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Priming integrin alpha 5 promotes the osteogenic differentiation of human periodontal ligament stem cells due to cytoskeleton and cell cycle changes

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ABSTRACT

To seek a potential target for periodontal tissue regeneration, this study aimed to explore the role of Integrin alpha 5 (ITGA5) in human periodontal ligament stem cells (PDLSCs). Transwell assay, Cell Counting Kit 8 (CCK8) assay, cell cycle assay, alkaline phosphatase (ALP) activity, alizarin red staining, and western blot were used to investigate the effects of ITGA5 on PDLSC migration, proliferation and osteogenic differentiation. The in vivo effect was investigated by nude mice subcutaneous transplantation with cell and hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) complex. The involved mechanism was explored by the iTRAQ proteomic technique and validated by western blot and immunofluorescence. We found that ITGA5forced expression enhanced the proliferation, migration, and osteogenic capacity of PDLSCs, while inhibited ITGA5 expression had the opposite effects. The phosphorylation of focal adhesion kinase (FAK), phosphatidylinositide 3-kinases/protein kinase B (PI3K/AKT), and mitogen-activated protein kinase kinase/extracellular signal-regulated protein kinases 1 and 2 (MEK1/2/ERK1/2) were crucial in this process. Forced expression of ITGA5 in PDLSCs increased osteoid and PDL-like tissue formation in vivo. Proteomic and bioinformatic analysis revealed that cytoskeleton and cell cycle changes were involved. Keratin, type II cytoskeletal 6B (KRT6B) and desmin (DES) may distinguish this process and serve as new markers of PDLSC differentiation.

Significance: Periodontitis is highly prevalent and can impair PDL and teeth functioning. One of the most promising therapies to periodontitis therapies is PDL regeneration by utilizing PDLSCs. While many obstacles remain to be resolved, the regulation of PDLSC osteogenic differentiation is a main concern. The present study demonstrated the potential clinical value of an ITGA5 priming peptide, which may be utilized in PDL tissue repair and regeneration. The mechanism elucidated in this study would help to fuel its application.

1. Introduction

Periodontal ligament stem cells (PDLSCs) are undifferentiated mesenchymal cells that lie in PDL tissue, which possesses high regenerative capacity and a rapid turnover rate [1,2]. PDLSCs function as one of the most important regenerative cell resources under unfavorable conditions. Thus, it is one of the most promising cell sources in periodontal tissue regeneration, the key of which is to promote osteogenic differentiation of PDLSCs and to form functional periodontal tissue. However, the mechanism of PDLSC osteogenic differentiation has not been fully clarified. Thus, it is necessary to explore certain molecules that are crucial for regulating this process.

Previously, we found that knocking down integrin alpha 5 (ITGA5) expression in human dental pulp stem cells (DPSCs) by using a lentiviral vector weakened proliferation and migration capacity [3] while enhancing odontogenic differentiation [4]. Interestingly, this was distinct from human mesenchymal stromal cells (MSCs), as priming ITGA5 promoted the osteogenic differentiation of MSCs [5]. PDLSCs share similarities and differences with DPSCs and MSCs, but the role that ITGA5 plays in the process of PDLSC osteogenic differentiation is still unknown.

Integrins are one of the major families of cell adhesion receptors [6,7]. All integrins are non-covalently-linked and heterodimeric molecules, which contain an alpha (α) and a beta (β) subunit. These two subunits are type I transmembrane proteins, mostly containing large extracellular domains and short cytoplasmic domains [8,9].

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Functionally, the cytoplasmic domain of integrin coordinates the assembly of cytoskeletal polymers and signaling complexes intracellularly, while the extracellular domain engages either counter-receptors on adjacent cell surfaces or extracellular matrix macromolecules. Integrin family members are key to many pivotal processes, such as tissue integrity, cell trafficking, and cell differentiation [10,11]. ITGA5 is a subunit of the integrin α5β1 heterodimer, which functions by integrating with integrin β1 [12]. Moreover, and the phosphorylation of focal adhesion kinase (FAK), phosphatidylinositide 3-kinases/protein kinase B (PI3K/AKT), and mitogen-activated protein kinase kinase/extracellular signal-regulated protein kinases 1 and 2 (MEK1/2/ERK1/2) has been reported to play a role in the process of ITGA5-mediated MSC osteogenic differentiation [5].

Considering the crucial regulatory effects of ITGA5 on DPSCs and MSCs and the important functions of integrins, we hypothesized that ITGA5 may play an important role in PDLSC osteogenic differentiation. However, the exact effect and the involved mechanism must be elucidated. Hence, the present study aimed to investigate the effect of ITGA5 on PDLSC migration, proliferation and osteogenic differentiation by adopting lentivirus-mediated ITGA5 knockdown and overexpression, and an ITGA5 priming synthetic cyclic peptide. Importantly, we aimed to reveal the underlying mechanism using an iTRAQ proteomic approach and subsequent bioinformatic analysis.

2. Materials and methods

2.1. Isolation and culture of human PDLSCs

Human PDL tissue from intact and healthy tooth samples were isolated and cultured as described previously [13]. The third molars or premolars were collected from donors aged 12 to 25 years, who were undergoing tooth extraction due to impacted teeth or orthodontic treatment in the Oral and Maxillofacial Surgery Department of Nanfang Hospital in Guangzhou, China. Specifically, the PDLSCs for the iTRAQ proteomics experiment were from a single donor, and the PDLSCs for the other experiments in the present study were all from 3 other donors. Informed consents were obtained from the patients and this subject was approved by the Ethics Committee of Nanfang Hospital.

PDL tissue was minced into pieces and then digested. A limited dilution technique was used to obtain single cell–derived colonies, as described by Seo et al. [1]. All primary cells were used at 3 to 4 passages, and the same passage of PDLSCs was used for each experiment.

2.2. Construction of ITGA5i and ITGA5 shRNA lentivirus and cell infection

The ITGA5-targeting (ITGA5i) oligonucleotide sequence (CTCCTA TATGTGACCAGAGTT), scrambled sequence and lentiviral work vector were designed as previously described [4]. The ITGA5 overexpression lentivirus containing the only and full transcript of ITGA5 gene and control lentivirus used a lentiviral work vector (Ubi-MCS-3FLAG-SV40-EGFP-IRES-puromycin, GeneChem, Shanghai, China). Following procedures see Cui et al. [4]. PDLSCs were infected with the above lentivirus at a multiplicity of infection of 20.

2.3. Synthetic cyclic peptide (SCP)

The cyclic peptide GA-CRRETAWAC-GA was synthesized (GL Biochem, Shanghai, China) according to Fromigue et al. [14] using standard automated continuous-flow SPPS methods. The cyclic peptide was purified and characterization of the peptide was performed by mass spectrometric on a Q-TOF Ultima Global hybrid quadrupole/time-of-flight (Q-TOF) instrument. The predicted and observed high resolution masses (HRMS) for GA-CRRETAWAC-GA ($C_{54}H_{84}N_{20}O_{17}S_2$) were 1349.5844 and 1349.5778, respectively.

2.4. Cell proliferation assay

For Cell Counting Kit 8 (CCK8) assay, PDLSCs were seeded into 96-well plates at 1000 cells in 100 μL of complete culture medium per well. At the indicated time points, the medium was replaced by a kit solution (TransDetect cell counting kit, Transgene, China) and complete culture medium at a ratio of 1:9, and the samples were incubated for 1 h at 37 °C. The absorbance of each sample was analyzed at 450 nm using a microtiter plate reader (Tecan, Switzerland). The assay was repeated in triplicate.

For cell cycle assay, cells were digested and fixed by 75% cold ethyl alcohol for 1 h, followed by the addition of RNase A at 37 °C for 30 min and adding propidium iodide at room temperature for 30 min (cell cycle detection kit, KeyGEN, China), and then detected by flow cytometry (BD, USA). The assay was repeated 3 times.

2.5. Transwell migration assay

The migration capacity of PDLSCs was evaluated using Transwell chambers (8 μm pore size, BD Falcon, USA). Following overnight serum starvation, cells were harvested and re-suspended in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) without fetal bovine serum (FBS), then added to the upper Transwell chamber as a volume of 200 μL. The migration ability of PDLSCs was stimulated by adding 600 μL of DMEM supplemented with 20% FBS (Gibco, USA) to the lower chamber. Following 24 h culture, the cells that transversed through the membrane were fixed in methanol and stained with hematoxylin (Baso, China) for 30 min. Cells were counted in 10 random fields visualized under a light microscope and expressed as the average number of cells per field. The assay was repeated in triplicate.

2.6. Alizarin red staining and ALP activity staining

PDLSCs were seeded in 500 μL of complete culture medium at 24-well plates and cultured to 70% confluence. Differentiation was induced by culturing cells in complete medium supplemented with 10 mmol/L of β-glycerol phosphate, 50 μg/mL of ascorbic acid, and 10^{-7} mol/L of dexamethasone (Sigma-Aldrich, USA) for 3 weeks for alizarin red (Sigma-Aldrich, USA) staining and 10 days for alkaline phosphatase (ALP) activity staining (BCIP/NBT Alkaline Phosphatase Color Development Kit, Beyotime, China). The MEK1/2/ERK1/2 inhibitor U0126 (10 μM, Beyotime, China), and the PI3K/AKT inhibitor LY294002 (25 μM, Beyotime, China) were added to the medium once 3 days before ALP staining and three times 12 days, 16 days and 19 days before alizarin red staining. The induced cells were fixed in 4% paraformaldehyde for 20 min at room temperature and then stained. The assay was repeated 3 times.

2.7. Western blot analysis

PDLSCs were cultured in 6-well plates until 80% confluence. Cells were collected and lysed (cell lysis buffer kit, KeyGEN, China) after 14 days. U0126 and LY294002 were added to the medium 2 days before collection. Cell extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) at 200 mA for 40 min to 2 h (according to the molecular weight, i.e. 1 min for 1 kDa). The membranes were then incubated overnight at 4 °C with their respective primary antibodies, including ITGA5 (1:2000, Abcam, UK), RUNX2 (runt-related transcription factor 2; 1:500, Bioworld, USA), ON (osteonectin) and COL1A1 (Collagen type I alpha 1 chain) (1:1000, Abcam, UK), GAPDH (glyceraldehyde 3-phosphate dehydrogenase; 1:1000, GNI, Japan), FAK (focal adhesion kinase 1; phosphor Y297), AKT (protein kinase B; phospho T308), ERK1 (extracellular signal-regulated kinase 1; pT202/pY204) + ERK2 (pT185/Py187) and ERK (1:1000, Abcam, UK), followed by incubation with horseradish peroxidase conjugated anti-