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Research Article

Cytoskeletal binding proteins distinguish cultured dental follicle cells and periodontal ligament cells

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ABSTRACT

Human dental follicle cells (DFCs) and periodontal ligament cells (PDLCs) derived from the ectomesenchymal tissue, have been shown to exhibit stem/progenitor cell properties and the ability to induce tissue regeneration. Stem cells in dental follicle differentiate into cementoblasts, periodontal ligament fibroblasts and osteoblasts, these cells form cementum, periodontal ligament and alveolar bone, respectively. While stem cells in dental follicle are a precursor to periodontal ligament fibroblasts, the molecular changes that distinguish cultured DFCs from PDLCs are still unknown. In this study, we have compared the immunophenotypic features and cell cycle status of the two cell lines. The results suggest that DFCs and PDLCs displayed similar features related to immunophenotype and cell cycle. Then we employed an isobaric tag for relative and absolute quantitation (iTRAQ) proteomics strategy to reveal the molecular differences between the two cell types. A total of 2138 proteins were identified and 39 of these proteins were consistently differentially expressed between DFCs and PDLCs. Gene ontology analyses revealed that the protein subsets expressed higher in PDLCs were related to actin binding, cytoskeletal protein binding, and structural constituent of muscle. Upon validation by real-time PCR, western blotting, and immunofluorescence staining. Tropomyosin 1 (TPM1) and caldesmon 1 (CALD1) were expressed higher in PDLCs than in DFCs. Our results suggested that PDLCs display enhanced actin cytoskeletal dynamics relative to DFCs while DFCs may exhibit a more robust antioxidant defense ability relative to PDLCs. This study expands our knowledge of the cultured DFCs and PDLCs proteome and provides new insights into possible mechanisms responsible for the different biological features observed in each cell type.

1. Introduction

Several dental derived stem/progenitor cells have been isolated from humans: dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHED), stem cells from apical papilla (SCAP), dental papilla cells (DPCs), dental follicle cells (DFCs), and periodontal ligament cells (PDLCs) [1]. The aforementioned cell types share a common origin with neural crest cells and provide a source for tissue regeneration. Although the exact mechanism of origin is still not completely clear, similarities and differences in immunophenotype, protein expression pattern, capacity of multilineage differentiation and self-renewal have been observed in dental derived stem/progenitor cells [2].

During tooth root development, the dental follicle (DF) gives rise to cementum, periodontal ligament (PDL) and alveolar bone. Thus, DFCs and PDLCs are derived from different developmental stages of the periodontal tissue. While DFCs and PDLCs display similar stem cell characteristics [1], differences exists between the two cell types. For instance, DFCs showed a higher expression level of Stro-1 and CD146 [3] and lower CD44 expression than that of PDLCs [4]. DFCs can differentiate into hepatocyte-like cells [5], but this remains to be determined in PDLCs. Human PDLCs have the potential to generate tendon *in vivo* [6], this has yet to be reported in DFCs. A systematic

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comparison of DFCs and PDLCs will aid in a better understanding of the different biological behaviors of these cell types.

Basal molecular difference between the two cell types most likely accounts for the observed differences in biological function both *in vitro* and *in vivo*. Large-scale molecular analyses of dental stem cells have been carried out at the transcriptomic level [7–9]. Comparative proteomic analysis has also been employed to distinguish between different types of dental derived stem cells and facilitate their definition through cellular phenotypic profile [5,10–12]. However, the protein profile of human DFCs and PDLCs has yet to be evaluated. A gel-based proteomics approach, 2-Dimensional Electrophoresis (DE)-MS, has been used by many studies; however, gel-free quantitative proteomics techniques have so far been underutilized in dentistry [13].

To better evaluate the basal differences in DFCs and PDLCs post translationally, we compared cultured DFCs and PDLCs at the proteomic level. An isobaric tag for relative and absolute quantitation (iTRAQ)-based quantitative proteomics strategy was employed.

2. Materials and methods

2.1. Cell culture

Isolation of human DFCs ($n=10$ individual donors, 12–25 years of age) and PDLCs ($n=10$ individual donors, 15–25 years of age) have been described previously [14]. Briefly, for the DFC isolation, dental follicles were separated and washed with PBS. Then the tissue was minced using a sterilized scissors and digested with a 0.1 U/ml collagenase type I and 1 U/ml dispase solution (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 °C. The suspension was centrifuged, seeded into 60 mm plates and cultured in α minimum essential medium (α-MEM; HyClone, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/ml penicillin (Sigma, St. Louis, MO, USA) and 100 μg/ml streptomycin (Sigma) in a incubator at 37 °C with 5% CO_2. The medium was changed every three days.

For PDLC isolation, PDL was scraped from the middle third of the root surface using a surgical scalpel after washing twice with PBS. PDL from the coronal and apical region were not used to avoid contamination by gingival and pulpal cells. Then the tissue was minced using a sterilized scissors and digested in a solution of 3 mg/mL collagenase type I (Sigma, St. Louis, MO, USA) and 4 mg/mL dispase (Sigma, St. Louis, MO, USA) for 1 h at 37 °C. The suspension was centrifuged, seeded into 60 mm plates and cultured in α minimum essential medium (α-MEM; HyClone, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/ml penicillin (Sigma, St. Louis, MO, USA) and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA) in a incubator at 37 °C with 5% CO_2. The medium was changed every three days.

Cells from different individuals were cultured separately and used in the following experiments at passage three or four. All experiments were conducted in accordance with the protocol approved by the Committee of Ethics of the Sichuan University with informed patient consent as required.

2.2. Flow cytometry

Both DFCs and PDLCs were trypsinized and washed with PBS. Then the cells were stained with primary antibodies labeled with allophycocyanin (APC) fluorescein isothiocyanate (FITC), phycoerythrin (PE) or PE-Cy5 fluorochromes before flow cytometry analysis. These antibodies included CD3, CD4, CD8, CD14, CD19, CD31, CD33, CD34, CD45, CD73, CD90, CD105, CD106, CD146 and HLA-DR (all purchased from BD Biosciences, San Jose, CA). The expression levels of these markers are expressed as the mean ± standard deviation (S.D.) for $n=3$ donors. The flow cytometry was performed using BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA) and analyzed by FlowJo software (TreeStar, San Carlos, CA, USA).

2.3. Cell cycle analysis

Both DFCs and PDLCs were synchronized with serum-free medium for 24 hours following cultured in α-MEM supplemented with 10% FBS for three days. Then the cells were detached with trypsin and fixed in 70% ethanol overnight. For DNA content analysis, the cells were incubated with RNase A at 37 °C for 30 min and then propidium iodide (PI) at 4 °C for 30 min. The flow cytometry was conducted as described above.

2.4. Protein extraction

DFCs from two patients (DFCs-1 and DFCs-2) and the PDLCs from another two patients (PDLCs-1 and PDLCs-2) were used for iTRAQ analysis. Both cell populations were washed with ice-cold phosphate buffered saline (PBS) and lysed in extraction buffer (4% SDS, 1 mM DTT, 150 mM Tris–HCl, pH 8.0) following ten rounds of sonication (10 s sonication with 15 s interval). The crude extract was then incubated in boiling water and clarified by centrifugation at 16,000*g* at 25 °C for 10 min. The protein content was determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA).

2.5. iTRAQ labeling

Protein digestion was performed according to the Filter Aided Sample Preparation (FASP) procedure described previously [15] and the resulting peptide mixture was labeled using the 4-plex iTRAQ reagent according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Briefly, 200 μg of proteins for each sample were reduced with 30 μl 4% SDS, 150 mM Tris–HCl pH 8.0, and 100 mM DTT buffer. The samples were washed using UT buffer (8 M Urea, 150 mM Tris–HCl, pH 8.0) by repeated ultrafiltration (Microcon units, 30 kD). Reduced cysteine residues were then blocked with 100 μl 50 mM iodoacetamide in UT buffer and the samples were incubated for 30 min in the dark. The filters were washed with 100 μl UT buffer twice before washing twice with 100 μl DS buffer (50 mM triethylammoniumbicarbonate, pH 8.5). Finally, the protein was digested with 5 μg trypsin (Promega, Madison, WI, USA) in 40 μl DS buffer at 37 °C overnight, and the resulting peptides were collected as a filtrate. The peptide content was determined by UV light spectral density at 280 nm.

The samples were labeled with iTRAQ reagent as follows: (PDLCs-1)-114, (PDLCs-2)-115, (DFCs-1)-116, and (DFCs-2)-117, and were multiplexed and vacuum dried.

2.6. Ultra high pressure easy Liquid Chromatography (LC)-Electrospray Ionization (ESI) Tandem MS (MS/MS) analysis by Q Exactive

Reversed phase chromatography was performed using the Thermo EASY-nLC 1000 system. Samples were loaded onto a Thermo Scientific EASY-column (2 cm, ID 100 μm, 5 μm, C18-A1) resin in solvent A (0.1% Formic acid) and then separated with a Thermo Scientific EASY-column (10 cm, ID 75 μm, 3 μm, C18-A2) at a flow rate of 250 nl/min with a 4-h gradient of solvent B (80% acetonitrile and 0.1% Formic acid) as follows: 0–45% B from 0 to 210 min, 35–100% B from 210 to 220 min, and 100% B from 0 to 220 to 240 min. The LC was coupled to a Q Exactive mass spectrometer. Survey scan (300–1800 mass/charge) were acquired in