Proteomic profiling of human placenta-derived mesenchymal stem cells upon transforming LIM mineralization protein-1 stimulation

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Abstract Human placenta-derived mesenchymal stem cells (hPDMSCs) can differentiate into different types of cells and thus have tremendous potential for cell therapy and tissue engineering. LIM mineralization protein-1 (LMP-1) plays an important role in osteoblast differentiation, maturation and bone formation. To determine a global effect of LMP-1 on hPDMSCs, we designed a study using a proteomic approach combined with adenovirus-mediated gene transfer of LMP-1 to identify LMP-1-induced changes in hPDMSCs on proteome level. We have generated proteome maps of undifferentiated hPDMSCs and LMP-1 induced hPDMSCs. Two dimensional gel electrophoresis revealed 22 spots with at least 2.0-fold changes in expression and 15 differently expressed proteins were successfully identified by MALDI-TOF-MS. The proteins regulated by LMP-1 included cytoskeletal proteins, cadmium-binding proteins, and metabolic proteins, etc. The expression of some identified proteins was confirmed by further Western blot analyses. Our results will play an important role in better elucidating the underlying molecular

mechanism in LMP-1 included hPDMSCs differentiation into osteoblasts.

Keywords Proteomics · LMP-1 · hPDMSCs · Mass spectrometry

Introduction

The placenta is a temporary organ formed during pregnancy, which is essential for the normal development of embryo and fetus. The main functions of the placenta are gaseous exchange between the fetus and mother, the provision of maternal nutrients to the fetus and the excretion of waste products from the fetus. Human placenta-derived mesenchymal stem cells (hPDMSCs) can be expanded in culture and differentiate into a variety of cell types such as osteoblast, hepatocyte, vascular endothelial, cartilage, and neural-like cells in response to different microenvironmental cues (Alvarez-Silva et al. 2003; Chien et al. 2006; Fukuchi et al. 2004; Huang 2007; Matikainen and Laine 2005; Miao et al. 2006; Parolini et al. 2008; Portmann-Lanz et al. 2010; Strakova et al. 2008; Wei et al. 2008; Yen et al. 2005; Zhang et al. 2006). Mesenchymal stem cells (MSCs) from bone marrow (BM) and placenta share similar characteristics, and possess immunomodulatory properties. Both have been found suitable for allogeneic stem cell therapy

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eliminating the need of immunosuppression (Zhang et al. 2004). The merits of using hPDMSCs lie in that they are free from ethical concern in procurement and that relatively large amount of placenta-derived MSCs can be readily obtained.

LIM mineralization protein-1 (LMP-1) is an intracellular LIM-domain protein that is directly involved in osteoblast differentiation (Boden et al. 1998). Previously, LMP-1 was identified to be a signalling molecule that acts by stimulating the synthesis and secretion of other osteoinductive factors such as bone morphogenetic protein 2 and 7 (BMP-2 and BMP-7), core binding factor alpha 1 (Cbfa1) and so on (Bunger et al. 2003; Kim et al. 2003; Minamide et al. 2003; Yoon and Boden 2002; Yoon et al. 2004). Adenovirus-based gene therapy strategies with *LMP-1* induce new bone formation in vitro and in vivo (Yoon et al. 2004) and produce an osteoinductive effect in ex vivo therapy for spinal fusion (Kim et al. 2003). Although there has been a potential application of LMP-1 in bone regenerative medicine, the physiological roles of LMP-1 in MSCs remain to be established.

Following the coming of the postgenomic era, the use of systematic approaches is required to obtain a holistic view and understanding of complex biological process. Proteome, the comprehensive analysis of the protein complement of the genome, determines cell phenotype and functions. Proteomic analysis has been widely applied to various fields of life sciences, such as drug discovery (Guido and Oliva 2009), cancer (Kelleher et al. 2009) and stem cell research (Hughes et al. 2011a). Recently, proteomic patterns of various types of stem cells were established by using two dimensional gel electrophoresis (2-DE) (Faca 2012; Hughes et al. 2011b). Changes of expression levels of proteins in stem cells during differentiation were monitored by 2-DE followed by protein identification (Park et al. 2007; van Hoof et al. 2012). Therefore, proteomics provides a systematic approach to the analysis of differentiation mechanism by resolving proteome.

The objective of this study was to compare the proteome of hPDMSCs cultured with or without LMP-1. We transiently overexpressed the human *LMP-1* gene in hPDMSCs using the adenoviral expression system based on gateway technology and investigated the regulation of hPDMSCs proteins during their differentiation induced by LMP-1. Therefore, in this report, we used 2-DE and MS-based proteomic

approaches to investigate the differential protein expression patterns in undifferentiated hPDMSCs and LMP-1 induced hPDMSCs. 15 proteins were identified by MS which were up- or down-regulated during the osteogenic differentiation. All these results may be useful to elucidate the precise molecular mechanism of hPDMSCs osteogenic differentiation.

Materials and methods

Culturing of placenta-derived mesenchymal stem cells

Full term normal human placentas (≥ 37 gestation ages) were collected from healthy donor mothers. To isolate placenta-derived MSCs, the chorioamniotic membrane was peeled off and separated from the chorionic plate of placenta. They were chopped into small pieces, then washed with phosphate-buffered saline (PBS), and initially digested with 0.5 % collagenase IV (Life Technologies, Carlsbad, CA, USA) in PBS at 37 °C for 1 h, followed by vigorous shaking for 15 min at 15-min intervals over 90 min in 0.25 % collagenase IV at 37 °C. An equal volume of Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, Grand Island, NY, USA) containing 10 % fetal bovine serum (FBS; Gibco-Invitrogen) was added and centrifuged at 1,000 rpm for 5 min to get the cell pellet. The harvested cells were resuspended in the culture medium and then incubated at 37 °C in an incubator with 5 % CO₂.

Flow cytometry

To confirm that hPDMSCs maintain their phenotype the cells from 4 to 6 passages were subjected to flow cytometry analysis. The cells were detached by 0.05 % trypsin (Life Technologies, Carlsbad, CA, USA) treatment, followed by centrifugation and washing with PBS. Then, the cells were fixed in chilled 70 % ethanol, and incubated in mouse anti-human FITC/PE conjugated antibodies against CD29, CD33, CD34, CD44, CD45, CD73, CD90, CD105 and CD117 (1:100 dilution) for 1 h on ice (all antibodies were purchased from Becton–Dickinson, San Diego, CA, USA). Finally, the cells were counted using a flow cytometry laser 488 nm, and the data were analyzed using BD Cellquest Pro software.

Construction of recombinant Ad vector DNA containing human LMP-1

A human *LMP-1* (accession number AF345904) cDNA construct was kept in our lab. The *LMP-1* was cloned into a pENTR TOPO/D vector and subsequently was transferred into the Adeno pAd/CMV/V5-DEST adenovirus vector by homologous recombination according to the manufacturer's protocols (Invitrogen).

Producing viral stocks and transducing MSCs

In this phase of the study, the HEK-293 cells (OriCell, Cyagen Biosciences, Guangzhou, China) were infected either with AdLMP-1 or with adenovirus without a transgene (AdLacZ) as the negative control. The 293A cells were transfected with *PacI* linearized recombinant or control adenoviruses. The day before transfection, the 293A cells were trypsinized, counted, and plated at 5×10^5 cells per well in a six-well plate. The transfection reaction was carried out using the lipofectamin reagent as described by the manufacturer (Invitrogen). In order to harvest the viruses, the cells were lysed with three consecutive freeze–thaw cycles, and then the viruses were collected from supernatant. Next, the adenoviruses were amplified by infecting additional 293A cells with the crude viral lysate, and then subjected to viral titer determination by plaque assay on 293A cells. Subsequently, for transduction of hPDMSCs with pAd/CMV/V5-LMP-1, 8×10^5 cells/well were seeded in 6-well plates in growth medium containing DMEM-LG (Gibco) and 10 % FBS and incubated for 12 h. To analyze the expression level of LMP-1 after adenovirus-mediated gene transfer, protein samples were probed with an anti-LMP antibody (1:500 dilution; ProteinTech Group Inc., Chicago, IL, USA).

Reverse-transcription PCR

Expression of osteoblast marker genes was determined by quantitative real-time PCR. Primers for osteocalcin were (forward) 5'-CCACCGAGACACCATGAGAG and (reverse) 5'-TCGTCACAGTCCGGATTGAC. Primers for type II collagen were (forward) 5'-AAC TGGCAAGCAAGGAGACA and (reverse) 5'-AGT TTCAGGTCTCTGCAGGT. Primers for cyclophilin A, a housekeeping gene, were (forward) 5'-CAG

CCGGTTCAGAGCAAACC and (reverse) 5'-GCCA GTCCTCTGTGTTCTCC. Total RNA was isolated from cells using RNeasy kit (Qiagen, Hilden, Germany). Real-time RT-PCR was carried out in 25- μ l reaction volume containing 12.5 μ l of SYBR Green I Mix (Takara, Otsu, Shiga, Japan), 0.5 μ M of each of the primers and 0.5 μ l ROX Reference Dye II (5 $\times$) and cDNA obtained from 10 ng of total RNA. The two-step amplification protocol consisted of a 2 min at 94 °C followed by target amplification via 40 cycles at 94 °C for 15 s, 61 °C for 40 s and 72 °C for 15 s. Detection of fluorescent product was carried out after the last step of each cycle. Following the final amplification cycle, a melting curve was acquired by one cycle of heating to 95 °C, cooling to 65 °C at 20 °C s⁻¹ and slowly heating to 95 °C at 0.1 °C s⁻¹ with continuous measurement of fluorescence at 520 nm. Relative mRNA abundances were quantified as Ct (cycle threshold value) and normalized to the Ct of cyclophilin A with the assumption that the efficiency of each RT and PCR was similar. Expression is shown as fold change in mRNA levels as calculated by the $2^{-\Delta\Delta C_t}$ method.

Osteogenic differentiation of hPDMSCs

To evaluate the occurrence of osteogenic commitment and differentiation of cells upon LMP-1 overexpression, hPDMSCs were alternatively transfected with either AdLMP-1 or AdLacZ. Osteogenic differentiation was assessed after 2 weeks by von Kossa staining, as previously described (Halvorsen et al. 2001). Briefly, cells were fixed overnight and subjected to dehydration using ethanol gradients of 70, 90, and 100 %. The plates were rinsed in distilled water and stained with 5 % silver nitrate solution by placing in front of a UV light source until calcium deposits turned black. Plates were then rinsed in distilled water with three changes and treated with 5 % sodium thiosulfate for 5 min, rinsed in distilled water and allowed to dry. Plates were stored in the dark for analysis.

Preparation of protein sample

hPDMSCs within the 4th culture passage were infected with pAd/CMV/V5-LMP-1 or control adenoviruses at the appropriate MOI. The total cells were harvested after 72 h induction. The harvested cells were washed

with ice-cold PBS three times, and the cellular pellets were dissolved in a lysis buffer containing 7 M urea, 4 % CHAPS, 40 mM Tris–Cl (pH 7.5), 1 mM EDTA, and 0.2 % Bio-Lyte 3/10 ampholyte. Fresh 1 mM dithiothreitol (DTT) and a protease inhibitor mixture containing 1 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml pepstatin A, and 10 mg/ml aprotinin, and then sonicated at 4 °C for 2 min. Finally, the sample was centrifuged under 12,000 rpm, 4 °C for 30 min. Protein concentrations in the samples were determined by an RC/DCTM protein assay after rehydration. All chemicals and kits were supplied by BioRad (Hercules, CA, USA) unless otherwise specified.

Two-dimensional gel electrophoresis

2-DE experiments were carried out according to the manufacturer's instruction (Bio-Rad). The manufacturer's recommended volume (350 µl, containing approximately 150 µg proteins) of recovered proteins was applied to 17 cm IPG strips (Immobiline Dry-Strips), pH 3–10, for overnight active rehydration. The IPG strips were then subjected to isoelectric focusing using a protean IEF cell. Focusing was performed as follows: 250 V for 30 min, 1,000 V for 30 min, 8,000 V for 4 h and then up to 40,000 Vh for approximately 3 h. Current did not exceed 50 mA per strip.

After isoelectric focusing, the IPG strips were equilibrated for 15 min in equilibration buffer I [6 M urea, 2 % SDS, 0.375 M Tris–HCl (pH 8.8), 20 % glycerol and 2 % (w/v) DTT] followed by 15 min in buffer II (same as buffer I but containing 2.5 % iodacetamide instead of DTT). For the second dimension, IPG strips were placed across a criterion precast gel, overlaid with agarose. Electrophoresis was run with a constant voltage, 120 V, until the bromophenol blue dye front had completely migrated out of the bottom of the gel. Gels were fixed overnight in a solution of 50 % ethanol and 10 % acetic acid, stained with Coomassie Brilliant Blue R-250 (BioRad) for at least 1 h and washed in water (Dyballa and Metzger 2009). Three independent samples were analyzed for each treatment.

Image analysis

Gel images were scanned with a Bio-Rad SanMaker 9700XL imaging system and image analysis was

performed using PDQuest (version 7.2.0; Bio-Rad). Protein spots detection and matching were performed automatically and matches reviewed manually and edited where necessary in order to remove false spots, include missed spots, and to distinguish spots within clusters. Protein spots that were determined to be differentially expressed (n -fold >2.0 or <0.5) by using the automatic analyses were verified manually.

In gel digest and protein identification

From the image analysis of controlled and induced cells, spots of interest were selected for protein identification by mass spectrometric analysis. Bands were trimmed as close as possible to minimize background polyacrylamide material. The gels were washed in 50 % methanol and 5 % acetic acid for several hours. The gel bands were dried with acetonitrile and reconstituted with 10 mM DTT solution to reduce the cysteines. 55 mM Iodacetamide (in 25 mM ammonium bicarbonate) was added to alkylate the cysteines, and the gel was washed again with cycles of 50 % acetonitrile and 50 mM ammonium bicarbonate. Gel slices were digested overnight with 12.5 ng/µl trypsin diluted 1:20 in 50 mM NH_4HCO_3 at 37 °C. After they were dried in a SpeedVac for 10 min, peptide extraction was performed with 1 % TFA. Extracted peptides were analyzed using a time-of-flight (TOF) delayed-extraction MALDI mass spectrometer (AnchorChipTM, Bruker Daltonics, Billerica, MA, USA).

Protein identification using peptide mass fingerprinting was performed by the MASCOT search engine (<http://www.matrixscience.com>) against the NCBI nonredundant protein database. The parameters were set to allow one possible missed cleavage for trypsin digestion and a peptide mass tolerance of 100 ppm. Modifications were considered of carbamidomethylation of cysteine and oxidation of methionine.

All identified proteins were manually categorized using the information provided by Uniprot database (<http://www.uniprot.org/uniprot/>), the Gene Ontology project (Ashburner et al. 2000) and related information from the scientific literature.

Western blot

The Western blot was performed as described by Towbin et al. (1979). The protein samples used in

2-DE were separated by SDS–PAGE, followed by transfer to PVDF membrane. Briefly, the membrane was blocked in 4 % skimmed milk powder dissolved in PBST (PBS containing 0.2 % Tween 20), immunoblotted with the antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a dilution ratio of 1:5,000, washed with PBST, incubated with AP-Goat Anti-Mouse IgG (zsbio, Beijing, China) in a dilution ratio of 1:2,000, washed with PBST, and visualized using BCIP/NBT method.

Results

Characterization of hPDMSCs

Flow cytometry analyses indicated that the population of hPDMSCs was positive for MSC markers CD29, CD44, CD73, CD90, CD105, CD117 and negative for hematopoietic markers CD33, CD34 and CD45, which was consistent with the characteristics of MSCs (Fig. 1), suggesting that expanded hPDMSCs maintain their phenotype. To verify that hPDMSCs had pluripotent differentiation potential, we analyzed their ability to differentiate into osteocytes and adipocytes. After a 28-days induction, it was shown that the hPDMSCs differentiated into osteocytes (detectable increase in alkaline phosphatase levels) and adipocytes (detectable by Oil Red O staining) (data not shown).

Adenovirus-mediated gene transfer

Western blot analysis of total cell lysates of LMP-1-transduced hPDMSC detected a ~50 kDa band using a specific antibody against LMP-1. No signal was detected in control-transduced cells (Fig. 2a). The hPDMSCs cell line was used to test the osteogenic potential of the adenovirus-mediated gene transfer construct. AdLMP-1- and control vector-transduced cells were harvested after 72 h. Quantitative real-time PCR analysis revealed that, relative to control cells, LMP-1 transduced cells showed an 12-fold increase in *osteocalcin* gene expression ($P < 0.05$) and a 7.5-fold increase in *type II collagen* expression ($P < 0.05$) (Fig. 2b), indicating that LMP-1 enhanced osteoblast differentiation. Von Kossa staining demonstrated accelerated nodule formation and mineralization in cells transduced with LMP-1 compared to control transduced (Fig. 2c). Taken together, these data indicated that the

AdLMP-1 produced by the adenovirus based vector was capable of stimulating osteogenesis.

LMP-1 induced proteome changes in hPDMSCs

The proteins in the cell lysates of undifferentiated hPDMSC and LMP-1 induced hPDMSC were separated via IEF as the first dimension and SDS–PAGE as the second dimension followed by Coomassie staining. To ensure reproducibility of the samples in 2-DE gels, we performed the experiments in triplicate and found to be highly reproducible. The protein profiles of 2-DE PAGE are shown in Fig. 3 as revealed by scanning, digitizing, and image analysis. More than 800 protein spots with pI values between 3 and 10 were detected on each of the 12 % 2-DE gels, containing 150 µg of protein loading. In general, protein spots from the 2-DE gels displayed excellent separation and focusing, and the overall pattern of protein expression appears to be similar for the two groups, suggesting that most protein levels did not change significantly after LMP-1 stimulation.

To study the protein patterns in the process of osteoblast differentiation of hPDMSCs induced by LMP-1, we compared the protein expression profiles of AdLMP-1-induced cells and the control (Fig. 3). Figure 3 shows one set of 2-DE gels (AdLMP-1 vs. AdLacZ), where differentially expressed proteins are indicated with arrows. Among the 22 spots, 15 proteins were identified that were either up-regulated or down-regulated by MS analysis and database searching. Table 1 gives a detailed overview of these 15 proteins with their respective spot ID, protein name, NCBI ID, molecular weight and pI value. Of the analyzed spots, nine protein spots expressed up-regulation (>twofold) and six protein spots showed down-regulation (<0.5) by comparing the protein profiles between the undifferentiated and induced cells.

Functional groups of regulated proteins

The proteins identified from 2-DE were grouped into different categories according to their functions as documented in Swiss-Prot and NCBI Database. Based on the analysis, the proteins were divided to several groups according to their biological process. The largest group was composed of proteins involved in cellular metabolic processes. Moreover, three proteins, annexin A2 (spot 15 and spot 17) and peroxiredoxin-1 could be

Fig. 1 Flow cytometry analysis of cell-surface markers in hPDMSCs. Cell surface analysis of hPDMSCs revealed homogenous mesenchymal population stained positive for CD29, CD44, CD73, CD90, CD105 and CD117, but negative for CD33, CD34 and CD45

related to skeletal system development. We also grouped proteins based on their molecular functions and cellular components (Table 1). The largest category in molecular function was protein binding, which contained 8 proteins. This was followed by calcium ion binding, nucleotide binding, ATP binding and structural molecule activity. For cellular component classification, the major class was cytoplasm, which was composed of 12 proteins. Two

other proteins, vimentin and heat shock protein beta-1 (HSPB1), belonged to cytoskeleton.

Confirmation of the proteomic data by Western blot analysis

To confirm the expression level of the identified proteins from hPDMSCs after AdLacZ or AdLMP-1

Fig. 2 AdLMP-1 stimulates osteoblast differentiation. **a** A Western blot showing LMP-1 protein expression in hPDMSC transduced with AdLMP-1. The ~50 kDa band for LMP-1 was detected in AdLMP-1-transduced cells but not in control-transduced cells. **b** Expression of osteoblast marker genes in

hPDMSCs overexpressing LMP-1 compared to control levels. LMP-1 transduction resulted in significant increases in osteocalcin and type II collagen (* $P < 0.05$). **c** Von Kossa staining of AdLMP-1 and control cultures demonstrating increased mineral deposition after 2 weeks of culture in osteogenic medium

Fig. 3 A two-dimensional reference map for hPDMSCs showing up and down regulated protein spots after LMP-1 treatment. Left: AdLacZ-transfected hPDMSCs; Right: AdLMP-1 transfected hPDMSCs. For first dimension separation an IPG strip with a nonlinear pH gradient 3–10 was used and the

second dimension was performed with 12 % SDS PAGE. Upward arrows indicate up-regulation, and downward arrows indicate down-regulation. Protein load was 150 μ g for the gels and spots were visualized by Coomassie staining. For details of protein identification and expression changes, see Table 1

treatment, we selected six differentially expressed proteins that have been reported to be relevant for bone biology, PCNA, vimentin, annexin A1, annexin A2,

eEF2 and peroxiredoxin-1. Figure 4 illustrates the results obtained by Western blot analysis. Western blot analysis, using tubulin as an internal control for

Table 1 Protein identification after tryptic in-gel digest with MALDI-TOF MS and database search

Spot no	Protein name	MW (kDa)	PI	Score	NCBI accession No	LMP-1 induced change (fold)	Biological process	Molecular function	Cellular component
1	Heat shock 70 kDa protein 1A/1B	70.0	5.4	85	gi1194248072	0.2	mRNA metabolic process Response to stress Negative regulation of apoptotic process Protein refolding	ATP binding Nucleotide binding Protein binding	Cytoplasm Mitochondrion Nucleus
2	eEF2 (Eukaryotic translation elongation factor 2)	95.3	6.6	158	gi14503483	4.0	GTP catabolic process Cellular protein metabolic process Translation	GTP binding Translation elongation factor activity nucleotide binding GTPase activity Structural molecule activity	Cytoplasm Ribonucleoprotein complex
3	Vimentin (variant)	53.7	4.9	135	gi162896523	3.1	Not classified	Structural molecule activity	Intermediate filament
4	Vimentin	53.6	4.9	102	gi162414289	2.5	Muscle filament sliding Cellular component movement	Structural molecule activity Protein binding	Cytoplasm Cytoskeleton
7	T-complex protein 1 subunit beta isoform 1	57.5	6.2	83	gi15453603	3.3	Apoptotic process Cellular protein metabolic process	Structural constituent of cytoskeleton ATP binding	Cytoplasm
9	Hypothetical protein	22.3	5.3	190	gi16624942	0.2	Protein folding Chaperone-mediated protein complex assembly	Nucleotide binding Protein Binding	Nucleus Microtubule
14	Annexin A1	38.6	7.7	92	gi14826643	0.2	Oxidation–reduction process Cell surface receptor signaling pathway	Oxidoreductase activity Calcium ion binding	Mitochondrion Cytoplasm
15, 17	Annexin A2	38.6	7.7	179 (spot15), 121 (spot17)	gi145786109	3.2	Response to interleukin-1 Estrous cycle phase Collagen fibril organization Fibrinolysis	Protein binding Structural molecule activity Cytoskeletal protein binding Calcium ion binding	Plasma membrane Membrane Cytoplasm Early endosome
16	Annexin A5	35.9	4.8	88	gi14502107	4.0	Skeletal system development Blood coagulation Signal transduction Hemostasis	Protein binding Calcium ion binding Eukaryotic cell surface binding	Protein complex Cytoplasm
18	Nicotinamide N-methyltransferase	30.0	5.56	167	gi15453790	2.8	Small molecule metabolic process Organ regeneration	Transferase activity Methyltransferase activity Nicotinamide N-methyltransferase activity	Cytoplasm Cytosol

Table 1 continued

Spot no	Protein name	MW (kDa)	PI	Score	NCBI accession No	LMP-1 induced change (fold)	Biological process	Molecular function	Cellular component
19	Heat shock protein beta-1	27.0	6.2	126	gi4504517	0.4	mRNA metabolic process Response to stress Response to unfolded protein Cell proliferation	Ubiquitin binding Protein kinase C binding Protein binding Antioxidant activity	Cytoplasm Cytoskeleton Nucleus Cytoplasm Nucleus
20	Peroxiredoxin-1	22.1	8.1	129	gi32455264	0.2	Skeletal system development Oxidation–reduction process	Protein binding Heme binding	Cytoplasm Nucleus
21	Proliferating cell nuclear antigen (PCNA)	28.7	4.4	82	gi33239451	4.1	Nucleotide-excision repair Translation synthesis	DNA binding DNA polymerase processivity factor activity	Cytoplasm Nucleoplasm
22	Chloride intracellular channel protein 1	23.8	5.1	96	gi895845	0.2	DNA replication Positive regulation of osteoblast differentiation Transport Ion transport	Protein binding Voltage-gated chloride channel activity Protein binding	Cytoplasm Mitochondrion Nucleus

normalization, clearly confirmed the significant increase in expression of PCNA, vimentin, annexin A2 and eEF2 after LMP-1 treatment. We also demonstrated that the expression of annexin A1 and peroxiredoxin-1 was decreased with Western blot analysis. The protein expression level analyzed by Western blot corresponded to the results obtained by 2-DE.

Discussion

MSCs have a wide range of promising applications in the treatment of autoimmune diseases, tissue repair, and regeneration. Although BM has been used as the main source for the isolation of multipotent MSCs, however, the harvest of BM is a highly invasive procedure. The number, differentiation potential, and maximal life span of BM-MSCs decline with increasing age of donors (Stenderup et al. 2003). Recently, a population of MSCs was isolated from human placenta. hPDMSCs that are similar to BM-MSCs in cell surface marker and multilineage differentiation potency but can be harvested much easier and at much higher numbers than BM-MSCs (Miao et al. 2006) will undoubtedly play a key role in the development of regenerative medicine.

There are several advantages to the adenoviral expression system such as transient and high expression levels, and the simplicity of infection conditions (Conget and Minguell 2000). Furthermore, considering the fact that a majority of hPDMSCs die a few days after transplantations, it would be reasonable to use the adenoviral expression system to take advantage of the short-term expression of LMP-1 in hPDMSCs.

Since their original isolation from rat calvarial bone in 1998 (Boden et al. 1998), LMP-1 has been employed to induce bone formation/healing in diverse animal models along with the osteogenic differentiation of mesoderm-derived cell types in vitro (Fei et al. 2007; Strohbach et al. 2008; Wang et al. 2011; Yoon and Boden 2002). Although the effects of LMP-1 on the MSCs have been widely studied, protein profiling of MSCs regarding the effect of LMP-1 has not been investigated comprehensively. In the present study, hPDMSCs were induced to osteoblast differentiation by LMP-1 treatment and changes of expression levels of proteins during hPDMSCs differentiation were monitored by 2-DE. In this study, using proteomic

Fig. 4 Results of Western blot analysis showing differentially expressed proteins in hPDMSCs cultured with AdLacZ or AdLMP-1. Western blot analysis showing the up-regulated proteins (annexin A2, vimentin, PCNA and eEF2) and the down-regulated proteins (annexin A1 and peroxiredoxin-1) in hPDMSCs cultured with AdLMP-1. Tubulin was used as a control

approaches based on 2-DE electrophoresis and MALDI-TOF MS, 15 differentially expressed proteins were identified.

Previous reports demonstrated that calcium-binding proteins, especially annexins, play an important role in the bone development (Kirsch et al. 1997; Wang and Kirsch 2002). Among the identified proteins, some have already been noted for roles in osteogenesis. Annexin A2 and annexin A5 are highly expressed in skeletal tissues (Gillette and Nielsen-Preiss 2004; Kirsch et al. 1997). Both of them serve as Ca^{2+} channels in the plasma membrane and in matrix vesicles that have the critical role of initiating the mineralization process in cartilage and perhaps in bone (Wang and Kirsch 2002). The overexpression of annexin A2 can lead to an increase in alkaline phosphatase activity as well as an increase in osteoblastic mineralization. In our study, two isoforms of annexin A2 (spots 15 and 17) were expressed in MSCs and showed different expression during hPDMSCs differentiation. Therefore, it is possible that a post-translational modification of annexin A2 might accompany a functional modification. As to annexin A5, it builds Ca^{2+} channels, leading to mineralization of bone matrix in the growth plates during osteogenesis. An increased expression of annexin A5 enhances these processes of mineralization. In this study, expressions of annexin A2 and annexin A5 were increased, further confirming their roles in osteogenesis. In addition, another member of annexin family, annexin A1, was identified in this study. Expression of this protein showed down-regulation both on proteomic analysis and western blot. It has been proposed to be involved in the regulation of cell growth and

differentiation, apoptosis, and inflammation (William et al. 1988; Ye et al. 2006). Previous studies demonstrated that overexpression of annexin A1 significantly suppressed the expression levels of cytoskeletal proteins (Yi et al. 2013; Zhang et al. 2007). Our results suggest that annexin A1 might have an important role in regulation of hPDMSCs differentiation into osteoblasts.

As shown in Table 1, 2 of the 15 identified proteins were categorized as cytoskeleton proteins. Generally, reassembling of cytoskeletal proteins is an important feature of morphological changes during differentiation. Moreover, cytoskeletal proteins may serve as scaffolding for molecular signaling cascades, providing sites for localization, and anchoring of signaling molecules (Walker and Spinale 1999). As a key component of the intermediate filaments cytoskeleton, vimentin contributes to the maintenance of cell shape, migration, and response to mechanical stress (Kim et al. 2005). Vimentin was found to be rich in stem cells (mesenchymal cells, BM-MSCs, skeletal muscle derived cells) (Morsczeck et al. 2005; Panepucci et al. 2004), which are able to differentiate into several cell lines under lineage specific stimulation including osteoblasts (Annunen-Rasila et al. 2007; Margaritescu et al. 2005). In a previous study, the osteoblast-like cell line MC3T3-E1 showed higher expression of vimentin during the differentiation and mineralization phase than in the proliferation phase, suggesting that vimentin may have a role in bone maturation (Kitching et al. 2002). According to the expression in this study and its known functions, vimentin is an extremely attractive candidate in osteoblast differentiation from hPDMSCs. Another cytoskeleton protein identified in

this study is HSPB1, also known as heat shock protein 27 (Hsp27), which is a capping protein that binds to the barbed end of actin filaments to stabilize the filament. Increasing evidence suggests that the HSPB1 may play a role in the formation and function of the cytoskeleton (Cooper and Schafer 2000; Liang and MacRae 1997; Margaritescu et al. 2005).

Proliferating cell nuclear antigen (PCNA) is an auxiliary protein of DNA polymerase delta and is involved in control of eukaryotic DNA replication by increasing the polymerase's processibility during elongation of the leading strand. It also acts as a landing pad for many other proteins involved in DNA metabolism (Moldovan et al. 2007). The expression of PCNA in the nucleus is tightly associated with cell cycle. PCNA, which is only present in normal proliferative and tumor cells, plays an important role in initiating cell proliferation and is a better index for reflecting the vegetative state of cells (Dirim et al. 2008; Mathews et al. 1984; Prelich et al. 1987). In the present experiment, we detected up-regulation of PCNA in AdLMP-1 induced cells during the differentiation of hPDMSCs into osteoblast cells. hPDMSCs have strong proliferative capacities, which grow logarithmically 2 days after the passage culture. These results conform to the trend of PCNA expression.

Eukaryotic translation elongation factor 2 (eEF2) was identified in this study. eEF2 is a single protein molecule, playing a key role in the elongation step of protein synthesis (Nygard and Nilsson 1990). It catalyses the translocation of the two tRNAs and the mRNA after peptidyl transfer on the 80 S ribosome (Ryazanov et al. 1991). eEF2 is crucial for the synthesis of peptides and proteins. Previous studies have proven that eEF2 is significantly expressed during the stages of differentiation and mineralization in MC3T3-E1 osteoblastic cells (Kitching et al. 2002). Up-regulation of eEF2 was also found by a proteomic approach in human BM MSCs cultured with fibroblast growth factor and TGF- β 1 (Kurpinski et al. 2009; Lee et al. 2009). In this study, we describe an increased expression of eEF2 in association with increased LMP-1 activity as compared to control. Increased expression of eEF2 would probably result in a stimulation of protein synthesis and contributes to the forming phenotype of cytoskeletal proteins.

Peroxiredoxin family of proteins, as anti-oxidation-like proteins, plays an important role in elimination of

peroxides from metabolism. This function is achieved through the reduction of peroxide or super oxide by thioredoxin (Rhee et al. 2001). In addition to their antioxidant activity, peroxiredoxins have been implicated in a number of cellular functions such as cell proliferation and differentiation, enhancement of natural killer cell activity, protection of radical-sensitive proteins, metabolism, and intracellular signaling (Nelson et al. 2011; Rhee et al. 2001). Our data showed that the expression of peroxiredoxin-1 was significantly decreased in treatment group, which suggest that peroxiredoxin-1 may play a role during the hPDMSCs differentiation into osteoblast cells.

Among the identified proteins, some of them are function-unknown proteins such as T-complex protein 1 subunit beta isoform 1, chloride intracellular channel protein 1, nicotinamide *N*-methyltransferase and hypothetical protein, which might have important roles in osteoblast differentiation.

In summary, we examined protein expression profiles in osteoblast differentiation from LMP-1-induced hPDMSCs. 15 proteins were identified that were either up-regulated or down-regulated in osteogenesis from hPDMSCs by MS analysis and database searching. They are potentially important players for osteoblast differentiation. The data derived from this study will lead to more focused and in-depth research on the effects of LMP-1-on cellular functions and MSC differentiation. The information obtained from this study will not only have significant impact on stem cell biology but also have profound implications in stem cell therapy and tissue regeneration.

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