Gene Expression Profile Associated with the Differentiation of Osteoblasts from Human Mesenchymal Stem Cells

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Human mesenchymal stem cells (hMSCs) in bone marrow (BM) can be induced to differentiate into a variety of mesenchymal tissues, including adipocytes, osteoblasts and chondroblasts, under the influence of certain growth or environmental factors. In this study, we analyzed the differentiation process and the associated gene expression profiles inherent to the process by which hMSCs differentiate into osteoblasts. We conducted a comparison of gene expression profiles of the normal human BM MSCs, using human 8K cDNA microarray, incubated in media containing either a combination of β-glycerol phosphate, L-ascorbic acid, and dexamethasone, or in medium lacking these osteogenic supplements. During the osteoblastic differentiation process, 36 genes were determined to be up-regulated, and 59 genes were shown to be down-regulated. *Osteoprotegerin, LRP5, and metallothionein 2A*, all of which are associated with the osteogenetic process, were up-regulated, and genes associated with the differentiation of MSCs into other lineages, including muscle, adipose tissue and vascular structure were down-regulated. The set of differentially expressed genes reported in this work should contribute to our current understanding of the processes inherent to the differentiation of MSCs into osteoblasts.

Key words – Gene expression, mesenchymal stem cell, osteoblast

Introduction

The development of bone and the mineralization of the extracellular matrix have been shown to be accompanied by a series of complex changes in the expression levels of a variety of genes(2,3,8). For instance, *sialoprotein* is known to be up-regulated during the mineralization phase of osteoblastic differentiation[4]. *Osteocalcin* and *osteopontin*, both of which are known inhibitors of the mineralization process, are up-regulated during the final stage of osteogenic differentiation[4]. Therefore, it seems reasonable to suggest that the identification of genes which are expressed during osteoblastic differentiation would result in a furthering of our knowledge regarding the molecular mechanisms underlying osteogenesis. cDNA microarrays have long been considered a rapid and exact method for the characterization of gene expression in a variety of MSC differentiation stages[5,12]. In the present study, we attempted to profile and characterize the patterns of gene expression occurring during the differentiation of hMSCs into osteoblasts, by cDNA microarray studies.

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Materials and Methods

hMSCs culture

Mononuclear cells were isolated from BM obtained from the iliac crests of single healthy donor, using Ficoll-Hypaque density gradient centrifugation. These cells were seeded at a density of 1×10^7 cells/30 ml in 175 cm^2 polystyrene flasks, and cultured in Dulbecco's Minimal Essential Medium (GIBCO-BRL, Grand Island, NY, USA), supplemented with 10% (vol/vol) fetal bovine serum (GIBCO-BRL). The MSCs were then allowed to adhere for 72 hrs. During the incubation, any attached fibroblastic cells which formed visible colonies were isolated, and the nonadherent cells were removed via medium replacements, which took place every 3 or 4 days. The isolated MSCs were then replated in 175 cm^2 polystyrene flasks, at a density of 1×10^6 cells/30 ml.

After at least three passages, the identification of the cultured hMSCs was confirmed via the moprphological observation of spindle-shaped fibroblast-like cells, which had

been stained with hematoxylin & eosin (H & E). Moreover, the isolated cultured hMSCs were analyzed with regard to surface antigen expression, via flow cytometry using CD45, CD34, CD14, SH2, and SH4. In order to generate mouse monoclonal antibodies, human MSC hybridoma cells expressing either SH2 or SH4 (1×10^7 cells) (Osiris Therapeutics, Baltimore, MD, USA) were injected into the abdominal cavities of experimental mice, and the ascites were collected and passaged through a protein G-sepharose column. After three such passages, the presence of MSC was verified via flow cytometry, using monoclonal antibodies against SH2, SH4, CD45, CD14, and CD34. The CD14-fluorescein isothiocyanate (FITC), CD45-FITC, and CD34-FTIC antibodies were purchased from PharMingen (San Diego, CA, USA). The MSCs were incubated for 10 min in 1 ml of 70% ethanol, then washed with PBS supplemented with 2% BSA. The MSC were then incubated with anti-SH2 or SH4 antibodies for 30 min at 4°C, washed twice, and incubated with FITC-conjugated whole anti-mouse IgG (Sigma, St Louis, MO, USA) for 30 min at 4°C. The samples were then analyzed with the FACSCalibur system (Becton-Dickinson, San Jose, CA, USA), and the data were analyzed with CellQuest software (Becton-Dickinson).

Induction of osteogenic differentiation

The cultured hMSCs were then incubated at 37℃ in a humidified atmosphere containing 95% air and 5% CO_2, with osteogenic supplements (OS) containing 10% fetal bovine serum, 10 mM β-glycerol phosphate, 50 μM L-ascorbic acid and 0.1 μM dexamethasone, in order to induce osteoblastic differentiation. We employed hMSCs which had been cultured under the same conditions, with the exception that OS had been added as a control. The media was changed twice a week, and we regularly analyzed changes in morphology and mineralization.

Histochemistry

In order to assess matrix mineralization, we conducted a histochemical assay for alkaline phosphatase at 0, 7, and 21 days after the construction of osteogenic culture. The cultured cells were fixed on ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA), using 2% formaldehyde, and were then allowed to react with FRT (Fast Red TR salt) for 10 min at room temperature. We employed hematoxylin as a counterstain.

cDNA microarray

Labeling of cDNA targets

The total RNAs extracted from culured cells were then labeled using a 3DNA indirect labeling 50 kit, and all reactions were conducted according to the manufacturer's instructions (Genisphere, Hatfield, PA). The total RNA was reverse-transcribed with reverse transcription (RT) primers, which had been tagged with either Cy3- or Cy5-specific 3DNA capture sequences. This mixture was incubated for 10 min at 80℃ and chilled on ice, then mixed with 19 μl of reverse transcription labeling mixture; 8 μl of 5x first-strand buffer, 2 μl of dNTP mixture (10 mM dGTP, 10 mM dATP, 10 mM dTTP and 10 mM dCTP), 4 μl of 0.1 M dithiotreitol (DTT), 3 μl of RNase-free water, and 2 μl of Superscript Ⅱ reverse transcriptase (Invitrogen, Carlsbad, CA). The resulting 40 μl-mixture was incubated for 2 hrs at 42℃. The synthesized tagged cDNAs were then fluorescently labeled with either Cy3-3DNA or Cy5-3DNA, on the basis of the complementary capture sequence, using 3DNA capture reagents (Genisphere, Hatfield, PA).

Microarray hybridization and signal detection

40 μl of the 3DNA labeled targets (Genisphere, Hatfield, PA) containing 2x hybridization buffer [20 μl SDS-based buffer, 2 μl array 50dT blocker, 10 μl cDNA concentrated by amicon microcon YM-30 (Millipore, Bedford, MA)] and 1 μl of Cot-1 DNA (1 μg/μl) were incubated for 10 min at 98℃, followed by a further incubation for 20 min at 60℃, prior to being applied to prehybridized slides (HSVC v1.0 Genomictree, Inc, Daejeon, Korea). In order to maintain the level of humidity within the chamber, we dropped 20 μl of DEPC water into the two reservoir wells. This chamber was then sealed tightly and incubated at 6 4℃ overnight, in a thermo-hybridization oven. For dendrimer detection, 2.5 μl of Cy3 and Cy5 labeled dendrimers (Genisphere, Hatfield, PA) were suspended in hybridization mixture, and then layered onto the slide. A coverslip was slowly positioned over the solution, and incubated at 65℃ for 5 hrs in a hybridization chamber (GenomicTree, Inc, Daejeon, Korea). After hybridization, we conducted a series of washes at 64℃ in 2x SSC, and 0.2% SDS for 15 min at room temperature in 2x SSC for 15 min, and then at room temperature in 0.2xSSC for 15 min. The hybridized slides were then scanned at a resolution of 10 microns under a green laser (543 nm) for Cy3 labeling, and then under a red laser (633 nm) for Cy5 labeling, using a Scan Array Express scanner (Packard Biochip tech-

nology, Boston, MA) with the appropriate gains on the photomultiplier tube (PMT), in order to acquire the highest intensity without saturation.

Microarray image and data analysis

The images which had been generated by the scanning of the glass slide microarrays were then analyzed using Imagene 5.1 software (BioDiscovery, Blvd, LA). The average expression ratio for all genes on the array was then normalized to 1.0. For background corrections, those data were calculated as negatives, in which the average intensity of the spot was smaller than two times the average background of the same area. Each of the experiments was repeated three times, using both fluorescent dyes to label both the control and the sample. We then conducted a significance test at a confidence level of 95%, in order to reduce the number of false positive or false negative ratios derived from the possible uneven incorporation of fluorescent dyes during labeling, or from any other experimental variables introduced by hybridization, washing conditions, or array features. The log base 2 of each value was also determined, in order to equalize the magnitude of the deflection of up-regulated and down-regulated genes, and the differences in gene expression were ranked on the basis of absolute value.

Real-time quantitative reverse transcription polymerase chain reaction (RQ-PCR)

The validation of gene expression from the microrrays was conducted via RQ-PCR for 5 selected genes. The primer (Bioneer, Choungwon, Korea) list is shown in Table 1. Total RNA was isolated at 0, 7 and 21 day timepoints after the addition of OS. First strand cDNAs were synthesized at 42℃ for 30 min in a 20 μl reverse transcriptase reaction from 1 μg of total RNA, using a Superscript II pre-amplification kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PCR was performed using the LightCycler-FastStart DNA Master SYBR Green system (Roche Molecular Biochemicals, Mannheim, Germany). PCR was carried out in a final volume of 20 μl using 0.5 μl of each primer (10 μM), 1.2 μl MgCl (25 mM), 2 μl of the supplied enzyme mix, 13.8 μl H_2O and finally 2 μl of the template. RNA expression levels for genes of interest were normalized to the level of *β_2-microglobulin* expression. PCR was performed in a Light-Cycler (Roche) with a 10 min pre-incubation at 95 ℃ followed by 50 cycles of 10 sec at 95 ℃ (denaturation), 8 sec at 58 ℃ (annealing) and 14 sec at 72 ℃ (amplification). PCR products were subjected to melting curve analysis using the light cycler system to exclude the amplification of unspecific products. Finally, the PCR products were analyzed by conventional agarose gel electrophoresis.

Results

Characteristics of cultured hMSCs

We observed spindle cell shaped fibroblast like cells in primary culture under a microscope. Following the seeding of 2×10^7 MNCs in the primary cultures, the median MSC harvest on day 24 was 6.2×10^6 cells (range, 2.0-8.6×10^6 cells) (range, days 17-69). The immunophenotype study, which utilized flow cytometry, indicated that these cells expressed mesenchymally-related antigens (SH2 and SH4), but not hematopoietic stem cell-related antigens (CD14, CD34, or CD45). The cultured MSCs exhibited similar phenotype and expression of SH2 and SH4, but this was not the case with regard to CD14, CD34, and CD45 (data not shown).

Table 1. Primers used for real time quantitative RT-PCR

Gene ID	Gene name	Primer sequence	Prodeuct size (bp)
AA156031	*Metallothionein 2A*	F 5′-GTCACGGTCAGGGTTGTACATAA-3′ R 5′-CTCCTGCAAATGCAAAGAGTG-3′	217
AA194983	*Osteoprotegerin*	F 5′-GAGGCATTCTTCAGGTTTGC-3′ R 5′-CTGGGTTTGCATGCCTTTAT-3′	356
T60048	*Actin γ 2*	F 5′-CGTGACCTCACGGACTACCT-3′ R 5′-CTTCTGCATCCTGTCAGCAA-3′	399
AA143331	*Matrix metalloproteinase 1*	F 5′-AGGGGATGCTCATTTTGATG-3′ R 5′-ACCGGACTTCATCTCTGTCG-3′	449
	β2-microglobulin	F 5′-ACCCCCACTGAAAAAGATGA-3′ R 5′-ATCTTCAAACCTCCATGATG-3′	115

F: forward, R: reverse

Differentiation of MSCs into osteoblasts

The osteoblasts cultured in the presence of OS manifested strong alkaline phosphatase activity, according to the results of staining and developing with FRT on 0, 7, and 21 days under the ostegenic condition (Fig. 1). However, the MSCs cultured in the control medium only stained faintly or mildly, indicating low alkaline phosphatase activity as compared with the cells which had been cultured in the OS.

Analysis of gene expression profiles during osteoblast differentiation

cDNA microarray analysis

We hypothesized that genes might be differentially regulated during osteoblast differentiation in a stage-specific pattern. Therefore, after the results were normalized, we selected genes which had been up- or down-regulated on days 0, 7, and 21 in the presence of OS, but not in the control medium. 95 out of 8000 genes showed a greater than 2 fold difference in expression levels, through statistical analysis using a significance test at 95 % confidence level, in at least one of the 3 different time course responses to OS treatment. Among 95 genes, 36 were up-regulated and related to the development of matrix mineralization nodules, while 59were down-regulated. The selected genes were then categorized on the basis of their known or suspected functions, and are listed in Tables 2 and 3.

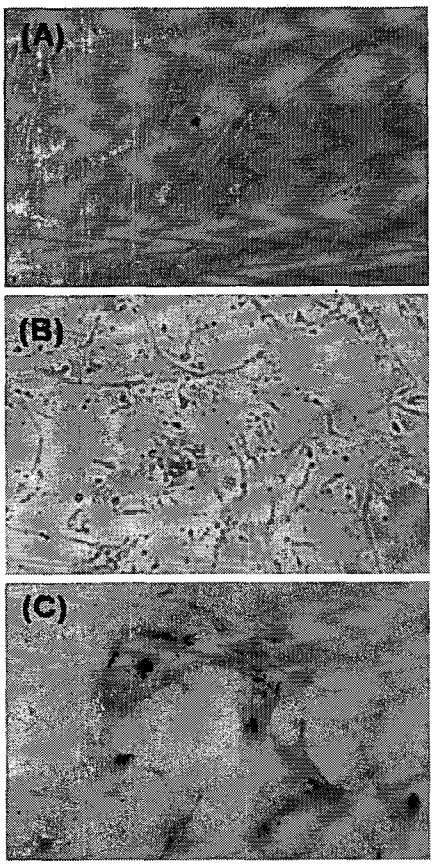

Fig. 1. Mesenchymal stem cells (MSCs) in primary culture showing in morphology of spindle-shaped fibroblast-like cells (A). MSC were tested for the ability to differentiate *in vitro* to osteoblastic lineage cells (B), alkaline phosphatase activity was visualized by the staining developed with FRT (Fast Red TR salt)(C).

RQ-PCR analysis

In order to confirm the data generated in the microarray studies, we performed RQ-PCR on the total RNA derived from the cultured cells for the selected 4 genes (*metallothionein 2A, OPG, actin γ2 and matrix metalloproteinase 1*), using *ß2 microglobulin* as a control. We used LIGHT CYCLER data analysis software for the analysis of the copy numbers and the linear regression curves. The correlation coefficient r was -1.00, the error was 0.0317, and the slope was found to be -3.508.

Metalloproteinase 2 gene expression was 15.9 higher than the control value on day 7, and was 8.1 times higher on day 21. *OPG* was overexpressed 67.5 and 5 times higher than the control level on days 7 and 21. *Matrix metalloproteinase I* was expressed 16.7 and 2.5 times higher than the control level on days 7 and 21. Conversely, on day 7, *actin γ 2* expression was less than 80% of the expression levels measured on day 0. Therefore, our RQ-PCR results were consistent with cDNA microarray data (Fig. 2). We also conducted gel electrophoresis with the PCR products generated from the Light Cycler PCR, and verified identical results (data not shown).

Discussion

Each of the stages of the differentiation of osteoblasts from proosteoblast to mature osteoblasts is characterized by a series of distinct protein markers. Research regarding this process is currently proceeding fairly actively[5,11].

Angiopoietin-like 4 (ANGPLT4, T54298) is an angiopoietin-related protein, which is also commonly referred to as PPARG (peroxisome proliferator-activated receptor-γ). Although the role of this protein currently remains unknown, it appears to be somehow related to adipocyte development[18]. We determined that the expression of this

Table 2. Genes up-regulated in osteogenesis induction medium

A. Up-regulated genes on day 7 only

7 day	21 day	Gene ID	UniGene No.	Gene name
Not associated with osteogenesis				
2.31365	0.59765	T54298	Hs.9613	*angiopoietin-like 4*
Unknown				
2.19695	1.01275	R93124	Hs.391800	*pseudo-chlordecone reductase*
2.13295	1.52745	AI161252	Hs.75794	*endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2*
2.05695	1.22495	AA663549	Hs.8128	*phosphatidyl serine decarboxylase*

B. Up-regulated genes on day 21 only

7 day	21 day	Gene ID	UniGene No.	Gene name
Associated with osteogenesis				
1.20585	5.83335	AA253464	Hs.40499	*dickkopf homolog 1 (Xenopus laevis)*
0.36465	3.49375	AA143331	Hs.83169	*matrix metalloproteinase 1 (interstitial collagenase)*
0.53565	2.69375	AA194983	Hs.81791	*tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)*
1.30585	2.35455	AA705308	Hs.81073	*fetuin B*
-0.88995	2.27615	AA279114	Hs.6347	*LRP5 = low density lipoprotein receptor-related protein 5*
Not associated with osteogenesis				
1.33085	3.06285	AA974052	Hs.271966	*antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5*
-1.48395	2.58815	H63096	Hs.320793	*ESTs, Highly similar to RNG7_HUMAN Class II histocompatibility antigen, M beta chain precursor*
1.72305	2.20155	W56300	Hs.81328	*nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha*
0.08655	2.29825	AA598526	Hs.197540	*hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)*
1.09955	2.21915	AA456636	Hs.433737	*RAN, member RAS oncogene family*
0.33595	2.12035	N27190	Hs.77917	*ubiquitin carboxyl-terminal esterase L3*
1.00565	2.22705	H13623	Hs.2132	*epidermal growth factor receptor pathway substrate 8*
0.92165	2.18985	H09614	Hs.251871	*CTP synthase*
1.88895	2.13865	N80129	Hs.380778	*metallothionein 1L*
Unknown				
1.12555	2.21505	AA136125	Hs.89718	*spermine synthase*
1.99755	2.06375	AA446120	Hs.394	*adrenomedullin*
1.63655	2.01035	N55269	Hs.93675	*decidual protein induced by progesterone*

C. Up-regulated genes on both day 7 and day 21

7 day	21 day	Gene ID	UniGene No	Gene name
Associated with osteogenesis				
2.31605	2.52525	H53340	Hs.334409	*EST, Highly similar to metallothionein 1G [H. sapiens]*
2.11565	2.22945	AA156031	Hs.418241	*metallothionein 2A*
Not associated with osteogenesis				
2.82255	4.09045	W46900	Hs.789	*chemokine (C-X-C motif) ligand 1*
2.56735	2.29275	AA448478	Hs.265827	*interferon, alpha-inducible protein (clone IFI-6-16)*
3.08555	2.39165	AI346878	Hs.37129	*sodium channel, nonvoltage-gated 1, beta*
2.49125	2.45475	AI360772	Hs.406409	*myosin IF*
2.33695	2.93895	AA682386	Hs.77729	*oxidized low density lipoprotein (lectin-like) receptor 1*
2.56125	2.23175	AA476272	Hs.211600	*tumor necrosis factor, alpha-induced protein 3*
2.52695	2.68535	AA488084	Hs.177781	*Homo sapiens, clone IMAGE:4711494, mRNA sequence*
2.25635	2.31165	W92764	Hs.29352	*tumor necrosis factor, alpha-induced protein 6*
Unknown				
2.30985	3.39445	AI631772	Hs.133083	*dihydrodiol dehydrogenase (dimeric)*
2.29375	2.07325	AA41925	Hs.503860	*6-pyruvoyltetrahydropterin synthase*
2.09275	2.1013	AA44827	Hs.17013	*forkhead box O1A (rhabdomyosarcoma)*
2.06695	2.60975	AA872383	Hs.433205	*Similar to RNA helicase-related protein [H. sapiens],*
2.19705	2.23515	T73556	Hs.154890	*fatty-acid-Coenzyme A ligase, long-chain 2*

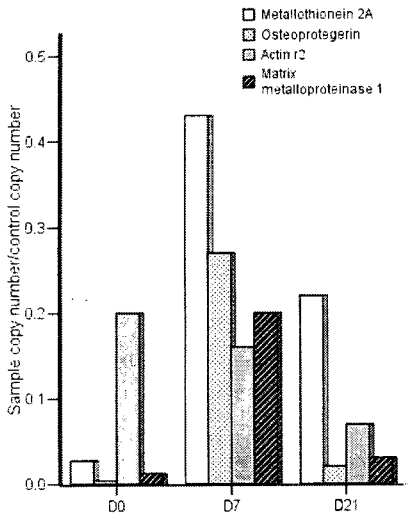

Fig. 2. Expression of the specific genes of interest. Calculation of the copy numbers are based on external standard (control: *β2-microglobulin*).

protein was elevated on day 7, and diminished on day 21 (Table 2A).

Some genes which were determined to have been either up- or down- regulated, were already known to be associated with the process of bone formation. *OPG* (*osteoprotegerin, osteoclastogenesis inhibitory factor, AA194983,* Table 2B) was expressed abundantly on day 21 as seen on the cDNA microarray. It is secreted from the osteoblasts, and can inhibit the maturation of the osteoclasts. As is well known, it is able to protect the bones from routine osteoclast remodeling, and produce an increase in both bone density and bone volume[13]. According to Doi *et al.*, the concentration of *OPG* increased on culture day 15 and was maintained at the final stage of differentiation[5]. In the present study, the expression of *OPG* was elevated on day 21, as evidenced by cDNA microarray. *LRP5 (AA279114)* encodes for the low-density lipoprotein receptor-related protein, and is also involved in the Wnt-mediated signaling pathway in pluripotent MSCs. This can be induced 2 weeks after osteogenic differentiation[7]. It has also been demonstrated that the Wnt antagonist, Dickkopf 1 protein (DKK1, AA253464) interferes with Wnt signaling, via binding to *LRP5*. Previous reports have shown that short-term exposure to low DKK1 levels induce a moderate degree of MSC proliferation into osteoblasts, whereas the long-term exposure to high DKK1 levels induced a loss of cell viability, elicited osteoblastic differentiation, and promoted os-

Table 3. Genes down-regulated in osteogenesis induction medium

A. Down-regulated genes on day 7 only

7 day	21 day	Gene ID	UniGene No.	Gene name
-4.11215	-0.67955	AA465203	Hs.923	*single-stranded DNA binding protein*
-4.23835	0.64755	W81318	Hs.75307	*H1 histone family, member X*
-3.27135	1.87175	AA521483	Hs.24340	*centaurin, beta 2*
-2.19355	-0.90625	AA477400	Hs.300772	*tropomyosin 2 (beta)*
-2.23165	-0.66955	AA485677	Hs.380230	*thyroid hormone receptor interactor 6*
-2.90155	-0.34935	AA504211	Hs.115770	*tumor necrosis factor (ligand) superfamily, member 11*
-2.81865	-0.82075	AA634006	Hs.377755	*Unnamed protein product [Homo sapiens]*
-2.32205	-0.63925	T60048	Hs.516105	*actin, gamma 2, smooth muscle, enteric*
-2.12745	-0.21295	T98612	Hs.327412	*PRO3121 [Homo sapiens], mRNA sequence*
-2.18005	-0.19755	N27159	Hs.727	*inhibin, beta A (activin A, activin AB alpha polypeptide)*
-2.64935	-0.36365	R39239	Hs.289114	*tenascin C (hexabrachion)*
-2.86425	-0.07685	AA452535	Hs.172609	*nucleobindin 1*
-2.48015	-0.80735	AA427561	Hs.211573	*heparan sulfate proteoglycan 2 (perlecan)*
-2.54885	-0.64045	AI299995	Hs.25338	*protease, serine, 23*
-2.53145	-0.89055	AA490172	Hs.179573	*collagen, type I, alpha 2*
-2.89215	0.23355	AA620580	Hs.82793	*proteasome (prosome, macropain) subunit, beta type, 3*
-2.09415	-0.92785	AA457238	Hs.169172	*calpain 6*
-2.11725	0.37875	AA284669	Hs.77274	*plasminogen activator, urokinase*
-2.38185	-0.52385	AA461456	Hs.82985	*collagen, type V, alpha 2*
-2.64515	0.09495	AA055504	Hs.236218	*tripartite motif-containing 32*
-2.19925	-0.80885	AA598653	Hs.136348	*osteoblast specific factor 2 (fasciclin I-like)*
-2.03515	-0.14005	AI668906	Hs.433399	*transgelin*
-2.23435	-0.86095	H09066	Hs.106674	*BRCA1 associated protein-1*
-2.26655	0.34725	R31701	Hs.30627	*ESTs, Highly similar to IgG Fc binding protein*
-2.29175	0.23575	R67275	Hs.82772	*collagen, type XI, alpha 1*

B. Down-regulated genes on day 21 only

7 day	21 day	Gene ID	UniGene No.	Gene name
-1.66835	-2.93925	R59212	Hs.268515	*meningioma (disrupted in balanced translocation) 1*
-1.83105	-2.41595	AA496022	Hs.296049	*microfibrillar-associated protein 4*
-1.87895	-2.03395	AA488681	Hs.74576	*GDP dissociation inhibitor 1*
-1.65815	-2.01955	T50790	Hs.334345	*cytochrome P450, subfamily IIA, polypeptide 6*
-0.83145	-2.06145	AA447115	Hs.237356	*chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)*
-1.31535	-2.03695	AA459941	Hs.201776	*paternally expressed 3*
-1.94525	-2.12915	AA040856	Hs.153260	*SH3-domain kinase binding protein 1*
-1.31545	-2.45025	AA041388	Hs.3734	*ESTs, Moderately similar to hypothetical protein FLJ20378*
-0.80735	-2.10825	AA284856	Hs.433369	*Human fibroblast mRNA fragment with Alu sequence (pRHF11)*
-0.1001	-2.1626	AI243295	Hs.16743	*adrenomedullin receptor*

C. Down-regulated genes on both day 7 and day 21

7 day	21 day	Gene ID	UniGene No.	Gene name
-5.06305	-4.31665	H08561	Hs.380833	*Unknown (protein for IMAGE:4183312), mRNA sequence*
-4.22145	-2.80535	AA703652	Hs.145601	*slit homolog 3 (Drosophila)*
-3.64035	-2.13725	AA427801	Hs.2799	*cartilage linking protein 1*
-3.30475	-2.52955	AA459308	Hs.252418	*elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)*
-3.30155	-2.33715	N39452	Hs.251673	*DNA (cytosine-5-)-methyltransferase 3 beta*
-3.02855	-3.11955	N94385	Hs.1584	*EST, Weakly similar to COMP_HUMAN Cartilage oligomeric matrix protein precursor (COMP) [H.sapiens]*
-2.99075	-2.29545	AA496691	Hs.76111	*dystroglycan 1 (dystrophin-associated glycoprotein 1)*
-2.99015	-2.98145	AA436564	Hs.306178	*C-mer proto-oncogene tyrosine kinase (MERTK)*
-2.98655	-3.28085	AA455157	Hs.409034	*collagen, type XV, alpha 1*
-2.96955	-2.77825	AA046523	Hs.29463	*centrin, EF-hand protein, 3 (CDC31 homolog, yeast)*
-2.94595	-2.06205	N51018	Hs.821	*biglycan*
-2.38505	-2.22145	AA142943	Hs.103854	*docking protein 1, 62kDa (downstream of tyrosine kinase 1)*
-2.87355	-2.83705	AA149204	Hs.175783	*solute carrier family 39 (zinc transporter), member 2*
-2.25825	-2.50335	AA418852	Hs.22199	*ECSIT*
-2.70675	-2.24455	AA454668	Hs.88474	*prostaglandin-endoperoxide synthase 1*
-2.45345	-2.34095	AI378517	Hs.348710	*Homo sapiens, clone IMAGE:4047062, mRNA, mRNA sequence*
-2.22735	-2.41985	R52651	Hs.91622	*neuronal pentraxin receptor*
-2.01095	-2.10975	T59043	Hs.518808	*alpha-fetoprotein*
-2.26655	-2.02125	AA037229	Hs.218040	*integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)*
-2.72305	-2.23655	AI744681	Hs.2159	*aggrecan 1*
-2.43385	-2.56515	AA418747	Hs.98309	*interleukin 23, alpha subunit p19*
-2.10905	-2.20935	AI129421	Hs.83077	*interleukin 18 (interferon-gamma-inducing factor)*
-2.30055	-2.09915	AI973060	Hs.194679	*WNT1 inducible signaling pathway protein 2*
-2.24645	-2.43665	AA150487	Hs.401981	*EST, Weakly similar to hypothetical protein FLJ20378*

teoclast differentiation *in vitro*. The extent of expression or variation in both types of cells has been shown to be associated with both the presence of lytic bone lesions, and with hereditary osteoporosis[16]. In this study, we found these two genes to be expressed at elevated levels on day 21 (Table 2B). However, the duration of exposure and concentration were not assessed, and thus a re-evaluation will be warranted in the future.

Matrix metalloproteinase 1 (interstitial collagenase, AA143331, Table 2B) was expressed at elevated levels on day 21, as shown by cDNA microarray analysis. According to the report of Mizutani *et al.*, ascorbic acid promotes osteoblastic differentiation and the mineralization of mouse calvarial osteoblast MC3T3-E1 cells. Furthermore, when Ca^{2+} or Zn^{2+} was added to those cells, proteolysis was induced. They also reported that, in the early stages of differentiation in cells which had been exposed to ascorbic acid, the level of membrane type-matrix metalloproteinase 1 (MT1-MMP) mRNA was elevated, and then gradually decreased to control levels. During the early stages of osteoblastic differentiation, a variety of collagenase and matrix metalloproteinases were shown to destroy the osteoid membrane on the surface of the bones, and this resulted in the exposure of the mineralized matrix to the osteoclasts. As a consequence of this exposure, they are believed to induce bone absorption[10].

Metallothionein 2A (AA156031) exhibited abundant expression on day 7, and these elevated levels persisted into the final stage of differentiation, as was shown by our cDNA microarray analysis (Table 2C). This finding was consistent with the results of another previous study[5].

Metallothionein is a cysteine-rich, low molecular weight intracellular molecule. It exhibits a high degree of affinity for some metals, including cadmium and zinc. It can be induced by exposure to these heavy metals, chemicals, cytokines, and some hormones, and can also be used in the detoxification of heavy metal poisoning[1]. Miyahara *et al.* also determined that the addition of dexamethasone to MC3T3-E1 cells induced methallothionein synthesis in osteoblastic cells after 3 days[9].

Tropomyosin 2β (AA477400) and *actin γ 2 (T60048)*, both of which were suppressed in the early stage of osteoblastic differentiation in this study, were intimately linked with the development of muscle tissues. As the hMSCs can differentiate into myoblasts under the proper conditions, we became aware that osteoblasts could be elicited from hMSCs when myoblastic differentiation was inhibited during its early stages. In addition, as Splegelman *et al.* observed, the synthesis of actin occurred at low levels in cells which underwent adipose differentiation, and this was attributed to the loss of tubulin and actin mRNA sequences in the early stages[14]. Similarly, in the present study, we observed a morphological change in the spindle-shaped hMSCs, which transformed themselves into a wide cuboidal shape according to the decreases in the expression of genes related to the development of muscle tissue. Additionally, several genes which were suppressed throughout the entire differentiation process, were coupled with cartilage or muscle tissue development in the hMSCs. For example, *cartilage linking protein 1 (AA427801)* and *COMP_HUMAN Cartilage oligomeric (N94385)* are normally expressed during the development of cartilage[6]. According to Thur *et al.*, mutations in this gene can elicit both pseudoachoondroplasia and epiphyseal dysplasia[15]. The *collagen, type XV alpha 1 (AA455157)* is integral to the development of skeletal muscle and small vascular structures, and *Slit homologue 3 (drosophila, AA703652)* has been linked to the formation of nervous tissue. Also, *elastin (AA459308)*, is a well-known constituent of elastic fibers. The mutations in this gene have been shown to induce supravalvular aortic stenosis or Williams' syndrome[17].

In this paper, we have assessed and reported the stage-specific patterns of gene expression which occur during the differentiation of hMSCs into osteoblasts. Future research should then be directed at the determination of the manner in which those genes play their particular roles in the process of osteoblastic differentiation.

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초록 : 인간 중간엽 줄기세포로부터 골아세포로의 분화시 관찰되는 유전자 발현 분석

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인체의 골수내에 존재하는 중간엽 줄기세포는 성장인자나 환경적 요인에 의해 지방세포, 골아세포, 연골모세포 및 연골세포 등으로 분화됨이 알려져 있다. 본 연구에서는 정상 인체의 골수으로 얻어진 중간엽 줄기세포로부터 골아세포의 분화 가능성을 알아보고 이에 관여하는 유전자의 발현을 조사하였다. 정상 골수의 중간엽 줄기세포를 골유도성 자극보조제로서 β-glycerol phosphate, L-ascorbic acid 및 dexamethasone을 첨가하여 골아세포로의 분화를 유도한 세포와 골유도성 자극보조제를 첨가하지 않은 세포를 배양하여 일정 간격으로 cDNA microarray를 이용하여 각각의 단계에서 발현되는 유전자를 검사하고 이로 인해 얻어진 유전자의 발현량을 분석하기 위해 real time quantitative RT-PCR 을 시행하였다. 골유도성 자극보조제를 첨가한 군에서 첨가하지 않은 군에 비하여 정상적인 골아세포로의 성장이 유도되었고, 분화과정에서 36개의 유전자의 발현이 증가되었고, 59개의 유전자의 발현이 억제되었다. 주로 골 생성 과정과 연관이 있다고 알려진 *osteoprotegerin, LRP5* 및 *metallothionein 2A* 등의 유전자들이 분화과정에서 발현 증가되어 나타났고, 줄기세포로부터 분화될 수 있는 조직들 중 근육, 지방, 연골, 혈관 및 신경 조직과 연관된 유전자들은 분화 후기에 감소하거나 혹은 전분화 과정 동안 발현이 억제되었다. 본 연구에서는 골아세포의 분화와 연관된 유전자 발현을 확인함으로써 특정 조건하에서 중간엽 줄기세포로부터 골아세포로의 분화가 가능함을 확인할 수 있었고, 이 과정에 관련된 특정 유전자의 발현 양상을 밝힐 수 있었다.