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Genome-wide Screening by cDNA Microarray of Genes Associated with Matrix Mineralization by Human Mesenchymal Stem Cells *in Vitro*

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Using a culture system that facilitates osteogenic differentiation of bone marrow-derived human mesenchymal stem cells, we analyzed gene-expression profiles during the mineralization process by means of a cDNA microarray system consisting of 23,040 genes. We compared expression profiles of the cells at days 3, 15, and 27 of incubation in media containing either a combination of 0.1 μM dexamethasone, 0.05 mM ascorbic acid–2-phosphate, and 10 mM β-glycerophosphate, dexamethasone only, ascorbic acid–2-phosphate plus β-glycerophosphate, or medium without any of these osteogenic supplements. Histochemical analysis revealed osteogenic differentiation of cells incubated in the presence of all three agents, but not in the other cultures. Comparison of the expression profiles disclosed transcriptional stimulation of 55 genes and repression of 82 genes among more than 20,000 examined. A set of differentially expressed genes we report here should contribute to a better understanding of the process of mineralization in the matrix surrounding human mesenchymal stem cells. © 2002 Elsevier Science

Key Words: **cDNA microarray; dexamethasone; mesenchymal stem cells; mineralization; osteogenesis.**

The development of bone and mineralization of extracellular matrix is accompanied by complex changes in expression levels of various genes (1–3). For example bone sialoprotein, an initiator of matrix mineralization, is up-regulated during the mineralization phase of osteoblast differentiation (4, 5). Osteocalcin and osteopontin are also induced but only in the last phase of osteogenesis, so they are generally considered to be inhibitors of the mineralization process (6, 7).

Several previous studies revealed factors likely to be associated with growth and differentiation of osteoprogenitors and osteoblasts. However, since most of those studies used cell lines prepared from tumor tissues or cell lines from rat or other animals (8–12), they may not correctly reflect osteogenic changes in normal human mesenchymal lineages (13, 14). Mesenchymal stem cells (hMSCs) purified from human bone marrow can selectively differentiate into osteogenic, chondrogenic, or adipogenic lineages depending on the condition of the medium in which they are cultured (15, 16). In published experiments, addition of 0.1 μM dexamethasone (Dex), 0.05 mM ascorbic acid–2-phosphate (AsAP), and 10 mM β-glycerophosphate (bGP) induced osteogenesis of hMSCs and led to formation of matrix mineral nodules *in vitro* (17, 18).

Identification of proteins associated with normal growth and differentiation during osteogenesis and observation of their interactions can help to elucidate the molecular mechanisms underlying osteogenesis and mineralization. In recent years cDNA microarrays have been successful in identifying differentiation stage-specific gene expression in embryonic stem cells (19), preadipocytes (20), monocyte-derived dendritic cells (21), progenitors of oligodendroglia (22), and murine osteoblasts (12). We have developed and applied a cDNA microarray consisting 23,040 genes for analysis of expression profiles of cancer cells (23–26).

For the present study we invoked our cDNA microarray system to search for candidate genes possibly associated with differentiation and matrix mineralization in a recently established line of hMSCs characterized by the ability to differentiate into osteoblastic lineages. We report here a set of genes that represent candidates for involvement in osteogenesis and matrix mineralization.

MATERIALS AND METHODS

Cell cultures. Cryopreserved hMSCs from a single donor and their required basal medium and growth supplements (50 ml of

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TABLE 1

Primers Used for cDNA Microarray and RT-PCR

Clone ID	Gene name	Primer sequence	Product size (bp)
A4243	Metallothionein 2A	Forward 5′-GTCACGGTCAGGGTTGTACATAA-3′ Reverse 5′-CTCCTGCAAATGCAAAGAGTG-3′	217
A1095	Osteoprotegerin	Forward 5′-ATTAAGTGGACCACCCAGGA-3′ Reverse 5′-ACTGATTGGACCTGGTTAC-3′	1143
A2547N	S100 calcium-binding protein A10	Forward 5′-ACGTACTAAGGAAGGCGCACAG-3′ Reverse 5′-TGCTAAGTGTCCTGATCTGCTC-3′	527
B0830N	Inhibitor of DNA binding protein 4	Forward 5′-TGCCCAGTATAGACTCGGAAGT-3′ Reverse 5′-GAGATTGGACAGTAGCTTAGCG-3′	676
A2389	Procollagen C-endopeptidase enhancer	Forward 5′-CTACGATGCTCTGGAGGTCTTC-3′ Reverse 5′-CTCTTGCTTAGGTTGGTGAGGAT-3′	1032
A2053	Glyceraldehyde-3-phosphate dehydrogenase	Forward 5′-ATGGAAATCCCATCACCATCT-3′ Reverse 5′-GGTTGAGCACAGGGTACTTTATT-3′	1000

mesenchymal cell growth supplement, 10 ml of 200 mM L-glutamine, 0.5 ml of penicillin–streptomycin mixture containing 25 units of penicillin and 25 μg of streptomycin) were purchased from BioWhittaker Inc. (Walkersville, MD). Three supplements for inducing osteogenesis, Dex, bGP, and AsAP, were purchased from Sigma Chemical Co. (St. Louis, MO).

We passaged the hMSCs three times before induction, according to the suppliers' instructions. We plated hMSCs at densities of 3.1×10^3 cells/cm^2 in 0.2 ml/cm^2 of control medium and incubated the cultures at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. The following day (Day 0), we replaced the culture medium with medium containing 0.1 μM Dex, 1 mM stock in 100% ethanol, 0.05 mM AsAP, and 10 mM bGP (osteogenesis-induction supplements, OS). On the same schedule, hMSCs were cultured in parallel under three different conditions: control medium only, control medium plus 0.05 mM AsAP and 10 mM bGP, or control medium containing 0.1 μM Dex. The media were changed twice a week until 2 or 3 days before extraction of total RNA and analysis for alkaline phosphatase (AP).

Histochemistry. At days 3, 15, and 27, cultured cells or culture media were assayed as described below. Histochemical analysis for AP was performed for 1 h at 25°C using Sigma kit No. 85 (Sigma) according to the manufacturer's instructions. Mineralized matrix was stained by alizarin red S (Sigma). The cell layers were first washed with phosphate-buffered saline and fixed with 100% methanol for 15 min; then the culture dishes were washed with deionized water three times and incubated with alizarin red S solution for 10 min. Finally, the cell layers were washed thoroughly with deionized water three times and air-dried.

cDNA microarray. We selected 23,040 independent cDNAs from the UniGene database of the National Center for Biotechnology Information. The cDNA microarray experiments were performed as described previously (23, 24).

Normalization and selection of genes. Data from the microarray were normalized to the averaged signals of housekeeping genes that had been spotted as internal controls on each slide. The Cy5:Cy3 ratio for each sample was calculated by averaging the signals from duplicate spots. A cutoff value for each expression level was automatically calculated according to background fluctuation, which can be estimated as the variance of the log ratio of Cy5:Cy3 minus the variance of the log ratio of Cy5:Cy3 of highly expressed genes (the upper 30%, where the background fluctuation is so small that it can be ignored). We selected genes with an expression level (above about 10^5) where the background fluctuation was less than a critical value (1.0), as described previously (23). For comparison of expression levels, the relative expression of each gene was recorded in one of four categories: (a) up-regulated in supplement-treated hMSCs (Cy5:Cy3 signal ratio >2.0); (b) down-regulated in supplement-treated hMSCs (Cy5:Cy3 ratio <0.5); (c) unchanged in control or supplement-treated hMSCs; or (d) not expressed. Within each set of slides, the number of items in each category was recorded. To find genes associated with matrix mineralization, first we selected genes that were up-regulated on days 3, 15, and/or 27 in cultures containing OS (i.e., all three osteogenesis-inducing agents). Second, we deleted genes that were up-regulated on the stated days in all cultures including the control.

Semiquantitative RT-PCR. Semiquantitative RT-PCR experiments for six genes were performed to confirm the cDNA microarray results and the reliability of our approach. The same total RNAs as used for cDNA microarray hybridization were reverse-transcribed using Superscript II (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Each PCR was performed using cDNAs as templates in a 20 μl reaction volume containing the same gene-specific primers as those used for constructing the respective microarray targets (Table 1). The thermal profiles consisted of 95°C for 5 min, then 20–30 cycles (the number of PCR cycles was optimized in each case to ensure that the intensity of each product fell within the linear phase of amplification) at 94°C for denaturing, 58°C for annealing, and 72°C for extension. The signal of glyceraldehyde-3-phosphate dehydrogenase, one of the housekeeping genes, was used to normalize the results because its signal intensities were unchanged in all sets of slides.

RESULTS

Production of AP by hMSCs and Matrix Mineralization

AP activity in hMSCs was analyzed after 3, 15, and 27 days of culture under four different conditions. Cells grown in the control medium alone were stained only faintly or moderately, indicating low AP activity when compared with cells cultured in the other three (supplemented) media. Although hMSCs cultured in OS revealed moderate to strong staining, cells grown with Dex alone or with bGP plus AsAP also produced AP (Fig. 1).

However, staining with alizarin red S distinguished hMSCs cultured in the OS from those grown in the other three media. Nodules of mineralized matrix were strongly stained with alizarin red S at day 27 (Fig. 2).

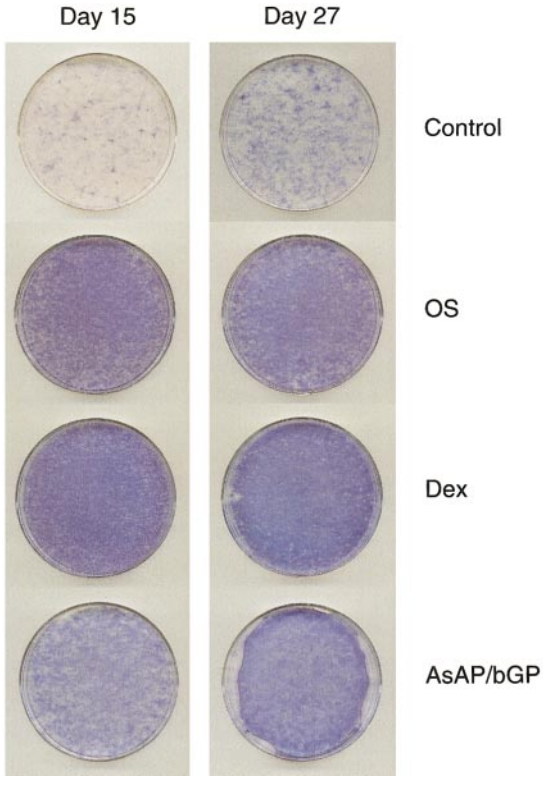

FIG. 1. Alkaline phosphatase activity was analyzed histochemically, using Sigma kit No. 58, in hMSCs cultured in either control medium alone, medium with three supplements (OS), with only one (Dex), or with two (AsAP/bGP). Concentrations of each supplement and cell densities were described under Materials and Methods.

These data suggest that OS is the minimal and essential supplementation for hMSCs to form matrix mineralization nodules, and that induction of these nodules *in vitro* requires about 3 to 4 weeks.

cDNA Microarray Analysis

We harvested hMSCs from each of the four different culture media on days 3, 15, and 27 and isolated total RNAs for analysis on the cDNA microarray. After normalization of the results (see Materials and Methods), we selected genes that were up- or down-regulated on days 3, 15, and/or 27 in cells cultured in the presence of OS, but not under the other three culture conditions. Among more than 20,000 genes analyzed, 55 were up-regulated more than two-fold at one or more time points in cells that developed matrix mineralization nodules, and 82 were down-regulated to less than half their normal level of expression. The selected genes and their known or suspected functions are listed in Tables 2 and 3. In accord with the morphological and histochemical changes we observed, several genes related to calcium-related or mineral metabolism were induced in hMSCs during osteogenesis. Others were ESTs of unknown function or genes already known to be associated with osteogenesis, such as osteoprotegerin (*OPG*) and insulin-like growth factor-binding protein 4 (*IGFBP-4*).

Several genes encoding proteins related to muscle structure were repressed at an early stage when hMSCs were cultured in the OS medium. Collagens and extracellular matrix proteins were also suppressed at an early stage of differentiation.

Semiquantitative RT-PCR

To verify the results obtained by the cDNA microarray analysis, we performed semiquantitative RT-PCR for six selected genes. GAPDH, a housekeeping gene, served as a quantitative control since its Cy3/Cy5 ratios were unchanged in all experiments. Figure 3 shows the results of representative RT-PCR experiments. Procollagen C-endopeptidase enhancer was down-regulated at day 3 in OS medium although in other media it was not. Methallothionein 2A (*MT2a*) and *OPG* were significantly up-regulated at days 15 and 27, specifically in cells that had formed matrix mineralization nodules. Inhibitor of DNA binding 4 (*Id4*) was significantly suppressed during matrix mineralization.

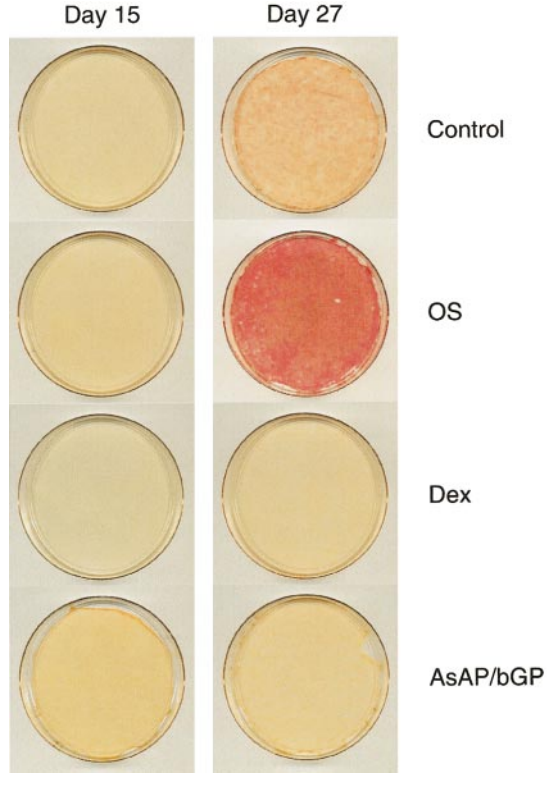

FIG. 2. Alizarin red S staining of calcified matrix around hMSCs cultured for 15 days and 27 days without (Control) and with defined supplements (OS, Dex, or AsAP/bGP) added to the culture medium.

TABLE 2

Genes Up-Regulated in Osteogenesis-Induction Medium (OS)

Fold change[a]						
Day 3	Day 15	Day 27	Clone ID	UniGene No.[b]	Gene name[c]	Known functions
Up-regulated at day 3						
2.17	0.78	1.02	A1904	Hs.78619	γ-Glutamyl hydrolase (GGH)	γ-glutamyl hydrolase
2.29	0.97	1.39	A8819	Hs.12107	Putative breast adenocarcinoma marker (BC-2)	Unknown
2.01	1.34	1.24	B7110	Hs.137556	MT-protocadherin	Unknown
2.44	1.42	1.32	B7655	Hs.13233	EST	Unknown
2.15	0.89	1.27	B8444	Hs.24608	DKFZP564D177 protein	Unknown
2.01	1.02	1.14	B9172	Hs.26358	DKFZP566K1924 protein	Unknown
2.11	1.57	1.32	C7639	Hs.71618	Polymerase (RNA) II (DNA directed) polypeptide L (POLR2L)	Cell growth and/or maintenance (transcription)
Up-regulated at day 15						
0.31	2.17	0.48	A0611	Hs.180383	Dual specificity phosphatase 6 (DUSP6)	Protein phosphatase
1.57	2.05	0.94	A2547N	Hs.119301	S100 calcium-binding protein A10 (S100A10)	Calcium-binding
1.15	2.21	1.34	A3603	Hs.76549	ATPase, Na+/K+ transporting, α 1 polypeptide (ATP1A1)	Sodium/potassium exchange
1.37	2.11	1.34	A5019	Hs.41693	DnaJ (Hsp40) homolog, subfamily B, member 4 (DNAJB4)	Unknown
1.28	2.29	1.66	A6322	Hs.202	Benzodiazapine receptor, peripheral (BZRP)	Benzodiazepine receptor
0.76	2.53	1.50	A6427	Hs.209614	Hypothetical protein MGC4415	Unknown
1.36	2.02	1.70	A8395	Hs.172103	EST	Unknown
0.89	2.30	1.62	A8949	Hs.289068	*Homo sapiens* cDNA FLJ11918 fis, clone HEMBB1000272	Unknown
1.31	2.05	1.66	B0480	Hs.116135	EST	Unknown
1.25	2.02	1.89	B1883	Hs.293964	EST	Unknown
1.47	2.02	1.59	B3795	Hs.173484	Hypothetical protein FLJ10337	Unknown
0.97	2.07	1.42	C5016	Hs.118126	Protective protein for β-galactosidase (PPGB)	Carboxypeptidase
1.77	2.07	1.65	C5032	Hs.151513	Mannosyl (α,3-)-glycoprotein beta, 2-*N*-acetylglucosaminyltransferase (MGAT1)	Metabolism (protein glycosylation)
1.39	2.03	1.06	C8154	Hs.75922	Brain protein I3 (BRI3)	Unknown
Up-regulated at day 27						
1.12	1.74	2.69	A0206	Hs.82932	Cyclin D1 (CCND1)	Cell cycle
0.58	1.21	2.07	A2076	Hs.75693	Prolylcarboxypeptidase (PRCP)	Serine carboxypeptidase
0.95	1.37	2.42	A2167	Hs.2253	Complement component 2 (C2)	Complement activation
0.60	0.57	2.93	A2310	Hs.75616	Seladin-1	Unknown
0.98	0.86	2.09	A2495	Hs.76941	ATPase, Na+/K+ transporting, beta 3 polypeptide (ATP1B3)	Ligand binding (ATPase)
1.56	1.81	2.14	A5038	Hs.24322	ATPase, H+ transporting, lysosomal (ATP6H)	Cell growth and/or maintenance (hydrogen transport)
1.45	1.54	2.05	A5409	Hs.6113	Staufen (STAU)	Cellular component (microtubule cytoskeleton)
1.20	1.18	2.42	A6507	Hs.7137	Clones 23667 and 23775 zinc finger protein (LOC57862)	Unknown
1.40	1.49	2.38	A7504	Hs.211586	Phophoinositide-3-kinase, regulatory subunit, polypeptide 2 (PIK3R2)	Growth signaling pathways
1.16	1.33	2.10	A8146	Hs.11112	EST	Unknown
1.28	1.25	2.19	A8213	Hs.273369	Uncharacterized hematopoietic stem/progenitor cells protein MDS027(MDS027)	Unknown
1.22	1.17	2.35	A9074	Hs.279583	CGI-81 protein (DREV1)	Unknown
1.26	1.95	2.10	A9826	Hs.76288	Calpain 2, (m/II) large subunit (CAPN2)	Calcium-activated neutral peptidase
0.92	0.79	3.96	B0201	Hs.106876	ATPase, H+ transporting, lysosomal (vacuolar proton pump), member D (ATP6D)	Iron–sulfur electron transfer carrier
1.48	1.67	2.35	B0886	Hs.110630	Human BRCA2 region, mRNA sequence CG006	Unknown
1.32	1.41	2.17	B2015	Hs.117031	EST	Unknown
1.05	0.90	2.11	B2098	Hs.287797	Integrin, β 1 (fibronectin receptor, β polypeptide, antigen CD29 includes MDF2, MSK12) (ITGB1)	Cell–cell adhesion
1.18	0.91	2.23	B2847N	Hs.285519	*Homo sapiens* cDNA FLJ11904 fis, clone HEMBB1000048	Unknown
1.00	1.15	2.34	B4142	Hs.258730	Heme-regulated initiation factor 2-α kinase	Unknown
0.86	1.10	2.05	B4231	Hs.3642	RAB1, member RAS oncogene family (RAB1)	GTPase

TABLE 2—*Continued*

Fold change[a]						
Day 3	Day 15	Day 27	Clone ID	UniGene No.[b]	Gene name[c]	Known functions
1.19	1.41	2.55	B5097	Hs.9006	VAMP (vesicle-associated membrane protein)-associated protein A (33 kD) (VAPA)	Cell growth and/or maintenance (vesicle transport)
1.99	1.31	2.31	B7634	Hs.76325	Step II splicing factor SLU7 (SLU7)	Nucleic acid binding
0.84	0.64	2.71	B7736	Hs.81942	Polymerase (DNA-directed), alpha (70 kD)	Unknown
1.66	1.02	2.31	B7824	Hs.69388	Hypothetical protein FLJ20505 (FLJ20505)	Unknown
1.03	1.38	2.43	B8281	Hs.23876	Vacuolar protein sorting protein 18 (VPS18)	Unknown
1.91	1.77	2.10	B9722	Hs.29079	EST	Unknown
1.66	1.23	2.14	C3754	Hs.8768	Hypothetical protein FLJ10849 (FLJ10849)	Unknown
Up-regulated at day 3 and day 15						
2.37	2.50	1.28	A4614	Hs.6721	Lysophospholipase-like (HU-K5)	Phospholipase
Up-regulated at day 15 and day 27						
0.76	4.12	2.71	A1095	Hs.81791	Osteoprotegerin (OPG)	Bone development and maintenance
1.67	2.10	4.08	A7672	Hs.1516	Insulin-like growth factor-binding protein 4 (IGFBP4)	Bone development and maintenance
1.36	2.07	2.09	B3889	Hs.50535	EST	Unknown
1.24	3.73	2.70	B3930	Hs.57548	EST	Unknown
Up-regulated at day 3, day 15, and day 27						
2.80	2.02	2.08	A4243	Hs.118786	Metallothionein 2A (MT2A)	Ligand binding (heavy metal)
2.58	2.35	2.38	B9040	Hs.25897	*Homo sapiens* mRNA; cDNA DKFZp434A2410 (from clone DKFZp434A2410); partial cds	Unknown

[a] Fold change represents a ratio of signal intensity (Cy5/Cy3).
[b] UniGene accession number.
[c] Abbreviations appear within parentheses after gene name are official gene symbols.

DISCUSSION

To identify genes involved in mineralization of extracellular matrix during differentiation of hMSCs, we performed cDNA microarray analysis using hMSCs that could be induced to differentiate into osteoblastic lineages (17, 18). In experiments using four different culture conditions, we clearly demonstrated that OS medium containing Dex, bGP, and AsAP could induce matrix mineralization, which was detected by virtue of strong staining with alizarin red S; media containing either Dex alone, or bGP plus AsAP, could not (Fig. 2).

Using a computer analysis we selected genes that showed altered expression in the cells cultured in the OS medium but not in the other three media. We identified 55 genes that were up-regulated more than two-fold and 82 that were down-regulated by more than 50% during the mineralization process. Among the genes selected, several had already been noted for roles in osteogenesis. For example *OPG*, a glycoprotein secreted from osteoblasts, binds its ligand at the osteoclast stage, inhibits genesis of osteoclasts, and increases bone mineral density and bone volume in rats (27, 28); *OPG*-deficient mice exhibit not only an osteoporotic phenotype but also pathological calcification in aorta and renal arteries (29). *IGFBP4* and *MT2a* are also reportedly up-regulated by Dex (30, 31), but up-regulation of these genes by Dex was not significant in our experiments unless bGP and AsAP were present as well. Under these conditions (i.e., OS medium), S100 calcium-binding protein A10 and calpain 2, thought to be associated with calcium-binding of minerals in extracellular matrix (32), were also among the up-regulated genes.

Expression of transcription factor 4, also known as *SEF2* or *ITF2/E2:2*, was up-regulated by day 15 in our experiments. A previous study reported up-regulation of this transcription factor during osteoblast differentiation in murine (MC3T3-E1) calvarial-derived cells (12), although its precise function in osteogenesis is still not defined. Collagen molecules (33) and fibronectin (34), initiators of the bone mineralization process, were not included in our list because they were also up-regulated under nonmineralizing conditions. Hence, we assume that collagen and fibronectin are required, but not specific, factors for the matrix mineralization process.

We identified other genes that were suppressed when hMSCs were cultured in OS medium. They included tropomyosin 2 (beta), myosin regulatory light chain 2, and others related to muscle structure. Since hMSCs can differentiate into myoblast lineages under particular conditions, we hypothesize that induction to osteoblasts requires suppression of genes that are essential for differentiation to myoblasts. During an early stage of differentiation to adipocytes in an experiment using 3T3-F442A preadipocytes, morphological

TABLE 3

Genes Suppressed for Osteogenesis-Induction Medium (OS)

Fold change[a]						
Day 3	Day 15	Day 27	Clone ID	UniGene No.[b]	Gene name[c]	Known functions
Down-regulated at day 3						
0.30	1.79	4.18	A0107	Hs.1116	Lymphotoxin β receptor (TNFR superfamily, member 3) (LTBR)	Signal transduction
0.42	2.78	3.37	A0640	Hs.83337	Latent transforming growth factor β binding protein 2 (LTBP2)	Cellular component (extracellular matrix)
0.45	1.13	1.74	A0791	Hs.750	Fibrillin 1 (Marfan syndrome)	Cellular component (extracellular matrix)
0.42	1.48	2.35	A0852	Hs.118126	Protective protein for β-galactosidase (galactosialidosis) (PPGB)	Carboxypeptidase
0.45	2.18	2.94	A0977	Hs.118174	Tetratricopeptide repeat domain 3 (TTC3)	Unknown
0.32	0.61	0.98	A1049	Hs.699	Peptidylprolyl isomerase B (cyclophilin B)	Cellular component (cytoplasm)
0.47	1.59	1.21	A1194	Hs.79706	Plectin 1, intermediate filament binding protein, 500 kD (PLEC1)	Epidermal development and maintenance
0.48	6.13	6.49	A1592	Hs.290070	Gelsolin (amyloidosis, Finnish type) (GSN)	Cellular component (cytoskeleton)
0.44	1.20	1.44	A1948	Hs.78979	Golgi apparatus protein 1 (GLG1)	Cellular component (Golgi apparatus)
0.31	1.60	1.41	A2009	Hs.79339	Lectin, galactoside-binding, soluble, 3 binding protein (LGALS3BP)	Immune response
0.47	1.38	1.77	A2107	Hs.76722	CCAAT/enhancer binding protein (C/EBP), δ (CEBPD)	Transcription factor
0.49	0.83	0.65	A2231	Hs.152936	Adaptor-related protein complex, 2, μ 1 subunit (AP2M1)	Intracellular transporter (vesicle)
0.31	1.96	1.64	A2388	Hs.202097	Procollagen C-endopeptidase enhancer (PCOLCE)	Collagen binding
0.09	0.78	1.68	A2442	Hs.102171	Immunoglobulin superfamily containing leucine-rich repeat (ISLR)	Unknown
0.45	0.82	1.42	A2482	Hs.102867	Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3 (SLC13A3)	Unknown
0.49	1.51	2.03	A2503	Hs.279518	Amyloid β (A4) precursor-like protein 2 (APLP2)	Unknown
0.49	0.91	1.20	A2543	Hs.300772	Tropomyosin 2 (β) (TPM2)	Structural protein of muscle
0.47	2.01	6.37	A2548	Hs.119529	Niemann–Pick disease, type C2 gene (NPC2)	Unknown
0.32	1.85	3.50	A2800	Hs.2706	Glutathione peroxidase 4 (phospholipid hydroperoxidase) (GPX4)	Unknown
0.36	0.86	1.26	A3078	Hs.816	SRY (sex determining region Y)-box 2 (SOX2)	Nucleic acid binding (transcription factor)
0.33	4.53	18.02	A3145	Hs.1584	Cartilage oligomeric matrix protein (pseudoachondroplasia, epiphyseal dysplasia 1, multiple) (COMP)	Cellular component (extracellular matrix)
0.45	2.68	6.46	A3288	Hs.5831	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor) (TIMP1)	Cell proliferation
0.35	1.98	3.13	A3738	Hs.89137	Low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor) (LRP1)	LDL receptor
0.27	3.99	2.80	A3739	Hs.119571	Collagen, type III, α 1 (Ehlers-Danlos syndrome type IV, autosomal dominant) (COL3A1)	Extracellular matrix component
0.35	1.55	2.58	A4197	Hs.172928	Collagen, type I, α 1 (COL1A1)	Extracellular matrix component
0.48	1.36	1.96	A4200	Hs.277477	Major histocompatibility complex, class I, C (HLA-C)	Defense/immunity protein
0.33	3.28	6.71	A4236	Hs.146360	Interferon induced transmembrane protein 1 (9–27) (IFITM1)	Cellular component (plasma membrane)
0.26	2.05	2.22	A4254	Hs.108885	Collagen, type VI, alpha 1 (COL6A1)	Extracellular matrix component
0.47	1.09	1.29	A4399	Hs.26002	LIM domain binding 1 (LDB1)	Developmental processes
0.39	1.28	1.42	A4641	Hs.9615	Myosin regulatory light chain 2, smooth muscle isoform	Structural protein of muscle
0.14	1.05	2.71	A5212	Hs.4194	*Homo sapiens* cDNA: FLJ21303 fis, clone COL02107	Unknown
0.42	1.82	2.32	A6080	Hs.7357	DKFZP586N1922 protein	Unknown
0.46	2.54	5.03	A6187	Hs.156667	KIAA1536 protein	Unknown

TABLE 3—*Continued*

Fold change[a]						
Day 3	Day 15	Day 27	Clone ID	UniGene No.[b]	Gene name[c]	Known functions
0.46	1.73	1.00	A7478	Hs.2399	Matrix metalloproteinase 14 (membrane-inserted) (MT1-MMP)	Metalloendopeptidase
0.39	1.48	0.69	A9316N	Hs.239307	Tyrosyl-tRNA synthetase (YARS)	Cellular component (extracellular space)
0.31	3.79	1.91	B2104	Hs.21858	Trinucleotide repeat containing 3 (TNRC3)	Unknown
0.38	0.59	0.57	B4143	Hs.77646	*Homo sapiens* mRNA; cDNA DKFZp761M0223 (from clone DKFZp761M0223)	Unknown
0.45	0.74	0.70	B7734	Hs.74335	Heat shock 90-kDa protein 1, β (HSPCB)	Unknown
0.43	1.78	0.82	B8438	Hs.24594	Ubiquitination factor E4B (homologous to yeast UFD2) (UBE4B)	Unknown
Down-regulated at day 15						
0.97	0.46	1.22	A2320	Hs.75353	KIAA0123 protein	Unknown
0.77	0.48	0.77	A2415	Hs.180878	Lipoprotein lipase (LPL)	Lipase
0.80	0.45	0.89	A2704	Hs.2985	Emerin (Emery–Dreifuss muscular dystrophy) (EMD)	Cell growth and/or maintenance
1.20	0.48	2.20	A2992	Hs.299465	Ribosomal protein S26 (RPS26)	40S ribosomal subunit
0.76	0.48	0.62	A4159	Hs.272897	Tubulin, α, brain-specific	Unknown
0.58	0.48	0.89	A4632	Hs.3622	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide II (P4HA2)	Ligand binding or carrier (electron transporter)
0.57	0.44	0.55	A5311N	Hs.288232	*Homo sapiens* cDNA: FLJ22642 fis, clone HSI06970	Unknown
0.97	0.48	2.96	A8156	Hs.296326	EST	Unknown
0.81	0.46	0.57	B3201N	Hs.119908	Nucleolar protein NOP5/NOP58	Unknown
1.14	0.49	0.68	B4532	Hs.296273	EST	Unknown
0.75	0.49	0.58	B7786	Hs.180566	Mucosa associated lymphoid tissue lymphoma translocation gene 1 (MALT1)	Unknown
0.57	0.42	1.01	B7887	Hs.22505	Hypothetical protein FLJ10159	Unknown
0.54	0.42	0.53	B8341	Hs.24119	*Homo sapiens* mRNA; cDNA DKFZp586G2222 (from clone DKFZp586G2222)	Unknown
0.76	0.49	0.99	C3611	Hs.272458	Protein phosphatase 3 (formerly 2B), catalytic subunit, α isoform (calcineurin A α) (PPP3CA)	Calmodulin activation of calcineurin
0.58	0.39	0.61	C3898	Hs.182429	Protein disulfide isomerase-related protein	Unknown
Down-regulated at day 27						
0.73	0.51	0.47	A0437	Hs.241520	Transcriptional coactivator	Nucleic acid binding (enhancer binding)
0.92	1.77	0.47	A1085	Hs.227751	Lectin, galactoside-binding, soluble, 1 (galectin 1) (LGALS1)	Apoptosis
1.09	1.04	0.48	A1214	Hs.77798	Histidyl-tRNA synthetase (HARS)	Unknown
1.27	2.32	0.29	A1852	Hs.77899	Tropomyosin 1 (alpha) (TPM1)	Structural protein of muscle
0.97	1.24	0.49	A9118	Hs.108873	EST	Unknown
0.81	0.59	0.46	B0829	Hs.330716	*Homo sapiens* cDNA FLJ14368 fis, clone HEMBA1001122	Unknown
0.98	0.69	0.48	B4746N	Hs.14376	Actin, γ 1 (ACTG1)	Structural protein of muscle
1.49	0.69	0.44	B4778N	Hs.121147	EST	Unknown
1.27	0.51	0.40	B4781N	Hs.279868	SUMO-1 activating enzyme subunit 1	Enzyme regulator (activator)
0.68	0.59	0.45	B5992	Hs.14846	*Homo sapiens* mRNA; cDNA DKFZp564D016 (from clone DKFZp564D016)	Unknown
0.89	0.64	0.41	B7491	Hs.12680	*Homo sapiens* cDNA FLJ10196 fis, clone HEMBA1004776	Unknown
0.92	0.96	0.48	B7880N	Hs.289008	*Homo sapiens* cDNA: FLJ21814 fis, clone HEP01068	Unknown
0.53	1.37	0.46	B8026	Hs.98135	Hypothetical protein FLJ20559	Unknown
0.68	0.58	0.48	B9603	Hs.28462	EST	Unknown
0.70	0.54	0.43	C3641	Hs.113503	Karyopherin (importin) β 3 (KPNB3)	Enzyme regulator (GTPase inhibitor)
0.91	0.84	0.41	C3762	Hs.7393	Hypothetical protein from EUROIMAGE 1987170	Unknown
1.28	1.17	0.48	C4707	Hs.49759	EST	Unknown
1.26	1.07	0.44	C7923	Hs.82202	Ribosomal protein L17 (RPL17)	60S ribosomal subunit
1.39	0.76	0.46	C8036	Hs.74047	Electron-transfer-flavoprotein, β polypeptide (ETFB)	Ligand binding or carrier

TABLE 3—*Continued*

Fold change[a]						
Day 3	Day 15	Day 27	Clone ID	UniGene No.[b]	Gene name[c]	Known functions
Down-regulated at day 3 and day 15						
0.47	0.43	0.69	A2405	Hs.289088	Heat shock 90-kDa protein 1, α (HSPCA)	Unknown
0.44	0.46	0.78	A3495	Hs.289101	Glucose regulated protein, 58 kDa (GRP58)	Phospholipase
Down-regulated at day 15 and day 27						
0.50	0.31	0.49	A2429	Hs.82030	Tryptophanyl-tRNA synthetase (WARS)	Cell growth and/or maintenance (protein biosynthesis)
0.52	0.45	0.41	A6712	Hs.8700	Deleted in liver cancer 1 (DLC1)	Unknown
1.44	0.15	0.43	B0830N	Hs.34853	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein (ID4)	Ligand binding or carrier (transcription co-repressor)
0.90	0.42	0.37	B4446N	Hs.17144	Short-chain dehydrogenase/reductase 1	Ligand binding or carrier (electron transporter)
0.67	0.38	0.36	B9836	Hs.24684	KIAA1376 protein	Unknown
0.68	0.49	0.43	C0341	Hs.180414	Heat shock 70-kDa protein 8 (HSPA8)	Unknown
Down-regulated at day 3, day 15, and day 27						
0.30	0.31	0.44	A2452	Hs.108080	Cysteine and glycine-rich protein 1 (CSRP1)	Cell growth

[a] Fold change represents a ratio of signal intensity (Cy5/Cy3).
[b] UniGene accession number.
[c] Abbreviations appear within parentheses after gene name are official gene symbols.

changes followed reduced expression of tubulin and actin (35). Our findings indicate that during osteogenesis in OS medium, reduced expression of several genes related to muscle structure occurred in association with morphological changes of hMSCs from spindle shapes to broad and cuboidal shapes. Matrix methalloproteinase 14 (*MT1-MMP*) was also down-regulated at day 3. Expression of *MT1-MMP* is suppressed by some growth factors (36); mice that lack *MT1-MMP* reveal craniofacial dysmorphism, arthritis, osteopenia, dwarfism, and fibrosis of soft tissues due to ablation of a collagenolytic activity that is essential for modeling of skeletal and extraskeletal connective tissues (37). Those observations in the knockout mouse, along with our findings, indicate that *MT1-MMP* expression is an important factor during osteogenesis.

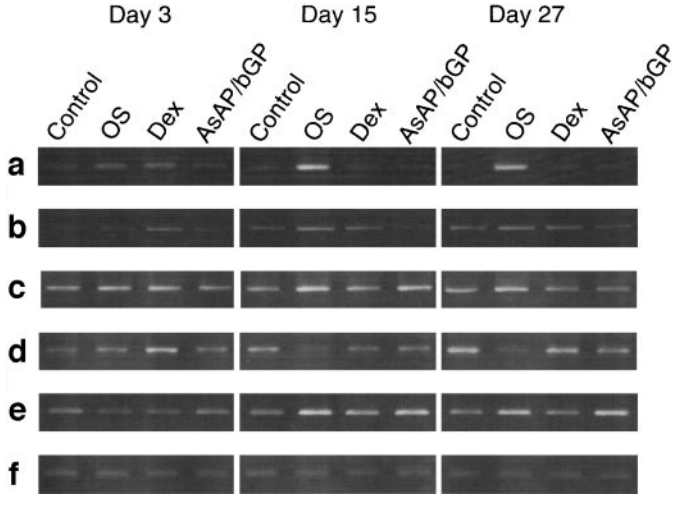

FIG. 3. Semiquantitative RT-PCR using RNAs from hMSCs. Each band shows amplified DNA from cells cultured in control medium (Control), control medium with OS (OS), control medium with Dex (Dex), and control medium with AsAP/bGP (AsAP/bGP) from left to right in each panel. Genes shown are (a) MT2a; (b) OPG; (c) S100 calcium-binding protein A10; (d) Id4; (e) procollagen C-endopeptidase enhancer. The integrity of each template was controlled through amplification of glyceraldehyde-3-phosphate dehydrogenase (f).

Id4 was down-regulated on days 15 and 27 of osteogenesis induction. *Id4* is a member of the Id helix-loop-helix family of proteins, which have been implicated in the control of growth and differentiation in a number of different cell types (38–40). *Id4* may interact with a basic helix-loop-helix protein important in the osteogenic process, to prevent it from binding to DNA and activating transcription (38). Although Dex alone can up-regulate *Id4* expression in 3T3-L1 preadipocytes (39) and *Id1* expression in osteoblastic cells (41), we found that the OS medium in fact suppressed *Id4* expression in hMSCs. We speculate that mechanisms of bone mineralization may be explained not only by the presence of initiation factors but also by the absence of inhibitors of mineralization, such as *Id4*, in the bone matrix.

Our identification of a set of genes that may be associated with mineralization of bone matrix provides important information toward a better understanding of the precise mechanisms of osteogenic differentiation as well as the molecular etiology of diseases in which biomineralization is impaired, e.g., osteoporosis, ectopic calcification at ligaments, and atherosclerosis.

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