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New Gene Groups Associated with Dissimilar Osteoblastic Differentiation Are Linked to Osteosarcomagenesis

FREDERICK LUK[1], YAN YU[1], HAI-TAO DONG[1†], WILLIAM R. WALSH[1] and JIA-LIN YANG[2]

[1]*Surgical and Orthopedic Research Laboratories, Prince of Wales Clinical School, Faculty of Medicine, University of New South Wales, Sydney, NSW, Australia;*
[2]*Surgical Oncology Research Group, Department of Surgery, Prince of Wales Clinical School, Faculty of Medicine, University of New South Wales, Sydney, NSW, Australia*

Abstract. *Background: Osteosarcoma has been recently redefined as a differentiation disease and its investigation is hampered by broad and complex genetic alterations. Gene expression analysis of two human osteosarcoma cell lines that are dissimilar in tumour differentiation status and osteogenic property would advance our understanding of osteosarcomagenesis. Materials and Methods: Gene ontology classification, hierarchical clustering, functional annotation analysis and inspection of transcription factors and their targets were used to examine differences between Saos-2 and U-2 OS cells. Microarray data were verified with real-time quantitative PCR and immunocytochemistry. Results: Genes from cell binding, cell adhesion and nervous system, as well as some well-known factors of bone formation and osteoblast characterization were identified as being differentially altered in this study. Conclusion: The osteogenicity of osteosarcoma or the disrupted osteoblast differentiation is correlated to cell binding, cell adhesion and the nervous system, as well as the osteogenic signalling system.*

Osteosarcoma is a malignant mesenchymal tumour and a common primary tumour of bone. It is frequently localized at the distal femur and proximal tibia region where rapid bone growth occurs. This disease mainly occurs in adolescence and the elderly, and it is the second highest cause of cancer-related death after lymphoma in the paediatric age group (1). Osteosarcoma has complex tumorigenesis, tumour progression and metastatic processes. Various cytogenetic and genetic abnormalities have been identified in osteosarcoma, including (i) dysregulation of tumour suppressor genes (tumor protein p53 (*TP53*), retinoblastoma 1 (*RB1*)), cyclin-dependent kinase (*CDK*) inhibitors ($p16^{INK4a}$, $p14^{ARF}$) and some signalling pathways (Wnt, Shh); (ii) activation of oncogenes (v-myc myelocytomatosis viral oncogene homolog (avian) (*MYC*), jun proto-oncogene (*JUN*), FBJ murine osteosarcoma viral oncogene homolog (*FOS*), v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (*ERBB2*)) and (iii) overexpression of certain types of genes/proteins (met proto-oncogene (*MET*), matrix metallopeptidases (MMPs), S100 calcium binding protein A6 (*S100A6*)) (1, 2). However, the process of osteosarcomagenesis remains unclear.

It has been recently suggested that osteosarcoma can be regarded as a differentiation disease, such that any disruption along the differentiation of mesenchymal stem cells to osteoblasts will lead to the development of osteosarcoma, thus making the treatment and the study of osteosarcoma very difficult (1, 3). Studies have demonstrated that restoring differentiation and/or overcoming differentiation defects was able to partially regulate the tumourigenicity of osteosarcoma cells. Hence, this has been exploited as a therapeutic approach combined with current osteosarcoma therapies (4-6). Current studies are focusing on osteogenic factors/regulators and transcription factors. For example, restoring expression of a well-known bone growth mediator, runt-related transcription factor 2 (*RUNX2*), and its downstream transcription factor, osterix (*SP7*), reduced tumourigenicity and increased antitumour properties in osteosarcoma cells (7, 8). However, treatment with other popular bone growth stimuli bone morphogenetic proteins (BMPs) did not assist restoration or promotion of terminal osteoblastic differentiation, but rather enhanced tumourigenicity of osteosarcoma (3). The controversial results further support the notion that osteosarcomagenesis is a complex process and may involves more genes or gene groups, thus more

†Visiting scholar from Institute of Biotechnology, Zhejiang University, Hangzhou, Zhejiang 310029, P.R. China.

Correspondence to: J.-L. Yang, Surgical Oncology Research Group, Department of Surgery, Prince of Wales Clinical School, Faculty of Medicine, University of New South Wales, Sydney, NSW, Australia. Tel: +61 293823319, Fax: +61 293822629, e-mail: j.yang@unsw.edu.au

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investigation of the relationship between osteoblastic differentiation and osteosarcoma is required.

Human osteosarcoma cell lines have been widely used in osteosarcoma related studies. These cell lines are osteoblast-like cells and known to be at different differentiation states, which have some distinctive characteristics. For example, several studies have shown that the two commonly used osteosarcoma cell lines, Saos-2 (more-differentiated) and U-2 OS (less-differentiated), expressed different level of osteogenic or osteoblastic factors, such as BMPs and Smads (3, 5, 9, 10). Our previous study also demonstrated that implantation of Saos-2 cells into the thigh muscle of nude mice induced ectopic bone formation, but a xenograft tumour was formed with the implantation of U-2 OS cells (9). Due to limitations of available technologies, previous studies were limited to small numbers of genes. Therefore, we hypothesized that studying the global gene expression of the two human osteosarcoma cell lines (Saos-2 and U-2 OS) with dissimilar differentiation status would enhance the understanding of the association between osteoblast differentiation and osteosarcoma tumorigenesis.

In this study, Affymetrix™ microarray, real-time PCR, protein/DNA array and immunocytochemistry were employed to study the gene profiles of the two human osteosarcoma cell lines, Saos-2 and U-2 OS. Identification of significantly and differently expressed genes will provide insight into the genetic attributes in relation to osteosarcoma differentiation and osteogenic properties, as well as advance our understanding of osteosarcomagenesis.

Materials and Methods

Cell lines and culture media. All culture media and reagents were purchased from Invitrogen (Carlsbad, CA, USA) unless otherwise stated. Human OS cell lines, Saos-2 and U-2 OS, and mouse myoblast cell line C2C12, were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and the normal growth medium was composed of high glucose DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin (100 unit/ml)/streptomycin (100 μg/ml) solution. All cultures were maintained at 37˚C regulated with 5% CO_2 in humidified air.

Cell conditioned media (CM) was prepared by replacing the growth medium with the high glucose DMEM (Invitrogen) at confluence and then culture for a further 3 days. The Saos-2 and U-2 OS CMs were collected, subsequently filtered (0.2 μm) to remove cellular debris, and diluted in the ratio of 1:1 and 1:3 with fresh high glucose DMEM and supplemented with 10% (v/v) FBS and penicillin (100 unit/ml)/streptomycin (100 μg/ml) solution for used in alkaline phosphatase activity and mineralization assay.

Osteogenic medium (OM) was prepared with the high glucose DMEM supplemented with 15% v/v FBS, 1X antibiotic-antimycotic [penicillin G (100 unit/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml)], dexamethasone (100 nM), L-ascorbate-2-phosphate (50 μM) and β-glycerophopshate (10 mM).

Detection of alkaline phosphatase (ALP) activity. C2C12 cells were cultured until confluence and replaced with fresh growth medium, diluted Saos-2 or U-2 OS CM, or OM and then cultured for a further 3 days. The cultured cells from each condition were washed twice with ice-cold 0.9% (w/v) NaCl buffered solution and lysed with 0.1% (v/v) Triton-X in 0.1 M Tris-HCl buffer (pH 9.8) for 10 min at –20˚C. Total cell lysate was collected and centrifuged at 10,000 rpm for 10 min at 4˚C. The supernatant was collected in a pre-chilled tube and total protein was quantified by DC Protein Assay (BioRad Laboratories, Hercules, CA, USA). ALP activity was assessed by measuring absorbance of 620 nm at room temperature (RT) in diethanolamine-$MgCl_2$ buffer (1 M diethanolamine, 1 mM $MgCl_2$, 3 mM NaN_3, pH 9.8) using *p*-nitrophenylphosphate (Sigma-Aldrich, St. Louis, MO, USA) as the substrate (3.8 mM) at 5 min intervals over a period of an hour. ALP activity was expressed as nanomole *p*-nitrophenol/mg of protein/min.

Detection of calcium deposition and mineralization. C2C12 cells were cultured until confluence and replaced with either fresh growth medium, or diluted Saos-2 or U-2 OS CM, or OM and then cultured for a further 42 days. The cultured cells were washed with phosphate-buffered saline (PBS) twice and fixed with 4% (v/v) formaldehyde for 15 min at RT. Excess fixative agent was rinsed off with deionized water. *(i) Alizarin red S staining:* Alizarin red S staining solution (40 mM) was added to the fixed cells, which were then incubated for 20 min at RT. Excess staining solution was rinsed off with deionized water. The number of positively stained, orange-red, calcium nodules were counted and recorded under a light microscope within 30 min after staining.

(ii) von Kossa's staining: Silver nitrate solution (1% w/v) was added to the fixed cells, which were then incubated under UV light for 30 min at RT and rinsed with deionized water. Sodium thiosulfate solution (5% w/v) was added and cells incubated for 5 min at RT before rinsing with deionized water. Stained cells were counterstained with nuclear fast red solution (0.1% w/v) for 5 min at RT and rinsed with deionized water, then serially dehydrated in 70% (v/v), 95% (v/v), and 100% (v/v) ethanol. The cells were dried and the number of positively stained, black, mineral nodules formed around the cells were counted and recorded under a light microscope.

RNA preparation and Affymetrix microarray processing. Saos-2 and U-2 OS cells were cultured for 5 days to reach confluence and their total RNAs were extracted using TRI Reagent® (Ambion Inc., Austin, TX, USA) in accordance with the manufacturer's protocol. Extracted RNAs were treated with DNase I (Invitrogen) to remove chromosomal DNA. The quantity and purity of RNA products were respectively determined by optical density measurement at 260 nm and the ratio of measurements 260/280 nm and 260/230 nm (Nanodrop ND-1000; Nanodrop Technologies, Wilmington, DE, USA). The quality of RNA products was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA) before the microarray experiment.

One-cycle target RNA labeling, hybridization (Affymetrix Fluidics Station 450), scanning (GeneChip® Scanner 3000) and raw data acquisition of the Affymetrix GeneChip Human Genome U133 plus 2.0 Arrays were performed by the Clive and Vera Ramaciotti Centre for Gene Function Analysis (UNSW, Sydney, Australia) following a standard procedure from Affymetrix.

Affymetrix microarray gene expression profiling. (i) Data processing: Fluorescence signal intensities data (Affymetrix CEL data files) and 'present/marginal/absent' call data (Affymetrix CHP data files) were

processed with GeneSpring GX 10.0.2 (Agilent Technologies Inc.). Differentially expressed genes were identified according to the guided workflow in the program. Briefly, probe level intensity data were processed according to the 'Robust Multichip Average' (RMA) algorithm for background adjustment, normalization and log-transformation of the perfect match values and then normalized to median for each gene before further analysis. Normalized data below the 20th percentile were removed and the independent sample (unpaired/asymptotic computation) *t*-test with Benjamini-Hochberg correction was used to distinguish genes that were statistically significantly differentially expressed ($p<0.05$) between the triplicate experiments of the two human OS cell lines. A list of the differentially expressed genes was generated by selecting genes with more than 3-fold expression changes and with 'present' call only.

(ii) Gene ontology and clustering analyses for data mining: Genes identified from the microarray analyses were subjected to gene ontology (GO) classification and then functional annotation clustering by the DAVID Bioinformatic Resources 2006 (National Institute of Allergy and Infectious Diseases, National Institute of Health, Frederick, MD, USA; http://david.abcc.ncifcrf.gov/tools.jsp) with the default 13 annotation categories, including GO terms, protein–protein interactions, bio-pathways and gene functional summaries. Unsupervised hierarchical cluster analysis with 'Euclidean Distance' was performed to identify specific genes. The gene expression profile identified from the microarray analyses was used to compare with the gene expression profile of mesenchymal stem cells, adipocytes, chondrocytes, fibroblast and osteoblasts obtained from the Gene Expression Omnibus (National Centre for Biotechnology Information (NCBI); http://www.ncbi.nlm.nih.gov/geo/) with accession number GSE9451 (11). A list of significantly differentially expressed genes was obtained by comparing the result acquired from the functional annotation clustering with the expression hierarchical clustering.

Microarray gene expression verification. (i) Real-time quantitative RT-PCR: Total RNA was reverse transcripted into cDNA according to the Superscript™ III first-strand synthesis system (Invitrogen). Real-time quantitative PCR (qRT-PCR) was performed in a Stratagene Mx3005P™ QPCR System. The primers were purchased from Sigma Life Science® (Sigma-Aldrich) and ABsolute™ Blue QPCR SYBR® Green Mix Plus Rox Vial (ABgene, Epsom, UK) was used for the PCR reactions.

(ii) Immunocytochemistry: All chemicals used in this section were purchased from Sigma-Aldrich unless otherwise stated. Cells were cultured in an 8-well chamber slide and fixed with 3.7% v/v formaldehyde in PBS containing 0.1% v/v Triton X-100 for 30 min at room temperature, followed by quenching with 0.3% v/v H2O2 and 50% v/v methanol in Milli-Q water for 10 min at room temperature. The chambers were washed twice with PBS and protein-specific primary antibody (0.1 μg/100 μl) diluted with 1% bovine serum albumin (BSA) in PBS was added for overnight incubation at 4˚C. The chambers were washed three times with 0.25% Tween-20 in PBS (PBST) for 5 min and horseradish peroxidise (HRP)–conjugated polymers (anti-rabbit or anti-mouse) from the EnVision™ system (DAKO, Glostrup, Denmark) was added for 60 min incubation at room temperature, followed by washing four times with PBST for 5 min. The reaction was visualized by incubating the slide with DAB+ chromogen staining buffer system (3,3'-diaminobenzidine in chromogen solution; DAKO) for 30 min at room temperature. The cells were then counterstained with filtered Harris's haematoxylin.

Protein/DNA array analysis and data mining. Nuclear protein was extracted and combined with labelled oligonucleotides to form protein/DNA complexes. Oligonucleotides were eluted from the complexes and hybridized onto TranSignal™ Protein/DNA Array I (Panomics Inc., CA, USA) according to the product manual. Information for the 54 distinct consensus binding elements on the array is available from Panomics (http://www.panomics.com). A chemiluminescence procedure with Hyperfilm ECL (Amersham Pharmacia Biotech, Uppsala, Sweden) was used to detect the signals on the array. Exposed films were imaged using UVI-Doc Mw version 99.03 (UVItec, Cambridge, UK). Information of genes associated with each transcription factor was obtained from the array product manual and the NCBI database (http://www.ncbi.nlm.nih.gov/). Information of genes that are regulated and targeted by a particular transcription factor was obtained from the Cold Spring Harbor Laboratory, 'Transcriptional Regulatory Element Database' (TRED) (http://rulai.cshl.edu/TRED).

Statistical analysis. Significantly differentially expressed genes between the two osteosarcoma cell lines from Affymetrix microarray experiments were initially selected using an independent *t*-test with Benjamini-Hochberg correction (GeneSpring GX 10.0.2 Program). The difference of ALP activity, calcium deposition and mineralization in C2C12 were analysed by unpaired (two-tail) Student's *t*-test (GraphPad Prism™). Statistical values of $p<0.05$ were considered significant.

Results

Confirmation of osteogenic induction by Saos-2. ALP activity and the degree of calcium deposition and mineralization are markers often used to show osteoblast phenotypic characteristics and bone formation. As shown in Figure 1A, the ALP activity of C2C12 was stimulated after culturing in the Saos-2 conditioned medium for 6 days and the formation of mineralized nodules (Figure 1B and 1C) in C2C12 was also increased after culturing for 42 days when compared to the normal growth medium and the osteogenic medium. These effects were concentration-dependent, as the concentration of Saos-2 conditioned medium increased from 25% (1:3) to 50% (1:1) the effects were enhanced. However, there were no significant changes for the C2C12 cells cultured in the U-2 OS-conditioned media. This result demonstrated that the factors in the conditioned media released by Saos-2 were able to induce the osteogenic characteristic of C2C12 and this was enhanced when the concentrations of these factors were increased. However, the factors in the conditioned media released by U-2 OS showed no or little effects on the osteogenic characteristic of C2C12. These results confirmed that the two osteosarcoma cell lines with the dissimilar differentiation status were still different in their osteogenic induction ability and, thus, suitable to be used for further analysis.

Identification of 75 genes significantly and differentially expressed in the dissimilar human osteosarcoma cell lines. Triplicate experiments on gene expression for each osteosarcoma cell line were performed in this study. Each data

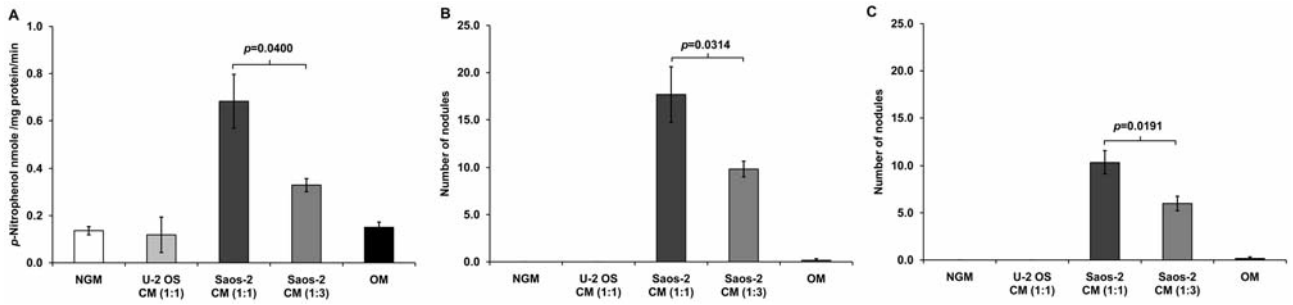

Figure 1. *Osteogenic induction properties of Saos-2 and U-2 OS cells. Subconfluent mouse myoblast cells, C2C12, were cultured for 6 days in the Saos-2 or U-2 OS conditioned medium (CM), normal growth medium (NGM) or osteogenic medium (OM) for quantitative analysis of alkaline phosphatase activity (A). Cells cultured for 42 days were used for quantitative analysis of calcium deposition (B) and mineralization (C). Different proportions of Saos-2-conditioned media were also tested. Triplicate (A) or duplicate (B and C) experiments with triplicate samples in each were performed and data are shown as mean values with error bars of standard deviation.*

set was confirmed with the criteria in the 'Affymetrix - Data Analysis Fundamentals Manual' for a good microarray experiment, including (i) scaling factors did not differ more than 3-fold, (ii) the signal background level was less than 100, (iii) the noise level did not differ more than 3-fold, and (iv) the 3'/5' GAPDH ratio was not more than 3, of all the different arrays in the comparison. The signal intensity of the genes in the six microarrays is shown in the scatter diagrams to demonstrate the consistency among the two groups of samples (Figure 2). Moreover, after normalization and transformation of the microarray data, expression of the spike control and box-whisker plot of signal intensity was used to further assess and confirm the quality of data among the six microarrays.

The gene expression profile was compared between Saos-2 and U-2 OS. In the comparison, U-2 OS was used as a baseline to identify any gene alteration in Saos-2. After the processing of data, 1,968 genes were selected as being differentially expressed and the distribution of these genes is shown in Figure 3. GO classification of these differentially expressed genes demonstrated that 162 biological processes, 26 cellular components and 35 molecular functions were involved with expression analysis systematic explorer (EASE) score less than 0.05 ($p<0.05$). Further selection with false discovery rate (FDR) less than 0.05 had showed that 12 biological processes, such as cell and organ development, cell differentiation and cell adhesion, and 1 molecular function, such as protein binding were highly related to the selected genes.

Functional annotation clustering was also used to categorize and group the differentially expressed genes by a novel algorithm that measures the relationships of the annotations involved and ranks them based on the 'enrichment scores'. Four different annotation clusters were selected with enrichment score more than 3, which contained 628 unique genes in total. The first cluster contained genes that are related to cell or organ development and cell differentiation. The second cluster contained genes that are related to cell adhesion and the 'cadherin' class of adhesion proteins. The third cluster contained genes that are related to negative regulation of cells. The fourth cluster contained genes that are related to structural patterning process. Additionally, hierarchical clustering analysis of signal intensity had identified ten clusters of 210 genes with a discrete expression pattern that was similar to that of osteoblast and chondrocyte.

Finally, the gene list obtained from functional annotation clustering analysis was matched with the gene list from the hierarchical clustering analysis. A total of 75 genes (46 up- and 29 down-regulated genes in Saos-2) were identified in both analyses and denoted as being significantly and differentially expressed genes in this study, as shown in Figure 4. Among these 75 genes, 27 (36%) are transcription factors, 15 (20%) are well-known bone-related factors or markers, other genes have known function in cell surface, nervous system and cell developments, and some other cellular functions.

Verification of microarray data by real-time PCR and immunocytochemistry. Five representative bone-related genes alkaline phosphatase liver/bone/kidney (*ALPL*), distal-less homeobox 5 (*DLX5*), fibroblast growth factor receptor 2 (*FGFR2*), *RUNX2* and *SP7* from 75 differentially regulated genes, and three other genes bone gamma-carboxyglutamate (gla) protein/osteocalcin (*BGLAP*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and runt-related transcription factor 3 (*RUNX3*), were selected to validate the microarray data by qRT-PCR. To normalize the qRT-PCR data, a relative expression value was obtained by comparing each gene expression with the β-actin transcript. Similarly, in the microarray, the expression of the selected genes was compared with the β-actin transcript to obtain a relative expression value. The β-actin-normalized relative expression ratio of the eight selected gene are presented in Figure 5, and the results showed that the expression trends of the selected genes were consistent in between the microarray and the quantitative RT-PCR.

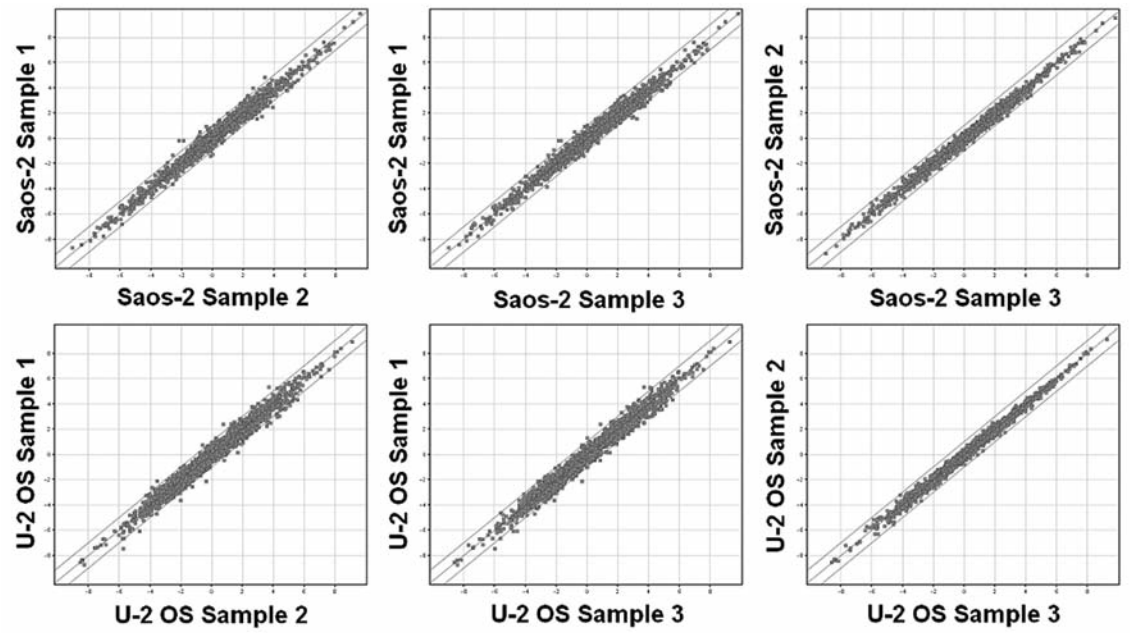

Figure 2. *Scatter plots of the signal intensity of genes on microarray. Quality of microarray hybridization was assessed by the distribution of signal intensity between triplicate experiments (labelled as sample 1, 2, and 3) of the two experimental groups (Saos-2 and U-2 OS). The graphs showed that most of the genes were within 2-fold change differences between the compared samples.*

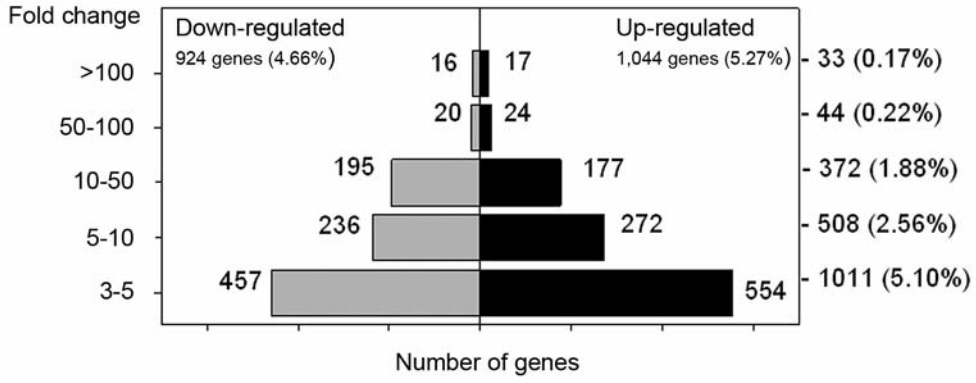

Figure 3. *Differentially expressed genes in Saos-2 cells were discovered by the utilization of the U-2 OS gene expression profile as a baseline for comparison. Selection of the genes that had a p-value less than 0.05 between the triplicate experiments and more than 3-fold change differences resulted in 1,044 up-regulated and 924 down-regulated genes (1,968 genes in total, i.e. 9.93% of genes on the microarray) in Saos-2 cells.*

Expression of four representative proteins (Figure 6), including ALP, GAPDH, RUNX2 and SP7, was also detected by immunocytochemistry and results were consistent with specific mRNA expression.

Differential transcriptional regulation in the dissimilar human osteosarcoma cell lines. Transcription factors are important in regulating gene expression in cells for any cellular activities. Therefore, analysis of the biochemical activities of transcription factors in the two human osteosarcoma cell lines provided extra information on their distinctive characteristics. Analysis showed that a total of 23 out of the 54 detectable transcription factors (19 in Saos-2 and 23 in U-2 OS) were found to be active in the Protein/DNA array. Among these 23 active transcription factors, 8 had shown differential activities: POU domain protein 3 (BRN-3), CCAAT binding factor (*CBF*), v-myb myeloblastosis viral oncogene homolog (avian) transcription factor (*c-Myb*) and myocyte enhancer factors (*MEF-1*) had higher activity in Saos-2, while signal transducer and activator of transcription 4 (*STAT4*), thyroid hormone receptor (*TR*), vitamin D (1,25-dihydroxyvitamin D_3) receptor (*VDR*) and heat-shock consenus elements (*HSE*) had higher activity in U-2 OS. These factors

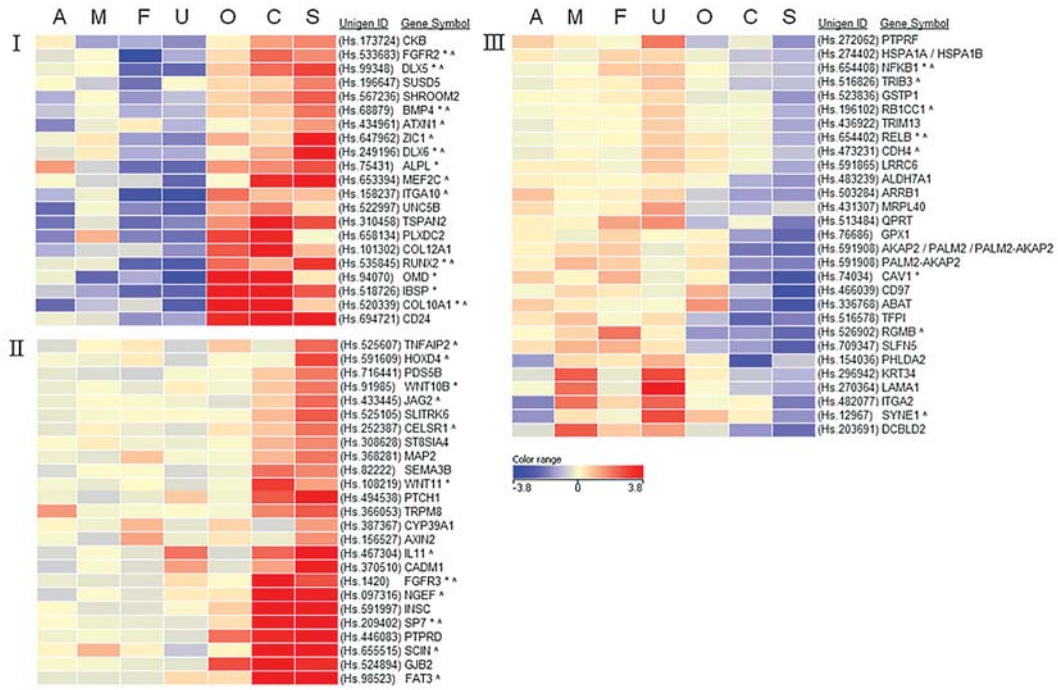

Figure 4. *The hierarchical clustering analysis showing the average signal intensity of the identified 75 differentially expressed genes in Saos-2 cells when compared to U-2 OS cells after several analyses. Distinctive gene expression patterns were recognized from the comparison between Saos-2 (S) and U-2 OS (U) cell lines and five normal human cells, including adipocyte (A), mesenchymal stem cells (M), fibroblast (F), osteoblast (O) and chondrocyte (C). Clusters I, II and III showed that the gene expressions in Saos-2 cells corresponded to the two bone forming cell types, osteoblast and chondrocyte, while gene expressions in U-2 OS cells were similarly expressed as adipocyte, fibroblast, and mesenchymal stem cells. *Bone markers, ^transcription factors.*

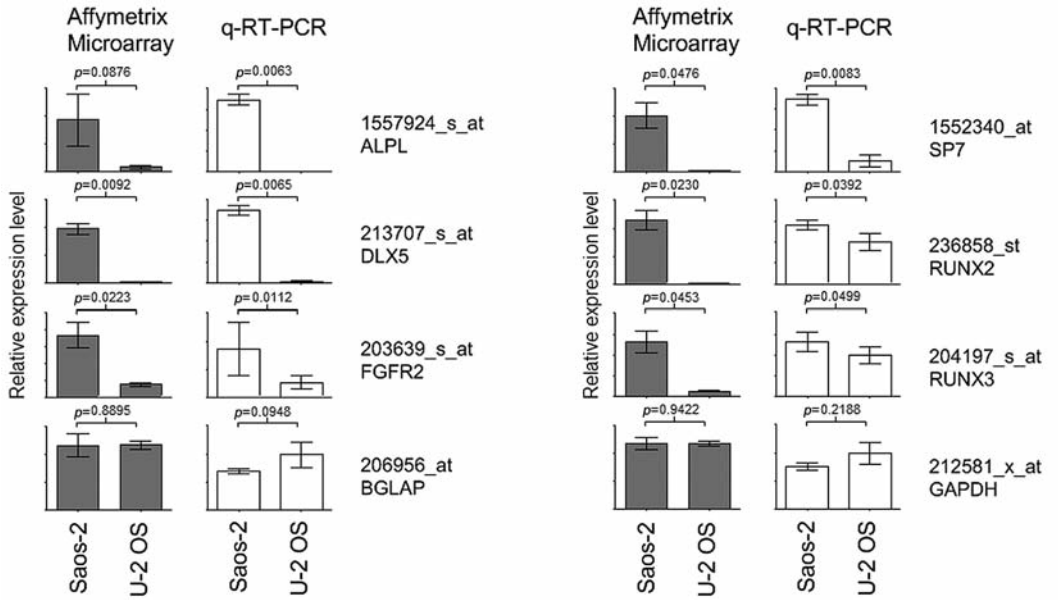

Figure 5. *Verification of microarray results by qRT-PCR. The β-actin–normalized relative gene expressions level (mean±standard deviation) of eight represented genes was shown. Significantly and differentially expressed gene was identified when $p<0.05$. Results showed that similar gene regulations between Saos-2 and U-2 OS cells were agreed in both microarray and qRT-PCR analysis. ALPL, Alkaline phosphatase; DLX5, distal-less homeobox 5; FGFR2, fibroblast growth factor receptor 2; BGLAP, bone gamma-carboxyglutamate protein (osteocalcin); SP7, Sp7 transcription factor (osterix); RUNX2, runt-related transcription factor 2; RUNX3, runt-related transcription factor 3; GAPDH, glyceraldehyde 3-phophate dehydrogenase.*

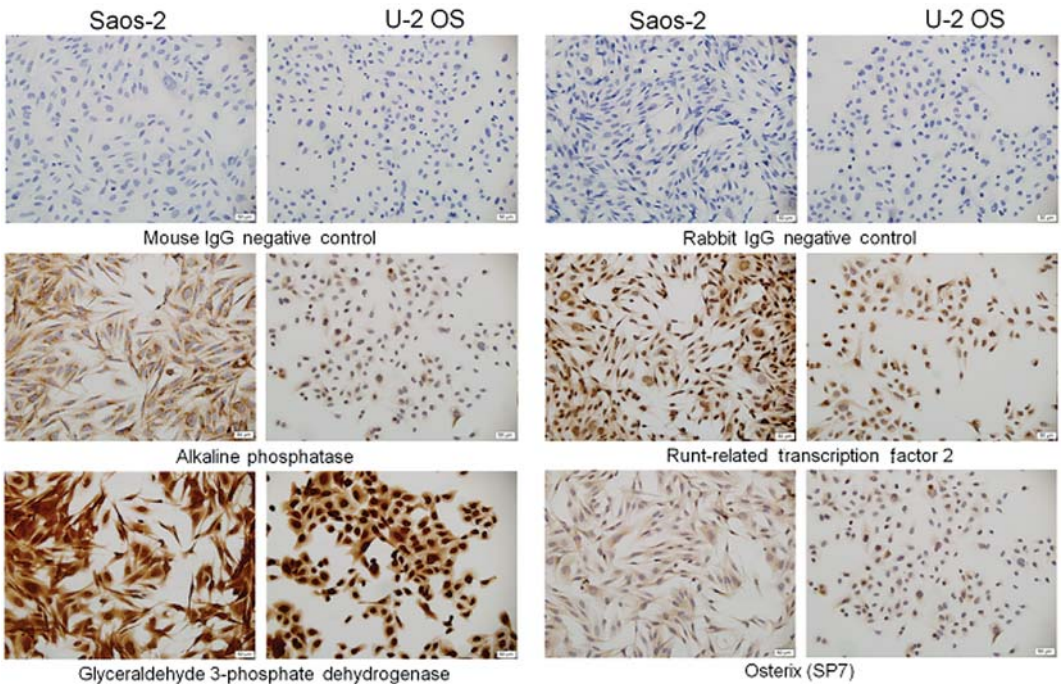

Figure 6. *Verification of microarray results by immunocytochemistry. Protein expression was consistent with the mRNA expression detected by qRT-PCR. Original magnification, ×20.*

were associated with 22 genes from the Affymetrix microarray (as shown in Table I). The highly regulated gene myocyte enhancer factor 2C (*MEF2C*) in Saos-2 was categorized as being significantly up-regulated in both microarray and transcription factor activity analysis.

Genes that are the known binding targets of transcription factors were retrieved and analysed. Twelve out of the 23 active transcription factors from the protein/DNA array in the two osteosarcoma cell lines were available in the TRED database, and in total, 774 unique transcription factor–targeted genes were obtained. Comparison between these genes to the 75 significantly differentially expressed genes identified 11 genes, of which cadherin EGF LAG seven-pass G-type receptor 1 (*CELSR1*), *RUNX2* and unc-5 homolog B (C. elegans) (*UNC5B*) were found to be up-regulated and caveolin 1 (*CAV1*), glutathione *S*-transferase pi 1 (*GSTP1*), heat-shock 70 kDa protein 1A (*HSPA1A*), integrin alpha 2 (*ITGA2*), mitochondrial ribosomal protein L40 (*MRPL40*), nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NFKB1*), v-rel reticuloendotheliosis viral oncogene homolog B (*RELB*) and tribbles homolog 3 (Drosophila) (*TRIB3*) were found to be down-regulated in Saos-2.

Discussion

Osteosarcoma arises from impaired differentiation of immature osteoblasts into more mature types. Evaluation of the genetic profile of the disrupted osteoblast differentiation in osteosarcoma was difficult due to the lack of appropriate samples and limited knowledge about the disruption of the osteoblast differentiation process.

The present study initially characterized the osteogenic induction ability of the two human osteosarcoma cell lines, Saos-2 and U-2 OS, using mouse myoblast cells, C2C12. Analysis revealed that the ALP activity and the mineralized nodule formation in C2C12 were increased after culturing in the Saos-2 conditioned media compared with the control media and the U-2 OS conditioned media. These results suggest that the two osteosarcoma cell lines, which were dissimilar in tumour differentiation status, maintained the difference in their osteogenic induction ability and, thus, were feasible to be used in the gene profiling studies.

Our previous study also verified that Saos-2 differentially expressed a profile of osteogenic factors compared with U-2 OS (9), justifying the different osteogenic induction abilities of the conditioned media obtained from both cell lines. In addition, this was also consistent with our previous *in vivo* study, which demonstrated that injection of osteosarcoma cells into mouse thigh muscle induced ectopic bone formation for Saos-2 and xenograft tumour formation for U-2 OS (9).

All of the above data support the notion that the more-differentiated Saos-2 cell line has distinctive osteogenic induction ability and osteogenicity compared with the less-differentiated U-2 OS. These differences were shown to be

Table I. *Expression of genes related to transcription factors. The protein/DNA array identified eight differentially active transcription factors between Saos-2 and U-2 OS cells. The genes related to these transcription factors were extracted from Affymetrix microarray analysis along with their regulation.*

Transcription factor	Related genes in microarray	Gene title	Unigene ID	Gene regulation in Saos-2 compared to U-2 OS
BRN-3	*POU4F1*	POU class 4 homeobox 1	Hs.654522	Up
	POU4F2	POU class 4 homeobox 2	Hs.266	Up
	POU4F3	POU class 4 homeobox 3	Hs.553499	Down
CBF (CCAAT	*NFYA*	Nuclear transcription factor Y, alpha	Hs.10441	Down
binding factor)	*NFYB*	Nuclear transcription factor Y, beta	Hs.84928	Up
	NFYC	Nuclear transcription factor Y, gamma	Hs.233458	Down
c-Myb	*MYB*	V-myb myeloblastosis viral oncogene homolog (avian)	Hs.654446	Up
MEF-1	*MEF2A*	Myocyte enhancer factor 2A	Hs.268675	Up
	MEF2B	Myocyte enhancer factor 2B	Hs.153629	Up
	MEF2C	Myocyte enhancer factor 2C	Hs.653394	Up
	MEF2D	Myocyte enhancer factor 2D	Hs.314327	Down
HSE (Heat-shock	*HSF1*	Heat-shock transcription factor 1	Hs.530227	Down
consenus elements)	*HSF2*	Heat-shock transcription factor 2	Hs.158195	Up
	HSF2BP	Heat-shock transcription factor 2 binding protein	Hs.406157	Down
	HSF4	Heat-shock transcription factor 4	Hs.512156	Down
	HSF5	Heat-shock transcription factor family member 5	Hs.380061	Up
	HSFX1/HFSX2	Heat-shock transcription factor family, X-linked 1/X-linked 2	Hs.592255	Up
STAT 4	*STAT4*	Signal transducer and activator of transcription 4	Hs.80642	Down
TR	*NR1D1/THRA*	Nuclear receptor subfamily 1, group D, member 1/ Thyroid hormone receptor, alpha	Hs.724	Down
	THRA	Thyroid hormone receptor, alpha	Hs.724	Down
	THRB	Thyroid hormone receptor, beta	Hs.187861	Down
VDR	*VDR*	Vitamin D (1,25- dihydroxyvitamin D3) receptor	Hs.524368	Down

consistent in both *in vitro* and *in vivo* conditions, as well as in the current passage of cells. Evidence from other studies also supported the osteogenicity of Saos-2 (12, 13) and tumourigenicity of U-2 OS (5).

Microarray studies were then applied to investigate the genetic profile of the two dissimilar osteosarcoma cell lines followed by subsequent analysis. One of our key investigations was to compare the different expression profiles obtained from our study against normal human cell lines.

The study by Kubo *et al*. (11) utilized microarray to identify the transcription factors that could be used as specific markers for human mesenchymal cells during the differentiation of human mesenchymal stem cells into adipocytes, chondrocytes, fibroblasts and osteoblasts. Our study employed the same technology as that of Kubo *et al*., thus the microarray expression results between both studies are directly comparable. This particular comparison assisted in the identification of differentially expressed genes that are important to the osteogenic characteristic of Saos-2 in regards to the differentiated and non-differentiated cell conditions, and eliminated the genes that differed only between the two human osteosarcoma cell lines.

Gene ontology analysis suggested that genes involved in organ development, cell binding and adhesion and nervous system signalling are important in differentiating the osteogenic properties of the two human osteosarcoma cell lines. Given that development of osteosarcoma involves dysregulation of the differentiation process, it implies that the differentially expressed genes are associated with multiple organ developmental processes. Furthermore, cellular organization is an important step during organ developmental processes. It also explains that the differentially expressed genes are also correlated with cellular adhesion processes.

An increasing number of investigations have shown the emerging relationships between the human nervous system and bone, and regarded it as 'neuroskeletal biology' (14). Important discoveries include the decrease in bone mass resulting from leptin stimulation of β-adrenergic signalling system and the increased expression of the main osteoblast differentiation mediator *RUNX2* resulting from stimulation of *N*-methyl-D-aspartate signalling system in osteoblasts (15-17). It was possible to reverse this bone mass reduction through the nervous system with the use of β-adrenergic receptor antagonist (also known as β-blocker) by eliminating the activation of β-adrenergic signalling system from the binding of leptin (18). The β-adrenergic receptor antagonist has attracted many studies with regards to bone metabolism, and has been reviewed for its potential clinical applications in osteoporosis and fracture healing (19). Bone abnormality such as osteoporosis is proposed to be a neuroskeletal disease (20).

Thus, it is possible that other factors related to the nervous system are involved in osteoblast differentiation and bone formation, or even in the development of osteosarcoma.

Many studies have also reviewed the correlation of cell adhesion and cadherin molecules with cancer progression and differentiation (21, 22). Expression of an epithelial cell adhesion molecule (C-CAM) has been found to correlate with cell differentiation during development of human prostate but not in prostate cancer cells (23). Induced expression of C-CAM in a human prostate cancer model has shown antitumour effects (24). Moreover, expression of neuronal cadherin (N-cadherin) was found to be up-regulated concomitantly with osteoblast differentiation (25), but was down-regulated in osteosarcoma (26).

Amongst the 75 differentially expressed genes in Saos-2 cells, osteoblast-related markers such as *FGFR2*, *DLX5*, distal-less homeobox 6 (*DLX6*), *MEF2C*, wingless-type MMTV integration site family member 10B (*WNT10B*), RUNX2 and SP7 were identified in our analysis. RUNX2 has been recognized as the major transcription factor controlling osteoblast differentiation. All these osteoblast-related markers contribute to the regulation of bone formation during skeletal development and post-natal life (27-29). Several other factors with known functions in bone formation and development were also detected in our analysis, which includes the bone markers such as alkaline phosphatase (*ALP*) and bone sialoprotein (*IBSP*), bone morphogenic protein 4 (*BMP4*), collagens (collagen type X alpha 1 (*COL10A1*), collagen type XII alpha 1 (*COL12A1*)), osteoclast regulators (*NFKB1* and semaphorin 3B (*SEMA3B*)) and a transcription factor (*MEF2C*) (30-32). Knockout of *CAV1* and *NFKB1* genes in mice have also been demonstrated to positively affect bone growth (33, 34).

Cell adhesion and cadherin are one of the significant gene clusters in this study after both gene ontology and functional annotation analysis. Factors such as cell adhesion molecule 1 (CADM1), signal transducer CD24 (CD24), signal transducer CD97 (CD97), cadherin 4 type 1 (CDH4), CELSR1, FAT tumor suppressor homolog 3 (Drosophila) (FAT3), gap junction protein beta 2 (GJB2), ITGA2, integrin alpha 10 (ITGA10), scinderin (SCIN) and tetraspanin 2 (TSPAN2) are different types of cell surface proteins involved in cell binding and adhesion. SCIN, also known as adseverin, belongs to the gelsolin family of actin-regulatory proteins. Its expression was found in mouse embryos restricted to endochondral bone formation and the development of adult outer renal medulla and intestine (35). In humans, the only function identified for this protein is the stabilization of actin (36, 37).

Gap junction proteins, also known as connexins, play an important role in signal transmission between cells. Gap junction protein alpha 1 (GJA1) expression is found in osteoblasts, osteocytes and chondrocytes, where it mediates osteoblast differentiation and bone formation (38). However, deficiency of GJA1 only showed incomplete but not totally ablated bone formation (39). Other gap junction proteins such as gap junction protein β2 (GJB2) may also play a role in transmitting signalling factors during bone formation.

CD24, a cell surface protein, was found to be the most significantly altered gene from our microarray analysis. CD24 is a heavily glycosylated short mucin-like protein, that anchors to the outer surface of the cytoplasmic membrane by glycosyl-phosphatidyl-inositol anchor protein but has no transmembrane domain. CD24 was initially identified as a surface antigen of B-cells and was correlated to the maturation of B-cells. Further studies demonstrated that it also participates in cell proliferation and differentiation, and may be related to carcinogenesis (40). Expression of CD24 protein was not only detected in normal haemopoietic cells, such as B-lineage cells and mature granulocytes, but also in breast, non-small cell lung and colorectal cancer (41-43). Although CD24 is suggested as a prognostic marker for these types of cancer, its biological role in this regard is not known.

Studies have demonstrated the cell signal transmission and cell-to-cell interaction of CD24 in B-cells (44, 45). It is also known that mammalian haematopoiesis occurs in the skeletal system. However, there is limited knowledge in the understanding of the relationship between the skeletal and the haematopoietic system. One piece of evidence demonstrating the relationship between the two systems is that the haematopoietic cytokine granulocyte colony-stimulating factor, which has an important function during haematopoiesis, is constitutively produced by osteoblast cells (46). Surface protein, such as CD24, may be involved in the communication between haematopoietic cells and osteoblasts, and may serve a role in controlling the osteoblast differentiation or bone formation.

Cell-to-cell communication has always been an important factor in cell regulation. Several recent reviews have highlighted the importance of communication between cells within the skeletal system and between cells in the skeletal and haematopoietic system (47, 48). Osteoblast differentiation and bone formation are complex cellular processes involving the regulation of many different cells. Although many factors are recognized as being important regulators for these processes, other regulatory factors and mechanisms remain unexplored. Further investigations are required to understand the relationship between overexpression of CD24 and disrupted osteoblast differentiation in osteosarcoma.

Another interesting finding from our results is the down-regulation of β-arrestin 1 (*ARRB1*). As discussed earlier, activation of β-adrenergic signalling system in osteoblasts can reduce bone mass, and can be reversed by β-blocker. The activation of the β-adrenergic signalling system requires a protein complex containing β-adrenergic receptor and activated c-Src. Src recruitment is mediated by ARRB1, which serves as an adaptor protein to bind both the c-Src and agonist-occupied β-adrenergic receptor (49). Thus, the down-regulation of *ARBB1* will hamper the activation of β-

adrenergic signalling system and may impair the normal function of osteoblasts.

A large proportion of the differentially expressed genes were found to be related to transcription factors. The activity of transcription factors and their targeted genes were investigated by the use of both protein/DNA array and Affymetrix™ microarray analysis. A list of the differentially active transcription factors and their targeted binding genes was constructed and matched with the 75 differentially expressed genes. This analysis identified a total of 12 significant genes, 1 gene of a transcription factor and 11 genes of transcription factor binding targets, in regards to the differentially active transcription factors between Saos-2 and U-2 OS cells. Genes identified from this analysis would not correspond directly to the active transcription factors at the current growth stage, as the gene expression of the active transcription factors should occur in the earlier stage of growth. Thus, it is reasonable that the majority of genes identified from this analysis were the transcription factor binding targets.

Amongst the 12 genes identified from transcription factor analysis, *CAV1, MEF2C, NKFB1, RELB* and *RUNX2* are related to osteoblast and/or bone formation (33, 50, 51), but not with the other 7 genes. Reviewing the annotations and functions of the other 7 genes from NCBI database showed that (i) CELSR1, ITGA2 and UNC5B are membrane proteins; (ii) CELSR1, GSTP1 and UNC5B are involved in the development and signalling of the nervous system; (iii) ITGA2 is involved in a cellular binding and adhesion, and extracellular stimulation responding system; (iv) MRPL40 is a ribosomal protein that helps in protein synthesis within the mitochondrion; (v) HSPA1A is a heat-response protein and plays a role in the conformation of protein; (vi) TRIB3 is a putative protein kinase that is induced by the transcription factor NF-κB and negatively regulates cell survival. Further investigation of these genes is required to understand their roles in osteoblast differentiation and osteosarcoma development.

In summary, analysis of the gene expression profile from two osteosarcoma cell lines with dissimilar differentiation status showed for the first time that genes from cell-adhesion and nervous system are expressed differentially between the cells. Moreover, genes involved in osteoblast differentiation were also identified in this study. The current findings provide a basis for further investigation of the interaction between these genes, which may help to advance our understanding of the mechanisms involved in the control of osteoblast differentiation and development of osteosarcoma.

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