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# Comparative proteomic profiling of human dental pulp stem cells and periodontal ligament stem cells under in vitro osteogenic induction

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ABSTRACT

*Objective:* This study aimed to compare the proteomic profiling of human dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) under in vitro osteogenic induction, which imitates the microenvironment during osteo-/odontogenesis of DPSCs and PDLSCs.

*Design:* The proteomic profiles of osteoinduced DPSCs and PDLSCs from a single donor were compared using the isobaric tag for relative and absolute quantitation (iTRAQ) technique and subsequent bioinformatics analysis.

*Results:* A total of 159 differentially expressed proteins in PDLSCs and DPSCs were identified, 82 of which had a higher expression level in PDLSCs, while 77 were more highly expressed in DPSCs. Among these enriched proteins, certain members from the collagen, heat shock protein and protein S100 families may distinguish osteoinduced PDLSCs and DPSCs. Gene ontology (GO) classification revealed that a large number of the enriched terms distinguishing PDLSCs and DPSCs are involved in catalytic activity, protein binding, regulation of protein metabolic processes and response to stimulus. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated several involved pathways, including the fatty acid biosynthesis pathway, pantothenate and CoA biosynthesis pathway, arachidonic acid metabolism pathway and PPAR signaling pathway. Further verification showed that the mineralization and migration capacities of PDLSCs were greater than those of DPSCs, in which heat shock protein beta-1, Protein S100-A10 and S100-A11 may play a part.

*Conclusions:* Less than 5% of the differentially expressed proteins make up the comparative proteomic profile between osteoinduced PDLSCs and DPSCs. This study helps to characterize the differences between osteoinduced PDLSCs and DPSCs in vitro.

## 1. Introduction

Human dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) are two of the most important adult mesenchymal dental-derived stem cells that can be isolated from adult dental pulp and periodontal ligament tissues, respectively. Although these cell types are both derived from neural crest and share a similar immunophenotype in vitro, DPSCs and PDLSCs are considered to be distinct from each other due to their disparate origins and functions. DPSCs lie in dental pulp tissue, which is surrounded by dentin and plays a vital role in tooth nourishment, inhibiting bacterial invasion and reacting to mechanical and chemical stimuli. As undifferentiated mesenchymal cells, DPSCs can differentiate into odontoblasts and form reparative dentin under an exogenous stimulus (Gronthos, Mankani, Brahim, Robey, & Shi, 2000). Periodontal ligament tissue possesses high regenerative capacity and a rapid turnover rate. Fibroblasts, comprising most of the PDLSCs, produce extracellular matrix and other substances, which are key to maintaining periodontal homeostasis (Hinz, 2013) and periodontal ligament width over the lifetime (Lim et al., 2014). Similar to dental pulp, the periodontal ligament also contains undifferentiated mesenchymal cells, PDLSCs (Ivanovski, Gronthos, Shi, & Bartold, 2006; Seo et al., 2004), which function as regenerative cell resources under unfavorable conditions.

One of the most widely adopted methods to study the osteogenic or odontogenic differentiation of dental-derived cells is in vitro osteogenic induction using osteogenic induction medium (Cui et al., 2014; Qu et al., 2016). As a well-recognized method, in vitro osteogenic induction, to a certain degree, imitates the microenvironment needed for osteogenesis/odontogenesis of mesenchymal cells. Under such conditions, although DPSCs and PDLSCs can form osteoid tissue, which is a

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trait of osteogenesis/odontogenesis, DPSCs differentiate into odontoblast-like cells (Gronthos et al., 2000) while certain PDLSCs differentiate into cementoblast-like cells, adipocytes, and collagen-forming cells (Seo et al., 2004). This finding suggests that they have distinct reactions towards in vitro osteogenic induction, and gaining insight into these reactions may contribute to further exploration and utilization of these two types of dental mesenchymal cells for clinical application.

Clinically, the loss of dental pulp tissue and periodontal ligament tissue due to pulpitis (Peng et al., 2017) and periodontitis (Slots, 2017), respectively, usually leads to poor prognosis of the affected teeth. The core idea of tissue regeneration of dental pulp and periodontal ligament lies in inducing DPSCs and PDLSCs to differentiate into odontoblasts and cementoblasts, thereby restoring the damaged tissue and ensuring its functionality. However, DPSCs and PDLSCs are difficult to isolate from patients with pulpitis or periodontitis patients, and cannot be replaced by each other or by other cell sources due to their unique differentiation characters (Miran, Mitsiadis, & Pagella, 2016). Therefore, we are less likely to find suitable autologous cell sources for tissue regeneration, let alone have the opportunity to isolate and expand DPSCs and PDLSCs for autotransplantation in patients who have lost their pulp or periodontal ligament tissue. Understanding out how DPSCs and PDLSCs are different and seeking a mutual replacement method for these two types of cells would benefit their clinical application.

Even though there has been intensive in vitro osteogenic induction researches on DPSCs (Wei et al., 2008) and PDLSCs, either collectively or separately, the difference in the underlying mechanisms between these two types of cells remains poorly understood. Understanding the exact differences in proteomic profiling and relevant pathways would help to elucidates their osteogenic mechanisms. However, to the best of our knowledge, only one comparative proteomic study regarding human DPSCs and PDLSCs has been reported (Eleuterio et al., 2013), and that study was conducted under a standard culture condition using a two-dimensional electrophoresis approach. Thus, this study aimed to compare the differentially expressed proteins in DPSCs and PDLSCs, along with the underlying mechanisms, under in vitro osteogenic induction using an iTRAQ proteomic approach and bioinformatics analysis.

## 2. Materials and methods

### 2.1. Isolation and culture of human DPSCs and PDLSCs

Human dental pulp and periodontal ligament tissue from healthy and intact tooth samples were isolated and cultured as described previously (Ma et al., 2012; Qu et al., 2016). Briefly, four premolars were collected from a single male donor (12 years of age), who was undergoing tooth extraction due to orthodontic treatment in the Oral and Maxillofacial Surgery Department, Nanfang Hospital, Guangzhou, China. Informed consent was obtained from the patient, and this subject was approved by the Ethics Committee of Nanfang Hospital.

Tooth samples were rinsed three times with phosphate buffered saline containing 100 units/mL penicillin and 100 mg/mL streptomycin. Dental periodontal ligament tissue was separated from the surface of the tooth and dental pulp tissue was isolated from the pulp chamber. Each tissue was minced into pieces and then later digested in 3 mg/mL collagenase type I (SigmaAldrich, USA) and 4 mg/mL dispase (Sigma-Aldrich, USA) at 37 °C for 1 h as described previously (Cui et al., 2014). Single-cell suspensions were obtained by passing the cells through a 70 mm strainer (Carrigtwohill Co, Ireland). Single-cell suspensions were seeded into six-well plates at a density of $10^4$ cells/well with Dulbecco's Modified Eagle's Medium supplemented with 1) 15% fetal calf serum (Gibco-BRL, Grand Island, NY); 2) 100 mmol/L ascorbic acid 2-phosphate; 3) 2 mmol/L glutamine; 3) 100 U/mL penicillin; and 4) 100 mg/mL streptomycin, at 37 °C in 5% carbon dioxide. The medium was changed every 2 days, and a limited dilution technique was used to obtain single cell–derived colonies (Seo et al., 2004). Periodontal ligament and dental pulp cells of passage 1 at logarithmic phase were selected. The multiple proportion dilution method was performed to modulate cell density to 10 to 15 cells/mL. Cells were seeded into 96-well plates with a volume of 100 μL/well, and the medium was changed every 2 to 3 days. After reaching approximately 80% confluence, single cell–derived colonies were subcultured using 0.25% (w/v) trypsin-0.02% ethylenediamine tetraacetic acid digestion. All primary cells were used at 3 to 4 passages, and the same passage of PDLSCs or DPSCs were used for each experiment.

### 2.2. Protein preparation

Samples of DPSCs and PDLSCs were cultured in one of the most widely adopted recipes for osteogenic induction for 14 days until protein preparation: complete medium supplemented with 10 mM/L β-glycerol phosphate, 50 mg/mL ascorbic acid, and $10^{-7}$ M dexamethasone. For protein digestion, cells were centrifuged at 1,200*g* and washed in ice-cold phosphate buffered saline three times. Subsequently, frozen cell pellets were resuspended in 8 M urea, 6 M Gua, 6 M urea/2 M Gua, 1% RG, 2% sodium deoxycholate, or 0.5% RG/0.5% sodium deoxycholate (all in 100 mM $NH_4HCO_3$) in the presence of 5 mM Tris (2-carboxyethyl) phosphine in triplicate experiments. Following sonication, all urea-containing samples were incubated for 1 h at 37 °C, whereas all other samples were incubated at 60 °C for 30 min. After the extraction and reducing steps, all samples were incubated with 10 mM iodoacetamide at 25 °C for 30 min. Following bicinchoninic acid measurement, the protein samples were quenched using 20 mM *N*-acetylcysteine, and 50 μg of total protein was used for protein digestion. After diluting the chaotropic salt concentration to 6 M and diluting sodium deoxycholate to 1% using 100 mM NH4HCO3, 0.5 μg LysC was added to the protein samples. LysC was allowed to cleave for 4 h at 37 °C, followed by overnight digestion at 37 °C using 1 μg of trypsin. For the second digestion step, the LysC-digested samples were further diluted to a chaotropic salt concentration of 1.6 M. Before liquid chromatography–mass spectrometry analysis, sodium deoxycholate and RG were precipitated using 1% trifluoroacetic acid, and all samples were desalted using C18 microspin columns (Harvard Apparatus, USA) according to the manufacturer's instructions. For digestion, we followed the previous published method of Wisniewski et al. (Wisniewski, Zougman, Nagaraj, & Mann, 2009). For the filter aided sample preparation experiments, cells were reconstituted in 5% sodium deoxycholate and 4% sodium dodecyl sulfate, respectively, sonicated and incubated for 15 min at 95 °C in the presence of 5 mM Tris (2-carboxyethyl) phosphine. Following an additional sonication step, the samples were enabled to cool and further incubated with 10 mM iodoacetamide at 25 °C for 30 min. A total of 50 μg of cleared protein isolate was later transferred into spin filters (Microcon YM-30, Millipore, USA). Sodium dodecyl sulfate – containing samples were mixed with 8 M urea and centrifuged for 15 min at 14,000*g*. Two additional urea wash/centrifugation cycles were performed, and tandem digest was performed as described earlier. Upon protein digestion, the filter was rinsed twice with 0.1% trifluoroacetic acid, and flow throughs were collected in the same tube. For sodium deoxycholate – filter aided sample preparation, the protein isolate was washed once with 1% sodium deoxycholate prior to tandem digest. Postdigest sodium deoxycholate was precipitated using 1% trifluoroacetic acid.

### 2.3. iTRAQ labeling of peptides

The supernatant containing precisely 100 μg protein of each sample was digested with Trypsin Gold (Promega, USA) at 37 °C for 16 h. After trypsin digestion, peptides were dried by vacuum centrifugation. Desalted peptides were labeled with iTRAQ reagents (sigma, iTRAQ@ Reagent-8PLEX Multiplex Kit, 4381663) according to the manufacturer's instructions (AB Sciex, CA). For 100 μg peptide 1units of labeling