Journal of Cellular Biochemistry 92:733–744 (2004)

Gene Expression Profile of Human Bone Marrow Stromal Cells Determined By Restriction Fragment Differential Display Analysis

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Abstract Using an in vitro osteogenic culture system, we carried out a restriction fragment differential display (RFDD-PCR) to identify genes expressed by these cells in their undifferentiated stage and not expressed, or expressed at a lower level, in a closely related but distinct cell type: bone marrow stromal cells (BMSC)-derived osteoblasts (BDO). Forty-seven candidate regulated genes, selected by RFDD, were analyzed by RT-PCR analysis in three cell clones and in primary cultures from seven different donors. A subset of three genes were confirmed as upregulated in BMSC relative to BDO in every primary culture and cloned population examined: βIG-h3, IGFbp3, and LOXL2. Their differential expression was confirmed by Northern analysis and the corresponding proteins were detected by immunolocalization in BMSC. J. Cell. Biochem. 92: 733–744, 2004.

Key words: human bone marrow stromal cells; differential display; gene expression profile; βIG-h3; IGFbp-3; LOXL2; cancer

Human bone marrow stromal cells (BMSC) represent a phenotypically and functionally heterogeneous population of cells, which reside in the marrow cavity and provide support for hematopoiesis [Dexter et al., 1977]. BMSC show properties shared by stem cells: they have a self-renewal potential and can differentiate into several cell lineages including osteoblasts, chondrocytes, adipocytes, myelosupportive stroma, and myoblasts [Prockop, 1997]. The multipotential property of BMSC made them appealing candidates for use in tissue repair, cell replacement in diseased tissues and for drug/toxin screening. In spite of this, BMSC remain poorly characterized at both the cellular and molecular level. Previous efforts to understand the biology of human BMSC have led to the identification of a few phenotypic markers such as Stro1, HOP-26, CD49a, and CD166 [Stewart et al., 2003]. Recently, efforts to characterize human BMSC have focused on the analysis of gene expression profiles either of a few selected lineage-specific markers [Shur et al., 2001; Frank et al., 2002] or of a large number of genes [Doi et al., 2002; Qi et al., 2003] or, finally, of the entire complement of expressed transcripts of BMSC [Tremain et al., 2001; Jia et al., 2002]. However, these studies still did not provide an univocal molecular description of human BMSC, neither identified any BMSC-specific molecular marker. To gain a better understanding of BMSC biology, we decided to identify genes expressed by these cells in their undifferentiated stage and not expressed, or expressed at a lower level, in closely related but distinct cell types.

Since the BMSC osteogenic differentiation process can be easily induced in culture by appropriate treatment [Maniatopoulos et al.,

Massimiliano Monticone and Yi Liu contributed equally to this work.

Grant sponsor: Consiglio Nazionale delle Ricerche, Rome (Italy); Grant sponsor: P.F. Biotecnologie; Grant number: 99.00524.PF49; Grant sponsor: Italian (ASI) and European (ESA-ERISTO) Space Agencies.

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Received 25 September 2003; Accepted 1 March 2004

DOI 10.1002/jcb.20120

1988], and the same process can arise spontaneously with time from BMSC in vitro cultures [Banfi et al., 2002], it has been suggested that osteogenesis could be the default differentiation pathway of BMSC [Muraglia et al., 2000]. Therefore, we chose BMSC induced toward the osteoblastic lineage as a closely related but distinct cell type to use as a read out system to identify a BMSC characteristic gene expression pattern. With this aim, we took advantage of the restriction fragment differential display technique (RFDD-PCR) to select genes that are expressed by human BMSC and not expressed, or expressed at a lower extent, by BMSC-derived osteoblasts (thereafter referred as BDO). By using this approach, we identified a restricted set of genes downregulated upon osteoblastic differentiation of BMSC. To validate this result, semiquantitative RT-PCR was performed. In order to rule out a bias due to individual variations in gene expression, we extended the analysis to cultures obtained from several donors. Furthermore, due to the heterogeneous nature of the BMSC primary culture [Malaval et al., 1994], single-colony-derived BMSC were also examined in order to exclude genes whose expression occurred in contaminating non-BMSC possibly present in the cultures. By using this combined and multistep screening strategy, we identified three genes that are consistently upregulated in all BMSC primary cultures and clones, and four additional genes upregulated in most BMSC primary cultures tested when compared to corresponding BDO. We also confirmed at the protein level the expression of the three genes found always upregulated in cultured BMSC. A possible use of these genes as "stemness" markers of BMSC and similarity in their expression regulation with that observed in cancer cells are discussed.

MATERIALS AND METHODS

Cell Culture and Cloning

To obtain primary cultures, human BMSC were derived from iliac crest marrow aspirates of healthy donors after informed consent. The bone marrow transplantation (BMT) procedures, approved by the institutional ethics committee, were carried out at S. Martino Hospital and G. Gaslini Pediatric Hospital at Genova in Italy. All donors were white Caucasians and aged ranging from 15 to 55 years. BMSC cultures were established by methods described previously [Galotto et al., 1999] using the following growth medium: COON's modified Ham's F12 medium (F12, Peprotech, Rocky Hill, NJ) supplemented with 1 ng/ml of human recombinant fibroblast growth factor-2 (FGF-2, Peprotech). The BMSC were selected for their ability to adhere to the plastic dish. When they reached the confluence (P0), BMSC were detached with 0.05% trypsin-0, 01% EDTA (Sigma-Aldrich, St. Louis, MO). Cells were replated at a concentration of 5×10^5 cells/10-cm Petri dish. An equal number of dishes were then cultured for 2 weeks in growth medium or in differentiation medium.

Single colonies of BMSC were obtained from bone marrow aspirates. About 2×10^5 nucleated cells were plated per 10-cm Petri dish. The appearance of stromal colonies was examined daily by microscopic analysis. After 15–20 days, single colonies were individually trypsinized and each colony was replated in a 6-well plate (P1). After P1 cells reached confluence, they were again trypsinized and plated at a concentration of 2×10^5 cells/6-cm Petri dish. As for primary cultures, an equal number of dishes were then cultured for 2 weeks in growth medium or in differentiation medium.

Osteoblastic differentiation medium was obtained by supplementing the growth medium with 50 μg/ml ascorbic acid, 1.5 mg/ml β-glycerophosphate (Sigma), and 10^{-8} M dexamethasone (Sigma). Growth and differentiation medium were changed twice a week and ascorbic acid was added to the differentiation medium every other day.

Histochemistry

Alkaline phosphatase was revealed by immunoenzymatic kit (Sigma diagnostic Alkaline phosphatase). The cells, 30–40% confluent, were fixed for 30 s in a citrate–acetone–formaldehyde solution. Cells were then washed in distilled water and ALP expression was evaluated following the kit's directions. Deposition of a calcified matrix was evidenced by Alizarin Red staining. Cells were fixed for 10 min in 4% formalin at room temperature, washed in distilled water and stained for 10 min with Alizarin Red. Free dye was removed with one wash of 100% ethanol followed by distilled water.

Total RNA Extraction

Total RNA was extracted from cell cultures by using TRIzol reagent (Invitrogen, San

Diego, CA) according to the manufacturer's protocol.

Restriction Fragment Differential Display-PCR (RFDD-PCR)

DNA-free total RNA was extracted from cells cultured in both growth medium (BMSC sample) and osteogenic differentiation medium (BMSC derived osteoblast: BDO). One milligram of each RNA was reverse transcribed by using the $T_{25}V$ primer and the display THERMO RT-PCR system (Display Systems Biotech, Vista, CA). The double-stranded cDNAs were subjected to RFDD by using a displayPROFILE kit (Display Systems Biotech). Briefly, the cDNA was digested with *TaqI* restriction enzyme and ligated with two different DNA adaptors. Ligation products were amplified by using a conventional primer and a set of 64 selection primers, which allow selection of different cDNA subpopulation in 64 PCR reactions. Amplified BMSC and BDO fragments were separated side-by-side on a polyacrylamide gel. Fragments preferentially expressed in BMSC were excised from dried gels, reamplified using the same primer combinations, and cloned into a TA-cloning vector (Invitrogen). The cloned PCR products were sequenced from both ends with an automated 377 Perkin-Elmer sequencer. All sequencing reactions were performed with the BigDye terminator cycle sequencing kit (Perkin-Elmer, Wellesley, MA). DNA homology searches, with nucleotide sequences included in the GeneBank database, were performed using the BLAST protocol provided by the NCBI (http://www.ncbi.nlm.nih.gov/).

Semi-Quantitative RT-PCR Analysis

Starting from about 5 μg of total RNA, cDNA was synthesized by using an $Oligo(dT)_{12-18}$ primer and a Superscript first-strand synthesis system for RT-PCR (Invitrogen). cDNAs were diluted 10–50 times, then subjected to semi-quantitative PCR analysis. Specific sets of primers were designed by GeneWorks software (IntelliGenetics, Campbell, CA). Amplicon sizes ranged from 100 to 700 bp. The expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal standard. The PCR program consisted of 1 cycle at 95°C for 1 min and 30 s, then 26–40 cycles (the number of PCR cycles and the annealing temperature were optimized in each case to ensure that intensity of each amplicon fell within the linear phase of amplification) at 95°C for 30 s for denaturing, 58–60°C for 30 s for annealing, and 72°C for 45 s for extension, and finally 1 cycle at 72°C for 7 min. PCR products were separated on 1.5–2% agarose gel. Digital images were documented by GelDoc 1000 (Bio-Rad, Hercules, CA). Light intensities of specific amplicons were measured by using the Molecular Analyst software (Bio-Rad). Areas of equivalent sizes outside amplicon bands were defined as background. Relative gene expression was calculated by a ratio, (BDO amplicon-background)/(BMSC amplicon-background), and subsequently corrected by the relative expression of GAPDH. Information about the most relevant, forward and reverse, specific primers used in RT-PCR is listed in Table I.

Statistical Analysis

The overall consistency between measurements of gene expression in three BMSC clones and in BMSC primary cultures was evaluated by the Cronbach's alpha coefficient. The index ranges between 0 and 1, and the reliability is considered satisfactory when it is 0.7 or more [Bland and Altman, 1997]. To analyze the expression of individual genes in BMSC primary cultures, after transforming original values into their logarithms to stabilize their variance [Armitage and Berry, 1987], a *z*-test was performed by plotting the corresponding normalized mean of fold changes (NFC) and their related 95% confidence intervals (CI). A gene was considered either under- or over-expressed whenever the 95% CI did not include the expected fold change value under the null hypothesis of no difference between BMSC and BDO (i.e., 0 in logarithmic scale). Furthermore, NFC between −0.405 and 0.405, corresponding to a fold change (BMSC and BDO) between 0.67 and 1.5, were not considered as meaningful.

Northern Blot Analysis

For each sample 15 μg of total RNA were separated on a 1% denaturing formaldehyde/agarose gel, then transferred to a Hybond-N nylon membrane (Amersham Biosciences, Piscataway, NJ) and cross-linked by UV irradiation. DNA probes were labeled with [α^{32}P]-dCTP (Amersham) by using the Random

TABLE I. Most Relevant Oligonucleotide Primers Used for RT-PCR Analysis

Gene	Accession number	Sequence	Position (nt)
Alkaline phosphatase (ALP)	NM_000478	5′-ACGTGGCTAAGAATGTCATC-3′ 5′-CTGGTAGGCGATGTCCTTA-3′	392 → 867
Osteocalcin (OC)	X53698	5′-GCAGCGAGGTAGTGAAGAGAC-3′ 5′-TGGAGAGGAGCAGAACTGG–3′	143 → 370
Bone morphogenetic protein-2 (BMP-2)	NM_001200	5′-GGAAGAACTACCAGAAACGAG-3′ 5′-AGATGATCAGCCAGAGGAAAA-3′	677 → 1,333
Lysyl oxidase-like 2 (LOXL2)	NM_002318	5′-TTCTCCAGTGACACGTGGAC-3′ 5′-ACACATGGTGCTCAGTGAGC-3′	2,931 → 3,380
TGF beta-induced (βIG-h3)	NM_000358	5′-CCAGCAGATCATTGAGATCG-3′ 5′-AGATGTGGTTGTTCAGCAGG-3′	776 → 981
IGF binding protein 3 (IGFbp3)	X64875	5′-GAATGCTCACCACATGTTGG-3′ 5′-GGAAGATTCCTGAAGAGGAGG-3′	1,308 → 1,706
Vascular cell adhesion molecule1 (V-CAM1)	M60335	5′-GGCTTCAGGAGCTGAATACC-3′ 5′-TGCTGCAAGTCAATGAGACG-3′	5 → 244
Myristoylated ala-rich protein kinase c substrate (MARCKS)	M68956	5′-AGCCTCAATCAAGCCTGC-3′ 5′-CACTCCAACCAAACAAACGG-3′	1,221 → 1,369
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AF261085	5′-ACCACAGTCCATGCCATCAC-3′ 5′-TCCACCACCCTGTTGCTGTA-3′	629 → 1,080

Primed DNA Labeling Kit (Roche, Indianapolis, IN) and purified with MicroSpin™ S-300 HR columns (Amersham). Only probes having a specific activity $>8 \times 10^8$ cpm/μg of labeled DNA were used for hybridization. Blot hybridization washing and stripping were performed following Amersham's instructions. Membranes were exposed to Cyclone storage phosphor screens, signals were collected by a Cyclone Phosphoimager (Packard Instruments, Meridien, CT) and analyzed by the OptiQuant image analysis software (Packard Instruments).

Fluorescence Analysis

Confluent cultures of BMSC at P0 passage were trypsinized and plated in wells of chamber slides at the density of 7×10^4 cells/well. After 24 h cells were fixed and permeabilized as previously described [Marchisio et al., 1993]. Unspecific binding sites were saturated with 10% donkey serum in PBS; samples were then incubated with primary antibodies for 1 h at room temperature. The rabbit antiserum for βIG-h3 (REGEN Biotech, Seoul, Korea) was used at about 10 μg/ml in donkey serum diluted in PBS. The goat antisera for IGFbp3 and for LOXL2 were diluted to about 10 μg/ml in donkey serum. Antibody binding was detected by 30 min incubation at 0°C. Secondary antibodies were fluorescein-conjugated anti-rabbit (βIG-h3), Cy2-conjugated anti-rabbit (LOXL2), and TRICT-conjugated anti-goat (IGFbp3). Negative controls were performed by using non-immune rabbit and goat sera.

RESULTS

Identification of Genes Upregulated in BMSC Versus BDO by Differential Display Analysis

To identify genes preferentially expressed in BMSC, we performed a RFDD-PCR using total RNA extracted from the primary culture BM473 maintained either in growth medium or in differentiation medium. The osteogenic potential of BM473 was demonstrated by alkaline phosphatase activity and alizarin staining of calcified matrix (Fig. 1). To facilitate the comparison of gene expression profiles, the RFDD-PCR products from both RNA preparations were separated side-by-side by polyacrylamide

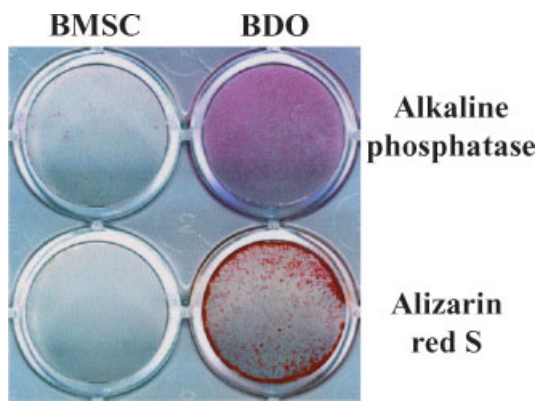

Fig. 1. Alkaline phosphatase activity and Alizarin red S staining were determined in primary culture BM473. Bone marrow stromal cells (BMSC) were cultured both in growth medium (BMSC) and in osteoblastic differentiation medium (BDO).

gel electrophoresis (data not shown). One hundred bands, either present only in BMSC samples or more abundant in BMSC samples than in BDO samples, were excised from gels. Approximately 50% of the re-amplified PCR products could be sequenced directly, whereas, other fragments needed to be cloned into vector pCR2.1 (Invitrogen) prior to the sequencing. The 92 resulting sequences, whose sizes varied between 53 and 290 bp, were subjected to BLAST search for gene identification. This analysis revealed 53 individual genes, of which 41 (77%) encoded for known proteins, six (11%) belonged to novel/uncharacterized genes, and six (11%) were false positives (e.g., short sequences or Alu-repeats). We classified these 41 genes with known identities into eight functional categories: cytoskeletal proteins, extracellular matrix proteins, proteases and protease inhibitors, protein modifiers, translation apparatus, signaling molecules, and miscellaneous function (Table II).

TABLE II. Gene Expression Profile of Three Bone Marrow Stromal Cells (BMSC) Clones

Gene	Accession number	Fold change* BM688 C2-P3	BM688 C3-P3	BM688 C9-P2
Markers osteogenesis				
ALP	NM_000478	0.242	0.113	0.100
BMP-2	NM_001200	1.475	1.439	0.141
Osteocalcin	X53698	0.532	1.225	0.690
Cytoskeletal proteins				
Adducin (γ)	U37122	4.717	10.753	1.376
Caldesmon I (CALD1)	M64110	3.322	8.621	0.968
Vimentin (VIM)	XM_042950	2.033	1.597	0.863
Matrix proteins				
Collagen, type I, alpha 1	NM_000088	1.520	0.914	0.776
Collagen, type III, alpha 1	NM_000090	5.376	1.541	0.978
Collagen type XII alpha-1	U73778	1.443	1.550	0.810
Lysyl oxidase-like 2 (LOXL2)	NM_002318	3.436	9.901	7.246
SPARC/osteonectin	J03040	1.468	1.842	1.010
TGF beta-induced (βIG-h3)	NM_000358	5.348	3.115	3.268
Miscellaneous				
Cbl-b	U26710	2.451	3.040	1.374
Glutaminase	AF327434	0.951	2.294	0.842
IGF binding protein 3 (IGFbp3)	X64875	3.215	7.692	8.130
Tumor rejection antigen 1 (TRA1)	XM_083864	2.710	15.152	1.212
URB	AF506819	1.447	2.398	1.094
V-CAM1	M60335	5.587	8.130	1.621
Proteases and protease inhibitors				
Aminopeptidase puromycin sensitive	XM_032201	0.796	2.012	0.893
Disintegrin metalloprotease (ADAM9)	NM_003816	1.437	3.802	0.347
Glia-derived nexin (GDN)	M17783	2.088	1.988	2.571
SERPINE1	M16006	2.114	0.732	0.749
Protein modifiers				
GRP78	AF216292	2.066	1.733	0.968
UDP-*N*-acetyl-alpha-D-galactosamine	U41514	2.421	4.587	0.828
Translation apparatus				
Glutamyl-prolyl-tRNA synthetase	NM_004446	1.245	6.944	1.667
Glycyl-tRNA synthetase (GARS)	U09587	0.320	1.280	1.988
Neoplasm-related C140 (RPL6)	D17554	2.004	3.436	0.962
Ribosomal protein L26 (RPL26)	NM_000987	1.225	1.613	0.775
Ribosomal protein L7 (RPL7)	NM_000971	1.965	6.849	0.983
Signaling molecules				
MARCKS	M68956	1.185	1.938	1.425
Vesicle membrane protein (RNP24)	NM_006815	0.943	1.486	1.300
Novel/unknown genes				
E.S.T. from osteosarcoma	BG028035	1.563	1.541	0.751
E.S.T. xo47h07.x1	AW513636	0.792	2.611	0.437
DC2 protein (DC2)	XM_034710	1.996	3.636	0.704
KIAA0203 gene product (KIAA0203)	NM_014781	3.040	8.772	0.678
HP1-BP74 (Histone1 like)	AF113534	12.346	1.876	0.792
Similarity to FGF receptor4b (*Xenopus*)	AL353940	1.477	9.259	3.367

*Fold change represents the ratio of gene expression in BMSC versus BDO.
Genes consistently down-regulated in all donors are shaded in light gray.

Validation of the Gene Expression Profile of BMSC by Semiquantitative RT-PCR Analysis of BMSC Clones

In order to verify whether the gene expression profile determined by RFDD was also observed in cloned BMSC, the 47 selected genes were subjected to semiquantitative RT-PCR analysis using RNA extracted from three BM688 single-colony derived cell cultures (C2-P3, C3-P3, and C9-P2) maintained in growth and differentiation medium. An example of RT-PCR performed with some of these genes is shown (Fig. 2).

The expression level of each gene was normalized to that of GAPDH used as a reference gene. The normalized fold change (NFC) corresponds to the ratio of a specific gene expression level in BMSC versus BDO (Table II). Genes were considered not regulated when the ratio of relative gene expression in BMSC to relative gene expression in BDO was between 0.67 and 1.5.

Although gene expression pattern was not identical among these three BM688 clones, six genes were found to be consistently up-regulated in all clones: two genes coding for matrix proteins (*LOXL2* and *βIG-h3*); one gene for the secreted factor IGF-bp3; one for an adhesion molecule V-CAM1; one for the protease GDN (Glia-derived nexin); and one uncharacterized gene similar to FGF receptor 4b from *Xenopus*. Of the remaining genes, 16 were found to be preferentially expressed in two clones and 12 genes in only one of the three clones (Table II).

Thirteen genes (27%), that were never found to be preferentially expressed in BMSC in any of the three clones (Table III), were classified as probable RFDD-PCR false positives and were excluded from subsequent analyses.

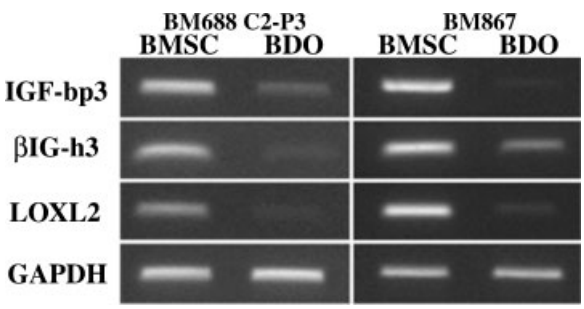

Fig. 2. Semiquantitative RT-PCR of a group of restriction fragment differential display-PCR (RFDD-PCR) derived genes. Each band corresponds to cDNA amplified from total RNA of cell cultured either in growth medium (BMSC) or in differentiation medium (BDO). Primers for GAPDH have been used to normalize the results. On the left results obtained with a cell clone, on the right a BMSC primary culture.

TABLE III. Genes not Regulated in Ctr Versus Osteo Cultures in any of the Three BM688-Clones

Gene	Accession number
ATP synthase gamma-subunit	D16563
Initiation factor 4b	AB076839
NADH dH subS	AF339086
RalA binding protein 1 (RALBP1)	NM_006788
RhoA-GTP bI.-protoonc. multidrug res. pr.	L25080
Ribosomal protein L23	NM_000978
Ribosomal protein L3 (RPL3)	NM_000967
Ribosomal protein L23a (RPL23A)	NM_000984
Ribosomal protein L34 (RPL34)	XM_034712
Ribosomal protein S13 (RPS13)	BC006772
Ribosomal protein S8 (RPS8)	NM_001012
Peroxiredoxin 4 (PRDX4)	U25182
Tyr 3-monoxygenase/trp 5-monoxygenase activ. prot, z polypeptide (YWHAZ)	BC003623

Genes were considered not regulated when the ratio (=relative gene expression in BMSC culture/relative gene expression in BDO culture) was between 0.67 and 1.5.

The overall consistency of gene expression profile was evaluated by Cronbach's alpha index. When all three clones were compared, the reliability coefficient value was 0.5771, which was below 0.7, the generally considered satisfactory minimal score [Bland and Altman, 1997]. The low alpha score suggests poor concordance in the expression profile among these three clones. No better concordance was seen when they were compared in pairs, as shown by their alpha scores (0.5303 for BM688 C2-P3 and C3-P3, 0.3201 for BM688 C2-P3 and C2-P9, 0.5384 for BM688 C3-P3 and C9-P2).

Validation of the Gene Expression Profile of BMSC by Semiquantitative RT-PCR Analysis of BMSC Primary Cultures Obtained From Seven Different Donors

To extend our investigation on the expression of the 34 genes shown to be preferentially expressed in at least one BMSC clone to different donors, BMSC cultures obtained from seven healthy donors were analyzed by semiquantitative RT-PCR (Table IV). Again cells were cultured for two weeks in growth or in differentiation medium and RNA was extracted in order to evaluate relative gene expression.

A *z*-test was performed to analyze individual gene expression (not shown). The gene expression was considered either up- or down-regulated whenever the 95% CI did not include the expected (log-transformed) fold change value under the null hypothesis of no difference between BMSC and BDO (line crossing the point of ordinate 0 in Fig. 3). Furthermore, NFC

TABLE IV. Gene Expression Profile of Seven BMSC Primary Culture

	Fold change*						
	BM688	BM802	BM953	BM867	BM932	BM991	BM1022
Age	15 (M)	33 (F)	35 (F)	50 (F)	42 (F)	51 (M)	17 (F)
Osteogenesis markers							
ALP	103.137	7.915	2.792	1.539	1.722	14.245	6.042
BMP-2	0.209	4.164	1.575	1.533	1.260	1.093	1.378
Osteocalcin	0.828	0.544	0.577	0.550	0.826	1.059	1..507
Cytoskeletal proteins							
Adducin (γ)	0.146	1.298	0.812	1.094	1.041	1.266	1.275
Caldesmon I (CALD1)	0.296	1.442	1.036	0.692	0.728	1.005	0.871
Vimentin (VIM)	0.210	1.612	1.081	0.828	1.214	0.912	1.466
Matrix proteins							
Collagen, type I, alpha 1	0.264	1.340	0.237	0.519	0.577	1.453	0.590
Collagen, type III, alpha 1	0.225	1.110	0.949	0.649	1.099	1.106	1.073
Collagen type XII alpha-1	0.356	1.335	0.766	0.718	0.416	1.526	1.112
Lysyl oxidase-like 2 (LOXL2)	0.063	0.328	0.422	0.168	0.328	0.274	0.293
SPARC/osteonectin	0.155	0.917	0.360	0.217	0.780	0.866	0.928
TGF beta-induced (βIG-h3)	0.171	0.445	0.551	0.270	0.500	0.604	0.277
Miscellaneous							
Cbl-b	0.571	1.066	0.987	0.742	0.674	0.808	1.073
Glutaminase	0.364	1.641	1.379	1.187	0.658	1.216	1.689
IGF binding protein 3 (IGFbp3)	0.093	0.383	0.182	0.102	0.330	0.366	0.369
Tumor rejection antigen 1 (TRA1)	0.112	1.922	0.500	0.089	1.383	0.921	0.854
URB	0.255	0.940	0.682	0.513	0.723	1.203	0.591
V-CAM1	0.019	0.507	0.717	0.114	0.366	0.779	0.509
Proteases and protease inhibitors							
Aminopeptidase puromycin sensitive	0.246	1.230	0.923	1.034	0.499	1.352	1.372
Disintegrin metalloprotease (ADAM9)	0.173	2.442	0.775	4.285	1.364	1.311	1.828
Glia-derived nexin (GDN)	0.358	1.149	0.938	0.373	0.874	0.642	0.944
SERPINE1	0.248	3.549	3.686	13.498	1.111	0.910	1.512
Protein modifiers							
GRP78	0.415	1.216	1.133	0.893	0.756	1.186	1.112
UDP-*N*-acetyl-alpha-D-galactosamine	0.105	0.941	0.850	0.418	0.906	1.100	1.467
Translation apparatus							
Glutamyl-prolyl-tRNA synthetase	0,382	2.061	1.126	1.988	0.638	1.034	1.606
Glycyl-tRNA synthetase (GARS)	0.652	1.499	2.011	1.153	0.803	0.945	1.182
Ribosomal protein L26 (RPL26)	0.375	0.903	0.294	1.335	0.908	0.590	1.025
Neoplasm-related C140 (RPL6)	0.085	1.236	1.055	1.084	1.015	1.449	1.247
Ribosomal protein L7 (RPL7)	0.130	1.821	0.702	3.811	1.241	1.356	1.396
Signaling molecules							
MARCKS	0.348	0.949	0.246	0.397	0.724	0.825	0.501
Vesicle membrane protein (RNP24)	0.394	1.132	1.338	0.888	0.727	0.946	0.731
Novel/unknown genes							
E.S.T. from osteosarcoma	0.412	1.201	0.613	0.516	1.043	1.051	1.113
E.S.T. xo47h07.x1	0.734	0.984	0.757	0.907	0.829	1.166	1.087
DC2 protein (DC2)	0.136	1.151	0.899	1.019	1.134	2.327	1.020
Similarity to FGF receptor4b (*Xenopus*)	0.149	0.656	1.080	0.246	1.022	1.043	0.758
HP1-BP74 (Histone1 like)	0.344	0.910	1.132	0.642	0.659	1.123	0.974
KIAA0203 gene product (KIAA0203)	n.a.	n.a.	0.334	12.464	0.862	0.959	1.473

*Fold change represents the ratio of gene expression in BDO versus BMSC (n.a. = no amplification).
Genes consistently down-regulated in all donors are shaded in black; light gray shows genes identified by a *z*-test that are significantly down-regulated in most donors.

between −0.405 and 0.405 were not considered as meaningful (the area between the two dashed lines in Fig. 3). Among the six genes found up-regulated in every one of the three cloned BMSC examined, versus BDO, a subset of three genes, referred to as pool A (*LOXL2*, *TGFBI*, and *IGF-bp3*), were also consistently up-regulated in all seven BMSC cultures (Table IV and Fig. 3). The mean gene expression in BMSC cultures for pool A was respectively 4.271, 2.704, and 4.438 folds higher than in their BDO counterparts. Among the remaining 31 genes selected for differential regulation in at least one BMSC clone versus the corresponding BDO, a subset of four, referred as pool B, (*SPARC/osteonectin*, *Urb*, *V-CAM1*, and *MARCKS*) were also significantly up-regulated in BMSC (Table IV and Fig. 3).

The mean gene expression in BMSC cultures for pool B was, respectively, 2.033-, 1.567-,

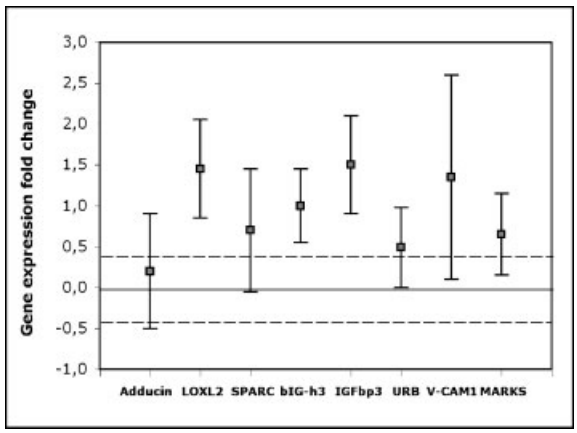

Fig. 3. Gene expression profile analyzed by a *z*-test. RT-PCR products were separated on agarose gels and documented. Using the gene *GAPDH* as an internal reference, the relative gene expression under BDO conditions and BMSC conditions were calculated separately for each gene. The relative gene expression in BMSC conditions divided by the relative gene expression in BDO conditions gives rise to the gene expression fold change. The mean value of each gene expression fold change among seven BMSC donors is plotted as Y-axis after logarithmic transformation. The solid line corresponds to value 0, indicating no fold change between the two conditions; whereas, the two dashed lines correspond to value ± 0.405, indicating the thresholds of meaningful gene expression fold change. Boxes represents the mean values of the fold change, error bars represent the 95% confidence intervals (CI). Area between these two dashed lines represents the range of no meaningful variation. Adducin is an example of gene without significant fold change: its mean value falls in the area marked by the two dashed lines. The other genes show instead a fold change significantly higher than value 0, therefore, they are considered as preferentially expressed genes in BMSC. In particular, the mean fold changes for LOXL2, TGFβI and IGF-bp3 are greater than 1, implying that the expression in BMSC conditions is 2.7-folds higher than that in BDO conditions.

3.657-, and 1.941-folds higher than in their BDO counterparts. Indeed, these seven primary cultures showed a good concordance when only these seven genes were analyzed (alpha = 0.9251).

Although the alpha score for the overall gene expression profile among these seven BMSC primary cultures was rather high (alpha = 0.8374), BM688 clearly behaved differently from the others. In BM688 primary culture, 32 out of 34 analyzed genes were downregulated in induced-osteogenic conditions, whereas only 5–15 were found in any of the other six BMSC primary cultures. Indeed when the overall expression profile among these six BMSC primary cultures was analyzed, we observed a higher reliability coefficient value (alpha = 0.8499).

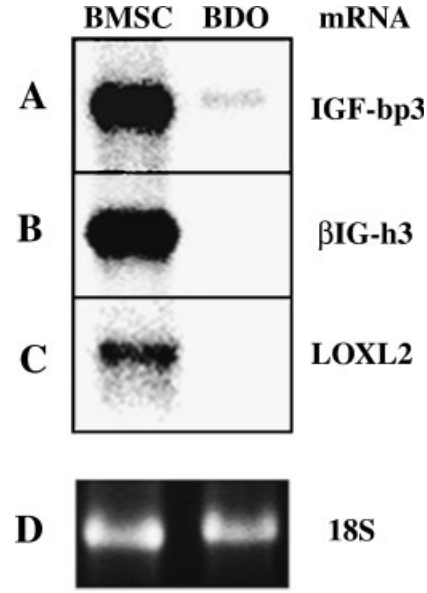

Fig. 4. Osteoblastic differentiation of BMSC primary cultures induces down-regulation in expression of LOXL2, TGFBI, and IGF-bp3. Total RNA from BMSC and BDO were blotted and subsequently hybridized with cDNA probes (**A**–**C**). An ethidium bromide-stained 18S ribosomal RNA band demonstrates equivalent loading across lanes (**D**).

Demonstration of Differential Expression of IGF-bp3, βIG-h3, and LOXL2 by Northern Blot and Immunofluorescence Analysis

To investigate upregulation of genes belonging to pool A by an alternative method, a Northern blot analysis of RNA extracted from BM1022 cultured in both growth and differentiation medium was performed. This analysis confirmed the results obtained for these genes by RFDD and RT-PCR (Fig. 4).

To verify whether the expression of pool A genes occurred also at the protein level, an indirect immunofluorescence analysis was performed. Double labeling experiments showed that 80% of the BMSC, cultured in growth medium, displayed a signal for LOXL2 and βIG-h3 proteins higher than negative control performed with non-immune sera (not shown). The same result was obtained when BMSC were double labeled with IGFbp3 and βIG-h3 antisera (not shown). Representative fields with double labeled BMSC and negative control BMSC are shown in Figure 5. No positive cells were detected when BDO were examined after incubation with LOXL2, IGFbp3, and βIG-h3 specific antisera and compared with negative control cells: BDO challenged with non-immune sera (data not shown).

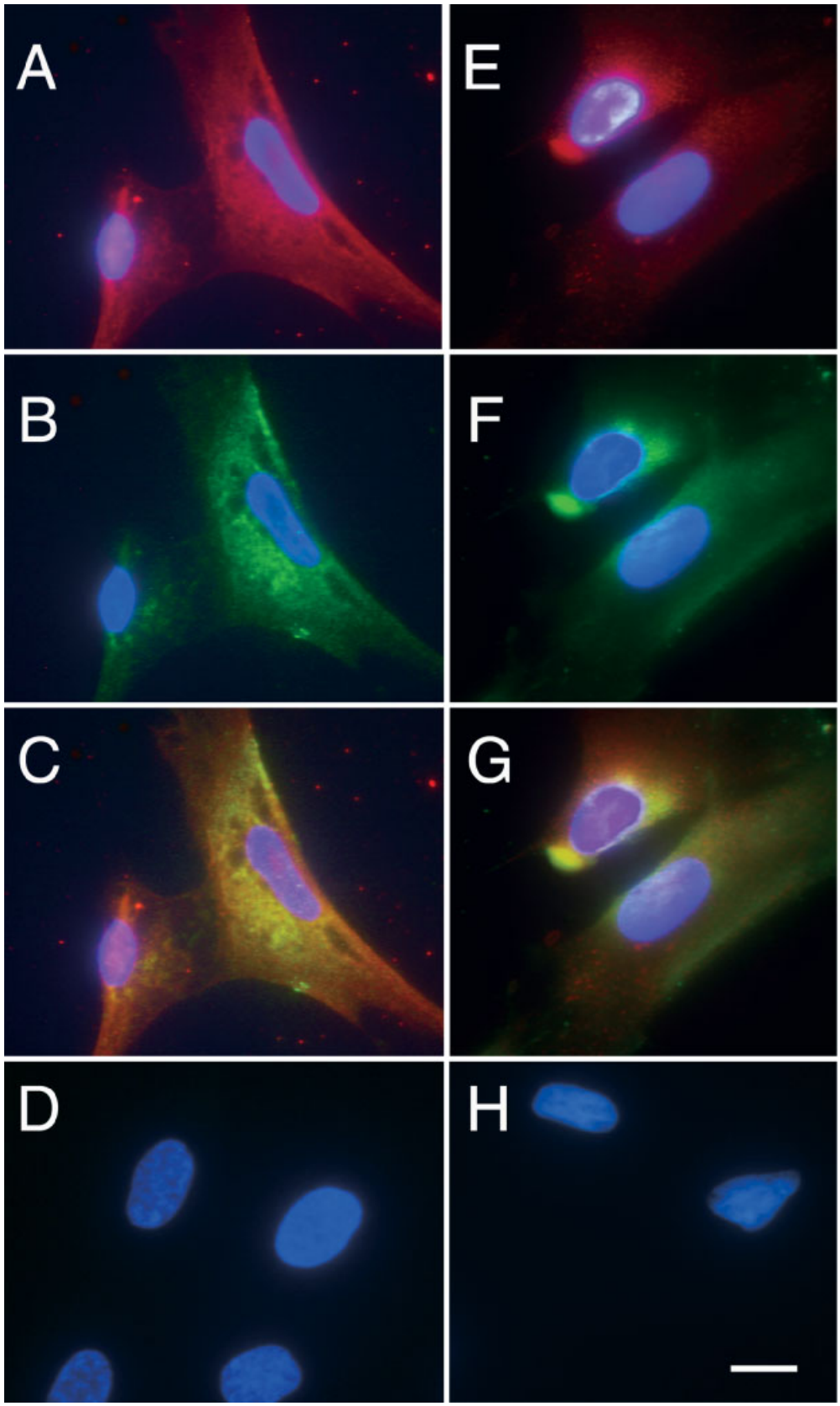

Fig. 5. Protein expression of IGF-bp3, bIG-h3, and LOXL2 in BMSC cultures. IGF-bp3 was detected with a TRITC-conjugated secondary antibody (**A**, **C**, **E**, and **G**), βIG-h3 was visualized with a fluorescein-conjugated secondary antibody (**B** and C) and LOXL2 with a Cy2-conjugated secondary antibody (**F** and G). The degree of spatial overlap in the distribution of the proteins is shown by the respective merged image (C and G). Negative controls for A and B (**D**) and for E and F (**H**). Scale bar 13.5 μm.

DISCUSSION

This report concerns the characterization of a gene expression profile of human BMSC compared with a closely related cell type, BDO using a differential display procedure. The specific goal was to identify genes preferentially expressed by BMSC versus BDO. Unlike similar, recently published studies, we examined cloned BMSC in addition to primary cell cultures, and compared preparations from a larger number of donors than done previously.

Our results indicate that 34/47 of candidate regulated genes, selected by RFDD, were confirmed by RT-PCR analysis to be preferentially expressed in BMSC, in at least one out of three cell clones examined. Among these restricted set of genes, a subset of three genes (pool A) were confirmed as up-regulated in BMSC versus BDO in every single primary culture and cloned population examined from seven different donors. Furthermore, we have also identified a second subset of four genes (pool B) significantly up-regulated in most but not all primary cultures. Northern analysis further confirmed the differential expression of pool A genes and, in addition, the corresponding encoded proteins were detected by immunolocalization in BMSC bringing evidence of their expression at the translational as well as transcriptional level.

Analysis of our data show a substantial discrepancy in gene expression profiles among primary BMSC cultures obtained from different individuals. This may reflect normal variation in gene expression in a healthy population as also reported recently for different cell types [Yan et al., 2002; Whitney et al., 2003] and BMSC [Qi et al., 2003]. In addition, we also show discrepancy in relative gene expression among cloned BMSC obtained from the same donor. We suggest that this may be explained by the fact that BMSC cultures, from which clones are obtained, are a heterogeneous population of different and still uncharacterized cell types [Kuznetsov et al., 1997; Phinney et al., 1999; Muraglia et al., 2000] and/or they may represent a populations of cells at different stages of differentiation, although derived from a single cell type. However, our data suggest extreme caution in evaluation of differential gene expression data from primary BMSC cultures derived from single or from several donors. We are also aware that age and sex related differences might account for the observed individual variations in BMSC gene expression profiles. However, the eight different individuals (including the primary cultures used to perform RFDD) we examined were not enough to address this specific point.

Nevertheless, our study identified, among 47 genes selected by RFDD, three genes (pool A) consistently upregulated in BMSC respect to BDO: *IGF-bp3*, *βIG-h3/TGFBI*, and *LOXL2*.

IGF-binding protein 3 is known to be the major IGF-binding protein secreted by human marrow cells [Rosen et al., 1997]. It is well known to regulate, with other IGFBPs, the effects of insulin like growth factors (IGFs) I and II on the stimulation of osteoblast differentiation, proliferation, and matrix protein expression [Frolik et al., 1988; Hock et al., 1988]. It has been proposed that IGF-bp3 is an inhibitory IGFBP in osteoblasts [Mohan et al., 1989]. βIG-h3 interacts with several matrix proteins such as type I collagen, laminin, and fibronectin [Kim et al., 2002b] and cell adhesion molecules [Kim et al., 2000b]. These observations suggested that βIG-h3 could mediate important cell functions as differentiation and cell adhesion [Kim et al., 2002a]. In particular, βIG-h3 seems to be involved, playing a negative role, as a regulator of differentiation in early stages of bone formation [Kim et al., 2000a]. LOXL2 belongs to the Lysyl oxidase family. The better characterized member of this family, LOX, is thought to be involved in many important biological functions, such as cell motility, cell growth, and differentiation [reviewed in Csiszar, 2001]. LOXL2 function is not understood yet, but it has been reported that LOXL2 probably participates in formation of collagen fibrils and insoluble elastin fibers [Csiszar, 2001].

Several studies demonstrate upregulation of these three genes (pool A) in cancer cells when compared to their counterpart normal cells [Skonier et al., 1992; Kitahara et al., 2001; Zajchowski et al., 2001; Kirschmann et al., 2002; Sasaki et al., 2002; Akiri et al., 2003; Schmid et al., 2003]. Our data and these studies suggest a similarity between these cells with respect to their cell–matrix interactions and their ability to colonize distant tissues that deserve further investigation.

In the pool B genes, we found *V-CAM 1*, *Urb*, *osteonectin*, and *MARCKS*. It should be noted that most of the genes we identified (5/7), in both pools A and B, encode proteins essential for the biogenesis of connective tissues or relevant to

cell–cell and cell–matrix interactions. Therefore, our study provides strong support for the importance of these processes in mediating BMSC functions [Gronthos et al., 2001]. Our data are in agreement with very recent reports: in fact LOXL2, and some of genes belonging to pool B (*V-CAM 1*, *osteonectin*) were among those described as respectively characteristic of BMSC and stem cells gene expression profiles [Qi et al., 2003]. Moreover, βIG-h3 and, again, osteonectin were found to be inhibited in dexamethasone-induced osteogenic differentiation [Dieudonne et al., 1999].

The herein reported identification of a restricted set of genes upregulated in human BMSC respect to BDO should contribute toward a better understanding of the molecular mechanisms of BMSC differentiation and provide a starting point for thorough examination of similarities between these cells and cancer cells.

ACKNOWLEDGMENTS

We thank Prof. O. Greengard and Prof. M. Porotto for editing the manuscript; L. Benazzi for technical assistance. We also acknowledge the generosity of the Prof. I.-S. Kim and of the REGEN Biotech (Seoul, Korea) for their gift of anti βig-h3 antibody; Prof. G. Neufeld and Dr. G. Akiri for anti-LOXL2 antibody.

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