

Differential Expression Profiling of Membrane Proteins by Quantitative Proteomics in a Human Mesenchymal Stem Cell Line Undergoing Osteoblast Differentiation

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ABSTRACT

One of the major limitations for understanding the biology of human mesenchymal stem cells (hMSCs) is the absence of prospective markers needed for distinguishing them from other cells and for monitoring lineage-specific differentiation. Mass spectrometry (MS)-based proteomics has proven extremely useful for analyzing complex protein expression patterns and, when applied quantitatively, can be used to resolve subtle differences between samples. Thus, we used MS to characterize changes in expression of membrane protein markers before and after short-term induction of osteoblast (OB) differentiation in a cell model of hMSCs established by overexpression of human telomerase reverse-transcriptase gene. We identified 463 unique proteins with extremely high confidence, including all known markers of hMSCs (e.g., SH3 [CD71], SH2 [CD105], CD166, CD44, Thy1, CD29, and HOP26 [CD63]) among 148 integral membrane or membrane-anchored proteins and 159 membrane-associated

proteins. Twenty-nine integrins and cell adhesion molecules, 20 receptors, and 18 Ras-related small GTPases were also identified. Upon OB differentiation, the expression levels of 83 proteins increased by at least twofold whereas the levels of another 21 decreased by at least twofold. For example, alkaline phosphatase (ALP), versican core protein, and tenascin increased 27-, 12-, and 4-fold, respectively, and fatty acid synthase decreased sixfold. The observed increases in versican and ALP were confirmed using immunocytochemistry and cytochemistry. Quantitative real-time reverse transcription-polymerase chain reaction confirmed the presence of mRNA of these membrane proteins. However, with the exception of ALP, no concordance was detected between the changes in levels of gene and protein expression during OB differentiation. In conclusion, MS-based proteomics can reveal novel markers for MSCs that can be used for their isolation and for monitoring OB differentiation. *STEM CELLS* 2005;23:1367-1377

INTRODUCTION

Human mesenchymal stem cells (hMSCs) are multipotent stem cells found in the bone marrow stroma and possibly the stroma of many other organs [1]. These cells are able to differentiate

into multiple mesoderm-type cells, including osteoblasts (OBs), adipocytes, and chondrocytes. Due to their versatile growth and differentiation potential, hMSCs are ideal candidates for use in regenerative medicine and cell transplantation protocols [2]. Tra-

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ditionally, hMSCs have been isolated from bone marrow using physicochemical properties (adhesions to plastic or other extracellular matrix), and only a small number of surface markers are currently available for their isolation [3–5]. Also, study of their differentiation potential (e.g., into OB) has relied on monitoring changes in a small number of proteins [6–8]. Thus, identification of marker proteins that can be used to identify and distinguish hMSCs from other cell types, as well as to monitor their differentiation progress, is needed to make use of the full therapeutic potential of hMSCs. In addition, comprehensive profiles of MSC membrane proteins can provide novel biological insights into the proliferation and differentiation of these cells, including the potential for identifying therapeutic targets.

Few studies have tried to identify new molecular markers of hMSCs because most investigations have used hybridoma technology to obtain monoclonal antibodies against subsets of MSCs [4, 5, 9]. Although informative, this method is too focused to provide a global understanding of changes in the membrane phenotype of MSCs during differentiation. Mass spectrometry (MS)-based proteomics tools can be applied qualitatively to gain a more holistic view of biological systems, as seen in recent studies of keratinocytes [10] and the osteoclast secretome [11]. However, quantitative proteomics methods allow dynamic changes in cell differentiation stages to be followed on the same broad scale, potentially revealing much more insight into the system of interest. Of particular relevance to this study, Conrads et al. [12] have used isotope-coded affinity tags to examine the effects of inorganic phosphate on the murine OB proteome. The choice of MS instrument and database search parameters can also have a large impact on the reliability of protein identifications [13], leading to unacceptably high levels of false-positive identifications if criteria are not stringent enough.

Application of proteomics to human stem cell biology has been hampered by the lack of physiologically relevant cell models that can be expanded to generate the levels of material required for such studies. We have recently developed a cell model for hMSCs by overexpressing the human telomerase reverse transcription (hTERT) gene in normal hMSCs and thus created a cell line termed hMSC-TERT that maintains the phenotypic characteristics and hormonal responsiveness of normal hMSCs despite extensive cell proliferation [14, 15]. To gain some understanding of the process of OB differentiation of hMSCs, we have enriched membrane proteins from hMSC-TERT before and after induction of OB lineage commitment in short-term culture *in vitro*. Using quantitative, MS-based proteomics, we have identified 463 proteins with very high confidence and have measured their changes in expression induced by OB differentiation. Several marker proteins of stem cells and OB were identified, along with several new candidate proteins with putative roles in stem cell proliferation and differentiation. For those gene products changing most dramatically, we have also measured the concomitant changes in their mRNA expression levels

by reverse transcription (RT) real-time polymerase chain reaction (PCR) and confirmed the protein level changes for three of them by cytochemistry and confocal immunofluorescence microscopy.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade or the highest purity available. Cell culture reagents, including Cell Dissociation Buffer (CDB), were obtained from Gibco Invitrogen (Tastrup, Denmark, <http://www.invitrogen.com>). Calcitriol (1,25 dihydroxy vitamin D3, vit. D3) was obtained from Leo (Ballerup, Denmark, <http://www.leo-pharma.com/w-site/leo/docs.nsf>). Complete protease inhibitor cocktail tablets were obtained from Roche Diagnostics GmbH (Mannheim, Germany, <http://www.roche.com>). Sequencing-grade modified porcine trypsin was purchased from Promega (Madison, WI, <http://www.promega.com>). Endopeptidase LysC was purchased from Wako Pure Chemical Industries (Osaka, Japan, <http://www.wako-chem.co.jp/english>). Primary and secondary antibodies were used and obtained as follows: mouse anti-transferrin receptor (1:200 dilution; Zymed Laboratories, San Francisco, <http://www.zymed.com>), rabbit anti-versican (1:100 dilution; Affinity Bioreagents, Golden, CO, <http://www.bioreagents.com>), rabbit anti-collagen $\alpha 1$ (1:100, a kind gift from Dr. Juha Risteli [16]), Alexa488-conjugated chicken anti-mouse (1:250; Molecular Probes Inc., Eugene, OR, <http://probes.invitrogen.com>), and Cy3-conjugated donkey anti-rabbit (1:250; Jackson ImmunoResearch Laboratories, West Grove, PA, <http://www.jacksonimmuno.com>).

Cell Culture

hMSC-TERT [14] cells were maintained in 145-cm² Petri dishes in minimal essential medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For induction of OB differentiation [8, 15], cells were seeded at 3.0×10^5 cells per 9.6-cm² Petri dish and left to attach overnight. The media was then changed to either control media (as above with added vehicle) or OB differentiation media (control media supplemented with calcitriol [10 nM]), and 48 hours later, the media was replenished. The cells were harvested on day 4 by washing them twice with phosphate-buffered saline (PBS), allowing them to detach in CDB according to the manufacturer's instructions, and then pelleting them at 1000g.

Subcellular Fractionation

The cell pellet from two 145-cm² dishes was washed twice in PBS, resuspended in a hypotonic buffer (20 mM KCl, 10 mM HEPES, pH 7.4) for 2 minutes, and collected again by centrifugation (1000g, 5 minutes). The resulting pellet was resuspended in sucrose homogenization buffer (255 mM sucrose, 20 mM HEPES, 1 mM EDTA, pH 7.4, 4°C) containing complete protease inhibitor

tablets and transferred to a 7-ml glass homogenizer (Wheaton Science Products, Højbjerg, Denmark, <http://www.wheatonsci.com>), where the cells were broken with five strokes with the loose pestle and 35 strokes with the tight pestle [17]. The number of strokes was determined empirically during preliminary experiments under the described conditions by observing via phase-contrast microscopy when more than 95% of the cells were broken. The cell lysates were transferred to microfuge tubes and centrifuged at 14,000g for 10 minutes, after which the resulting supernatant was transferred to 4-ml polycarbonate tubes and centrifuged at 245,000g for 2 hours at 4°C. The pellet from this step was resuspended in 100 mM Na₂CO₃ (pH 11) and disrupted mechanically using a 25-gauge syringe before incubating with rotation for 90 minutes at 4°C. Finally, the membranes were collected by centrifugation for a further 90 minutes at 245,000g. Each preparation from two 145-cm² dishes of cells yielded approximately 50 µg of protein. All fractionation steps were carried out at 4°C or on ice.

Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)

The membrane pellet was solubilized in deionized 6-M urea/2-M thiourea buffered by 20 mM Tris (pH 8.0), reduced, alkylated, and digested to peptides with trypsin and endopeptidase LysC as described [18]. Ten micrograms of total peptide mass from each sample was desalted/concentrated/filtered as described [19] and transferred to 96-well sampling plate.

Peptides were eluted from the analytical column by three-step, 140-minute gradient running from 2%–80% Buffer B and sprayed directly into the orifice of a QSTAR-Pulsar quadrupole time-of-flight (TOF) hybrid mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada, <http://www.sciex.com>) or an LTQ-FT linear ion trap-Fourier transform mass spectrometer (Finnigan, Bremen, Germany, <http://www.thermo.com>) [20]. Both mass spectrometers were set up in a data-dependent acquisition mode in which multiply charged peptides were selected for fragmentation. In the QSTAR, fragmentation was accomplished by collision-induced dissociation and the fragments were measured in the TOF region, whereas in the LTQ-FT, fragmentation was carried out in the ion-trap and resulting fragments were measured using the ion-trap detectors. The QSTAR acquired four fragment spectra for every survey scan, giving a cycle time of approximately 5 seconds. The LTQ-FT was set to acquire five-fragment spectra in the ion trap, while at the same time the FT detector was measuring a full m/z range survey scan at 100,000 resolution, resulting in a cycle time of approximately 1.5 seconds.

Protein Identification

Fragment spectra were searched against the Human International Protein Index (IPI) database (March 1, 2004) (<ftp://ftp.ebi.ac.uk/pub/databases/IPI/current/>) by using Mascot Server (Matrix Science, London, <http://www.matrixscience.com>). The

following search parameters were used in all Mascot searches: tryptic cleavage rules with a maximum of one missed cleavage, cysteine carbamidomethylation, methionine oxidation, fragmentation rules appropriate for the instrument (ESI-QUAD-TOF for QSTAR, ESI-TRAP for LTQ-FT), and mass accuracies appropriate for the instrument (a maximum 0.2-Da error tolerance for the QSTAR in both the MS and MS/MS data and a maximum 9 ppm for MS and 0.5-Da error tolerance for MS/MS for the LTQ-FT). Peptides identified using these criteria were then filtered further after iterative mass recalibration. For QSTAR data, peptides with an IonsScore >40 were accepted without further verification whereas those with IonsScores between 30 and 40 were manually verified by criteria consistent with Q-TOF-type fragmentation (a minimum length of eight amino acids, a consecutive y-ion series of at least three ions, or the presence of an intense terminal proline ion). For LTQ-FT data, peptides were accepted only if they had an IonsScore greater than 25, containing more than seven amino acids, and had a relative mass error of less than 9 ppm. In total, 4,200 unique peptides that met these conditions were identified, so the fragment spectra were also searched against the reversed human IPI database [21] to establish statistical rates of false-positive identifications. In this way, 46 peptides with similar criteria were identified, resulting in a false-positive rate of 46/4,200, or 1.1%, on the peptide level. Therefore, proteins were considered identified only if they were observed in at least two of the three pairs of samples analyzed and if two or more unique peptides meeting the above criteria were sequenced, giving a potential false-positive rate of approximately 1 in 10,000.

To completely eliminate redundant protein identifications in the final protein list reported here, the peptides were researched against the most recent human IPI database (v2.37) using the PAM30 scoring matrix for BLAST (Basic Local Alignment Search Tool) searches with short sequences (<ftp://ftp.ncbi.nih.gov/blast/executables/>) and the results were compiled into protein hits. Finally, any protein hit whose set of identifying peptides was equal, to or a subset of, another hit was considered redundant and removed. In this way, distinct protein isoforms were retained only if they were distinguished from others by at least one unique peptide. Manual verification of spectral assignments from Mascot result files, iterative mass recalibration, elution time correlation, and extraction of quantitative data from the raw data files was performed using MSQuant (<http://msquant.sourceforge.net>) [22]. When a peptide was not identified by tandem MS, its elution time was predicted from the correlation of the control and differentiated samples to extract the correct ion current [23]. Due to limitations in the linear dynamic range of QSTARs when measuring differences greater than 10-fold, we report ratios greater than this limit as > (i.e., >27) with no SD [18]. The average R² value for the correlation of elution times between two consecutive LC/MS/MS experiments was greater than 0.99, indicating the reliability of elution time prediction. Peptide ion volumes

(time \times ion count \times mass window [Da-s]) were calculated by integration of the XIC (eXtracted Ion Chromatogram), from which the ratios of OBs differentiated to hMSCs were calculated. Assignments of all extracted ion chromatograms were manually verified. All protein and corresponding peptide data were stored in an SQL 2000 (Microsoft, Redmond, WA, <http://www.microsoft.com>) relational database. Gene Ontology (GO) annotations (<ftp://ftp.geneontology.org/pub/go/gene-associations/>) were assigned to each identified protein by an automated, in-house script using the human cross-reference table (<ftp://ftp.ebi.ac.uk/pub/databases/GO/goa/HUMAN/xrefs.goa>). Transmembrane domains were predicted using the TMPred algorithm (http://www.ch.embnet.org/software/TMPRED_form.html). Sequence coverage (supplemental online Fig. 1) was mapped using the coverage tool from Proteios (<http://www.proteios.org/proj/coverage>).

RT and Real-Time PCR

Total RNA was isolated by RNAElute kit (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) according to the manufacturer's instructions, after which the RNA samples were treated with DNase I (Roche Molecular Biochemicals, Basel Switzerland, <http://www.roche-applied-science.com>). The cDNA was prepared from 2 μ g of total RNA with cDNA synthesis Kit (Bio-Rad, Hercules, CA, <http://www.bio-rad.com>) in a final volume of 20 μ l. The primers for all assayed genes were designed using the PrimerSelect program of the Lasergene software package (DNA-STAR, Madison, WI, <http://www.dnastar.com/web/index.php>). For a single PCR amounting to 20 μ l, 0.1 μ l of cDNA was used. SYBR Green I supermix (Bio-Rad) was used to visualize PCR products in real time. A three-temperature cycling, consisting of a denaturation step at 95°C for 15 seconds and annealing/extension step at 50°C to 68°C for 20 seconds, 72°C for 20 seconds, was carried out in an iCycler instrument (Bio-Rad). Optimal annealing temperatures were determined for each gene, the lengths of the PCR products were verified by agarose gel electrophoresis, and melting curve analysis was used to assess the specificity after each PCR. β -Actin was used as an endogenous standard to normalize for input load of cDNA between the samples. For the quantitative analysis of the individual genes, two independent cDNA samples were prepared and each of the cDNAs was tested in duplicate.

Staining for Alkaline Phosphatase Activity

Cells were washed in PBS twice and fixed in methanol/formalin (9:1) for 1 minute. The cells were incubated with alkaline phosphatase (ALP) substrate solution (5 mg naphthol AS-TR phosphate in 25 ml water plus 10 mg Fast red TR in 24 ml of 0.1 M Tris buffer, pH 9.5) for 1 hour at room temperature. Mayers-hematoxylin was used as a counter-stain. Cells were photographed using an Olympus IX 50 inverted microscope (Olympus, Albertslund, Denmark, <http://www.olympus.com>) equipped with an Olympus C3040 zoom digital camera (magnification \times 40).

Immunocytochemical Staining

Control and OB-differentiated cells were grown on coverslips and fixed in 4% formaldehyde in PBS (all PBS solutions contained 1.04 mM MgCl₂ and 0.9 mM CaCl₂) for 10 minutes on ice. Excess formaldehyde was reacted with 50 mM NH₄Cl in PBS for 5 minutes, and then nonspecific immunoglobulin-G binding sites were blocked for 20 minutes with 3% bovine serum albumin in PBS. Primary and secondary antibody incubations were each 1 hour, and coverslips were washed four times with PBS after each. Coverslips were mounted on glass slides using Dako Fluorescent Mounting Solution (DakoCytomation, Glostrup, Denmark, <http://www.dakocytomation.com>) and imaged using an Axiovert 200M laser-scanning confocal microscope 510 (Carl Zeiss, Jena, Germany, <http://www.zeiss.com>). Pinhole settings were adjusted to give a confocal depth of approximately 1 μ m.

RESULTS

The primary aim of this study was to identify and quantify changes in plasma membrane proteins, as well as those of the biosynthetic and endosomal pathways in hMSCs during OB differentiation. We first enriched integral membrane proteins and membrane-associated proteins by eliminating the highly abundant nuclear and mitochondrial proteins before collecting the remaining membranes by an ultra-high-speed centrifugation step. Resuspending the membranes in Na₂CO₃ at pH 11 reduced the potential masking effects of highly abundant cytosolic proteins bound loosely to the membrane pellet. Pairs of membrane samples prepared in this way (three control and three OB-differentiated cells) were digested to peptides and analyzed by information-dependent acquisition on two different classes of hybrid mass spectrometers: QSTAR Pulsar quadrupole TOF instruments or a LTQ-FT linear ion-trap/Fourier transform ion cyclotron resonance instrument [20]. In each case, the instrument software selected peptide ions from the survey scan (Fig. 1A) to be fragmented (Fig. 1B). The total ion chromatogram in each pair of samples (Fig. 1C) looked superficially similar but was far too complex to serve as a basis for quantitative comparison. Therefore, using an adaptation of MSQuant [22, 23], we first correlated the retention times (see Materials and Methods) of high-confidence peptides (IonsScore >40) identified in both members of each pair of hMSC and OB-differentiated cell membranes (Fig. 1D) before extracting the ion chromatograms for each monoisotopic ion for all identified peptides (Fig. 1E). By making use of the retention time correlation and high-mass accuracy to locate unsequenced peptide ions in the opposing sample, this strategy only required a peptide to be identified in one of the two samples. The effect of OB differentiation was determined by dividing the results obtained in OB-differentiated samples by that in control samples (OB/control ratios) for each protein based on the average for all peptides observed from that protein across all replicates. The OB/control ratios measured for these proteins ranged from 6-fold downregulation to 27-fold upregulation (Fig. 1F).

Compilation of the data from all three analyses resulted in the identification of 463 proteins seen in at least two of the three analyses and with at least two unique peptides. This set of proteins represents an extremely high confidence profile of membrane proteins in hMSCs because, to be considered identified, proteins had to be observed in at least two different samples and had to have at least two sequenced peptides detected with high mass accuracy. This means that it is extremely unlikely that there is even one false-positive identification among the 463 (see Materials and Methods). A complete list of all proteins

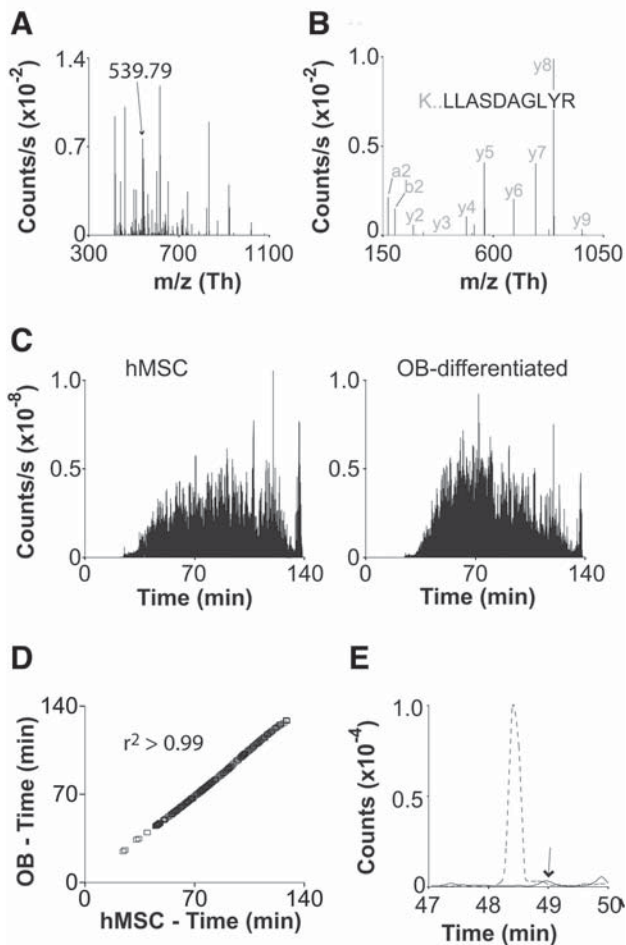


Figure 1. Liquid chromatography/tandem mass spectrometry. (A): Survey scans of peptides eluted from reversed-phase columns were acquired as described in Materials and Methods and used to select peptide ions for fragmentation. Fragment spectra (B) of the 539.79-m/z ion from (A) was found to match the indicated peptide from versican core protein. (C): Representative total ion chromatograms from hMSCs and OB-differentiated samples. (D): Plot of elution times for peptides sequenced in both samples of a pair. The calculated correlation coefficient is greater than 0.99. (E): Representative ion chromatograms for peptide LLASDAGLYR of versican core protein measured in hMSCs (solid line, near baseline) and OB-differentiated (dashed line) samples. (F): Plot of ratios for all proteins quantified here, ordered from largest positive change with OB differentiation toward largest negative change. Abbreviations: hMSC, human mesenchymal stem cell; OB, osteoblast.

and peptides identified in this study is presented in the online supplementary material.

Classification of the subcellular localizations of 463 proteins based on annotations in the UniProt Knowledgebase (<http://www.ebi.uniprot.org/index.shtml>) and the Gene Ontology Consortium revealed several classes of enriched proteins (Fig. 2A). Sixty-six percent are integral membrane proteins, proteins with known or predicted membrane anchors or proteins known to interact with other membrane proteins. These included all known markers of MSCs, including ALP, 5'-nucleotidase (CD73), Thy-1 glycoprotein (CD90), neprilysin (CD10), myeloid plasma membrane glycoprotein (CD13), endoglin (CD105), activated leukocyte-cell adhesion molecule (CD166), HOP26 (CD63), integrin β 1 (CD29), integrin α 5 (CD49e), integrin α 4 (CD49d), phagocytic glycoprotein I (CD44), fibronectin, collagen type VI, and epidermal growth factor receptor [3, 24–26]. Other CD antigens detected were CD98, CD59, CD51, CD107b, CD107a, CD91, CD99, CD71, CD47, and CD108. Also, several members of the integrins, integrin alpha 11, integrin beta-5, integrin alpha-2 (CD49b), integrin alpha-6 (CD49f), integrin alpha-V (CD51), and integrin alpha-3 (CD49c), were detected (see supplemental material).

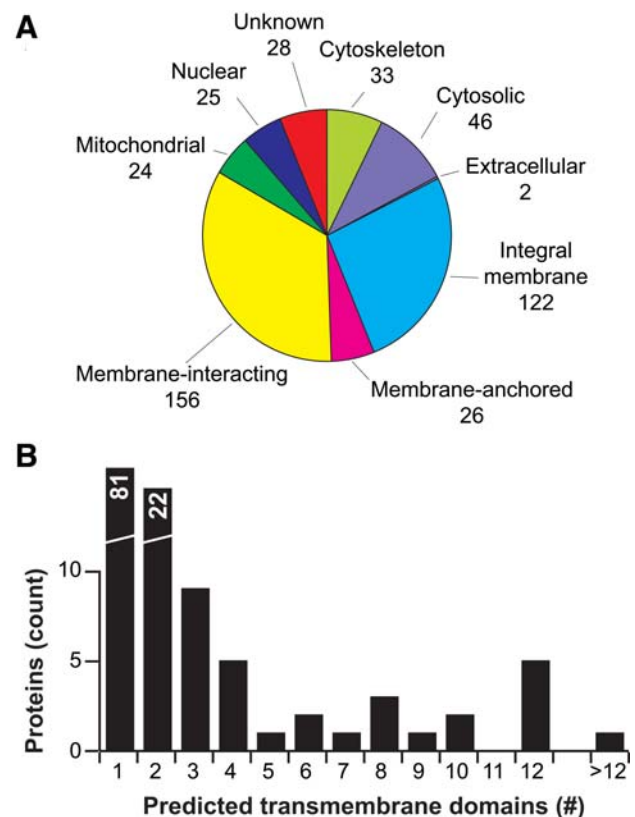


Figure 2. Distribution of identified proteins. (A): Subcellular distribution of all identified proteins according to their annotation in the Swiss-Prot Knowledgebase. (B): Number of proteins with different numbers of transmembrane domains, predicted as described in Materials and Methods.

Membrane proteins, particularly of the plasmalemma, are important in defining the unique characteristics of stem cells. To better understand the nature of the MSC membrane proteome, we took amino acid sequences of all proteins identified here and predicted their membrane topology using publicly available bioinformatics tools. Interestingly, 133 of the 463 proteins had at least one α -helical transmembrane domain (TMD) predicted in their primary sequence, 11 more than were classified as integral membrane based on their UniProt annotations. Most proteins contained either one or two TMDs, but many proteins with several TMDs were also detected (Fig. 2B and supplemental online material). Likewise, the success of the membrane enrichment is also reflected by the observation that only 11% of the identified proteins originated from nuclei or mitochondria.

Induction of OB differentiation of hMSC-TERT cells in our experiments was confirmed by the presence of increased expression of four OB-specific genes: *ALP*, collagen type I (*COL1*), osteopontin and osteocalcin, and bone sialoprotein 2 (*BSP2*) (Fig. 3A). Our working hypothesis in this study was that markers of OB differentiation should show an OB/hMSC ratio greater than or less than 1. As expected, ALP, detected here with 9% sequence coverage, displayed the highest OB/hMSC ratio (>27, Fig. 1C and supplemental online material) of all the proteins quantified in this study. The expression levels of several other proteins also increased or decreased with OB differentiation. To determine a significance threshold for these changes, we first considered the measurement errors in this analysis. The average relative SD of OB/hMSC ratios measured here was 47%, so we considered any protein whose expression level changed by at least this, or twofold in either direction, to be of potential interest. Based on these criteria, we identified 83 proteins that increased at least twofold and 21 proteins that decreased at least twofold (Table 1 and supplemental online material).

We next sought to verify some of the proteins that showed the largest changes during early-stage OB differentiation. By staining cells for ALP activity, we observed significantly increased levels of the enzyme in OB-differentiated cells compared with control cultures (Fig. 3B). Furthermore, we used dual-color immunofluorescence to double-label CD71/versican and CD71/COL1 in nonpermeabilized hMSC-TERT and OB-differentiated cells. We selected CD71 (transferrin receptor) as a control in these experiments because a portion of it is found on the plasma membrane and, in our quantitative analysis described above, it did not change upon induction of OB differentiation (1.0 ± 0.5). Using CD71 staining intensities as a baseline, COL1 and versican were both more abundant in the OB-differentiated cells compared with control cells (Figs. 3C, 3D).

Although the quantitative analysis of hMSC and OB membranes revealed several proteins whose expression levels changed dramatically, our dataset also contains a great deal of

additional information. We asked if there are any functional classes of proteins identified in this study that are coregulated during OB differentiation. Mapping GO terms (see Materials and Methods) to the proteins identified here revealed several functional classes of proteins displaying very close coregulation (Table 2). In particular, all nine proteins annotated in the cell-matrix adhesion and integrin receptor signaling pathway GO categories displayed remarkably consistent changes in their expression levels, increasing an average of 2.0 ± 0.5 -fold with OB differentiation. All five heteronuclear ribonuclear proteins (hnRNPs) increased even more dramatically with OB differentiation (6.9 ± 4.0 -fold).

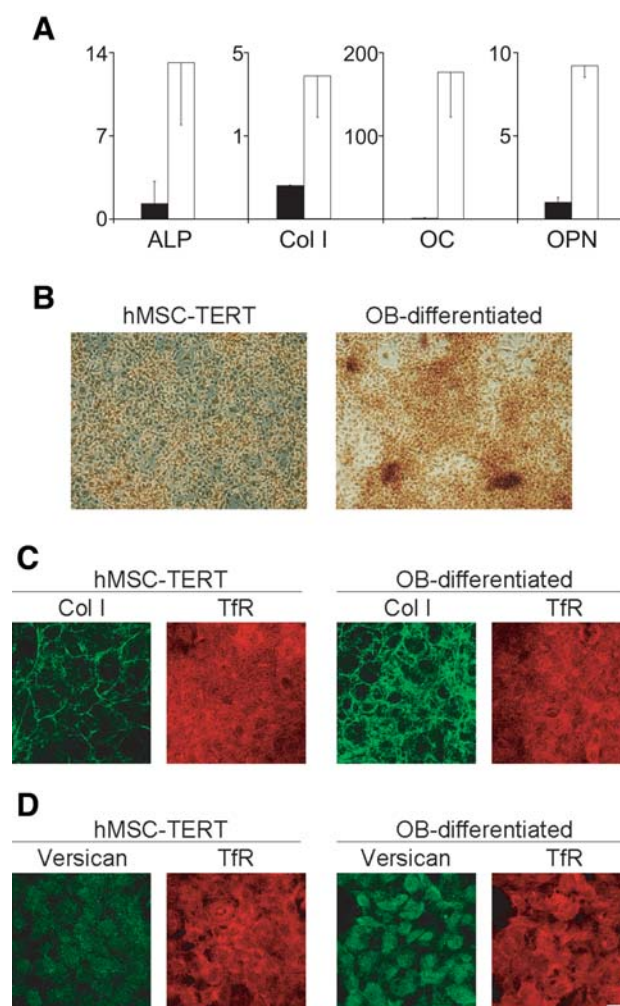


Figure 3. Marker proteins. **(A):** Fold changes in mRNA levels of Runx2 (CBFA-1), alkaline phosphatase (ALP), collagen type I (Col I), osteopontin (OPN), osteocalcin (OC), and bone sialoprotein 2 (BSP2) between human mesenchymal stem cells (hMSCs) (black bars) and osteoblast (OB)-differentiated cells (open bars). **(B):** ALP enzyme activity in hMSCs and OB-differentiated cells, detected as described in Materials and Methods. **(C):** Collagen type I $\alpha 1$ (Col I) and transferrin receptor (TfR) levels on hMSCs and OB-differentiated cells. **(D):** Versican and TfR levels on hMSCs and OB-differentiated cells. Scale bar = 50 μ m.

Table 1. Proteins changing expression during differentiation

UniProt	Name	Coverage ^a	OB/hMSC ^b	
			Protein	mRNA
P05186	Alkaline phosphatase	9%	27 ^c	13 ± 5
P84089	Enhancer of rudimentary	21%	15 ^c	1.0 ± 0.1
P13611	Versican core protein	2%	13 ^c	1.1 ± 0.2
P22087	Fibrillarlin	13%	12 ^c	ND
P07910	hnRNP C1/C2	14%	11 ^c	1.0 ± 0.1
P63245	RACK1	10%	10 ^c	1.0 ± 0.1
P38159	hnRNP G	15%	8.7 ± 4.4	0.8 ± 0.3
Q00839	hnRNP U	12%	8.7 ± 1.5	1.2 ± 0.1
P04040	Catalase	11%	6.4 ± 0.6	0.9 ± 0.1
Q86VM3	MYB-binding protein 1A	7%	5.3 ± 2.6	1.1 ± 0.2
P41252	Iso-tRNA synthetase	11%	5.1 ± 1.9	1.0 ± 0.3
Q9P2E9	Ribosome-binding protein 1	17%	5.1 ± 1.7	1.06 ± 0.04
P51659	Multifunctional enzyme 2	10%	4.9 ± 1.9	0.9 ± 0.1
Q08211	RNA helicase A	6%	4.2 ± 1.1	ND
P24821	Tenascin	3%	4.1 ± 0.1	0.9 ± 0.1
Q9BZE4	GTP-binding protein NGB	8%	4.1 ± 0.9	1.1 ± 0.2
Q86V81	THO complex subunit 4	19%	4.0 ± 1.2	ND
Q8N765	KIAA0776	4%	3.8 ^d	1.0 ± 0.3
Q9NR30	RNA helicase II	7%	3.7 ± 2.7	ND
P62070	R-Ras2	33%	3.7 ± 2.3	1.4 ± 0.2
Q9UBG0	uPAR-associated protein	3%	3.4 ± 2.5	2.3 ± 0.5
P35232	Prohibitin	30%	3.4 ± 2.6	ND
Q9NRG3	GK001	8%	3.3 ± 1.7	1.1 ± 0.2
O75165	DnaJC13	5%	3.2 ± 1.6	0.9 ± 0.3
P61604	10-kDa HSP	42%	3.2 ± 0.9	0.8 ± 0.2
O75643	U5200KD	5%	3.2 ± 0.8	ND
Q96AG0	EBNA-2 coactivator	12%	3.2 ± 2.5	ND
O60506	hnRNP Q	4%	3.2 ± 0.6	ND
P06576	ATP synthase β	17%	3.1 ± 0.7	1.3 ± 0.4
P35998	26S ATPase subunit 2	19%	3.1 ± 0.7	0.9 ± 0.1
Q9UKX5	Integrin α11	6%	3.0 ± 2.3	1.1 ± 0.1
P12109	Collagen α 1(VI)	8%	3.0 ± 2.5	1.2 ± 0.1
P62282	Ribosomal protein S11	31%	3.0 ± 2.9	1.2 ± 0.1
Q9HBH5	Retinol dehydrogenase 14	10%	3.0 ± 0.9	1.0 ± 0.3
Q00610	Clathrin heavy chain 1	21%	0.3 ± 0.6	1.4 ± 0.1
Q9UHQ4	BAP29	13%	0.3 ± 0.1	0.9 ± 0.1
P62841	Ribosomal protein S15	46%	0.3 ± 0.3	0.9 ± 0.2
Q8WUZ1	Hypothetical protein	7%	0.3 ^d	0.9 ± 0.1
O75326	CD108	5%	0.3 ^d	ND
P67936	Tropomyosin α4	14%	0.2 ± 0.2	1.0 ± 0.2
P49327	Fatty acid synthase	4%	0.16 ± 0.01	1.1 ± 0.1

^aSequence coverage observed for protein.

^bFold change and SD measured for each protein or its corresponding mRNA upon OB differentiation.

^cSD not calculated (see Materials and Methods).

^dToo few peptides were quantified to calculate an SD.

Abbreviations: hMSC, human mesenchymal stem cell; ND, not determined; OB, osteoblast.

Although proteins are the primary effectors of biological function, large-scale alterations in the levels of gene expression are often diagnostic of the changing roles of cells or tissues. We used quantitative real-time PCR to ask if the changes in levels of protein expression were mirrored by changes in

gene expression. The mRNA expression levels for the genes corresponding to the 41 proteins whose expression levels change more than threefold were measured, but only the level of ALP mRNA changed significantly with OB differentiation (supplemental online table).

Table 2. Coregulated functional families

GO no.	GO description	UniProt	Protein name	OB/hMSC ^a
0006814	Sodium transport	P05023	Na ⁺ /K ⁺ -transporting ATPase α 1 chain	1.4 \pm 0.7
		P54709	Na ⁺ /K ⁺ -transporting ATPase β 3 chain	1.2 \pm 0.6
		Q9UP95	Solute carrier family 12 member 4	1.3 \pm 0.5
00151992	Proton transport	Q9Y487	V-ATPase 116-kDa subunit a isoform 2	1.8 ^b
		P25705	ATP synthase alpha chain	2.3 \pm 1.5
		P12953	V-ATP synthase subunit d	2.9 ^b
		Q13488	V-ATPase 116-kDa subunit a isoform 3	2.4 \pm 0.2
		P06576	ATP synthase beta chain	3.1 \pm 0.7
0030163	Protein degradation	Q03527	26S protease regulatory subunit 4	2.4 \pm 1.7
		Q92524	26S protease regulatory subunit S10B	1.7 \pm 1.1
		P47210	26S protease regulatory subunit 8	2.4 \pm 1.7
0007160	Cell-matrix adhesion	P05556	CD29	1.6 \pm 0.6
0007229	Integrin receptor signaling pathway	P13612	Integrin alpha-4	1.4 \pm 0.5
		P23229	CD49f	1.7 \pm 0.5
		Q13418	Integrin-linked protein kinase 1	1.9 \pm 1.4
		P17301	Integrin alpha-2	2.0 \pm 1.0
		P06756	Integrin alpha-V	1.5 \pm 0.4
		P18084	Integrin beta-5	2.0 \pm 1.1
		Q9UKX5	Integrin alpha-11	3.1 \pm 2.3
P08648	Integrin alpha-5	2.5 \pm 1.2		
0008436	Heterogenous nuclear ribonuclear protein	P07910	hnRNP C1/C2	1.1 ^c
		O60506	hnRNP Q	3.2 \pm 0.6
		Q00839	hnRNPU	8.7 \pm 1.5
		P61980	hnRNPK	2.8 \pm 2.5
		P38159	hnRNP G	8.7 \pm 4.4
0005125	Cytokine	Q12904	Multisynthetase complex component	1.8 \pm 0.4
		P14174	Macrophage migration inhibitory factor	1.7 \pm 0.5

GO codes and descriptions for functional classes of proteins coregulated during OB differentiation. UniProt accession codes and descriptions are included for all proteins in each functional class identified.

^aFold change and SD measured for each protein upon OB differentiation.

^bToo few peptides were quantified to calculate an SD.

^cSD not calculated (see Materials and Methods).

Abbreviations: GO, Gene Ontology; hMSC, human mesenchymal stem cell; ND, not determined; OB, osteoblast.

DISCUSSION

In this study, we present for the first time a global view of the dynamic changes undergone by the hMSC membrane proteome before and after short-term OB differentiation. Our study reveals several new surface markers that can potentially be used for isolation of hMSCs and for monitoring OB differentiation. By identifying 463 proteins with near certainty, our data represent a valuable source of knowledge about the hMSC membrane proteome. For this level of confidence in identifications, the depth of coverage achieved here is also remarkable. Several typically low-abundance signaling proteins were identified with ease, such as epidermal growth factor receptor (11 peptides, 10% sequence coverage), R-ras (7 peptides, 32% sequence coverage), and TKT receptor tyrosine kinase (4 peptides, 5% sequence coverage), suggesting that this approach can be applied to other stem cells to obtain a more detailed analysis of changes in cell membrane proteome during differentiation.

To obtain the large number of cells needed to conduct an in-depth, proteomic characterization, we used the hMSC-TERT cell line that expresses a high level of telomerase due to ectopic expression of hTERT gene as a model for normal hMSCs [14, 15]. The

cells express CD105 and CD166, as well as Stro-1, the markers currently used to identify multipotential hMSCs [3, 26]. In addition, the data presented here show that hMSC-TERT expresses other surface markers known to reside in normal hMSCs [3, 24–26], indicating that the telomerase overexpression did not affect the phenotypic make up of the cells.

Several proteins were identified in this analysis that have not been described previously in MSCs and may play an important role in hMSC biology. Among these were several integrins (e.g., Cd49f, CD49c, integrin α 11, integrin α 2, and integrin-linked protein kinase) and CD antigens (e.g., CD98, CD147, and CD99). Previous reports concerning the functions of these proteins may suggest an important role for them in hMSC biology. For example, CD98 is known for its possible role for cell growth and amino acid transport in placenta [27], CD147 is an extracellular metalloproteinase inducer expressed largely in endothelial cells, CD99 is involved in cell adhesion, and CD47 (integrin-associated protein) plays a role in increased intracellular calcium upon cell adhesion to extracellular matrix. Thus, more detailed studies are needed to determine the role of these proteins in hMSC biology.

Traditionally, few protein markers are used to monitor OB differentiation based on studies performed on cultured OB from rat calvaria in vitro [6, 7]. Only ALP, as a glycosylphosphatidylinositol-anchored protein, is expected to be associated with membranes. In addition to the upregulation of ALP observed here, several candidate marker proteins for OB differentiation have also been identified, and one, versican core protein, has been confirmed by immunocytochemistry. Versican, or chondroitin sulphate proteoglycan, is known to be secreted later in the bone development process but was not previously considered a marker of early OB differentiation. We also identified two of the proteins that versican is known to interact with in the extracellular matrix, CD44 and tenascin. Tenascin likely functions to stop cell migration [28], so it is interesting to note that its expression was also significantly increased upon OB differentiation (4.1 ± 0.1). The observed coregulation of these two known binding partners, versican and tenascin, may suggest that these proteins work together to stop cell migration and allow OB differentiation to proceed, but this hypothesis needs experimental confirmation.

The concomitant upregulation of entire functional classes of proteins during differentiation suggests that these proteins are intimately involved in the differentiation process and that their regulation proceeds through similar regulatory mechanisms. Our observation that integrins and adhesion molecules increase during OB differentiation (Table 2) corroborates previous observations [29] and extends the family of proteins known to exhibit this role. In light of a recent report linking hnRNPs to cell adhesion [30], our finding that five members of this family are upregulated as cells differentiate into OB lends additional support to the critical role of adhesion in the differentiation process.

While most proteins that changed during OB differentiation were found to increase, several proteins were also observed to be downregulated. Most strikingly, FAS decreased upon OB differentiation. FAS is a homodimeric, multifunctional protein that catalyzes the synthesis of long-chain fatty acids and is expressed during adipocyte differentiation [31] as well as in proliferating cells of the bone marrow [32]. Its downregulation during OB differentiation likely reflects the decreased lipid metabolism and the nonproliferative nature of cells committed to the OB lineage.

Because this study was conducted in cultured cells, a stable isotope labeling (SIL) method such as SILAC (SIL with Amino acids in Cell culture)[29, 30] would be a viable alternative to the ion intensity-based quantitation we have used here. However, our method has the advantage that it can be extended to the proteomic analysis of human biopsies, in which the cells cannot be effectively labeled using metabolic means and the amount of available sample is extremely limiting. Chemical SIL methods generally require several hundred micrograms or milligrams of starting material, as do extensive cell fractionation methods to further enrich plasma membranes [10, 33, 34]. For these reasons, we have

avoided extensive subcellular fractionation and SIL methods here to develop effective procedures for analyzing limited normal human material.

The advent of large-scale methods for detecting both protein and mRNA abundance has revealed a surprising lack of correlation between the message and the product [35, 36] for as-yet-unknown reasons. A similar situation was observed here, although there are at least two potential reasons explaining the lack of concordance between changes in protein and mRNA levels during differentiation: Changes in mRNA levels may have peaked and returned to normal levels before our measuring them, and gene expression data reflect changes on the level of the whole cell while membrane proteomics does not necessarily represent the entire complement of a protein within a cell. Our procedure should represent all integral membrane proteins and those anchored to membranes by a lipid tail because we analyzed all postnuclear, postmitochondrial membranes. However, for proteins associating with membranes via protein-protein interactions, or, in other terms, those whose association is easily reversed, the changes measured here may represent changes in the levels of association with the membrane rather than changes in protein expression levels.

Our study has some limitations. First, we used the hMSC-TERT as a model for normal hMSCs. Although the hMSC-TERT cells exhibit surface marker expression and differentiation potential similar to that of normal hMSCs, hTERT overexpression may affect other biological functions of the cells, and thus the new surface markers identified in our study need to be confirmed in cultures of normal hMSCs. Second, we used short-term treatment with calcitriol in the presence of fetal calf serum to induce the OB phenotype. This method succeeded in inducing several known mRNA OB-specific gene markers, indicating commitment to OB lineage. However, histochemical staining for ALP revealed cellular heterogeneity with respect to the degree and intensity of staining, suggesting that not all the cells were synchronized with respect to OB differentiation. Experiments using a stronger osteogenic-differentiation mixture that contains β -glycerophosphate, ascorbic acid, and dexamethasone may have resulted in a better OB-phenotype induction. Because this differentiation mixture can also induce mineralization in vitro, it can be used to obtain mature matrix-mineralizing osteoblastic cells for analysis. Also, alternative methods for obtaining homogenous populations of differentiated cells using hMSC lines expressing reporter genes at specific OB differentiation stages may have overcome the issue of cellular heterogeneity observed in our cultures. This approach has been applied to smooth muscle cell differentiation [37] and hMSCs [38]. Finally, several of the detected proteins are not specific for hMSCs and are present in other cell types. It remains to be determined whether combining several of these new markers will increase the sensitivity for identification and isolation of hMSCs and their differentiated progeny.

In conclusion, we have reported a very high confidence profile of the membrane proteome of hMSCs during OB differentiation. We have identified several new potential molecular markers of hMSCs at both the undifferentiated and OB differentiated stage that can be potentially useful in understanding the biology of hMSCs. The increased expression levels of 16 proteins known or implicated in cell adhesion (nine cell matrix adhesion proteins, five hnRNPs, versican, and tenascin) suggests the importance of OB adhesion to the underlying matrix in the process of OB differentiation.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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