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Original article

Gene expression characteristics of osteoblast differentiation in human umbilical cord mesenchymal stem cells induced by demineralized bone matrix

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Background: Mesenchymal stem cells are multipotential cells capable of differentiating into osteoblasts, chondrocytes, adipocytes, tenocytes, and myoblasts. Wharton's jelly contains stem cells that are a rich source of primitive multipotent mesenchymal cells. Demineralized bone matrix (DBM) has been extensively utilized as a biomaterial to promote new bone formation.
Objective: To isolate and characterize umbilical cord mesenchymal stem (UCMS) cells derived from Wharton's jelly and examine the biological activity of DBM in this cell line.
Methods: Osteoblast differentiation of the UCMS cells was determined using alkaline phosphatase activity assay. To examine differential gene expression during osteoblast differentiation, total RNA was isolated from UCMS cells in the absence or presence of DBM on day 7 and analyzed using osteogenesis cDNA gene array. The selected genes were verified using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.
Results: Wharton's jelly derived cells could differentiate along an osteogenic lineage after treatment of DBM. Alkaline phosphatase activity assay showed that human UCMS cells could differentiate into osteogenic lineage. Gene expression of human UCMS cells treated with DBM for 7 days was analyzed by using cDNA array and RT-PCR analyses. We found that expression of runx2 and smad2 was upregulated whereas smad7 expression was downregulated as confirmed by RT-PCR.
Conclusion: UCMS cells from a Wharton's jelly of human umbilical cord could express osteogenesis genes for treatment with DBM. Wharton's jelly from umbilical cord is a new source of mesenchymal stem cells that are readily available for application to bone tissue engineering.

***Keywords*:** Demineralized bone matrix, gene expression, mesenchymal stem cells, osteoblast, RT-PCR.

Human mesenchymal stem cells (MSC) are multipotent cells which can typically differentiate into the osteogenic, chondrogenic, and adipogenic lineages [1]. Because of their multipotency and well-established *in vitro* culturing protocols, MSC have been widely used to study the osteogenic mechanism. Although some signaling pathways and gene expression have been identified in the process of osteoblast differentiation [2, 3], the comprehensive and exact molecular mechanism controlling MSC differentiation is still poorly understood.

Demineralized bone matrices (DBM) obtained from human cortical bone are prepared by removing the minerals while allowing the active bone inductive growth factors, collagenous proteins and other organic constituents to remain [4, 5]. DBM should be used in conjunction with a transplant or implant displaying mechanical strength. DBM can augment cortical grafts used for bridging gaps or defects and lengthening procedures by increasing the connectivity of the structural graft with the host bone. Another useful application is providing a biologic boost to patients who have less-than-ideal physiology. Because DBM has higher concentrations of available bone morphogenic proteins, it can aid in the incorporation of other grafts. Other uses include delayed unions, nonunions, packing joints for arthrodesis, filling resected cysts, and filling gaps of debrided infected bone. DBM are potentially attractive scaffolds for use in tissue engineering since DBM are able to support and promote osteogenesis of matrix-incorporated osteoprogenitors *in vivo*.

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The potential of DBM to induce osteoblast differentiation in mesenchymal stem cells leads to the application of DBM in biomaterials for the treatment of bone defects. In addition, the osteoprogenitor cell source can be derived from a Wharton's jelly of umbilical cord. Previous studies have demonstrated that human mesenchymal stem cells derived from Wharton's jelly comprise pluripotent or multipotent stem cells that are able to differentiate into osteoblasts [6, 7]. However, the studies and data regarding the osteoinductive activity in DBM to activating osteoblast differentiation in human UCMS cells are limited. Therefore, the objectives of this research are to determine the osteoinductive potential of DBM to induce human UCMS cells to differentiate into bone-forming cells and to investigate gene expression profiling of osteoblast differentiation in human UCMS cells using cDNA array and RT-PCR analyses.

Materials and methods

Preparation of demineralized bone matrix

Freeze-dried human cortical and cancellous bone from donors (the age of 15-65 years) was ground by impact fragmentation and separated using sized sieves. Ground bone matrix (particle size less than 1,000 microns) was demineralized by exposure to diluted hydrochloric acid. Briefly, ground bone matrix was exposed to 0.5 N HCl (100 mg DBM to 10 ml 0.5 N HCl) and demineralized bone matrices of variable calcium content were obtained by removing the bone matrix from the acid for 8 hours. The variably demineralized bone matrices were washed, freeze-dried, and stored at -80 °C.

Cell line initiation

Initiation of umbilical cord mesenchymal stem (UCMS) cells was accomplished as previously described [8] with some modifications. Human umbilical cords were obtained from full-term Caesarian section births with informed consent. Umbilical vein and arteries were removed, and the remaining tissues were washed 3 times with alpha-minimal essential medium (alpha-MEM, Gibco BRL) containing 200 units/ml penicillin and 100 μg/ml streptomycin, cut into small fragments, 1.0 mm x 1.0 mm pieces, placed on the surface of the T-25 flasks and cultured in alpha-MEM supplemented with 10 % fetal bovine serum (FBS) and penicillin (100 units/ml)/streptomycin (50 g/ml) in a 5 % CO_2 incubator at 37 °C. The outgrowing cells were combined and transferred into T-75 flasks by detachment with a 0.025 % trypsin and 0.05 % EDTA solution and cultured in the same medium and incubator. When UCMS cells reached confluence, they were split into new T-75 flasks at split ratio 1:4. Cells were continually passaged until sufficient numbers of cells had been generated to provide an opportunity to create a cell bank where cells of a uniform passage number were cryopreserved and stored for use in subsequent studies.

Cell culture and differentiation of mesenchymal stem cells

UCMS were thawed and cultured to sufficient cell numbers and seeded into T-25 flasks at 5.0 x 10^5 cells per flask (or 2.0 x 10^4 cells/cm^2). These cells were maintained in alpha-MEM supplemented with 10 % FBS and penicillin (100 units/ml)/streptomycin (50 μg/ml) until reaching confluence. The alpha-MEM with 10 % FBS was then changed to alpha-MEM supplemented with 2 % FBS in the absence (as a control group) or presence (as a DBM group) of 5 mg DBM. Alkaline phosphatase activity was subsequently assayed at days 0, 3, 5, 7, and 10.

Alkaline phosphatase activity

Alkaline phosphatase activities were determined using a modification of the procedure as previously described [8]. Basically, treated cells were quickly rinsed twice with 3 ml of deionized water, scraped with a cell scraper (Fisher Scientific, Pittsburgh, PA) in 3 ml of deionized water and sonicated at 30 % intensity for 30 seconds. Next 200 μl aliquot samples were transferred to each well of a 96-well plate and were mixed with 40 μl of 100 mmol/ml p-nitrophenyl phosphate in 0.15 M 2-amino-2-methyl-1-propanol buffer, pH 10.4, and incubated for 20 minutes at 37 °C. The reaction was stopped by the addition of 10 μl of 10 N NaOH into each well and absorbance determined at 405 nm using a microplate reader (Multiskan Ascent, Labsystems, Franklin, MA). Alkaline phosphatase activity (expressed as pmols of converted p-nitrophenol/min) was normalized by total protein content. Protein concentrations of the samples were determined by using the BCA protein assay (Pierce, Rockford, IL). The alkaline phosphatase activity was expressed as pmols of p-nitrophenol/min/ g of protein).

Isolation of RNA

Total RNA was prepared from UCMS cells after 7 days of culture with or without DBM using the Rneasy Mini Kit (Qiagen, USA) according to the instructions provided by the manufacturer. The purity and amount of isolated RNA were assessed by spectrophotometric measurement at 260 and 280 nm.

cDNA array hybridization and signal analysis

A cDNA probe was synthesized from total RNA isolated as described above. Equal amounts of total cellular RNA (5 μg) were reverse transcribed to cDNA at 42 °C for 90 minutes in a volume of 20 μl containing the following reagents: 3 μl GEA primer mix (Buffer A); 4 μl 5x GEA labeling buffer (Buffer BN); 1 μl RNase inhibitor (Promega, Madison, WI), 2 μl 1 mM Biotin-16-dUTP (Roche), and 50 U MMLV reverse transcriptase (Promega, Madison, WI). After terminating the reaction by adding 2 μl 10x of stop solution (Buffer C), the biotin labeled cDNA probes were denatured by heating at 94 °C for 5 minutes and chilled quickly on ice. Human osteogenesis GEArray Q series cDNA expression array (Super Array Bioscience, USA) nylon membranes, containing over 96 cDNA fragments from genes associated with bone development, were hybridized with the biotin labeled cDNA probes. The membranes were washed, scanned, and analyzed using ScanAlyze and GEArray Analyzer software. Genes with ratio number above 2 or under 0.5 (2 fold induction or repression) were selected and subjected to further analysis. Each signal was normalized against the signal of a housekeeping gene to compare data from two different arrays. The relative specific intensity within each signal was calculated as the ratio of the density of each signal in the DBM treatment divided by the density of the corresponding signal in the control.

RT-PCR analysis

RNA was isolated from UCMS cells that were cultured with or without DBM for 7 days and was reverse transcribed to cDNA at 42 °C for 60 minutes in a volume of 20 μl containing the following reagents: 0.5 mM dNTP mix; 1x ImProm-II Reaction Buffer; 6 mM $MgCl_2$; and 20 U of Recombinant RNasin Ribonuclease Inhibitor. The reactions were then terminated at 70 °C for 15 minutes. Aliquots of the cDNA were amplified in 100 μl of a PCR reaction mixture which contained 1 μM primer sets, 1x thermophilic polymerase reaction buffer (BioRad), and 5 U of iTaq DNA polymerase (BioRad). Using a thermocycler, DNA amplification was performed including an initial denaturation at 94 °C for 2 minutes, followed by 25 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute, and extension at 72 °C for 2 minutes. The final cycle included 5 minutes for extension. Glyceraldehyde phosphate dehydrogenase (gapdh) primers were added in all reactions as an internal control. Furthermore, cDNAs were also amplified using specific primers (**Table 1**) by RT-PCR to generate products corresponding to mRNA encoding for runx2, smad2, smad7, and gapdh after 7 days of culture.

Detection of PCR products

The PCR products were analyzed on 2 % agarose gels and visualized with ethidium bromide. The density of each band was quantified using NIH Image software. The relative gene expression was determined by dividing the densitometric value of treatment by that of the control.

Table 1. Oligonucleotide primers used for RT-PCR analyses.

No.	Name	Sequences (5' -3')	product	Tm
1.	runx2 (cbfa1)	Foward : 5' CCCCACGACAACCGCACCAT 3' Reverse: 5' CACTCCGGCCCACAAATC 3'	270 bp	64 °C
2.	smad2	Foward : 5' AGAGAGTTGAGACACCAGTTTTGC 3' Reverse: 5' ATAGTCATCCAGAGGCGGAAGTT 3'	86 bp	60 °C
3.	smad7	Foward : 5' GAATCTTACGGGAAGATCAACCC 3' Reverse: 5' CGCAGAGTCGGCTAAGGTG 3'	67 bp	60 °C
4.	gapdh	Foward : 5' ACCACAGTCCATGCCATCAC 3' Reverse: 5' TCCACCACCCTGTTGCTGTA 3'	370 bp	60 °C

Statistical analysis

The data were expressed as means with error bars representing standard deviation (SD). Analysis of variance (ANOVA) was used to determine the significant differences among treatment groups. An unpaired *t* test was performed to determine the significant difference between control and experimental groups. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Passage 3-5 of UCMS cells was used for 0, 3, 5, 7, and 10 days of culture with DBM. **Table 2** demonstrates the alkaline phosphatase activity of both the control and DBM treated cell cultures. During the first 5 days of cell culture, the alkaline phosphatase activity in the UCMS cells did not significantly change for the control or DBM groups. There was a significant difference only in the DBM treated group on day 7. After reaching a maximum at day 7 in the DBM treated culture flasks, the alkaline phosphatase activity decreased at day 10. Additionally, the control cells possess a spindle shape whereas the DBM treated cells appeared shortened and flattened after 7 days of incubation (data not shown).

Table 3 represents typical hybridization results where the cDNA probe was derived from non-treated cells and from DBM treated cells. Using a cutoff value of two fold, which is conventionally used in similar studies [9-11], the highly upregulated genes were runx2, vdr, tgfb2, cd36, flt1, and smad2. The downregulated genes (level of gene expression < 1.0) are shown. Among downregulated genes, smad7 displayed a >2-fold decrease in the level of transcript.

Table 2. Alkaline phosphatase activity in UCMS cells by DBM with various time points.

Day	Alkaline phosphatase activity (*p* mol/min/ g protein)	
	Control	DBM
0	1.76 ± 0.36	1.76 ± 0.36
3	2.12 ± 0.40	2.08 ± 0.44
5	0.99 ± 0.45	2.05 ± 0.26
7	2.12 ± 0.35	87.75 ± 12.2*
10	2.68 ± 0.38	2.34 ± 1.35

*$P<0.0001$

Table 3. Representative gene expression 7 days after culture.

Level of gene expression (Ratio DBM/Control)	Genes
>2.0	runx2, vdr, tgfb2, cd36, flt1, smad2
1.0-2.0	itga1, smad4, col16a1, pdgfa, egf, spp1, itga2, igf2, col4a3, fn1, col11a1, egfr, sparc, itgb1, msx1, col4a5, col5a1, col12a1, mmp10, ctsk, tnf, anxa5, nfkb1, col10a1, smad1, bgn, bmp6, smad5, alpl, itga3, dcn, sox9, smad9, col4a4, csf3, igf1, mmp9
<1.0	tgfbr2, col15a1, rsl1d1, bmp5, scarb1, col18a1, fgfr2, itgav, smad7, bmp4, col17a1, igf1r, vcam1, mmp8, bmp8b, bglap, tgfb1, arse, twist1, msx2, bmp7, fgf1, smad6, itgam, col19a1, col9a2, fgfr3, col1a1, bmp3, gdf10, bmpr1a, fgf2, col2a1, icam1, fgfr1, col7a1, csf2, col14a1, casr, col3a1, bmp2, fgf3, bmp1

To verify the cDNA array results, RT-PCR analysis was performed using RNA prepared under the same condition used in cDNA array studies. Among the upregulated genes, runx2 and smad2 found to be highly upregulated gene were selected. Gene encoding for smad7 was chosen for a downregulated gene. A housekeeping gapdh gene, whose expression was unchanged during osteogenic differentiation, was chosen to serve as an internal control. RT-PCR analysis of parallel samples was consistent with the cDNA array data in that there was an increase in the level of runx2 and smad2 expression after day 7 of DBM treatment as shown in **Fig. 1**. Also, in agreement with the cDNA array analysis, expression of the gene encoding for smad7 decreased after day 7 of DBM addition (**Fig. 1**). **Figure 2** shows the normalized gene expression levels of RT-PCR bands for runx2, smad2 and smad7. There was a significant increase in the expression level of runx2 (0.565 ± 0.018 vs. 0.276 ± 0.018, $P<0.001$) and smad2 (0.948 ± 0.082 vs. 0.714 ± 0.021, $P<0.001$) after day 7 of DBM treatment in comparison with the control. Expression of the gene encoding for smad7 in the DBM group was lower than that of the control (0.575 ± 0.018 vs. 0.646 ± 0.017, $P<0.05$). The RT-PCR results demonstrated identical expression patterns for selected genes as revealed by cDNA array analysis.

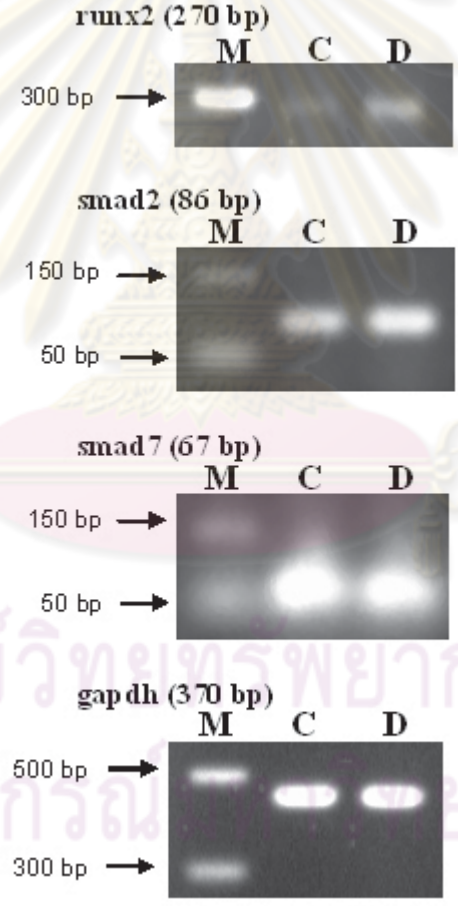

Fig. 1 RT-PCR showing the upregulation of runx2 and smad2 and the downregulation of smad7 in UCMS cells. Gapdh was used as an internal control. (M=Molecular weight marker, C=Control, D=DBM addition).

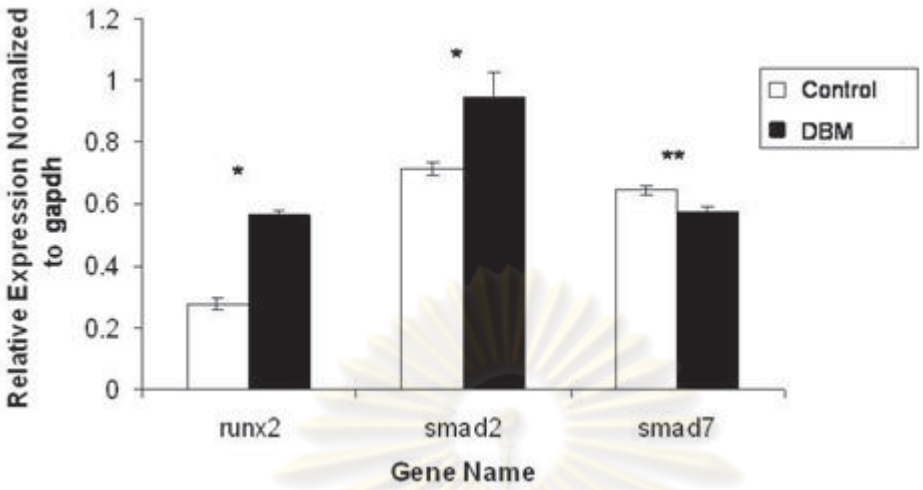

Fig. 2 Densitography of the gel electrophoresis from runx2, smad2, and smad7 mRNA expression of UCMS cells in the absence (control) or presence of DBM on day 7. The data were normalized with that of the housekeeping gene gapdh. **P*<0.001, ***P*<0.05: control *vs* the DBM group.

Discussion

The mesenchymal cell induction process particularly associated with osteoinductivity of DBM is assessed by changes in the alkaline phosphatase activity of cells being used in the study. The changes of alkaline phosphatase activity over time were studied for the purpose of examining the effects of DBM on human umbilical cord mesenchymal stem cell induction. The alkaline phosphatase activity did not change during the first 5 days of cell culture. The DBM treated cell cultures had significantly higher alkaline phosphatase activity than the comparable control cell cultures on day 7. The alkaline phosphatase level declined to base line level on day 10. This decline was presumably associated with a decrease in available nutrients and a reduction in cell metabolism.

In this study, gene expression profiling osteogenic differentiation in human UCMS cells was also examined. Osteogenic differentiation is a complex process and involves distinct genotypic changes that are accompanied by specific phenotypic alterations. Recent advances in cDNA gene expression array technology allow for simultaneous monitoring of a number of known and unknown genes in parallel. cDNA expression arrays are rapidly becoming the method of choice to investigate gene expression profiles in various specialized systems. In the current study, human osteogenesis gene arrays were utilized to differentially display bone-related genes that may play crucial roles in osteoblast development. This study investigated the expression of over 96 genes involved in the regulation of osteogenic differentiation.

Among the upregulated genes during osteogenic differentiation, runx2 coding for transcription factor protein is a prominent osteoblastic marker that has been associated with the development and function of osteoblasts and has been shown to play an important role in bone formation. Interestingly, vdr, tgfb2, cd36, flt1, and smad2 were also upregulated. Vitamin D receptor (VDR) exerts biological effects via an interaction of the vitamin D and plays a central role in the regulation of the early stages of human osteoblast differentiation [12]. TGF-β2, one of TGF-β isoforms within bone matrix, modulates the differentiation of osteoblasts and the proliferation of osteoprogenitor cells [13]. CD36 has been known as a marker of progenitor for erythroid and myeloid lineages in human bone marrow and its function in osteoblast differentiation remains unclear. FLT-1, vascular endothelial growth factor receptor, plays an important role in the regulation of bone remodeling by attracting endothelial cells and stimulating osteoblast differentiation [14]. In contrast, expression of the gene encoding for Smad7 was downregulated. This decrease might have implications during the process of bone development because Smad7 is inhibitory in the bone morphogenetic protein signaling pathway.

Although bone is a highly specialized tissue, osteoblasts are very similar to fibroblasts in terms of cell morphology and gene expression [15, 16]. A characteristic feature of osteoblasts is the secretion and processing of extracellular matrix. The most

dramatic changes were observed in the cDNA array experiments for extracellular matrix genes, particularly the upregulation of runx2 or core binding factor a-1 (cbfa-1). Runx2 is an osteoblast-specific transcription factor that plays a role in the transcriptional process and can promote *in vitro* osteoblast differentiation [17]. Runx2 is thought to be the initial gene expressed during the differentiation process of osteoblasts [18]. The results of cDNA array analysis, and subsequent RT-PCR analysis of differentiating UCMS cells after exposure to DBM, revealed the upregulation of runx2 involved in osteoblast differentiation.

Osteoblasts are the cells that build and repair bone and Runx2 has a crucial role in osteoblast differentiation, function and consequently in bone biology. Runx2 at the early stage of embryogenesis determines the osteoblast lineage from multipotent mesenchymal stem cells, whereas it is inhibited at the late stage [19]. Ectopic expression of Runx2 in mesenchymal cell lines leads to up-regulation of osteoblast-specific genes like osteocalcin, alkaline phosphatase, collagenase-3 (matrix metalloproteinase-13, MMP-13), bone sialoprotein and collagen type Ialpha1 [20]. Mice with a mutated runx2 locus have a complete lack of ossification and both intramembranous and endochondral ossifications are totally blocked due to the maturational arrest of osteoblasts [21]. Accordingly, runx2-knockout mice display an absence of osteoblasts and bone, while the heterozygous mice show specific skeletal abnormalities that are characteristic of human heritable skeletal disorder [22].

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily, that includes TGF-βs, activins, inhibins, and Mullerian inhibiting substance [23, 24]. BMPs play multiple roles in the regulation of growth, differentiation, and apoptosis of various cell types. They exhibit important *in vivo* functions during embryonal development and tissue morphogenesis, including bone and cartilage formation. Members of the TGF-β superfamily exert their biological effects via binding to two types of serine/threonine kinase receptors (type I and type II), both of which are required for signaling activity. The type II receptor transphosphorylates the type I receptor, leading to the activation of intracellular substrates, including Smad (Sma and Mad) proteins. Since the type I receptor acts as a downstream component of the type II receptor, specificities of the intracellular signals are determined by the type I receptor. Smad proteins are signal transducers for the serine/threonine kinase receptors.

Eight Smad proteins (Smads 1 through 8) have thus far been isolated in mammals [25, 26]. Smads can be classified into three subtypes by structure and function, i.e., receptor-regulated (or pathway-restricted) Smads (R-Smads), common-mediator Smads (co-Smads), and inhibitory Smads (anti-Smads). R-Smads are the prototype of Smad proteins, which can be further classified into those activated by BMP receptors and those activated by TGF-β and activin receptors. Smad1, Smad5, and Smad8 (originally termed MADH6) are R-Smads activated by BMP receptors, whereas Smad2 and Smad3 are R-Smads activated by TGF-β and activin receptors. Smad4 is the only co-Smad identified in mammals. Smad6 and Smad7 are anti-Smads.

In the present study, the expression level of Smad2 increased markedly after DBM treatment suggesting that Smad2 mediates BMP signaling as downstream mediators in UCMS cells. It has been shown that Smad2 mediates TGF-β1 signaling as downstream mediators in rat bone marrow-derived mesenchymal stem cells [27]. In addition, altered expression of Runx2/Cbfa1 through upregulated TGF-β/Smad2 signaling may contribute to the impaired osteogenic differentiation of bone marrow stromal stem cells [28]. Smad proteins transduce signals from TGF-β superfamily ligands that regulate cell proliferation, differentiation and death through activation of receptor serine/threonine kinases. Along with other Smads, Smad2 are rapidly and specifically phosphorylated by TGF-β [29-31], and translocated to nuclei to be involved in regulation of gene expression. Smads are highly conserved across species and share conserved amino- and carboxyl-terminal regions termed MH1 and MH2 domains, respectively [32, 33]. The main active domains of the Smad proteins appear to be located in the carboxyl-terminal MH2 region. The activities of the MH2 domain are masked by the presence of an amino-terminal MH1 domain, whereas they are unmasked upon the removal of the inhibition by the MH1 domain upon activation, possibly by phosphorylation [33].

Taken together, the results of this study reveal for the first time that DBM induced the gene expression of osteoblast differentiation in UCMS cells by using cDNA gene array analysis. An observation of this study revealed that expressions of runx2

and smad2 were highly upregulated whereas the expression of smad7 was downregulated in the DBM treated cultures compared with the non-treated cultures. Gene arrays are rapidly becoming the method of choice to examine gene expression profiles in various specialized systems and to understand new bone-related genes which may play essential roles in osteoblast development.

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The authors have no conflict of interest to report.

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