vitamin D3 or dexametazone, induces embryonic stem cells to differentiate into mineralised osteoblasts in vitro.[3,4]

MC3T3-E1 mouse calvaria-derived cell line is a well-defined model of pre-osteoblast differentiation. During the first week of culture, MC3T3-E1 cells actively replicate but maintain a fusiform appearance. By the second week, the cultures display cuboidal morphology, attain confluence and undergo growth arrest. Downregulation of replication is associated with expression of osteoblast functions, including production of alkaline phosphatase (ALP), processing of procollagens to collagens and incremental deposition of a collagenous ECM. Mineralisation of ECM, which begins approximately 16 days after culture, marks the final phase of osteoblast phenotypic development. AA and beta-GP are both essential for the expression of osteoblast phenotype. AA is required for the deposition of collagen in the ECM and also induces ALP activity in mature cells but not in immature cells. Beta-GP displays synergistic actions with AA to further stimulate collagen accumulation and ALP activity in post-mitotic, differentiated osteoblast-like cells. Mineralisation of mature cultures requires the presence of beta-GP.[5]

AA is responsible for the sustained proliferation of MC3T3-E1 cells, and the effect is mediated through the synthesis of collagen.[6] The earliest effects of AA are the stimulation of type I procollagen mRNA and collagen synthesis (24 h after AA addition), followed by the induction of ALP (48—72 h) and osteocalcin (96—144 h) mRNAs.[7]

AA is carried into the cell by a specific Na(+)-dependent transporter.[8] Two Na(+)-dependent Vitamin C transporters exist, SVCT1 and 2. SVCT2 is expressed in pre-osteoblasts and is upregulated by dexamethasone.[9]

Alpha2beta1-Integrin is a major cellular receptor for type I collagen. A dose-dependent decrease in alpha2- and beta1-integrin mRNA levels was found in bone cultures deficient in AA.[10] Disruption of alpha2-integrin—ECM interactions blocked activation of the osteocalcin gene 2 promoter (OSE2). The latter is activated by an interaction between AA and Osf2 (also called Cbfa1/AML3/PEBP2alphaA), an osteoblast-specific transcription factor. Anti-alpha2-integrin blocking antibody reduced AA-dependent binding of Osf2 to OSE2, thus reducing the production of osteocalcin and bone sialoproteins.[11]

MC3T3-E1 cells constitutively express Bone Morphogenetic Protein (BMP)-2, -4 and -7, and BMP production is necessary for differentiation. Matrix responsiveness requires an alpha2beta1-integrin—collagen interaction and mitogen-activated protein kinase (MAPK) activity, which phosphorylates and activates the osteoblast-specific transcription factor Osf2/Cbfa1. Inhibition of extracellular-signal-regulated kinase phosphorylation blocked AA- or BMP-7/AA-dependent gene expression. Therefore, autocrine BMP production and integrin-mediated cell—collagen interactions are both required for osteoblast differentiation, and both these pathways require MAPK activity.[12]

Cbfa1/Runx2, a transcription factor essential for osteoblast differentiation, is activated by the MAPK pathway. This pathway is stimulated in at least two ways: (1) by binding of type I collagen to alpha2-beta1-integrins on the osteoblast surface and (2) by treatment of cells with the osteogenic growth factor, FGF2. Runx2 activity is also enhanced by factors known to stimulate specific signal transduction pathways such as parathyroid hormone (PTH)/PTH receptor (signals through protein kinase A (PKA) and PKC pathways) and BMPs (signal through Smad proteins). These findings suggest that Runx2 plays a central role in co-ordinating multiple signals involved in osteoblast differentiation.[13]

Additional early regulators of cell growth and differentiation are transforming growth factor beta 1 (TGF-beta1), tumour necrosis factor (TNF) and PTH. TGF-beta1 inhibits the differentiation of osteoblasts. During differentiation, there is a reduction in cell-surface TGF-beta receptors that is dependent on the interaction of cell-surface alpha2-beta1-integrin with matrix collagen synthesised by osteoblasts.[14] Also, TNF-alpha treatment inhibited the early phases of differentiation. TNF inhibited the expression of insulin-like growth factor I but had no effect on the expression of BMP-2, -4 or -6, or skeletal LIM protein (LMP-1).[15] In contrast, the expression of PTH/PTH receptor increases with osteoblastic differentiation.[16]

As AA has several functions and the mechanism by which AA stimulates pre-osteoblasts to growth and differentiation into osteoblasts is poorly understood, we attempted to address this question using a micro-array technique.

DNA micro-array is a molecular technology that enables the analysis of gene expression in parallel on a very large number of genes, spanning a significant fraction of the human genome. Gene expression is performed by a process of: (1) RNA extraction; (2) reverse transcription; (3) labelling of cDNA. Reference (i.e. untreated cells) and investigated (i.e. cells cultured with AA) cDNA are labelled with different dyes and then hybridised on slides containing cDNA fragments. The slides are scanned with a laser system, and two false colour images are generated for each hybridisation with cDNA from the investigated and reference cells. The overall result is the generation of a so-called genetic portrait.[17,18] This corresponds to up- or down-regulated genes in the investigated cell system.

In the present study, we defined the genetic effect of AA on pre-osteoblasts using MC3T3-E1 and micro-array slides containing 15,000 different oligonucleotides.

Materials and methods

Cell culture

A pre-osteoblast MC3T3-E1 cell line (ATCC CRL-2593) was cultured in 10 cm sterile Falcon wells (Becton Dickinson, New Jersey, USA), containing 10 ml of alpha-minimum essential medium with ribonucleosides and deoxyribonucleosides excluding AA, 2 mM L-glutamine, 1 mM sodium pyruvate supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, Mo, USA) and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml, Sigma). Cultures were maintained in a 5% CO_2 humidified atmosphere at 37 °C.

MC3T3 cells were collected and, before reaching confluency, a density of 1×10^6 cells per dish was seeded using 0.25% trypsin and 0.03% EDTA in phosphate-buffered saline for cell release. AA 50 μg/ml and 3 mM sodium phosphate were added to serum in one set of wells. Control wells contained 3 mM sodium phosphate in serum. After 24 h, when both cultures were subconfluent, cells were processed for RNA extraction.

DNA micro-array screening and analysis

RNA was extracted from the cells using RNAzol. Ten micrograms of total RNA were used for each sample. cDNA was synthesised using Superscript II (Life Technologies, Invitrogen, Milano, Italy) and amino-allyl dUTP (Sigma). Mono-reactive Cy3 and Cy5 esters (Amersham Pharmacia, Little Chalfont, UK) were used for indirect cDNA labelling. RNA extracted from untreated cells was labelled with Cy3 and used as a control against the Cy5-labelled cDNA treated with AA in the first experiment and then switched. Both steps were repeated twice. Mouse 15 K DNA micro-arrays were used (Ont. Cancer Institute, Toronto, Canada). For 15 K slides, 100 μl of the sample and control cDNAs in DIG Easy hybridization solution (Roche, Basel, Switzerland) were used in a sandwich hybridisation of the two slides constituting the 15 K set at 37 °C overnight. Washing was performed three times for 10 min with 1× saline sodium citrate (SSC), 0.1% sodium dodecyl sulphate 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 were switched. A GenePix 4000a DNA micro-array scanner (Axon, Union City, CA, USA) was used to scan the slides, and data were extracted with GenePix Pro. After removing the local background, genes with expression levels of <1000 were not included in the analysis, since ratios are not reliable at that detection level.

After scanning of the slides containing the 15 K genes in duplicate, the local background was calculated for each target location. A normalisation factor was estimated from ratios of medians. Normalisation was performed by adding the $\log_2$ of the normalisation 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 normalisation factor, and $\log_2$ ratios outside the 99.7% confidence interval (median $\pm$ 3 S.D. = 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 three-fold difference in expression between the treated and reference cells. GenePix Pro software was used to report genes above the threshold and with less than 10% difference in three different statistical evaluations of the intensity ratio, thus effectively enabling an automated quality control check of the hybridised spots. Furthermore, all the positively passed spots were visually inspected. GP3 perl script was used in order to post-process raw data files from the scanning procedure.[17,18] *Z*-score normalisation, a trimmed mean of 75% and a threshold value of three were used to filter the GenePix raw data.

Significance analysis of micro-array (SAM) was used to select the cDNAs which distinguish between two sample groups.[17,18] Specifically designed for use with micro-array data, SAM is a software program that reports the most statistically significant differentially expressed genes between two groups of samples. In addition, SAM reports an estimate of the median false discovery rate (FDR), which is the percentage of genes falsely reported as showing statistically significant differential expression. SAM uses an algorithm based on Student's *t*-test and also performs data permutations to determine the FDR.

FatiGO is a web interface (http://fatigo.bioinfo.cnio.es) that carries out simple data mining using gene ontology (GO) for DNA micro-array data. The data mining consists of the assignation of the most characteristic GO term to each cluster. GO terms are related to human, mouse and yeast genes and proteins. FatiGO implements Fisher's exact test for 2×2 contingency tables for comparing two groups of genes, and extracting a list of the distribution of GO terms among the groups is significantly different. A gene product can have one or more molecular functions, can be used in one or more biological processes, and may be associated with one or more cellular components. When we searched for the distribution of genes in a specific ontology (e.g. biological process), we selected the ontology and also the GO term's level (=3).

Results

We used micro-array technology to perform systemic analysis of expression profiles for thousands of genes simultaneously, and to provide primary information on transcriptional changes related to the effect of AA in pre-osteoblastic cells after 24 h of stimulation.

In total, 1884 clones had statistically significant differential expression levels (FDR < 0.01) (Fig. 1).

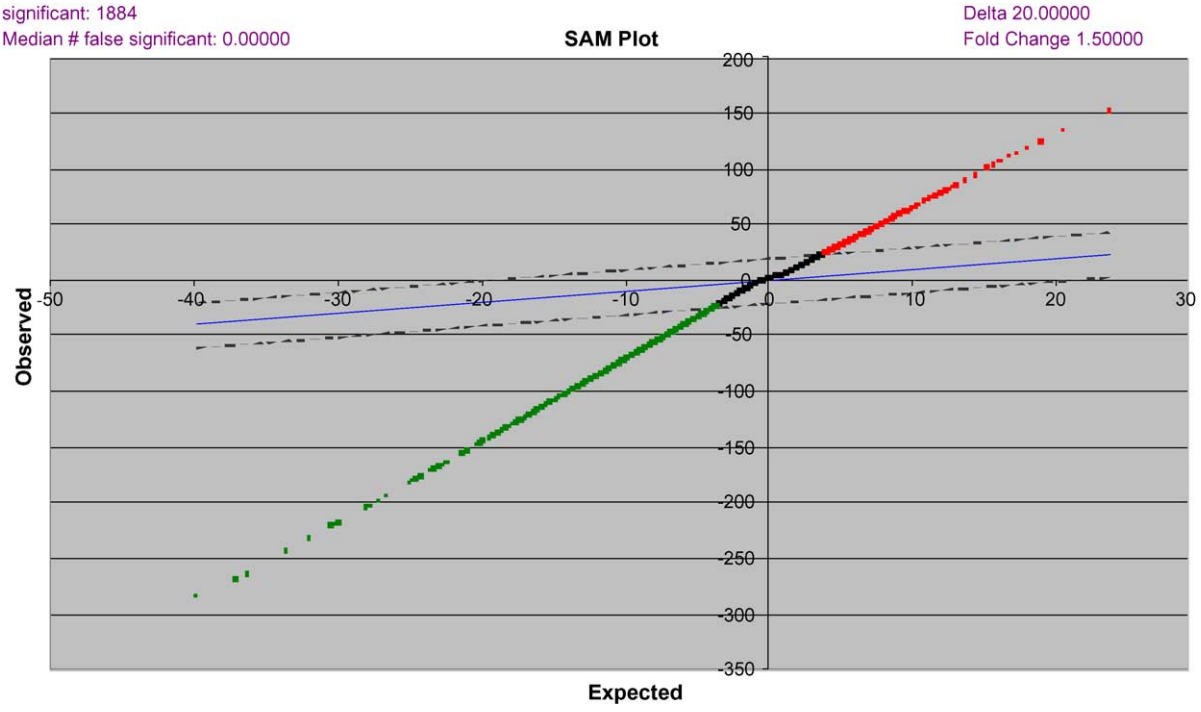

Figure 1 SAM plot of MC3T3-E1 treated with ascorbic acid for 24 h. The SAM program was used to compare observed d scores (*y*-axis) with expected d scores (*x*-axis), and evaluate significant genes based on user-defined thresholds. Positive significant genes are labelled in the upper-right graphic area (red points), whereas negative significant genes are plotted in the lower-left graphic area (green points). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Table 1 Ninety-seven up-regulated genes selected by the FatiGO program

Name	Symbol	Clone ID	Score (d)	Fold change
3-Mono-oxygenase/tryptophan 5-mono-oxygenase activation protein, gamma polypeptide	Ywhag	H3014H03	41,868844	1.85492
5′,3′-Nucleotidase, mitochondrial	Nt5m	H3148D02	36,538218	2.04827
5′,3′-Exoribonuclease 2	Xrn2	H3002C12	28,999403	3.99780
Adaptor protein complex AP-2, mu1	Ap2m1	H3012D08	34,200391	1.54553
Adaptor-related protein complex AP-1, mu subunit 1	Ap1m1	H3006H09	37,504414	2.77304
ADP-ribosylation factor-like 4	Arl4	H3081G08	75,715123	1.76902
Adrenomedullin	Adm	H3007E05	31,403188	3.97719
Aldehyde dehydrogenase 9, subfamily A1	Aldh9a1	H3019B04	26,619015	2.25837
Annexin A2	Anxa2	H3126C03	31,468735	1.55345
Annexin A7	Anxa7	H3154E10	28,218888	1.69883
Antigen identified by monoclonal antibody MRC OX-2	Mox2	H3157B02	34,944917	1.56975
Apolipoprotein B editing complex 1	Apobec1	H3108A03	28,234667	2.07014
Aquarius	Aqr	H3159F02	27,640477	2.52958
ATP-binding cassette, subfamily F (GCN20), member 2	Abcf2	H3089A11	40,405934	2.68750
Beta-site APP cleaving enzyme	Bace	H3083E02	65,324295	1.88410
Blood vessel epicardial substance	Bves	H3129F12	77,495627	3.68894
Bone morphogenetic protein 1	Bmp1	H3009B01	28,770041	3.30925
Bone morphogenetic protein 15	Bmp15	H3105B04	30,903693	1.55435
Brca1-associated protein 1	Bap1	H3028F01	33,70805	2.90928
Carnitine-deficiency-associated gene expressed in ventricle 3	Cdv3	H3043H02	31,145537	2.18257
Casein kinase II, alpha 2, polypeptide	Csnk2a2	H3076H10	24,38152	2.34915
Caspase 6	Casp6	H3157C09	52,219667	1.58545
Cathepsin 8	Cts8	H3018H08	25,013147	2.48265
CCCTC-binding factor	Ctcf	H3029E07	37,520347	2.57489
CDC-like kinase 2	Clk2	H3119D08	32,777081	2.34798
Cleavage stimulation factor, 3′ pre-RNA subunit 2, tau	Cstf2t	H3009G01	33,387532	2.70067
Coatomer protein complex, subunit gamma	Copg	H3121D09	30,841808	2.14275
Colony stimulating factor 1 (macrophage)	Csf1	H3057D05	73,859125	3.22314
Cyclin A2	Ccna2	H3079A09	39,01319	1.82238
Cyclin L1	Ccnl1	H3074C09	78,929641	1.92660
Cystathionine beta-synthase	Cbs	H3079G04	24,862955	3.17414
Defender against cell death 1	Dad1	H3151D08	26,07611	1.90392
Dystroglycan 1	Dag1	H3008B05	25,715875	2.80000
Gene trap locus F3b	Gtlf3b	H3153F03	28,150734	2.07238
General transcription factor II H, polypeptide 4	Gtf2h4	H3022F02	46,201803	2.13484
Glial-cell-line-derived neurotrophic factor family receptor alpha 1	Gfra1	H3110A02	29,378322	2.13450
GLI-Kruppel family member GLI2	Gli2	H3112E07	31,411864	2.29496
Glucosamine-6-phosphate deaminase 2	Gnpda2	H3115E03	34,690774	1.56250
Glutamate dehydrogenase	Glud	H3140E08	27,636018	1.57057
Golgi SNAP receptor complex member 2	Gosr2	H3035A10	33,291676	2.82152
Heat shock protein, A	Hspa9a	H3122D07	62,893555	3.16311
High-density-lipoprotein (HDL)-binding protein	Hdlbp	H3001G03	31,698297	2.24435
High mobility group AT-hook 2	Hmga2	H3125E10	25,607143	2.41441
Histocompatibility 2, class II antigen E beta	H2-Eb1	H3008F07	48,298132	2.20982
Integrin beta 4	Itgb4	H3003B08	38,54662	2.72659
Kangai 1 (suppression of tumourigenicity 6, prostate)	Kai1	H3139E03	37,912392	1.57036
Keratin complex 2, basic, gene 7	Krt2-7	H3007B05	27,577149	2.16231
Kinesin family member 23	Kif23	H3068A08	32,739043	2.37616
Kringle containing transmembrane protein	Kremen	H3078C08	32,408461	1.86836

Table 1 (*Continued*)

Name	Symbol	Clone ID	Score (d)	Fold change
Lectin, galactose binding, soluble 1	Lgals1	H3003A03	38,015824	1.88154
Leukaemia inhibitory factor	Lif	H3041H12	24,333384	3.22326
Ligase IV, DNA, ATP-dependent	Lig4	H3021F06	25,927906	2.83532
LIM domain binding 1	Ldb1	H3077H06	43,493903	5.14038
Lipase, member H	Liph	H3010F11	25,73085	8.04300
Ly1 antibody reactive clone	Lyar	H3155B08	33,538036	3.60309
Lymphocyte antigen 6 complex, locus E	Ly6e	H3027D05	24,186335	2.18642
Major vault protein	Mvp	H3038D05	26,057367	4.55413
Matrix metalloproteinase 7	Mmp7	H3093H12	27,93146	3.76463
Microtubule-associated protein, RP/EB family, member 1	Mapre1	H3055G01	27,376144	3.15682
Mid-1-related chloride channel 1	Mclc	H3069C03	37,859827	2.42135
Mini-chromosome maintenance deficient 6 (MIS5 homologue, S. pombe) (S. cerevisiae)	Mcm6	H3146C08	36,709199	1.86264
Myeloid cell leukemia sequence 1	Mcl1	H3108E12	44,172279	4.40036
MYST histone acetyltransferase (monocytic leukaemia) 3	Myst3	H3031D12	37,267338	2.05296
N-deacetylase/*N*-sulphotransferase (heparan glucosaminyl) 1	Ndst1	H3154B05	23,9702	3.55932
Nuclear receptor co-activator 2	Ncoa2	H3053G03	73,879719	1.88000
Nuclear protein family 6 (RNA-associated)	Nol6	H3003A04	31,945252	1.95344
Open reading frame 6	ORF6	H3103A09	27,125984	1.61375
Paraoxonase 3	Pon3	H3138D08	33,322	2.02539
Peptidylprolyl isomerase D (cyclophilin D)	Ppid	H3003B03	26,825865	4.26593
Phosphatidylinositol 3-kinase, C2 domain containing, alpha polypeptide	Pik3c2a	H3124D09	34,335953	3.33102
Phosphatidylinositol transfer protein	Pitpn	H3013F08	25,823081	3.90090
Phosphoinositol 3-phosphate-binding protein-3	Pepp3	H3112E05	27,601852	3.83072
Poly(A) binding protein, nuclear 1	Pabpn1	H3116G04	29,082583	1.91489
Polymerase (DNA directed), delta 2, regulatory subunit	Pold2	H3028E08	28,131429	1.54308
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 5	Psmd5	H3106F03	42,089208	2.07006
Proteasome (prosome, macropain) 28 subunit, alpha	Psme1	H3126C12	24,019733	2.04821
Protein kinase, cAMP dependent regulatory, type I, alpha	Prkar1a	H3145A03	46,957124	2.59080
Protein tyrosine phosphatase, receptor type, S	Ptprs	H3137G09	30,880359	1.52152
Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase 1	Pcmt1	H3011A03	43,197301	1.57077
Pyridoxal (pyridoxine, Vitamin B6) kinase	Pdxk	H3013B08	31,271665	2.62936
RAB10, member RAS oncogene family	Rab10	H3013D03	43,569805	1.97314
RAB11a, member RAS oncogene family	Rab11a	H3049H12	31,272245	3.00976
Retinol dehydrogenase 11	Rdh11	H3053B03	26,865619	3.16611
Ribosomal protein L18	Rpl18	H3126C02	26,340403	1.85946
Ribosomal protein S26	Rps26	H3120B03	28,937624	6.91202
Ring finger protein 34	Rnf34	H3144C08	36,618218	1.73069
Sideroflexin 3	Sfxn3	H3136H09	49,302873	11.44903
StAR-related lipid transfer (START) domain containing 5	Stard5	H3130F02	33,551126	1.99088
Thioesterase superfamily member 2	Them2	H3122A10	24,229056	2.14455
Thioredoxin reductase 1	Txnrd1	H3005B03	44,831954	4.30897
Transcription elongation factor A (SII) 1	Tcea1	H3139F02	30,577949	2.28731
Transcription elongation factor B (SIII), polypeptide 3	Tceb3	H3004A11	53,446664	3.46798
Transcription factor CP2	Tcfcp2	H3117A09	28,69496	1.74223
Transmembrane channel-like gene family 7	Tmc7	H3086B09	26,12686	2.15572
Transmembrane protein 2	Tmem2	H3026F09	28,059816	2.35524
Vinculin	Vcl	H3010D09	29,170476	3.44213
Von Hippel-Lindau syndrome homologue	Vhlh	H3119C01	25,397282	3.71377

Table 2 Three hundred and seventy-three down-regulated genes selected by the FatiGO program

Name	Symbol	Clone ID	Score (d)	Fold change
Actin-related protein 2/3 complex, subunit 1A	Arpc1a	H3135F05	−30,24410551	0.28271
Actin-related protein 2/3 complex, subunit 4	Arpc4	H3021C02	−28,36583323	0.34161
Actin, beta, cytoplasmic	Actb	H3002D02	−32,09027486	0.63681
Activating transcription factor 3	Atf3	H3053E12	−81,00516442	0.13993
Adaptor protein complex AP-1, beta 1 subunit	Ap1b1	H3122B10	−46,65460431	0.53413
Adaptor protein complex AP-1, gamma 1 subunit	Ap1g1	H3127D12	−41,39745145	0.15704
Adaptor protein complex AP-1, mu 2 subunit	Ap1m2	H3027D11	−37,33016985	0.54712
Adaptor-related protein complex 3, mu 1 subunit	Ap3m1	H3065B12	−30,33563636	0.29580
Adaptor-related protein complex AP-4, sigma 1	Ap4s1	H3075B04	−25,87111981	0.51703
Adenosine monophosphate deaminase 2 (isoform L)	Ampd2	H3117H07	−31,29336455	0.57307
Adenylate kinase 2	Ak2	H3156D05	−48,58295271	0.10340
Adenylate kinase 4	Ak4	H3076H06	−31,14716258	0.52973
ADP-ribosylation factor 4	Arf4	H3138F05	−72,07466578	0.21740
ADP-ribosylation factor GTPase activating protein 3	Arfgap3	H3059B11	−219,0987744	0.22422
Adrenergic receptor kinase, beta 1	Adrbk1	H3112H12	−61,44266434	0.25721
Aldo-keto reductase family 1, member B3 (aldose reductase)	Akr1b3	H3125D05	−50,6895577	0.45169
Aminoadipate-semialdehyde dehydrogenase–phosphopantetheinyl transferase	Aasdhppt	H3010C01	−37,02032317	0.42069
Amylo-1,6-glucosidase, 4-alpha-glucanotransferase	Agl	H3036B06	−89,04882968	0.32851
Angiotensin II, type I receptor-associated protein	Agtrap	H3027C07	−44,33648273	0.43765
Ankyrin repeat and FYVE domain containing 1	Ankfy1	H3127H11	−60,06231972	0.13513
Ankyrin repeat domain 10	Ankrd10	H3121C12	−73,61319108	0.26200
Annexin A3	Anxa3	H3092G05	−24,85609519	0.21696
Apoptosis, caspase activation inhibitor	Aven	H3092D11	−43,09120813	0.13526
Arachidonate 5-lipoxygenase activating protein	Alox5ap	H3117G12	−33,00601071	0.43034
ARP2 actin-related protein 2 homologue (yeast)	Actr2	H3002A08	−56,54551729	0.41873
ATP synthase, H + transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 1	Atp5g1	H3119A04	−38,2276587	0.39352
ATP synthase, H + transporting, mitochondrial F0 complex, subunit f, isoform 2	Atp5j2	H3118C01	−42,95086367	0.28914
ATPase, class II, type 9A	Atp9a	H3152H05	−38,6467714	0.37683
ATPase, H + transporting, V1 subunit B, isoform 2	Atp6v1b2	H3120H04	−27,11391843	0.65865
ATP-binding cassette, subfamily B (MDR/TAP), member 7	Abcb7	H3030D01	−37,40671481	0.37337
Basic transcription factor 3	Btf3	H3024F12	−63,76288019	0.14012
B-cell receptor-associated protein 29	Bcap29	H3153E06	−37,66791901	0.40969
B-cell translocation gene 4	Btg4	H3082H11	−41,68346919	0.30203
BCL2/adenovirus E1B 19 kD interacting protein like	Bnipl	H3119D04	−23,95131548	0.56873
Bcl2-like 10	Bcl2l10	H3103E12	−141,479161	0.15022
Beta-transducin repeat containing protein	Btrc	H3135B05	−56,89709506	0.18546
BMP2 inducible kinase	Bmp2k	H3024B10	−31,31084917	0.31659
Calbindin-28 K	Calb1	H3098H10	−24,35432186	0.49981
Calcium and integrin binding 1 (calmyrin)	Cib1	H3051F03	−26,88302446	0.46958
Calcium channel, voltage-dependent, beta 3 subunit	Cacnb3	H3117A06	−37,7405125	0.31737
Calcium channel, voltage-dependent, beta 3 subunit	Cacnb3	H3117A01	−44,55788865	0.51549
Calmegin	Clgn	H3002A01	−31,29068474	0.25750
Calmodulin 1	Calm1	H3156D12	−68,77006353	0.11504
Carnitine *O*-octanoyltransferase	Crot	H3107G12	−39,50007169	0.27003
Cartilage-associated protein	Crtap	H3010H04	−53,30088982	0.41161
CD164 antigen	Cd164	H3130D12	−54,66921757	0.13748
CD2 antigen (cytoplasmic tail) binding protein 2	Cd2bp2	H3106C05	−25,24168528	0.47324
CD24a antigen	Cd24a	H3109A05	−124,6921476	0.27589
CD44 antigen	Cd44	H3012H07	−75,54803746	0.18520
Cd63 antigen	Cd63	H3119E05	−64,45556051	0.34970
CDK2 (cyclin-dependent kinase 2)-associated protein 1	Cdkap1	H3122D05	−30,61606326	0.24207
CDP-diacylglycerol-inositol 3-phosphatidyltransferase (phosphatidylinositol synthase)	Cdipt	H3031C05	−41,96621532	0.56262

Table 2 (*Continued*)

Name	Symbol	Clone ID	Score (d)	Fold change
Centromere auto-antigen A	Cenpa	H3106B10	−32,71258418	0.38690
Centromere auto-antigen H	Cenph	H3102A05	−71,02557863	0.27833
Cerebellin 1 precursor protein	Cbln1	H3084H04	−36,57049596	0.28488
Chaperonin subunit 7 (eta)	Cct7	H3021F08	−31,96776795	0.45621
Chemokine orphan receptor 1	Cmkor1	H3148A05	−86,78698746	0.52512
Chemokine-like factor super family 3	Cklfsf3	H3006B01	−35,05360641	0.06004
Chloride intracellular channel 4 (mitochondrial)	Clic4	H3097C12	−28,54418183	0.31019
Chondroitin sulphate proteoglycan 6	Cspg6	H3113B11	−64,77597944	0.32904
Chromatin accessibility complex 1	Chrac1	H3155B11	−54,86011712	0.36819
c-myc Binding protein	Mycbp	H3130A06	−47,37389466	0.40377
Cryptochrome 1 (photolyase-like)	Cry1	H3080H12	−61,43953057	0.12766
c-src Tyrosine kinase	Csk	H3012F10	−33,47703237	0.39228
CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 1	Ctdsp1	H3096E12	−38,70294847	0.21983
Cullin 3	Cul3	H3104A12	−203,0410484	0.20259
Cyclin D2	Ccnd2	H3133B06	−29,63890475	0.40803
Cystatin C	Cst3	H3033F11	−28,64766534	0.52374
Cysteine-rich protein 1 (intestinal)	Crip1	H3108G04	−34,5396846	0.43100
Cytidine 5′-triphosphate synthase 2	Ctps2	H3143F12	−33,78782931	0.18427
Cytochrome P450, family 39, subfamily a, polypeptide 1	Cyp39a1	H3108A11	−37,06358421	0.35895
Cytoskeleton-associated protein 1	Ckap1	H3139A10	−46,22938086	0.51525
D-dopachrome tautomerase	Ddt	H3135H10	−24,80837265	0.66171
DEAH (Asp-Glu-Ala-His) box polypeptide 38	Dhx38	H3133H06	−104,2830614	0.28462
DEAH (Asp-Glu-Ala-His) box polypeptide 8	Dhx8	H3007G01	−34,65637521	0.43814
Death-associated protein	Dap	H3096A05	−69,03764715	0.25739
Desmoglein 2	Dsg2	H3045F04	−35,04558524	0.56877
Developmental pluripotency associated 5	Dppa5	H3104D05	−40,08698874	0.27131
Developmental pluripotency-associated 3	Dppa3	H3155A06	−77,7314124	0.25023
Developmentally regulated GTP binding protein 1	Drg1	H3131F06	−38,7914697	0.53730
Dipeptidylpeptidase 7	Dpp7	H3050G04	−33,82521527	0.50025
DNA fragmentation factor, beta subunit	Dffb	H3124D06	−125,5700031	0.20937
DNA methyltransferase 1-associated protein 1	Dmap1	H3105G05	−94,51379298	0.43115
Downregulator of transcription 1	Dr1	H3158B06	−36,13953693	0.48848
Dynamin 2	Dnm2	H3041F06	−26,89873168	0.30391
E1A-binding protein p400	Ep400	H3070A04	−32,87102623	0.51628
Ectonucleotide pyrophosphatase/phosphodiesterase 5	Enpp5	H3140D05	−29,5488946	0.27755
Electron transferring flavoprotein, alpha polypeptide	Etfa	H3140D12	−42,45846368	0.32513
Endoplasmic reticulum (ER) to nucleus signalling 1	Ern1	H3144B12	−69,47495042	0.13529
Endoplasmic reticulum protein 29	Erp29	H3123B04	−24,66246791	0.45456
Ephrin B3	Efnb3	H3120F06	−67,83348907	0.09596
Epidermal growth factor-containing fibulin-like extracellular matrix protein 2	Efemp2	H3107C11	−137,2062633	0.44001
Epimorphin	Epim	H3098H12	−67,03406284	0.26730
Estrogen receptor 1 (alpha)	Esr1	H3095A05	−127,875699	0.39183
Estrogen-related receptor, alpha	Esrra	H3011C04	−33,35988979	0.45533
Excision repair cross-complementing rodent repair deficiency,complementation group 5	Ercc5	H3130A10	−74,75721447	0.41170
Exportin, tRNA (nuclear export receptor for tRNAs)	Xpot	H3044E11	−57,05044567	0.24635
F11 receptor	F11r	H3121F06	−53,48014724	0.18018
Farnesyltransferase, CAAX box, alpha	Fnta	H3150F06	−52,18508766	0.17735
Fatty acid binding protein 3, muscle and heart	Fabp3	H3104D07	−30,52858073	0.41523
Fatty acid-coenzyme A ligase, long chain 5	Facl5	H3097F06	−40,00504389	0.58829
Fatty acid-coenzyme A ligase, long chain 4	Facl4	H3076C12	−95,79369691	0.20131
F-box only protein 3	Fbxo3	H3102A06	−26,49019084	0.40489
Fc receptor, IgG, alpha chain transporter	Fcgrt	H3153G01	−34,89277032	0.58326
Ferredoxin reductase	Fdxr	H3148B04	−53,52443791	0.51740
Ferritin heavy chain	Fth	H3008B06	−47,23454807	0.23618

Table 2 (*Continued*)

Name	Symbol	Clone ID	Score (d)	Fold change
Fidgetin-like 1	Fignl1	H3024H10	−40,74911787	0.43454
Filamin, beta	Flnb	H3021B06	−31,8708934	0.50800
Four and a half LIM domains 1	Fhl1	H3141F12	−36,05896679	0.17413
G protein-coupled receptor kinase 5	Gprk5	H3104A05	−23,64602971	0.28420
G protein-coupled receptor, family C, group 5, member C	Gprc5c	H3018F10	−27,17900232	0.38827
Ganglioside-induced differentiation-associated-protein 1	Gdap1	H3058B12	−24,96629047	0.55463
GATA binding protein 3	Gata3	H3049E04	−39,36518221	0.43122
Gem (nuclear organelle)-associated protein 4	Gemin4	H3130B05	−61,14240105	0.41432
Gene rich cluster, B gene	Grcb	H3123C04	−37,99979681	0.49017
General transcription factor III A	Gtf3a	H3119A12	−53,98700466	0.22994
Glia maturation factor, beta	Gmfb	H3148B05	−55,32832698	0.26219
Glioblastoma amplified sequence	Gbas	H3071C06	−181,9354745	0.15110
Glucose-6-phosphate dehydrogenase X-linked	G6pdx	H3032A12	−115,6033172	0.25821
Glutamate-ammonia ligase (glutamine synthase)	Glul	H3059G11	−60,33893316	0.35433
Glutathione S-transferase, mu 5	Gstm5	H3084E05	−48,80262603	0.44379
Glyceraldehyde-3-phosphate dehydrogenase	Gapd	H3025F11	−43,43478855	0.42396
Glyoxalase 1	Glo1	H3144C01	−37,5158194	0.46852
Glypican 1	Gpc1	H3144G12	−77,72055136	0.35352
GM2 ganglioside activator protein	Gm2a	H3035D04	−33,76010049	0.46292
Growth arrest specific 5	Gas5	H3113A12	−44,0300456	0.19037
Growth arrest specific 6	Gas6	H3077A11	−24,34522982	0.64724
Growth differentiation factor 3	Gdf3	H3038E05	−41,33026284	0.30106
H1 histone family, member 0	H1f0	H3136G12	−49,02077184	0.34256
H19 fetal liver mRNA	H19	H3140G12	−53,32487	0.58821
Heparan sulphate (glucosamine) 3-*O*-sulphotransferase 1	Hs3st1	H3001F12	−72,34704463	0.20535
Hexosaminidase A	Hexa	H3024C07	−26,62114055	0.40306
High mobility group 20A	Hmg20a	H3116A12	−66,45140912	0.25267
High mobility group box 1	Hmgb1	H3126A05	−70,44656456	0.29315
High mobility group box 3	Hmgb3	H3030H10	−69,77684382	0.37033
High mobility group nucleosomal binding domain 1	Hmgn1	H3120G04	−29,33448242	0.56585
Histidine-rich calcium-binding protein	Hrc	H3127D04	−41,0575724	0.48253
Histocompatibility 2, D region locus 1	H2-D1	H3141B11	−50,88240183	0.27965
Histone deacetylase 10	Hdac10	H3147G10	−29,83730576	0.48990
HLA-B-associated transcript 4	Bat4	H3059D08	−26,44385182	0.48918
HLA-B-associated transcript 5	Bat5	H3102D06	−30,81582096	0.16870
Homeodomain interacting protein kinase 1	Hipk1	H3152B05	−53,63017818	0.22802
Host cell factor C1	Hcfc1	H3011F01	−51,78609043	0.36207
Huntingtin interacting protein 1 related	Hip1r	H3148H06	−53,32823115	0.20939
Hydroxysteroid (17-beta) dehydrogenase 4	Hsd17b4	H3117D07	−30,2838659	0.54998
Hydroxysteroid dehydrogenase-1, delta<5>-3-beta	Hsd3b1	H3059B01	−95,4032878	0.35653
Importin 11	Ipo11	H3143A11	−62,3529375	0.29452
Importin 9	Ipo9	H3039H04	−33,23414698	0.60563
Insulin-induced gene 1	Insig1	H3055A01	−101,6483574	0.12080
Integrin beta 7	Itgb7	H3123H12	−42,38599744	0.16726
Integrin-linked kinase	Ilk	H3135C05	−40,7055775	0.32549
Interferon gamma inducible protein 30	Ifi30	H3040F11	−55,44194751	0.12615
Interferon regulatory factor 6	Irf6	H3079E06	−70,10453111	0.15337
Interleukin 2 receptor, gamma chain	Il2rg	H3022D06	−28,64739981	0.16915
Interleukin 25	Il25	H3001C11	−68,39642401	0.36906
Interleukin enhancer binding factor 3	Ilf3	H3090F12	−24,0659719	0.58889
Interleukin-1 receptor-associated kinase 1	Irak1	H3005F06	−37,52138248	0.46595
Lactotransferrin	Ltf	H3087A12	−283,9539678	0.14941
Laminin B1 subunit 1	Lamb1-1	H3147C04	−32,98914722	0.37976
Laminin receptor 1 (ribosomal protein SA)	Lamr1	H3115D04	−28,58729338	0.49463
Laminin, gamma 1	Lamc1	H3113E11	−45,90380642	0.24570
Leucine zipper protein 1	Luzp1	H3032D01	−28,05361547	0.45067
Limb region 1	Lmbr1	H3092G11	−170,9389371	0.31172

Table 2 (*Continued*)

Name	Symbol	Clone ID	Score (d)	Fold change
Low-density-lipoprotein-receptor-related protein 2	Lrp2	H3099D06	−28,58639561	0.25289
LPS-responsive beige-like anchor	Lrba	H3082F12	−60,15665246	0.21881
Lymphocyte-specific 1	Lsp1	H3150E06	−29,39362611	0.43758
Lysosomal apyrase-like 1	Lysal1	H3149D12	−51,60387642	0.48096
Lysosomal apyrase-like 2	Lysal2	H3023F06	−27,55622484	0.39750
Lysosomal-associated protein transmembrane 5	Laptm5	H3080C11	−56,07997228	0.21919
Lysozyme	Lyzs	H3054F05	−52,73779054	0.34533
Male enhanced antigen 1	Mea1	H3105F06	−65,53585604	0.37010
Mannosidase, alpha, class 1C, member 1	Man1c1	H3103F05	−38,17498608	0.26800
Mannoside acetylglucosaminyltransferase 2	Mgat2	H3010C12	−147,8902845	0.39921
Matrin 3	Matr3	H3144F11	−46,92382527	0.22599
Matrix gamma-carboxyglutamate (gla) protein	Mglap	H3134C05	−58,75272115	0.30682
Matrix metalloproteinase 2	Mmp2	H3158D11	−67,49094991	0.40525
Membrane metallo endopeptidase	Mme	H3051C12	−29,05639068	0.34387
Metallothionein 2	Mt2	H3013D11	−86,63525114	0.26932
Metallothionein-like 5, testis-specific (tesmin)	Mtl5	H3158G12	−36,68102668	0.57220
Microtubule-associated protein 4	Mtap4	H3058E07	−28,82252722	0.23022
Mitofusin 2	Mfn2	H3131D07	−28,63322301	0.51989
Modulator of apoptosis 1	Moap1	H3055C05	−66,29920398	0.37535
Mortality factor 4 like 1	Morf4l1	H3014G01	−46,136295	0.18126
Mpv17 transgene, kidney disease mutant	Mpv17	H3040F06	−42,17413189	0.27048
Msx-interacting-zinc finger	Miz1	H3094D05	−48,65937558	0.21190
Multiple endocrine neoplasia 1	Men1	H3134F11	−29,38118456	0.57242
MYB-binding protein (P160) 1a	Mybbp1a	H3008A10	−45,24696077	0.37965
Myelocytomatosis oncogene	Myc	H3076D10	−64,5093832	0.32912
Myeloid differentiation primary response gene 116	Myd116	H3027G12	−38,98048705	0.25049
Myosin IB	Myo1b	H3001A08	−33,89675552	0.35000
Myotubularin-related protein 2	Mtmr2	H3089G02	−33,73465298	0.20587
Myotubularin-related protein 3	Mtmr3	H3150D05	−68,3226587	0.15189
Myotubularin-related protein 9	Mtmr9	H3116F05	−34,81927728	0.31486
N-acetylneuraminate pyruvate lyase	Npl	H3100F11	−101,2724383	0.22284
NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10	Ndufb10	H3124H01	−27,77194444	0.45403
NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5	Ndufb5	H3086F10	−35,85350469	0.28383
Nascent polypeptide-associated complex alpha polypeptide	Naca	H3157F04	−47,95535393	0.61478
Nedd4 family interacting protein 2	Ndfip2	H3066A10	−34,05175359	0.44976
Neighbour of A-kinase anchoring protein 95	Nakap95	H3103H06	−61,55983751	0.07444
Neighbour of Brca1 gene 1	Nbr1	H3104H06	−25,78753045	0.06004
Neural precursor cell expressed, developmentally down-regulated gene 4	Nedd4	H3104B06	−36,21281766	0.12714
Nuclear cap binding protein subunit 2	Ncbp2	H3010A12	−29,03997582	0.28182
Nuclear protein 95	Np95	H3026E12	−83,84109682	0.37189
Nuclear receptor subfamily 2, group C, member 2	Nr2c2	H3133F10	−30,55471572	0.59413
Nuclear receptor subfamily 2, group E, member 3	Nr2e3	H3054C01	−49,85709392	0.15187
Nuclear respiratory factor 1	Nrf1	H3017B01	−51,56044491	0.43936
Nucleolar and coiled-body phosphoprotein 1	Nolc1	H3106A07	−34,56044898	0.49830
Nucleolar protein 5A	Nol5a	H3027H10	−99,38321163	0.36320
Nucleoporin 160	Nup160	H3020D05	−39,97278778	0.13993
Nucleoporin 37	Nup37	H3067A12	−58,84584351	0.22502
Ornithine aminotransferase	Oat	H3137B06	−53,36463448	0.17292
Oxoglutarate dehydrogenase (lipoamide)	Ogdh	H3133C05	−77,21598226	0.42454
P450 (cytochrome) oxidoreductase	Por	H3090E05	−60,19375	0.46167
Pericentrin 2	Pcnt2	H3103G11	−69,84244416	0.19813
Pericentriolar material 1	Pcm1	H3156A03	−24,87134356	0.63422
Perlecan (heparan sulphate proteoglycan 2)	Hspg2	H3156E04	−48,95218961	0.43105
Peroxisomal membrane protein 2	Pxmp2	H3124F10	−65,57824473	0.30201
Phosphatidylinositol-3-phosphate/ phosphatidylinositol 5-kinase, type III	Pip5k3	H3105A01	−32,26796825	0.55207

Table 2 (*Continued*)

Name	Symbol	Clone ID	Score (d)	Fold change
Phosphoglucomutase 1	Pgm1	H3015C06	−37,17132863	0.58359
Phosphoglucomutase 2	Pgm2	H3138C04	−87,88951552	0.34605
Phospholipase A2, group IVA (cytosolic, calcium-dependent)	Pla2g4a	H3103A05	−67,86662556	0.21666
Phospholipase C, delta	Plcd	H3115A05	−50,86924052	0.42842
Phospholipase C-like 2	Plcl2	H3072B11	−63,6481633	0.12197
Phosphomannomutase 2	Pmm2	H3019G05	−89,4246728	0.52588
Phosphorylase kinase beta	Phkb	H3159D05	−59,74078328	0.24170
Plakophilin 4	Pkp4	H3120E08	−59,16379612	0.19391
Platelet/endothelial cell adhesion molecule	Pecam	H3052F06	−52,81304001	0.13471
Pleckstrin homology domain containing, family C (with FERM domain) member 1	Plekhc1	H3082E10	−37,01398737	0.40610
Plexin B1	Plxnb1	H3108E04	−45,90890235	0.61097
Plexin domain containing 2	Plxdc2	H3065F07	−28,0721894	0.28385
Polyadenylate binding protein-interacting protein 1	Paip1	H3056A10	−64,29277051	0.44561
Polymerase (DNA directed), beta	Polb	H3028B10	−62,16082675	0.45006
Polymerase (DNA directed), epsilon	Pole	H3079E05	−78,15999163	0.34919
Polymerase (RNA) II (DNA directed) polypeptide J	Polr2j	H3077D11	−30,44831384	0.29937
Popeye domain containing 2	Popdc2	H3077C11	−47,07269198	0.47535
Potassium channel, subfamily K, member 1	Kcnk1	H3151G11	−167,3429266	0.55788
Pre B-cell leukemia transcription factor 1	Pbx1	H3140B12	−85,1883012	0.12329
Procollagen, type I, alpha 2	Col1a2	H3136A07	−33,93157827	0.30139
Procollagen, type III, alpha 1	Col3a1	H3124H10	−62,48228786	0.31191
Procollagen, type IV, alpha 5	Col4a5	H3159C08	−27,13942221	0.45359
Procollagen, type IX, alpha 3	Col9a3	H3135D05	−30,35593343	0.54885
Procollagen, type VI, alpha 1	Col6a1	H3151F07	−26,81295147	0.53892
Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha II polypeptide	P4ha2	H3003D12	−34,76470932	0.25862
Progressive ankylosis	ank	H3042C06	−71,18102914	0.23086
Proline-rich nuclear receptor coactivator 1	Pnrc1	H3116A06	−25,39804938	0.49470
Prolyl 4-hydroxylase, beta polypeptide	P4hb	H3125C05	−42,08184464	0.51132
Protease (prosome, macropain) 26S subunit, ATPase 1	Psmc1	H3134B06	−57,8844247	0.14322
Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting, 4 (parvulin)	Pin4	H3012E07	−36,91430603	0.36226
Protein kinase inhibitor, gamma	Pkig	H3126B12	−33,76844844	0.31888
Protein kinase, lysine deficient 1	Prkwnk1	H3026F04	−49,76730038	0.64857
Protein phosphatase 4, regulatory subunit 1	Ppp4r1	H3022D11	−40,13219837	0.18925
Protein regulator of cytokinesis 1	Prc1	H3155H02	−34,80884127	0.43053
Protein tyrosine phosphatase, non-receptor type 2	Ptpn2	H3156C04	−52,96178999	0.38801
Protein tyrosine phosphatase, receptor type, E	Ptpre	H3082E12	−62,05410008	0.41317
Protein tyrosine phosphatase, receptor type, F	Ptprf	H3147E11	−32,98975107	0.47277
PTK7 protein tyrosine kinase 7	Ptk7	H3130D11	−54,36221183	0.23356
Purinergic receptor P2X, ligand-gated ion channel 4	P2rx4	H3144F07	−27,93392455	0.41033
Pyruvate kinase, muscle	Pkm2	H3030D11	−29,48376752	0.46017
Rab acceptor 1 (prenylated)	Rabac1	H3105F01	−31,48924548	0.41026
RAB1, member RAS oncogene family	Rab1	H3002E05	−102,2514239	0.34627
RAB12, member RAS oncogene family	Rab12	H3143E11	−98,66408403	0.30732
RAB22, member RAS oncogene family	Rab22	H3124F07	−32,89516121	0.18260
Rab6 interacting protein 2	Rab6ip2	H3157H11	−35,67375959	0.26642
RAB9, member RAS oncogene family	Rab9	H3102H06	−39,62644717	0.10271
Rac GTPase-activating protein 1	Racgap1	H3148G06	−33,09890924	0.53238
RAS, dexamethasone-induced 1	Rasd1	H3108E05	−71,03983364	0.40255
RE1-silencing transcription factor	Rest	H3013F11	−95,52420923	0.31075
Regulatory factor X, 1 (influences HLA class II expression)	Rfx1	H3076E12	−47,72318845	0.17749
Rho GTPase activating protein 18	Arhgap18	H3103E10	−101,4111979	0.43477

Table 2 (*Continued*)

Name	Symbol	Clone ID	Score (d)	Fold change
Rho-associated coiled-coil forming kinase 1	Rock1	H3157H12	−43,89487645	0.49555
Rhophilin, Rho GTPase binding protein 2	Rhpn2	H3079C07	−26,08357245	0.26310
Ribonuclease/angiogenin inhibitor 1	Rnh1	H3123F11	−62,27428083	0.40424
Ribonucleic acid binding protein S1	Rnps1	H3121D06	−55,93178351	0.33174
Ribosomal protein 10	Rpl10	H3144C06	−34,24324451	0.42849
Ribosomal protein L12	Rpl12	H3113D05	−53,16605908	0.27633
Ribosomal protein L13a	Rpl13a	H3136G06	−48,24118244	0.21614
Ribosomal protein L15	Rpl15	H3027H06	−25,03352391	0.50356
Ribosomal protein L17	Rpl17	H3029F11	−50,48438137	0.39881
Ribosomal protein L22	Rpl22	H3134G12	−31,20710463	0.61529
Ribosomal protein L27	Rpl27	H3126D05	−44,06791284	0.24082
Ribosomal protein L27a	Rpl27a	H3009B05	−47,46412159	0.41972
Ribosomal protein S16	Rps16	H3112H11	−72,69771067	0.21275
Ribosomal protein S17	Rps17	H3118G05	−48,21150444	0.35614
Ring finger protein 144	Rnf144	H3113D11	−39,4380729	0.24827
Ring finger protein 38	Rnf38	H3070A06	−27,84698146	0.60478
Ring finger protein 41	Rnf41	H3074H10	−31,64355598	0.41812
Ring finger protein 7	Rnf7	H3146A01	−60,3712378	0.28235
Ring finger protein 8	Rnf8	H3046F10	−33,37733882	0.35643
RNA-binding motif protein 14	Rbm14	H3027A05	−80,74023735	0.40926
RNA-binding motif protein 8	Rbm8	H3131E01	−44,5762029	0.41693
RNA-binding motif protein 9	Rbm9	H3001E02	−125,4070366	0.48061
RNA-binding region (RNP1, RRM) containing 2	Rnpc2	H3143B05	−43,73045161	0.27576
S-adenosylhomocysteine hydrolase	Ahcy	H3144A12	−49,12123311	0.45887
Secreted acidic cysteine rich glycoprotein	Sparc	H3116A04	−32,90227677	0.60047
Secretory carrier membrane protein 2	Scamp2	H3142G07	−50,56847755	0.18275
Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4G	Sema4g	H3138E11	−57,01209196	0.23649
Serine (or cysteine) proteinase inhibitor, clade F), member 1	Serpinf1	H3138B11	−60,55942105	0.24955
Serine (or cysteine) proteinase inhibitor, clade H, member 1	Serpinh1	H3125A07	−36,11152716	0.32303
Serine hydroxymethyl transferase 2 (mitochondrial)	Shmt2	H3022C05	−60,00727651	0.62078
Serine protease inhibitor, Kunitz type 1	Spint1	H3037F06	−24,38793304	0.43151
Serine threonine kinase 31	Stk31	H3148B07	−27,22340106	0.52367
Serine/arginine repetitive matrix 1	Srrm1	H3037B12	−179,8111446	0.14876
Serine/threonine kinase 11	Stk11	H3052C12	−28,93102753	0.43164
Serine/threonine kinase 38	Stk38	H3143G10	−39,30346777	0.47987
SET domain, bifurcated 1	Setdb1	H3130E01	−39,93755379	0.27165
Seven in absentia 2	Siah2	H3092E12	−37,94498007	0.30111
Sex comb on midleg homologue 1	Scmh1	H3113B01	−46,73326105	0.29082
SH3-binding domain glutamic acid-rich protein	Sh3bgr	H3050D06	−101,5848689	0.24394
Signal transducer and activator of transcription 6	Stat6	H3142F05	−127,043571	0.32437
Signalling lymphocytic activation molecule family member 1	Slamf1	H3103C12	−139,5089405	0.19680
Single-strand selective monofunctional uracil DNA glycosylase	Smug1	H3050H05	−80,09472013	0.25153
Single-stranded DNA binding protein 3	Ssbp3	H3128H12	−39,22205592	0.06594
Small nuclear ribonucleoprotein E	Snrpe	H3112E01	−42,46657182	0.45210
Solute carrier family 12, member 7	Slc12a7	H3045B12	−70,79300589	0.14036
Solute carrier family 27 (fatty acid transporter), member 4	Slc27a4	H3109B11	−38,52014708	0.27981
Solute carrier family 34 (sodium phosphate), member 2	Slc34a2	H3101E05	−32,47928803	0.57495
Sorbin and SH3 domain containing 1	Sorbs1	H3114D04	−45,49942479	0.35626
Sorting nexin 2	Snx2	H3041B06	−25,83907817	0.33461

Table 2 (*Continued*)

Name	Symbol	Clone ID	Score (d)	Fold change
SPARC related modular calcium binding 1	Smoc1	H3120E06	−45,95942213	0.51213
Special AT-rich sequence binding protein 1	Satb1	H3080E12	−66,50787037	0.32452
S-phase kinase-associated protein 1A	Skp1a	H3023E01	−59,77834518	0.29839
Spindlin	Spin	H3051B07	−31,66197033	0.47321
Splicing factor, arginine/serine-rich 2 (SC-35)	Sfrs2	H3075D05	−28,20881959	0.33287
Splicing factor, arginine/serine-rich 6	Sfrs6	H3159G01	−40,09271484	0.35445
Sterol *O*-acyltransferase 1	Soat1	H3139F01	−29,45226178	0.42471
Striatin, calmodulin binding protein 4	Strn4	H3153A07	−32,83743848	0.55809
Superoxide dismutase 1, soluble	Sod1	H3130B11	−40,89927037	0.23007
Suppressor of K + transport defect 3	Skd3	H3027F10	−24,1156945	0.50338
Synaptogyrin 3	Syngr3	H3091E06	−57,37759127	0.16987
Synaptophysin-like protein	Sypl	H3008E05	−23,75907489	0.44484
Synaptosomal-associated protein	Snap29	H3078H04	−26,46912525	0.54160
Synaptotagmin-binding, cytoplasmic-RNA-interacting protein	Syncrip	H3028C11	−41,09975337	0.35120
Syndecan 1	Sdc1	H3013F05	−65,55825607	0.30135
Syndecan 4	Sdc4	H3138F12	−56,69482493	0.20586
Syntaxin 3	Stx3	H3025D06	−28,86514887	0.17604
Syntaxin-binding protein 2	Stxbp2	H3151C05	−44,96562579	0.46903
Telomeric repeat binding factor 2	Terf2	H3132H06	−62,77974389	0.18374
Tetratricopeptide repeat domain 11	Ttc11	H3132A04	−60,11961413	0.44337
Thiamine triphosphatase	Thtpa	H3091D06	−23,97263281	0.13975
Thyroid hormone receptor associated protein 6	Thrap6	H3066G07	−43,39159491	0.32151
Tissue inhibitor of metalloproteinase 4	Timp4	H3100A11	−95,71767022	0.30867
Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein	Tirap	H3003H12	−33,94933594	0.15595
Topoisomerase (DNA) II alpha	Top2a	H3139A05	−60,26221563	0.37328
Topoisomerase (DNA) III beta	Top3b	H3023E12	−61,35212946	0.66557
TRAF-binding protein	Trabid	H3066F12	−83,87167129	0.24997
Transcription factor E2a	Tcfe2a	H3137H01	−27,9541492	0.36665
Transcriptional regulator, SIN3A (yeast)	Sin3a	H3054G05	−124,3461212	0.23995
Transforming growth factor beta 1 induced transcript 4	Tgfb1i4	H3097C06	−50,63658462	0.32259
Transforming growth factor beta regulated gene 4	Tbrg4	H3004A05	−25,66020565	0.54561
Transforming, acidic coiled-coil containing protein 3	Tacc3	H3091G11	−54,97686811	0.34954
Transglutaminase 2, C polypeptide	Tgm2	H3137C06	−198,3935417	0.17555
Tropomodulin 3	Tmod3	H3084H05	−50,12687431	0.26519
Tropomyosin 1, alpha	Tpm1	H3035B02	−26,66148982	0.56339
Tropomyosin 2, beta	Tpm2	H3135D11	−37,79738589	0.35167
Tryptophanyl-tRNA synthetase	Wars	H3021A12	−28,02178147	0.42354
Tubulin cofactor a	Tbca	H3102D11	−51,08153563	0.21936
Tubulin, beta 5	Tubb5	H3029D06	−38,12550601	0.40335
Tuftelin interacting protein 11	Tfip11	H3115F04	−42,20741639	0.40815
Tumour-associated calcium signal transducer 1	Tacstd1	H3082F06	−23,66300338	0.23163
Tyrosine 3-mono-oxygenase/tryptophan 5-mono-oxygenase activation protein, beta polypeptide	Ywhab	H3080B03	−42,75773159	0.49558
Tyrosine hydroxylase	Th	H3053B07	−42,6001103	0.24045
Tyrosyl-tRNA synthetase	Yars	H3014H07	−27,06272432	0.49084
U2 small nuclear RNA auxiliary factor 1-like 4	U2af1l4	H3003B05	−29,762974	0.26264
U2af1-rs1 region 1	Murr1	H3137G06	−104,181046	0.16578
UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5	B3galt5	H3104D12	−58,18763597	0.07989
uroplakin 1A	Upk1a	H3076E06	−79,84965812	0.18145
v-maf Musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)	Mafb	H3119H05	−55,23763946	0.36651
Wiskott—Aldrich syndrome protein interacting protein	Waspip	H3048E12	−37,94768697	0.50428
Xanthine dehydrogenase	Xdh	H3151F06	−51,52391818	0.17242

Table 2 (*Continued*)

Name	Symbol	Clone ID	Score (d)	Fold change
Xylosylprotein beta1,4-galactosyltransferase, polypeptide 7 (galactosyltransferase I)	B4galt7	H3022E11	−243,8320351	0.33705
Zinc finger CCCH type domain containing 1	Zc3hdc1	H3060F10	−47,13922837	0.29145
Zinc finger protein 161	Zfp161	H3041D08	−30,07695935	0.59606
Zinc finger, A20 domain containing 1	Za20d1	H3092A04	−32,73641409	0.65275

Among these genes, 172 were upregulated and 580 were downregulated, as selected by SAM with 1.5 and −1.5 score values, respectively. We searched for the distribution of these clones in FatiGO, and found 97 up-regulated and 373 down-regulated genes (Tables 1 and 2).

Up-regulated genes

Notable genes overexpressed in AA-stimulated pre-osteoblasts and selected by GO analysis are those that encode for proteins involved in morphogenesis such as BMP-1 and -15, MMP7, Lif (a cytokine that induces macrophage differentiation but also acts in synergy with BMP-2 on primary fetal neural progenitor cells to induce astrocytes), Dag1 (a laminin-binding component of the dystrophin–glycoprotein complex that provides a linkage between the subsarcolemmal cytoskeleton and the ECM), Gfra1 (a glycosylphosphatidylinositol-linked cell-surface receptor that mediates activation of the RET tyrosine kinase receptor) and Lgals1 (belonging to the galectins, a family of beta-galactoside-binding proteins implicated in modulating cell–cell and cell–matrix interactions and LGALS-1 acts as an autocrine negative growth factor that regulates cell proliferation) (Table 1). Other genes are related to cell death, such as Mcl1 (belongs to the Bcl-2 family), Dad1 (an anti-apoptotic gene) and Rab10 (a member of the RAS oncogene family), and to cell communication such as Pik3c2a (belongs to the phosphoinositide-3-kinase family and is involved in integrin-dependent signalling), Ar-l4 (a member of the ADP-ribosylation factor family of GTP-binding proteins with nuclear localisation), Adm (a hypotensive peptide that shows a slight homology with the calcitonin-gene-related peptide), Ptprs (a member of the protein tyrosine phosphatase family) and Rab11a (belongs to the small GTPase superfamily (Rab family) and associated with both constitutive and regulated secretory pathways). Important genes with elevated expression participate in many aspects of cell growth, such as Bap1 (a tumour suppressor gene that functions in the BRCA1 growth control pathway), Ccna2 (a cyclin that promotes both cell cycle G1/S and G2/M transitions), Abcf2 (a member of the superfamily of ATP-binding cassette (ABC) transporters that transport various molecules across extra- and intra-cellular membranes), Vhlh (a protein involved in the ubiquitination and degradation of hypoxia-inducible-factor which is a transcription factor that plays a central role in the regulation of gene expression by oxygen) and Mcm6 (a highly conserved mini-chromosome maintenance protein essential for the initiation of eukaryotic genome replication).

Down-regulated genes

Relevant underexpressed genes selected by GO analysis encode for morphogenetic protein such as Sema4g (a semaphorin that serves as axon guidance ligands via multimeric receptor), Efnb3 (a member of the ephrin gene family that is important in brain development), Waspip (that plays an important role in the organisation of the actin cytoskeleton), Miz1 (a Msx-interacting-zinc finger protein) and TGF-beta1 induced transcript 4 (Table 2). Additional underexpressed genes are those of ECM proteins (i.e. laminin-beta1 and -gamma1, laminin receptor 1, procollagen type VI and IX, prolyl 4-hydroxylase beta polypeptide, procollagen-proline 2-oxoglutarate 4-dioxygenase alpha II, matrix metalloproteinase 2, and chondroitin sulphate proteoglycan 6), cell-adhesion molecules (i.e. integrin beta 7 and calcium and integrin binding 1) and cell-mobility proteins (i.e. tropomyosin 1 and 2, myosin IB, actin, beta, myotubularin-related protein 3 and 9, tubulin, beta 5, tubulin cofactor a, microtubule-associated protein 4 and cytoskeleton-associated protein 1). Among cytokines, some interleukin-related molecules are downregulated (i.e. interleukin-1-receptor-associated kinase 1, interleukin 2 receptor and interleukin 25). Additional notable underexpressed genes are oncogenes and apoptotic-related proteins such as Bcl-2l10 (that belongs to the BCL-2 protein family), Myc and C-myc-binding protein, some members of the RAS oncogene family (i.e. RAB1 and 9, and RAS-dexamethasone-induced 1)

and Mafb (or V-maf musculoaponeurotic fibrosarcoma oncogene family, protein B).

Discussion

AA is a cofactor required for the function of several hydroxylases. Its absence is responsible for scurvy, and it is a risk factor for periodontal disease.[1,2] Recently, it has been shown that AA induces embryonic stem cells and MC3T3 (a well-defined model of pre-osteoblast differentiation) to differentiate into mineralised osteoblasts in vitro.[3–5] AA is responsible for the sustained proliferation of MC3T3-E1 cells and the effect is mediated through the synthesis of collagen.[6,7]

How AA induces proliferation and commitment of pre-osteoblasts is poorly understood. Elucidation of the molecular mechanism has important clinical implications because it may facilitate the use of AA to accelerate bone regeneration. Here, we attempt to address this question by using MC3T3-E1 and micro-array slides containing 15,000 different oligonucleotides to study the early effect of AA on stem cells.

Relevant upregulated genes in AA-stimulated pre-osteoblasts are those involved in morphogenesis such as BMP-1 and -15. MC3T3-E1 cells constitutively express BMP-2, -4 and -7, and BMP production is necessary for differentiation.[12] Here, we add information regarding BMP-1 and -15 in the early phase of pre-osteoblast activation. Other enhanced morphogenetic proteins we found are Lif (a cytokine that acts in synergy with BMP-2), Dag1 (a laminin-binding component), Gfra1 (a receptor that mediates activation of the RET) and Lgals1 (a regulator of cell proliferation). Of interest is the upregulation of MMP7, an enzyme that degrades proteoglycans, fibronectin, elastin and casein. These data are in agreement with the fact that AA induces synthesis of MMP2 and -13 in MC3T3-E1 cells and that these enzymes have a role in the maturation of collagenous ECM.[19] Other morphogenetic genes are downregulated like Sema4g, Efnb3, Waspip, Miz1 and TGF-beta1. It is known that TGF-beta1 inhibits the differentiation of osteoblasts.[14] Moreover, during differentiation, there is a reduction in cell-surface TGF-beta receptors that is dependent on the interaction of cell-surface alpha2beta1-integrin with matrix collagen synthesised by osteoblasts.[14]

Among the overexpressed genes, some are involved in cell communication such as Arl4, Ptprs and Pik3c2a. The latter belongs to the phosphoinositide-3-kinase family and participates in integrin-dependent signalling. Integrins, expecially alpha2-beta1, are involved in the mechanism of action of AA on stem cells.[10–12] A dose-dependent decrease in alpha2- and beta1-integrin messenger RNA levels was found in bone cultures deficient in AA.[10] Anti-alpha2-integrin blocking antibody reduced the effect of AA on pre-osteoblasts,[11] and an interaction between integrin and collagen is required for activation of the MAPK pathway.[12] Here, additional information is reported on activation of the integrin pathway, expecially regarding integrin 4 (upregulated) and integrin 7 (downregulated).

Several ECM proteins, cell-adhesion molecules, cell-mobility proteins and interleukin-related molecules are downregulated in the early stage of AA stimulation. For example, several collagen proteins (i.e. Col1a2, Col3a1, Col4a5, Col9a3 and Col6a1) and some laminins (Lamb11 and Lamc1) are downregulated. In contrast, some ECM enzymes such as caspase 6 and cathepsin 8 are upregulated. Together, these data outline that there is a strength modulation of ECM during cell proliferation.

Previous reports have analysed several bone tissue markers during pre-osteoblast differentiation such as osteopontin, osteocalcin and osteonectin.[6,7,9] Modulation of ECM components was detected in the present study, but this did not include some of those described previously. The absence of several genes already associated with AA commitment is not surprising as: (1) some genes, such as osteopontin, osteocalcin and osteonectin, were not present on the array used in this study and (2) given that our study specifically addressed differences in gene expression during early stimulation of pre-osteoblasts by AA (i.e. mainly proliferation rather than differentiation), we would not necessarily expect to see the genes identified previously. Moreover, because no cDNA micro-array has a complete genome printed-up, more studies based on different systems are helpful for: (1) cross-validation of different results and (2) a wider comprehension of molecular pathways involved in AA cell stimulation and commitment.

In conclusion, AA is able to modulate a broad range of biological processes in pre-osteoblasts: (1) cell growth; (2) metabolism; (3) morphogenesis; (4) cell death; (5) cell communication. The precise interaction between the discussed genes is not yet clear.

It is evident that more investigations are needed. First, our results were derived from the MC3T3-E1 cell line (a mouse cell line). Second, an in vitro system differs from an in vivo system. In fact, a monolayer cell stratum differs significantly from bone tissue, where pre-osteoblasts are resident in the periosteum. The advantages of using a cell line instead of a primary culture are related to the fact that reproducibility of data is higher in a cell line

due to lack of variability. Primary cell cultures, on the other hand, provide a source of more normal, non-malignant cells, but they also contain a heterogenous 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 AA on mesenchymal cells. Third, we chose to perform the experiment after 24 h in order to get information on the early genetic effect of AA on pre-osteoblasts. Further investigations with more time points, different cell lines and primary cultures are needed. However, we believe that the present report provides new data on the genetic effect of AA on stem cells.

Acknowledgments

This work was supported by grants from the University of Ferrara, Fondazione CARIFE, Guyabioscience and Fondazione CARISBO. This work was also partially supported by the National Research Council, the Ministry of Education, University and Research, and by AROD (Research Association for Dentistry and Dermatology), Italy.

References

1. Leggott PJ, Robertson PB, Jacob RA, Zambon JJ, Walsh M, Armitage GC. Effects of ascorbic acid depletion and supplementation on periodontal health and subgingival microflora in humans. *J Dent Res* 1991;**70**:1531—6.
2. Nishida M, Grossi SG, Dunford RG, Ho AW, Trevisan M, Genco RJ. Dietary Vitamin C and the risk for periodontal disease. *J Periodontol* 2000;**71**:1215—23.
3. Buttery LD, Bourne S, Xynos JD, et al. Differentiation of osteoblasts and in vitro bone formation from murine embryonic stem cells. *Tissue Eng* 2001;**7**:89—99.
4. zur Nieden NI, Kempka G, Ahr HJ. In vitro differentiation of embryonic stem cells into mineralized osteoblasts. *Differentiation* 2003;**71**:18—27.
5. Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ. Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development. *J Bone Miner Res* 1992;**7**:683—92.
6. Harada S, Matsumoto T, Ogata E. Role of ascorbic acid in the regulation of proliferation in osteoblast-like MC3T3-E1 cells. *J Bone Miner Res* 1991;**6**:903—8.
7. Franceschi RT, Iyer BS. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *J Bone Miner Res* 1992;**7**:235—46.
8. Franceschi RT, Wilson JX, Dixon SJ. Requirement for Na(+)-dependent ascorbic acid transport in osteoblast function. *Am J Physiol* 1995;**268**:1430—9.
9. Fujita I, Hirano J, Itoh N, Nakanishi T, Tanaka K. Dexamethasone induces sodium-dependent Vitamin C transporter in a mouse osteoblastic cell line MC3T3-E1. *Br J Nutr* 2001; **86**:145—9.
10. Ganta DR, McCarthy MB, Gronowicz GA. Ascorbic acid alters collagen integrins in bone culture. *Endocrinology* 1997; **138**:3606—12.
11. Xiao G, Wang D, Benson MD, Karsenty G, Franceschi RT. Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. *J Biol Chem* 1998;**273**:32988—94.
12. Xiao G, Gopalakrishnan R, Jiang D, Reith E, Benson MD, Franceschi RT. Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. *J Bone Miner Res* 2002; **17**:101—10.
13. Franceschi RT, Xiao G, Jiang D, Gopalakrishnan R, Yang S, Reith E. Multiple signaling pathways converge on the Cbfa1/Runx2 transcription factor to regulate osteoblast differentiation. *Connect Tissue Res* 2003;**44**(Suppl. 1):109—16.
14. Takeuchi Y, Nakayama K, Matsumoto T. Differentiation and cell surface expression of transforming growth factor-beta receptors are regulated by interaction with matrix collagen in murine osteoblastic cells. *J Biol Chem* 1996;**271**:3938—44.
15. Gilbert L, He X, Farmer P, Boden S, Kozlowski M, Rubin J, et al. Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. *Endocrinology* 2000;**141**:3956—64.
16. McCauley LK, Koh AJ, Beecher CA, Cui Y, Decker JD, Franceschi RT. Effects of differentiation and transforming growth factor beta 1 on PTH/PTHrP receptor mRNA levels in MC3T3-E1 cells. *J Bone Miner Res* 1995;**10**:1243—55.
17. Carinci F, Francioso F, Rubini C, et al. Genetic portrait of malignant granular cell odontogenic tumour. *Oral Oncology* 2003;**39**:69—77.
18. Carinci F, Francioso F, Piattelli A, et al. Genetic expression profiling of six odontogenic tumors. *J Dent Res* 2003;**82**: 551—7.
19. Mizutani A, Sugiyama I, Kuno E, Matsunaga S, Tsukagoshi N. Expression of matrix metalloproteinases during ascorbate-induced differentiation of osteoblastic MC3T3-E1 cells. *J Bone Miner Res* 2001;**16**:243—9.