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Early osteogenic differential protein profile detected by proteomic analysis in human periodontal ligament cells

L. Wu[1]*, X. Wei[1]*, J. Ling[1], L. Liu[1], S. Liu[2], M. Li[2], Y. Xiao[3]

[1]Department of Orthodontics, Guanghua School of Stomatology, Sun Yat-Sen University, Guangdong, China, [2]Proteomics Laboratory, Zhongshan Medical College, Sun Yat-Sen University, Guangdong, China and [3]Institute of Health and Biomedical Innovation, Queensland University of Technology, Queensland, Australia

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Background and Objective: Human periodontal ligament cells play a pivotal role in maintaining periodontal ligament space, contain progenitors that are able to differentiate into cementoblasts/osteoblasts and have a tremendous potential to regenerate periodontal tissue. However, the exact molecular mechanisms governing the differentiation mechanisms of progenitors in periodontal ligament cells remain largely unknown. This study was carried out to investigate the differentially expressed proteins involved in the osteogenic differentiation of progenitors presented in periodontal ligament cells.

Material and Methods: Using two-dimensional gel electrophoresis, mass spectrometry and peptide mass fingerprinting, we analyzed the differential protein profiles of periodontal ligament cells undergoing mineralization.

Results: Compared with undifferentiated periodontal ligament cells, 61 proteins in periodontal ligament cells undergoing differentiation showed at least a 1.5-fold change in intensity, of which 29 differentially expressed proteins were successfully identified by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry. The expression of some of the identified proteins was further confirmed by western blotting and reverse transcription–polymerase chain reaction analysis. The identified proteins were cytoskeleton proteins and cytoskeleton-associated proteins, nuclear proteins and cell membrane-bound molecules.

Conclusion: Our results suggest that the proteins identified in this study may be associated with the unique function of periodontal ligament cells in maintaining periodontal tissue homeostasis, thus providing a comprehensive reference for understanding and investigating in greater detail the molecular mechanisms of periodontal ligament cells involved in periodontal regeneration.

Junqi Ling, DDS, PhD, Guanghua School of Stomatology, Sun Yat-sen University, Guangdong, China
Tel: +86 20 83862621
Fax: +86 20 83822807
e-mail: lingjunqi@mail.sysu.edu.cn

*Contributed equally to this study.

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The periodontal ligament is a narrow fibrous connective tissue that connects the alveolar bone to teeth and plays an important role in providing anchorage and supporting tooth function, maintaining homeostasis and repairing damaged tissue in response to periodontal diseases or mechanical trauma. Periodontal ligament contains a heterogeneous cell population at various stages of differentiation and lineage commitment. Previous studies indicate that periodontal ligament cells can form mineralized nodules *in vitro* and express bone-related proteins, such as

alkaline phosphatase, bone sialoprotein and osteocalcin (1–3). Recently, Seo *et al*. (4) reported that periodontal ligament cells contain stem cells with a high proliferative capacity, self-renewal properties, multilineage differentiation potential and the capability of forming cementum/periodontal ligament-like tissue *in vivo*. Thus, periodontal ligament cells play a pivotal role in maintaining periodontal ligament space and have tremendous potential to regenerate periodontal tissue. However, a comprehensive understanding of the molecular mechanisms controlling the osteogenic differentiation of progenitors in periodontal ligament cells remains poor.

Proteomic techniques such as two-dimensional gel electrophoresis, coupled with protein identification by mass spectrometry and bioinformatics, has led to a revolution in the ability of researchers to identify the temporal and spatial changes of the entire protein complement and their functions in a given cell type (5). The task of detecting changes in protein expression has recently been made possible by the introduction of the difference in gel electrophoresis (DIGE) technique (6). This method increases statistical confidence when combining experimental repetition with internal standards and facilitates the identification of quantitative and qualitative protein alterations during cell differentiation. Proteomics techniques are now widely accepted as important tools for identifying potential biomarkers or for studying the mechanisms of cell differentiation (7). In order to identify early differential proteins involved in the osteogenic differentiation of progenitors present in periodontal ligament cells, DIGE-based and mass spectrometry-based proteomics approaches were utilized to investigate early differentiation proteins in periodontal ligament cells after 7 d in osteogenic culture conditions. Twenty-nine proteins were identified using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, of which 12 were up-regulated and 17 were down-regulated during early osteogenic differentiation.

Material and methods

Isolation and culture of periodontal ligament cells

Healthy periodontal ligament tissues were collected from the middle-third area of the root of premolars obtained from patients (12–18 years of age) undergoing orthodontic treatment in the Department of Orthodontics, Sun Yat-sen University Dental Hospital. Informed consent was obtained from each subject and the research protocols were approved by the University Ethic Committee. Periodontal ligament tissues were minced and digested in a solution containing 3 mg/mL of type I collagenase (Worthington Biochem, Freehold, NJ, USA) and 4 mg/mL of dispase (Gibco-BRL Life Technologies, Breda, the Netherlands), at 37°C for 30 to 60 min, as previously described (4). The digested solution was filtered through a 70-μm strainer (Becton Dickinson, Sunnyvale, CA, USA) and the released cells were plated in six-well plates containing alpha-modified Eagle's minimal essential medium (Gibco-BRL) supplemented with 20% fetal bovine serum (Gibco-BRL), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Sigma, St Louis, MO, USA), and then incubated at 37°C in 5% CO_2. Cells from passages two to four were used in the following experiments.

Surface characterization of periodontal ligament cells using flow cytometry analysis

Periodontal ligament cells (1×10^5 cells) at passage 2 were harvested in phosphate-buffered saline containing 1 mM ethylenediamine tetraacetic acid, washed in phosphate-buffered saline and then incubated with 0.5 μg/100 μL of mouse anti-human STRO-1 (R&D Systems, Minneapolis, MN, USA) and CD146 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at 4°C. Cells were washed twice with cold phosphate-buffered saline containing 2% fetal bovine serum and incubated with 1 μg/100 μL of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin M or immunoglobulin G (Santa Cruz) for 30 min at 4°C. Mouse anti-human immunoglobulins to CD29–fluorescein isothiocyanate, CD44–-fluorescein isothiocyanate, CD 106phycoerythrin, and CD34–fluorescein isothiocyanate conjugates (BD Bioscience, Farmington, CT, USA) were also used for phenotype characterization. Mouse isotype antibodies (BD Bioscience) served as a control. Labeled cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). The experiments were repeated three times on all three patient samples.

Immunocytochemical staining and alizarin red staining

Cells at passage two were subcultured into 22 × 22-mm slides (2×10^4 cells/mL) for 24 h, fixed in 4% paraformaldehyde for 15 min, then blocked and incubated with monoclonal anti-vimentin or anti-pancytokeratin (Boster Biotechnology, Inc., Wuhan, China) at a 1:100 dilution, and rabbit anti-mouse immunoglobulin was used as the secondary antibody.

Osteogenic differentiation was verified by bone nodule formation after culture of periodontal ligament cells for 21 d in osteogenic media [alpha-modified Eagle's minimal essential medium supplemented with 10 mM beta-glycerophosphate, 0.2 mM ascorbic acid, 100 nM dexamethasone (Sigma) and 15% fetal bovine serum]. Controls were cultured in alpha-modified Eagle's minimal essential medium containing 15% fetal bovine serum. Mineral deposits were detected on day 21 by staining with 2% alizarin red, and osteocalcin expression was monitored on day 21 by immunocytochemical staining using polyclonal mouse anti-human osteocalcin (1:100 dilution; Santa Cruz Biotechnology).

Protein preparation

Periodontal ligament cells were cultured in osteogenic and control medium for 7 d. Prior to harvest, both differentiated cells and controls were incubated in serum-free medium for 24 h, after which the medium was removed and the cells were washed three times with ice-cold

Tris-buffered sucrose (10 mM Tris-base, 250 mM sucrose, pH 7.4). The cells were lysed with sodium dodecyl sulfate lysis buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) and 30 mM Tris (GE Healthcare, Fairfield, CT, USA), following which the lysates were sonicated on ice, then centrifuged (20,000 *g*, 30 min, 4°C). The protein samples were purified using the 2D Clean-Up Kit (GE Healthcare) and the protein content was determined using the 2D-Quant kit (GE Healthcare), according to the manufacturer's instructions.

CyDye labeling of extracted proteins and two-dimensional gel electrophoresis

Prior to two-dimensional gel electrophoresis, the protein extracts were labeled with fluorescent cyanine dyes developed for 2D-DIGE technology (GE Healthcare) following the manufacturer's recommendations. Fifty milligrams of differentiated and control proteins were labeled with Cy3 or Cy5, and 50 mg of the internal standard mixture was labeled with Cy2 (Table 1), followed by incubation on ice for 30 min in the dark. The reactions were quenched by the addition of 1 μL of 10 mM lysine for 10 min on ice in the dark. The quenched Cy3- and Cy5-labeled samples, and the Cy2-labeled internal standard, were pooled prior to analysis by two-dimensional gel electrophoresis. Two preparative gels, each containing 500 mg of unlabeled mixture proteins, were analyzed, and four DIGE gels were run to analyze differentiated and control samples.

Two-dimensional gel electrophoresis was performed according to the manufacturer's instructions (GE Healthcare). Briefly, immobilized dry strips (pH 4–7, 24 cm; GE Healthcare) were rehydrated for 12 h in 450 μL of rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (v/v) immobiline pH gradient (IPG) buffer (pH 4–7), 40 mM dithiothreitol and 0.002% (w/v) Bromophenol Blue], at 30 V for 12 h, using an Ettan IPGphor II IEF system (GE Healthcare). After rehydration, the samples were focused at 500 V for 1 h, at 1000 V for 1 h and at 8000 V for a total of 80,000 V/h at 20°C. The strips were incubated in Equilibration Buffer I containing 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate, 50 mM Tris–HCl (pH 8.8) and 1% dithiothreitol for 15 min at room temperature in 23°C and then soaked in Equilibration Buffer II for an additional 15 min. Buffer II was identical to Buffer I, except that it contained 2.5% iodoacetamide instead of 40 mM dithiothreitol. The equilibrated strips were embedded in 0.5% (w/v) agarose on top of 12.5% acrylamide slab gels. Second-dimension separations were performed on an Ettan DALT six electrophoresis system (GE Healthcare). The proteins were separated initially at 15°C, 2 W per gel, for 50 min, followed by 17 W per gel until the dye front reached the bottom of the gel. The two preparative gels were stained with deep purple total protein stain (RPN6306; GE Healthcare) following the manufacturer's instructions.

Table 1. Experimental design of difference in gel electrophoresis (DIGE) gels

Gel no.	Cy2	Cy3	Cy5
1	Pool[a]	Control 1	Treat 1
2	Pool	Control 2	Treat 2
3	Pool	Treat 3	Control 3
4	Pool	Treat 4	Control 4

[a]Pool is the internal standard of DIGE analysis.

Gel scanning and image analysis

Both sample and preparative gels were imaged using a Typhoon Variable Mode Imager 9400 (GE Healthcare) using the excitation filters Blue 488 nm (Cy2), Green 532 nm (Cy3), Red 633 nm (Cy5) and 532 nm (deep purple), with a resolution of 100 μm. The two-dimensional image of the gel from each treated sample was compared with that of the control sample via the internal standard sample. Protein expression analysis was performed for each of the four gels in parallel using the differential in-gel analysis module of DECYDER software 6.0 (GE Healthcare), using a value of 3000 as the initial estimation of the number of protein spots present. The intensities of individual protein spots of the proteomes of the differentiated samples were compared with those of the control. The differential in-gel analysis data were collated into a single analysis using the DECYDER biological variation analysis module, and final values for the expression ratio of specific protein spots between differentiated samples and the control were determined. In total, 2787–3039 protein spots were analyzed across all cell samples, and the Student's *t*-test and one-way analysis of variance were used to calculate significant differences in the relative abundance of individual protein spot features between differentiated samples and the control. A protein spot abundance ratio of $> +1.5$ or < -1.5 was considered to represent differential expression.

In-gel digestion and MALDI-TOF mass spectrometry

The selection of protein spots from the gels, in-gel digestion, peptide extraction, preparation of the samples for mass spectrometry and spotting on target slides were carried out automatically using an Ettan spot handling workstation (GE Healthcare). Identification of proteins by peptide mass fingerprinting was carried out on an Ettan MALDI-TOF mass spectrometer (GE Healthcare) operating in reflectron mode. Internal calibration was performed using the trypsin autodigestion peaks at m/z 842.509 and 2211.104. Mass spectra were obtained over the m/z range of 700–3000 Da. Peptide mass fingerprinting data were used to search the human National Centre for Biotechnology Information protein database using the Mascot search engine (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF; GE Healthcare). Search parameters were as follows: type of search, peptide mass fingerprint; enzyme, trypsin; fixed modification, carbamidomethylation (cysteine); variable modifications, oxidation (methionine); mass values, monoisotopic; peptide charge state, 1+; maximum missed cleavages, 1; and peptide mass tolerance, 100 p.p.m.

Western blot

Periodontal ligament cells on day 0 (at 80% confluence, before the addition of osteogenic induction medium) and day 7 of osteogenic induction were harvested and lysed in sodium dodecyl sulfate lysis buffer, sonicated and centrifuged at 12,000 *g* for 5 min. Equal amounts of protein (50 μg) were separated by electrophoresis on a 12.5% polyacrylamide gel and transferred to nitrocellulose membranes at 100 mA for 3 h. The membranes were blocked in 5% nonfat dry milk at room temperature in 23°C for 1 h, rinsed and incubated with primary antibodies (1:1000 dilution) of mouse anti-human heteronuclear ribonuclear proteins C1/C2 (Santa Cruz), mouse anti-human caldesmon, mouse anti-human tropomyosin, rabbit anti-human polyclonal annexin A4 (Lab Vision Corporation, Fremont, CA, USA), or mouse anti-human beta-actin (Santa Cruz), at 4°C overnight. The membranes were washed, then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 dilutions; Santa Cruz,) at room temperature in 23°C for 1 h and the proteins were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Quantitative real-time reverse transcription–polymerase chain reaction

Prior to osteogenic induction and after 7 d of induction, cells were lysed to permit extraction of total RNA using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The primers used for detection are listed in Table 2. Quantification of mRNAs was performed using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). One microgram of each RNA sample was subjected to reverse transcription using the omniscript RT kit (Qiagen) and the RNase-free DNase Set (Qiagen) in a total volume of 20 μL. The conditions for reverse transcription–polymerase chain reaction were 1 h at 37°C and 3 min at 95°C. Then, 5 μL of the reaction mixture was incubated with PCR master mix containing the double-stranded DNA dye SYBR Green I (Qiagen) in a total volume of 50 μL. All reactions were run with a hot-start pre-incubation step of 3 min at 93°C, followed by 40 cycles of 1 min at 93°C, 1 min at 55°C and 1 min at 72°C. The amount of template was quantified by using the comparative cycle threshold method according to the manufacturer's protocol. Measured mRNA levels were normalized against beta-actin mRNA expression. Experiments were performed in triplicate on three different cell samples.

Statistical analysis

All experiments were repeated three to four times, and the representative results are presented. The quantitative data are presented as mean ± standard deviation. One-way analysis of variance and the Bonferroni test were performed to compare differences in the mRNA expression between induced samples and controls. Differences were considered significant at a p-value of < 0.05. The SPSS software package (version 13.0; SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

Results

Cell characterization

Periodontal ligament cells expressed cell-surface epitopes associated with mesenchymal progenitors such as STRO-1, CD146, CD29, CD44 and CD106, but did not express CD34, a hematopoietic origin marker, as shown by flow cytometry. The expression of STRO-1, CD146, CD29, CD44 and CD106 was 15.3%, 47.6%, 99.9%, 99.8% and 76.8%, respectively (Fig. 1A–F, Table 3). The positive immunocytochemical staining for vimentin, the most widely distributed intermediate filament in fibroblasts, and negative staining for pancytokeratin, a marker for tissue of epithelial origin, indicated that the periodontal ligament cells isolated from periodontal ligament tissue in this study were of mesenchymal origin (Fig. 2A,B).

Alizarin red-positive nodules were seen in periodontal ligament cell cultures on day 21 of osteogenic induction, indicating that mineral deposition had taken place; however, no staining was detected in the controls (Fig. 2C). Furthermore, periodontal ligament cells showed expression of osteocalcin after 21 d of osteogenic induction (Fig. 2D). Quantitative real-time reverse transcription–polymerase chain reaction showed an up-regulation of

Table 2. Primers for quantitative real-time reverse transcription–polymerase chain reaction

Gene name	Primer sequence (forward and reverse)	Product size (bp)
ALP	5′-GCTTCACGGCATCCATGAG-3′ 5′-GAGGCATACGCCATGACGT-3′	63
OCN	5′-AGCAAAGGTGCAGCCTTTGT-3′ 5′-GCGCCTGGGTCTCTTCACT-3′	63
MEPE	5′-CCCTGGAAGAGAAGGAAACAG A-3′ 5′-TCTCCTTAATGTCACGCACGAT-3′	69
hnRNPC	5′-ATTGTGGGCTGCTCTGTTCAT-3′ 5′-CCCGGGCATTTCTCTCATTA-3′	67
CaD	5′-GCTCCCAAACCTTCTGACTTGA-3′ 5′-GTT CCG CTT GCT GGA TAC GT-3′	51
Tm	5′-GAA GAT GCC GAC CGC AAA T-3′ 5′-CGT TCC AGG TCG CTC TCA AT-3′	68
Annexin A4	5′-TAGCGTCCTTGCCTACCGC-3′ 5′-CGTCTATCAAGTCCCTGCCG-3′	86
Beta-actin	5′-TGAAACTCAACCTTCCCTTGGT-3′ 5′-GCGCGGCTACAGCTTCA-3′	59

ALP, alkaline phosphatase; CaD, caldesmon; hnRNPC, heterogeneous nuclear ribonucleoprotein C; MEPE, matrix extracellular phosphoglycoprotein; OCN, osteocalcin; Tm, tropomyosin.

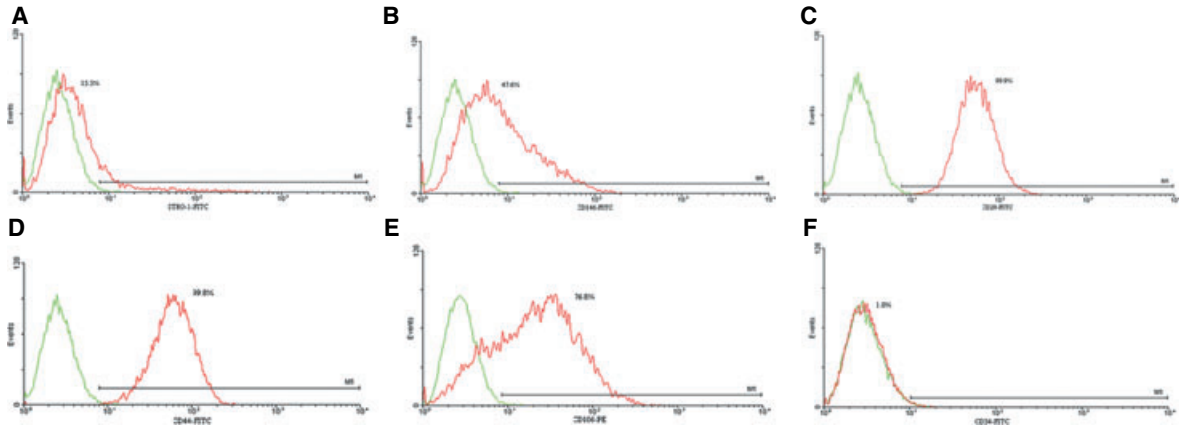

Fig. 1. Flow cytometry analysis of cell-surface markers in periodontal ligament cells at passage 2. The plots shown with a green line represent the isotype-control immunoglobulin profile, and the plots shown with a red line represent the specific antibody indicated. Periodontal ligament cells expressed cell-surface epitopes associated with the mesenchymal progenitors STRO-1 (A), CD146 (B), CD29 (C), CD44 (D) and CD106 (E), with positivities of 15.3%, 47.6%, 99.9%, 99.8% and 76.8%, respectively. Negative expression was observed for CD34, a hematopoietic origin marker (F). These results are representative of three samples from patients. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Table 3. Surface antigen expression of periodontal ligament cells, as analyzed by flow cytometry

Surface antigen	Percentage of positive cells (%)
STRO-1	14.4 ± 0.9
CD146	51.3 ± 4.2
CD29	99.3 ± 0.6
CD44	98.8 ± 1.0
CD106	76.8 ± 3.5
CD34	1.1 ± 0.1

the mRNA expression of alkaline phosphatase, osteocalcin and matrix extracellular phosphoglycoprotein under osteogenic culture conditions (Fig. 3). Significant differences were detected for alkaline phosphatase and osteocalcin on days 14 and 21, and for matrix extracellular phosphoglycoprotein on day 21, (ANOVA, $p < 0.05$) for each transcript. These results suggest that periodontal ligament cells possess progenitors that have the potential to differentiate into cementoblasts/osteoblasts *in vitro*.

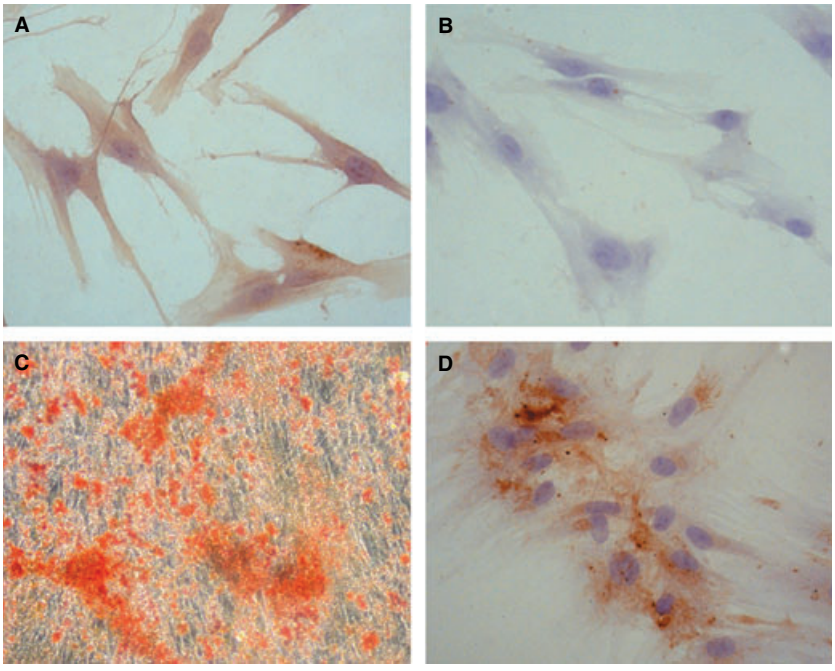

Fig. 2. Periodontal ligament cells stained positively for vimentin but negatively for pancytokeratin (A and B, original magnification ×200). Alizarin red-positive nodules were observed in periodontal ligament cell cultures on day 21 of osteogenic induction (C, original magnification ×100). Periodontal ligament cells showed expression of osteocalcin after 21 d after induction (D, original magnification ×200).

Osteogenic differential protein expression in periodontal ligament cells identified by 2D-DIGE and MALDI-TOF mass spectrometry

Protein profiles were compared between the osteogenic induced cells and controls. The DECYDER software detected more than 2787 protein spots across all four gels. Sixty-one protein spots showed a change in expression of at least 1.5-fold, whereby 14 proteins were up-regulated and 47 proteins were down-regulated ($p < 0.05$). The protein profile of the 2D-DIGE analysis is shown in Fig. 4 and Fig. 5. MALDI-TOF mass spectrometry identified 29 proteins in periodontal ligament cells by their peptide mass fingerprint, using the Swiss-Prot database. These proteins were classified into seven functional categories: cytoskeleton proteins, nuclear proteins, cell membrane-bound molecules, proteins involved in matrix synthesis, proteins involved in protein synthesis, metabolic enzymes and proteins involved in signal transduction (Table 4).

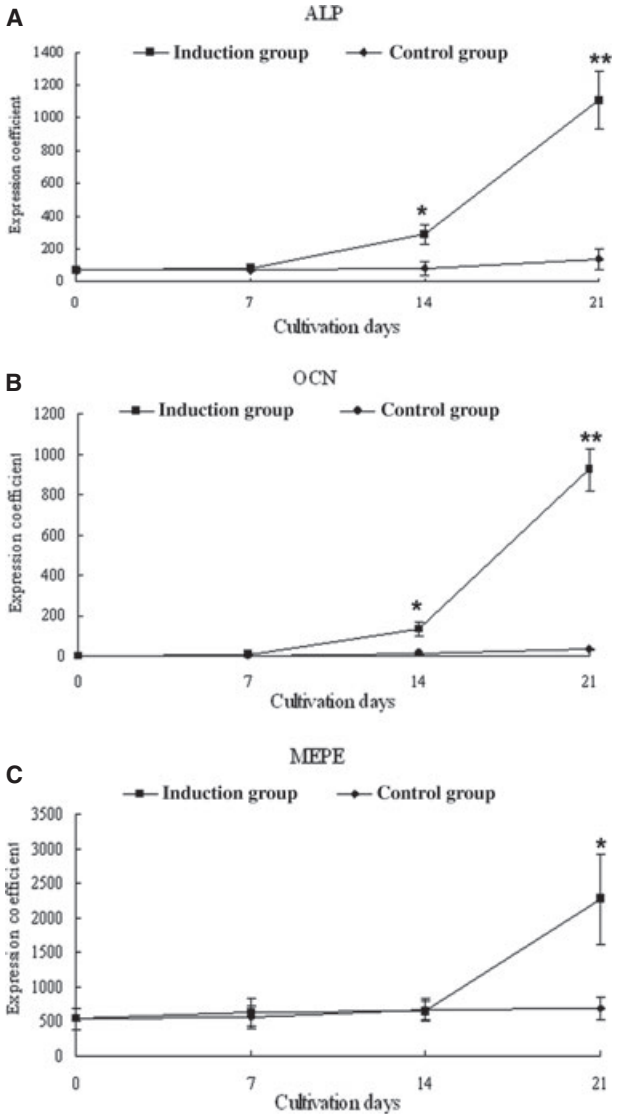

Fig. 3. Quantitative real-time reverse transcription–polymerase chain reaction analysis of alkaline phosphatase (A), osteocalcin (B) and matrix extracellular phosphoglycoprotein (C) gene expression in periodontal ligament cells. Periodontal ligament cells cultured in osteogenic induction medium were lysed on days 7, 14 and 21 of culture to extract total RNA, and cells cultured in alpha-modified Eagle's minimal essential medium containing 15% fetal bovine serum were used as controls. The relative mRNA level was calculated by dividing the absolute level of expression of each mRNA (alkaline phosphatase, osteocalcin and matrix extracellular phosphoglycoprotein) with the absolute level of expression of each mRNA of beta-actin and the resulting value was designated as the expression coefficient in the present study. Each point represents the mean value calculated from three different cell samples, with the same cell sample analyzed in triplicate. *Significant difference vs. the control at the same time-point, $p < 0.05$. **Significant difference vs. the control at the same time-point, $p < 0.001$. ALP, alkaline phosphatase; MEPE, matrix extracellular phosphoglycoprotein; OCN, osteocalcin.

Western blot and reverse transcription–polymerase chain reaction analysis of up-regulated or down-regulated proteins

To confirm the results obtained in 2D-DIGE gels, several proteins, such as the cytoskeleton-associated proteins caldesmon and tropomyosin, the heterogeneous nuclear ribonucleoprotein C and the calcium-binding protein annexin A4, were selected for further analyses by western blot and quantitative polymerase chain reaction (Fig. 6). The proteins and mRNA were extracted from periodontal ligament cells at days 0 and 7 of induction. Seven days after osteogenic induction of periodontal ligament cells, western blot analysis confirmed the down-regulation of caldesmon, tropomyosin and heterogeneous nuclear ribonucleoprotein C and the up-regulation of annexin A4. Furthermore, quantitative polymerase chain reaction analysis showed similar changes in the expression of mRNA for caldesmon, tropomyosin, heterogeneous nuclear ribonucleoprotein C and annexin A4 in osteogenic culture (Fig. 6).

Discussion

In this study, periodontal ligament cells isolated from periodontal ligament showed a profile of cell-surface epitopes which was similar to that reported by others (4,8,9). Seo *et al.* (4) showed that periodontal ligament stem cells are immunocytochemically positive for STRO-1 and CD146, which have been used to isolate and purify human bone marrow mesenchymal stem cells. In our flow cytometry analysis, it was found that the percentage of STRO-1-positive cells at passage 2 was higher than reported previously (8). Periodontal ligament cells expressed the mesenchymal stem cell-associated antigens CD44 and CD106, but were negative for the hematopoietic marker, CD34, which agreed with the report by Gronthos *et al.* (9). The osteogenic differentiation potential of periodontal ligament cells was demonstrated by the formation of mineralized matrix and the expression of the bone-related markers alkaline phosphatase, osteocalcin and matrix extracellular phosphoglycoprotein, when periodontal ligament cells were cultured in osteogenic media. These data indicated that cultured periodontal ligament cells possess progenitors that have the potential to differentiate into cementoblasts/osteoblasts *in vitro*.

Although some growth factors, genes and signaling molecules have been identified in the process of cementogenesis/osteogenesis using

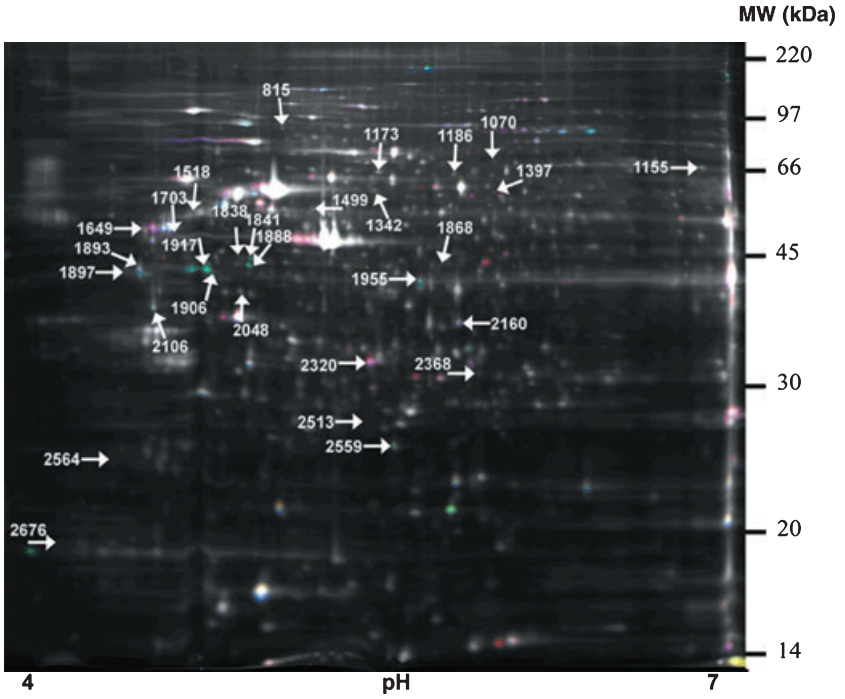

Fig. 4. Differential displayed proteomic profiling of periodontal ligament cells in osteogenic-induced medium for 7 d, as revealed using the two-dimensional difference in gel electrophoresis (2D-DIGE) technique. Computer-assisted comparative analysis using the biological variation analysis mode of the DECYDER software showed 14 protein spots that were up-regulated by more than 1.5-fold in intensity and 47 protein spots that were down-regulated by more than 1.5-fold in intensity in osteogenic-induced periodontal ligament cells compared with control cells ($p < 0.05$). Twenty-nine of the 61 proteins were identified through matrix-assisted laser desorption ionization (MALDI) peptide mass fingerprinting and database interrogation. The identified proteins are indicated by spot identification number (ID) referenced to Table 4.

conventional molecular biological approaches (10–12), mapping of the differential display of proteins in periodontal ligament cells during osteogenic differentiation will provide a better understanding of the underlying mechanisms. Proteomic profiling analysis, which provides a complementary and potentially more comprehensive approach, has been used to identify important protein candidates in the process of differentiation. In this study, periodontal ligament cells were cultured in osteogenic induction medium for 7 d, and the changes in protein expression in comparison with controls were determined using the highly sensitive 2D-DIGE and MALDI-TOF mass spectrometry technologies. In total, 29 differentially expressed proteins were identified in periodontal ligament cells during osteogenic differentiation. These proteins were mainly related to the cytoskeleton, nuclear regulation, cell membrane-biunding, matrix synthesis, metabolic enzymes and signal transduction.

It was noted that cytoskeleton proteins, including vimentin, caldesmon, tropomyosin and clathrin heavy-chain polypeptide, were down-regulated during cementoblastic/osteoblastic differentiation. The down-regulation of these cytoskeleton proteins is related to the re-organization of the cytoskeleton. Differentiation stage-specific re-organization of the cytoskeleton has been reported previously, and a significant down-regulation of vimentin has been identified in mesenchymal stem cells differentiating into cardiomyocytes (13). Vimentin exhibits a complex pattern of developmental and tissue-specific expression that is regulated by transforming growth factor-beta 1, which plays an important role in cell differentiation (14). Caldesmon contains actin-binding, myosin-binding, tropomyosin-binding, and Ca^{2+}-calmodulin-binding domains (15), and is often regarded as a differentiation marker of smooth muscle cells (16). Caldesmon was found to be down-regulated in mesenchymal stem cells during the process of neuron-like differentiation (17), providing further evidence of its involvement in cell differentiation. Caldesmon can be phosphorylated by p21-activated kinases, a dynamic process required to regulate actin dynamics and membrane protrusions in wound-induced cell migration (18). Tropomyosin is related to the collaborative interactions of the isoforms with different actin-binding proteins, such as caldesmon, myosin, Arp 2/3, cofilin, gelsolin and tropomodulin (19), and is a major regulator of F-actin functional specialization in migrating cells (20). Tropomyosins increase epithelial cell adhesion to matrix by enhancing actin fibers and focal adhesions, and play a role in transforming growth factor-beta 1-mediated cell motility and invasion (21). As natural partners of caldesmon, tropomyosins regulate actomyosin-based contractility, mediate the inhibitory effects of caldesmon on actomyosin and regulate the speed of cytokinesis and the membrane stability during cell division (22). As an inhibitor of the Arp2/3 complex and cofilin function, tropomyosin has been found to be localized in relation to actin filaments and to protect actin filaments from being severed by cofilin (23). Clathrin heavy-chain polypeptide, another actin filament-binding protein identified in this study, together with tropomyosin and annexin A4, has been identified to be involved in the osteogenic differentiation of mesenchymal stem cells (24). An earlier proteomics study of periodontal ligament fibroblasts found that the expression of actin, vimentin and tropomyosins was related to mechanical loading and remodeling of periodontal ligament (25). In our study, we found a down-regulation of vimentin, caldesmon, beta tropomyosin, skeletal muscle tropomyosin, clathrin heavy-chain polypeptide and related kinase PAK65 on day 7 of the osteogenic differentia-

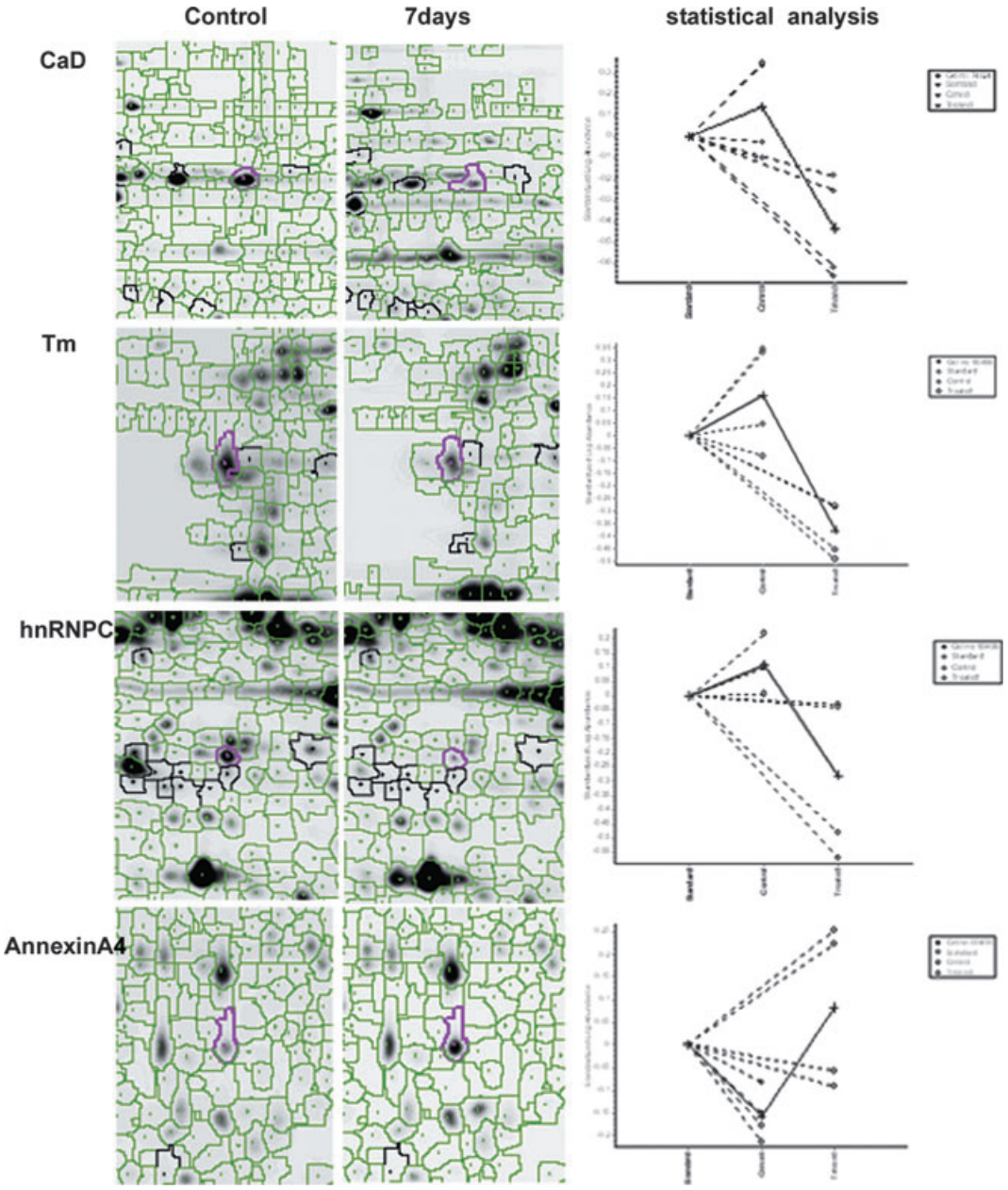

Fig. 5. The spot circled with the purple line in the control gels and in the 7-d gels represents caldesmon, tropomyosin, heterogeneous nuclear ribonucleoprotein C and annexin A4. Caldesmon, tropomyosin and heterogeneous nuclear ribonucleoprotein C were down-regulated, whereas annexin A4 was up-regulated in periodontal ligament cells after 7 d of osteogenic induction, as revealed using the two-dimensional difference in gel electrophoresis (2D-DIGE) technique. Graph view: the *y*-axis represents the standardized log abundance of protein expression. CaD, caldesmon; hnRNPC, heterogeneous nuclear ribonucleoprotein C; Tm, tropomyosin.

tion of progenitors present in periodontal ligament cells. Co-regulation of the functional class of proteins suggests that these cytoskeleton proteins are intimately involved in the differentiation process and that their regulation proceeds via similar regulatory mechanisms, possibly by regulating actin filament organization and dynamics. However, this hypothesis needs to be confirmed by further experiments.

The heterogeneous nuclear ribonucleoprotein C proteins (C1 and C2) belong to a class of proteins that bind to pre-mRNA in the cell nucleus. Increased expression of heterogeneous nuclear ribonucleoprotein C can induce the translation of p27 mRNA and trigger the inhibition of cell growth via p27-regulated cell cycle arrest (26). Forster and colleagues (24), applying a proteomic approach, observed the co-regulation of five members of the heterogeneous nuclear ribonucleoprotein family (hnRNP C1/C2, Q, U, K and G) during mesenchymal stem cell osteogenic differentiation. Altered expression of heterogeneous nuclear ribonucleoproteins has been regarded

Table 4. List of proteins with altered expression identified by two-dimensional difference in gel electrophoresis (2D-DIGE)/matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry during osteogenic differentiation of periodontal ligament cells

Spot ID	Protein name	NCBI accession no.	Coverage (%)	MW (kDa)	pI	Average ratio	*t*-test
Cytoskeleton proteins							
857	Caldesmon 1 isoform 4	gi|15149463	39.6	64.3	6.7	−3.67	0.016
1342	Vimentin	gi|37852	45.5	53.72	5.1	−1.83	0.0013
1893	Beta tropomyosin	gi|6573280	35	29.98	4.7	−3.66	0.0041
2106	Skeletal muscle tropomyosin	gi|339958	34.1	26.57	4.6	−4.15	0.016
1897	Clathrin heavy chain polypeptide	gi|1359719	5.2	189.1	5.5	−3.59	0.0065
Nuclear proteins							
1155	Lamin A/C isoform 3	gi|27436948	28.7	70.93	8.7	−1.6	0.0061
1838	Heterogeneous nuclear ribonucleoprotein C (C1/C2) (hnRNPC protein)	gi|13937888	18	33.64	5	−1.98	0.014
1888	Heterogeneous nuclear ribonucleoprotein C (C1/C2) (hnRNPC protein)	gi|14249959	22.5	32.43	5	−2.14	0.04
1906	Nucleophosmin 1, isoform 2	gi|16307090	25.3	29.62	4.5	−2.04	0.024
1917	Nucleophosmin 1, isoform 2	gi|16307090	32.1	29.62	4.5	−2.93	0.0095
Cell membrane-bound molecules							
2160	Annexin A4	gi|1703319	46.7	36.09	5.8	1.83	0.046
2564	Similar to erythrocyte membrane-associated giant protein antigen 332	gi|51459017	2.1	34.42	9.3	−1.95	0.0094
Proteins involved in matrix synthesis							
1173	Elastin microfibril interfacer 3	gi|71681796	8.2	83.68	8.5	−1.76	0.016
Metabolic enzymes							
815	Transglutaminase	gi|339521	10.2	78.38	5.2	−1.9	0.0095
1397	Chain H, the structure of human mitochondrial aldehyde dehydrogenase in complex with the antidipsotropic inhibitor daidzin	gi|49258360	12.6	54.94	5.7	2.57	0.0042
Protein synthesis							
2048	Eukaryotic translation elongation factor 1 delta isoform 1	gi|25453474	19.5	71.85	6	−1.7	0.00002
Signal transduction							
1186	1-hPAK65	gi|984305	27	55.18	6	−1.64	0.0007
1070	1-ERBB2	gi|71017495	50.8	7.08	8.3	−1.77	0.022
Others							
2513	Human H-chain ferritin	gi|2554682	28.4	21.38	5.2	−2.63	0.012
2559	Ferritin	gi|223574	37.4	20.04	5.5	−2.38	0.016
1841	SGT1B protein	gi|33286853	14.5	41.32	5.1	−2.11	0.018
1499	1-C1q-C	gi|33150626	30.6	26.1	9	2.5	0.0033
1518	Unnamed protein product	gi|21757045	48	52.48	5	2.83	0.016
1649	Reticulocabin precursor	gi|9963785	34.5	37.41	4.7	2	0.0016
1703	Unnamed protein product	gi|16552261	32.2	52.48	5	1.82	0.019
1868	Similar to ENSANGP00000000189	gi|37547159	3	23.64	10.4	3.71	0.022
2320	Similar to zinc finger protein 317; KRAB-containing zinc finger protein 317	gi|51467055	15	33.26	10	1.74	0.0069
2368	Chain A, crystal structure of the ternary complex of Eif4e-M7gpppa-4ebp1 peptide	gi|71041838	22.5	22.47	8.2	2.53	0.019

ID, identification number; MW, molecular weight; NCBI, National Centre for Biotechnology Information; PDLCs, periodontal ligament cells; pI, isoelectric point.

to be related to cell adhesion. The down-regulation of heterogeneous nuclear ribonucleoprotein C1/C2 found in our study further supports the critical role of this protein in cell adhesion during the osteogenic differentiation of progenitors presented in periodontal ligament cells. In addition to heterogeneous nuclear ribonucleoprotein C1/C2 proteins, the two nuclear proteins nucleophosmin 1 and lamins A/C were also down-regulated in the differentiation process. Nucleophosmin 1 serves as a shuttle protein for the nuclear transport of ribosomal components and presumably collaborates with other nucleolar proteins in ribosome assembly (27). Nuclear lamins A/C are intermediate filament proteins that form the nuclear lamina underlying the inner nuclear membrane (28). The absence of lamin A/C has been identified as a novel marker for undifferentiated embryoic stem cells, and lamin A/C expression has been identified as an early indicator of embryoic stem cell differentiation, indicating the functional role of lamin A/C in the maintenance of the differentiated state (29). The concomitant down-regulation of heterogeneous nuclear ribonucleoprotein C1/C2, nucleophosmin 1 and lamin A/C in our

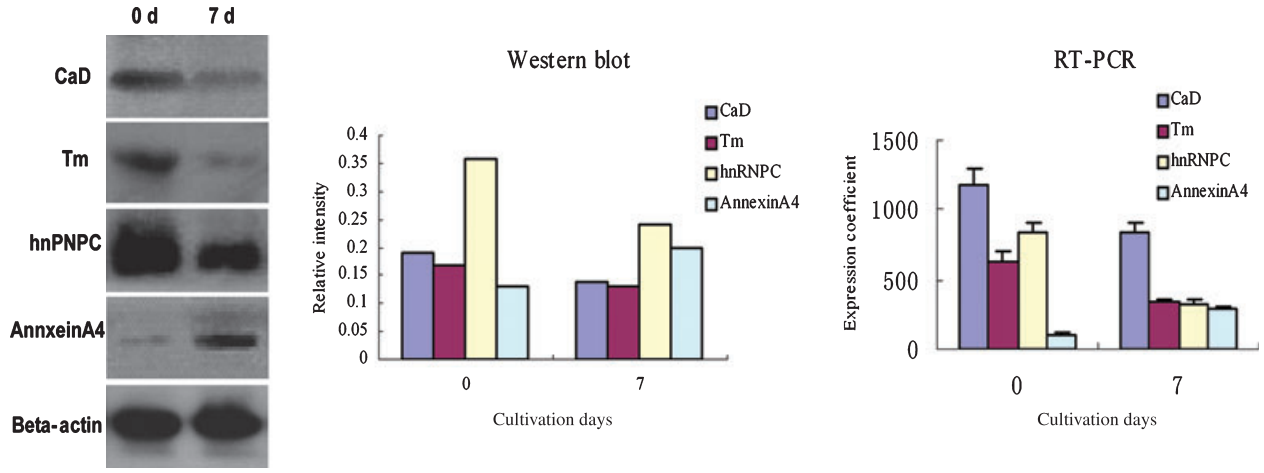

Fig. 6. Western blot and quantitative reverse transcription–polymerase chain reaction analysis of the protein and mRNA expression of caldesmon, tropomyosin heterogeneous nuclear ribonucleoprotein C and annexin A4 in periodontal ligament cells on days 0 and 7 of induction. Western blot analysis confirmed the down-regulation of caldesmon, tropomyosin and heterogeneous nuclear ribonucleoprotein C, and the up-regulation of annexin A4, 7 d after osteogenic induction of periodontal ligament cells. Quantitative reverse transcription–polymerse chain reaction analysis of caldesmon, tropomyosin, heterogeneous nuclear ribonucleoprotein C and annexin A4 expression also confirmed the DIGE findings. The mRNA levels of caldesmon, tropomyosin, heterogeneous nuclear ribonucleoprotein C and annexin A4 in the induction groups were significantly different from those of the the control ($p < 0.05$). These results were representative of three independent experiments. CaD, caldesmon; hnRNPC, heterogeneous nuclear ribonucleoprotein C; RT-PCR, reverse transcription–polymerase chain reaction; Tm, tropomyosin.

study suggest that these nuclear proteins may work together to allow osteogenic differentiation of the progenitors presented in periodontal ligament cells to proceed.

Annexins are a structurally related family of calcium-binding proteins that are strongly implicated in mediating a variety of cellular processes, including calcium handling, phospholipase A2 inhibition, adhesion, exocytosis and the regulation of interactions between membranes and the cytoskeleton (30). Several isoforms of annexins have already been found to play roles in cell differentiation and mineralization. Ye *et al*. (13) found, by two-dimensional gel electrophoresis and mass spectrometry, that three isoforms of annexin A1 are down-regulated to various degrees during rat mesenchymal stem cell differentiation into cardiomyocytes. Annexins A2, A5 and A6 are major components of matrix vesicles that enable the influx of Ca^{2+} into the vesicles and initiate the mineralization process of the growth plate cartilage (31). Annexin A4 is reported to regulate a calmodulin-dependent kinase II-activated (Ca^{2+}/CaMKII) chloride conductance and has been proposed to play a role in exocytosis, vesicle aggregation and trafficking in a Ca^{2+}-dependent manner (32–34). Annexin A6, together with alkaline phosphatase, is predominantly expressed by terminally differentiated, mineralizing chondrocytes, and are thus considered as late markers of chondrocyte differentiation (35). Previous proteomics studies have demonstrated the differential expression of annexins A1, A2, A4 and A5 in human mesenchymal stem cell osteogenic differentiation (36,37). In the current study, both the protein and mRNA expression of annexin A4 were up-regulated in the induced periodontal ligament cells, suggesting its involvement in the cellular processes of Ca^{2+} homeostasis and osteogenic differentiation. However, the exact role of this molecule in the osteogenic differentiation of progenitors presented in periodontal ligament cells requires further investigation.

It is worth noting that some typical bone-related proteins, such as alkaline phosphatase, osteocalcin and matrix extracellular phosphoglycoprotein, were not found to be up-regulated in the proteomic map during early differentiation, although these markers were significantly up-regulated in the late stage of osteogenic differentiation in progenitors presented in periodontal ligament cells, as confirmed by the quantitative polymerase chain reaction. Alkaline phosphatase is reported to be detectable during early osteogenic differentiation, but in this study neither mRNA levels nor protein levels showed any significant difference on day 7 of differentiation. One possible reason for this apparent variation may be the source of cells and differentiation protocols utilized in the various research laboratories. In addition, some major extracellular matrix proteins [such as type XII collagen, periostin, periodontal ligament-associated protein-1/asporin, and secreted protein, acidic and rich in cysteine (SPARC)] in periodontal ligament were also not detected, by the proteomic approach used in the present study, to be differentially expressed. Type XII collagen plays an integral role in the organization of the fibrils in the functional parallel arrangement by bridging the adjacent collagen fibrils and therefore is considered to serve as a specific molecular marker for periodontal ligament regeneration (38). Periostin can regulate type I collagen fibrillogenesis and thereby serve as an important

mediator of the biomechanical properties of fibrous connective tissues (39). Periodontal ligament-associated protein-1/asporin plays a negative role in regulating the mineralization of periodontal ligament cells, probably by inhibiting bone morphogenetic protein-2 functions to maintain periodontal ligament homeostasis *in vivo* (40). Secreted protein, acidic and rich in cysteine (SPARC, also named BM-40 or osteonectin), modulates collagen fibril assembly by binding several collagen types and regulates cell–extracellular matrix interactions that influence cell adhesion and migration (41). With regard to the lack of detection of these conventional bone-related and periodontal ligament-related proteins in the proteomic map, the reason is not clear, but three responsible technical factors have to be considered: first, owing to the limitation of current two-dimensional gel electrophoresis techniques, it may not be possible to separate some low-abundance proteins on gels; second, the amount of protein collected from the two preparative gels may be inadequate for mass spectrometry identification; and, third, the pH range (4–7) may also affect the number of identified proteins. Nevertheless, the early regulatory proteins identified in our study may provide some insights into the potential molecular interactions between early-stage and late-stage differential proteins. Future efforts will focus on better characterization of the proteins identified in the current study. Improved sensitivity is required, and other pH ranges should also be tested in future work to increase the sensitivity of protein detection.

Conclusion

In this study, we present for the first time the differential protein-expression profiles of periodontal ligament cells on day 7 of osteogenic induction. In total, 29 proteins were identified by mass spectrometry analysis to show differential expression during osteogenesis. These proteins mainly included cytoskeleton proteins and cytoskeleton-associated proteins, nuclear proteins and cell membrane-bound molecules, and may be associated with the unique function of periodontal ligament cells in maintaining periodontal tissue homeostasis, especially during the mineralization of periodontal ligament. Our results provide a comprehensive reference for understanding and investigating in greater detail the molecular mechanisms of periodontal ligament cells in periodontal regeneration.

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