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Original Article

IL-17 siRNA promotes human proliferation and osteogenic differentiation of human periodontal ligament stem cells via ERK1/2, JNK and p38 MAPK signaling

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Abstract: Objective: Inhibition of IL-17 on periodontal ligament stem cells (PDLSCs) proliferation osteogenic differentiation had been reported. This study was to investigate the effect of IL-17 on human PDLSCs (hPDLSCs). Methods: Isolated hPDLSCs from healthy fresh premolars were identified and then transfected with IL-17 silencing lentivirus plasmid. Then cell proliferation, colony formation, apoptosis, adipogenic and osteogenic differentiation was detected. Moreover, the activation of ERK1/2, JNK and p38 MAPK signaling was detected. Results: The isolated hPDLSCs were identified with > 99% percentage of STRO-1+/CD146+/CD44+/CD45- cells. When transfected with IL-17 silencing lentivirus plasmid, about 50% percentage was STRO-1+/CD146+/CD44+/CD45+ cells. Moreover, cell proliferation, colony formation, osteogenic differentiation, as well as expression of p-ERK1/2, p-JNK and p-p38 MAPK were significantly enhanced. On contrast, adipogenic differentiations were inhibited by IL-17 silencing. No difference was observed in cell apoptotic percentage before and after transfection. Conclusion: We concluded that IL-17 siRNA administration promoted cell proliferation, colony formation, osteogenic differentiation of hPDLSCs via the activation of ERK1/2, JNK and p38 MAPK signaling.

Keywords: Periodontal ligament stem cells, IL-17, osteogenic differentiation, MAPK signaling

Introduction

Periodontal disease is common directly caused by bacterial dental plaque in periodontium and is a common chronic inflammatory condition which accompanied by loss of periodontal attachment and tooth [1]. Periodontal disease severity often increases in pregnancy, diabetes mellitus, age, and arthritis [2-4]. In particularly, periodontal disease might be a potential risk factor for diabetes in women with prior gestational diabetes mellitus [2].

Periodontal ligament (PDL) tissue is one of component of periodontium and is a complex substance with characterization of fiber-reinforced, abundant blood vessels, providing oxygen to the periodontal cells, and containing several cells including endothelial cells, fibroblasts, and mesenchymal stem cells (MSCs) which show potential of somatic stem cells with the capable of differentiating into adipocytes, chondrocytes, osteoblast-like cells, neurocytes, and other types of cells *in vitro* with stimulation [5, 6]. Periodontal ligament (PDL) stem cells (PDLSCs) have been isolated from human PDL tissues of tooth for researches focusing on periodontal disease mechanisms or treatments because of its multipotentiality [7, 8]. For instance, periodontitis in swine [9], autoimmune encephalomyelitis in mice [10], and tendon tissue regeneration [11] could be treated with PDLSCs.

Multipotentiality of PDLSCs have been reported to associate with various inflammatory cytokines including interleukin (IL)-17, IL-1β, IL-6, and signaling pathways such as mitogen-activated protein kinases (MAPKs), extracellular signal-regulated protein kinase (ERK), Wnt, and nuclear factor (NF)-κB [5, 6, 12, 13]. For example, NF-κB modulated osteogenesis of PDLSCs in inflammatory microenvironments was reported to related to β-catenin [12]. The bone morphogenetic protein-9 induced osteogenic differentiation of PDLSCs mediated by ERK and p38 signaling pathways [14].

IL-17 is a Th17 cells-produced proinflamatory cytokine and is crucial in modulation of immune reactions and bone metabolism [6]. The dual role of IL-17 in periodontal disease and promotion to cell proliferation had been proposed [15, 16]. For instance, the inhibition of IL-17 on PDLSCs proliferation, migration, and osteogenic differentiation was assisted by activation of ERK1/2 and JNK MAPKs [6]. However, IL-17 secretion benefits to proliferation and invasion of human trophoblast cells, human retinal vascular endothelial cells, MSCs, or human ASM cells [17-20]. All these suggested the dual or controversial effect of IL-17 on proliferation and osteogenic differentiation of PDLSCs and intrigues the mechanisms related to these differences.

To investigate the effect of expression of IL-17 on the proliferation, adipogenic and osteogenic differentiation of PDLSCs and the mechanism behind it, we treated human PDLSCs (hPDLSCs) we IL-17 silencing plasmid and detected the proliferation, colony formation, ability of adipogenic and osteogenic differentiations as well. Moreover, activation of signaling pathways which had been reported to associate with adipogenic and osteogenic differentiation of PDLSCs were detected. This study would provide us with more direct information on the effect of IL-17 PDLSCs differentiation.

Materials and methods

Cells and culture conditions

All clinical procedures in this present studied were performed with approval from the Ethics Committee of The General Hospital of Jinan Military Commend. Informed consents were obtained from participants (n = 8) or their guardians. Healthy fresh premolars (n = 12) were removed from 8 patients (12-15 years old) during orthodontic treatments at the PLA Navy General Hospital. The isolation of hPDLSCs were performed according previous methods [21]. In brief, the periodontal membrane scrapped from tooth root was minced into as small pieces as possible, followed by digested with α-MEM (Sigma-Aldrich Co. St Louis, MO, USA) containing 3 mg/mL collagenase I and 4 mg/mL dispase II (Sigma-Aldrich) for 120 min at 37°C, with replaced medium every 30 minutes. Single-cell suspensions were obtained using a 70 μm nylon cell strainer (BD Falcon Labware, BD Labware, Franklin lakes, NJ, USA), and cell density was adjusted to 5×10^5 per T75 cell culture dishes, with α-MEM supplementing with 15% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 2 mM L-glutamine, 100 mM L-ascorbate-2-phosphate (Sigma-Aldrich), 100 μg/mL streptomycin/penicillin (Gibco) at 37°C, and 5% CO_2 in saturated humidity [21, 22]. The hPDLSCs were identified, separated and were then transferred to individual vessels. Cells at passage 3 were used in the experiments of this study.

Identification of hPDLSCs

For the identification of isolated cells from periodontal membranes, we used flow cytometry analysis to characterize the cell-surface markers of hPDLSCs [21, 23]. Cells cultured in T75 dishes were harvested by trypsin in PBS (Sigma-Aldrich) and transferred to a tube with 4% paraformaldehyde for fix for 15 min. Fixed cells were then separately incubated with the primary antibodies anti-STRO-1, anti-CD146, anti-CD44, and anti-CD45 for 1 hour at 37°C, and FITC-conjugated secondary antibody for 40 min, followed by subjection to the FACS Calibur, flow cytometer (BD Biosciences, Franklin lakes, NJ, USA). Cells with > 99% percentages of STRO-1+, CD146+, and CD44+ cells, and < 1% CD45+ cells were identified as hPDLSCs and used for the further studies.

Transfection of hPDLSCs

To construct a lentivirus plasmid knockdown the expression of IL-17 mRNA, the specifically shRNA (purchased from GenePharma, Shanghai, China) double-stranded oligonucleotides to IL-17 were inserted into the EcoRI site in the lentiviral vector GV217 (GeneChem), namely

IL-17 siRNA plasmid. Lentivirus titers were determined by Image J analysis software (NIH, Bethesda, MD, USA) with GFP/DAPI. For knockdown of IL-17 expression, IL-17 siRNA plasmid were transfected into the hPDLSCs in 24-well plates at 5×10^4 cells per well. Transfected cells were incubated in cultured conditions described above. Empty GV217 vectors packaged in lentivirus was used for negative transfection (Mock). Transfections of hPDLSCs were performed as previously described [23].

Cell proliferation assay

Cultured hPDLSCs were digested by trypsin (Sigma-Aldrich), harvested and 2×10^3 cells/well were seeded into 96-well plates and incubated in conditions described above overnight to adherent to well wall. At day 1, 2, 3, 4, and 5, 20 μL of 5 mg/mL MTT solution (Sigma-Aldrich) was added into each well and incubated for 4 h at 37°C, followed by additional incubation in 150 μL dimethyl sulfoxide for 15 min. The optical density at wavelength of 570 nm (OD570 nm) was determined using a Microplate spectrophotometer (Bio-Rad Labs, Hercules, CA, USA).

Soft agar colony formation assay

Transfected or control hPDLSCs cells were seeded into 6-well plates at density of 1×10^4 cells/well in α-MEM containing 15% FBS for 14 days, with replaced medium every 3 days. Soft agar colony formation assay were performed to evaluate the cloning formation ability of transfected or control hPDLSCs cells. Cells were washed with PBS and fixed by 10% paraformaldehyde for 30 s. Cell colonies were determined by crystal violet staining assay. Cell clones with more than 50 cells counted under microscopy. Each treatment was performed in triplicate.

Apoptosis analysis

Transfected or control hPDLSCs cells placed on 6-well plates for 48 h were allowed to confluent. Then cells were harvested by trypsin (Sigma-Aldrich) for apoptosis assay with the Annexin V apoptosis detection kit (Bender MedSystems, Vienna, Austria). Cell apoptosis analysis was performed according to manufactures' instructions of the kit. In brief, harvested cells were incubated with Annexin V and propidium iodide (PI) for 30 min in dark, and were subsequently subjected to Becton Dickinson FACS Calibur™ flow cytometry analysis (BD Biosciences).

Alizarin red S staining

To investigate the *in vitro* osteogenesis ability of hPDLSCs, Alizarin red S staining assay was performed [22]. A total of 1×10^4 cells/well hPDLSCs were seeded into 6-well plates and incubated in standard cultured conditions as described above to 85-90% confluence. Then cells were incubated with chondro-inductive medium (Sigma-Aldrich) for 21 days, with replaced fresh medium every 3 days to drive hPDLSC chondrogenic differentiation [24]. Then hPDLSCs were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) and were subsequently subjected to 2% alizarin red S (Sigma-Aldrich) for 30 min at 37°C. Plate samples were washed with distilled water, and matrix mineralization (calcium deposition) were observed under Olympus BX51 System Microscope. The mineralized matrix nodules (red colored) indicate matrix mineralization.

Oil Red O staining

To investigate the *in vitro* adipogenic differentiation of hPDLSCs, we preformed Oil Red O staining assay on hPDLSCs. A total of 1×10^5 cells/well hPDLSCs were placed into 6-well plates and incubated as described above to 85-90% confluence. Then hPDLSCs were transferred to the hMSC adipogenic differentiation medium (Sigma-Aldrich) for 28 days for adipogenesis induction. Then cells were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) and were subsequently subjected to Oil Red O staining systems to evaluated the lipid-drop accumulation [24].

Western blot analysis

Cellular proteins were extracted using lysis solution (RIPA, Beyotime Institute of Biotechnology, Shanghai, China) after transfection for 24 h. Cellular protein amount was determined using BCA protein determination kit (Pierce, Rockford, IL, USA). Then, cellular lysates of 100 μL were separated by 10% SDS-PAGE. Proteins were electro-transferred to PVDF membranes (Invitrogen Corp., Carlsbad, CA, USA), which were then blocked with 5% non-fat milk (BD Bio-

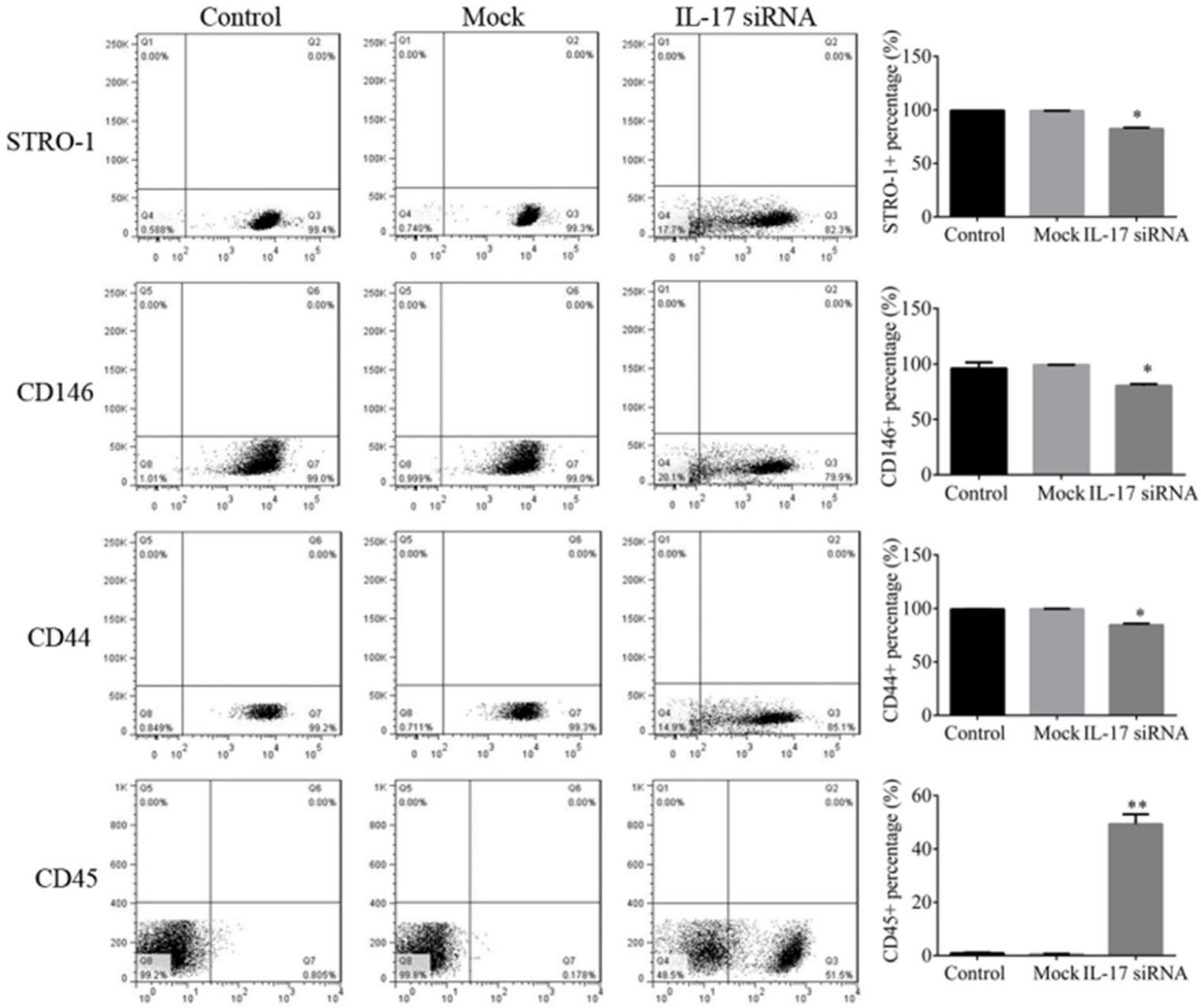
Control
Mock
IL-17 siRNA
STRO-1
CD146
CD44
CD45
STRO-1+ percentage (%)
CD146+ percentage (%)
CD44+ percentage (%)
CD45+ percentage (%)
Control
Mock
IL-17 siRNA

Figure 1. Characterization of hPDLSCs by flow cytometry. Isolated hPDLSCs showed > 99% percentages of STRO-1+/CD146+/CD44+/CD45- cells. IL-17 siRNA plasmid transfection elevated the percentage of CD45+ cells. *, and **indicates significant level at P < 0.05, and P < 0.01, vs. Control and Mock, respectively.

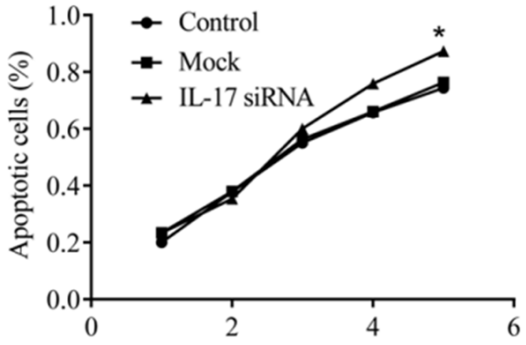

Figure 2. Cell proliferation assay of hPDLSCs. Isolated hPDLSCs were incubated for 1, 2, 3, 4, and 5 after IL-17 siRNA plasmid transfection, and cell viability was detected using MTT assay. *Indicates significant level at P < 0.05, vs. Control or Mock.

sciences) for 1 h, and probed with the specific primary antibodies anti-p-ERK1/2/ERK1/2, anti-p-JNK/JNK, anti-p-p38 MAPK/p-p38 MAPK, and anti-GAPDH (Abcam, dilution 1:1000 in TBST) at 4°C overnight. Additional incubation with HRP-conjugated secondary antibody (1:1000) was performed for 60 min at room temperature. Finally, enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL, USA) and AlphaEase software (Alpha, U. S. A.) were used for polypeptide bands analysis. Relative protein expression normalized to GAPDH (fold change/GAPDH) was calculated.

Statistical analysis

All cellular experiments were performed in six parallel wells. All results were statistically analyzed using the SPSS version 21.00. All quantitative data were presented as mean ± SEM (standard electronic modules), and the statistical differences among groups were analyzed using one-way ANOVA using GraphPad PRISM software package (GraphPad Software La Jolla, CA, USA). *P* value less than 0.05 (P < 0.05) was considered to be statistically significant.

Results

Characterization of hPDLSCs

Flow cytometer analysis showed that the isolated cells were hPDLSCs, with > 99% STRO-1+/CD146+/CD44+/CD45- cells (**Figure 1**). Cell percentage of CD45+ was < 1%. However, silencing of IL-17 expression elevated the percentage of CD45+ cells. HPDLSCs transfected with IL-17 siRNA plasmid were STRO-1+/CD146+/CD44+/CD45+ cells, with almost 50% CD45+ cells (P < 0.01), demonstrating the differentiation of hPDLSCs induced by IL-17 siRNA transfection.

IL-17 siRNA promote cell proliferation

The effect of IL-17 siRNA transfection on cell proliferation was detected to investigate whether IL-17 silencing impact cell proliferation of hPDLSCs. We found IL-17 siRNA enhanced cell proliferation and there was a difference in the cell viability between IL-17 siRNA and control or Mock group (P < 0.05, **Figure 2**). This suggested that the inhibition of IL-17 expression affected the proliferation of hPDLSCs.

IL-17 siRNA does not impact apoptosis of hPDLSCs

Figure 3 shows that the IL-17 silencing by siRNA technology did not impact the apoptosis ratio of hPDLSCs. No differences were observed between cell transfected with IL-17 siRNA plasmid and Control or Mock (P > 0.05, **Figure 3**). We demonstrated that the inhibition of IL-17 expression showed no influence on cell apoptosis of hPDLSCs.

IL-17 siRNA promotes colony formation assay

The effect of IL-17 siRNA on hPDLSCs colony formation ability was evaluated using the soft agar colony formation assay. We demonstrated that the silence of IL-17 gene in hPDLSCs promoted cell colony formation potential, with IL-17 siRNA transfected cells showed significantly higher colon numbers as compared to control and mock treated cells (P < 0.01, **Figure 4**). No difference was observed in colon number between control and mock group. This suggested that IL-17 siRNA promoted colony formation of hPDLSCs.

IL-17 siRNA inhibits the adipogenic differentiation of hPDLSCs

To investigate the effect of IL-17 on adipogenic differentiation of hPDLSCs, we performed Oil

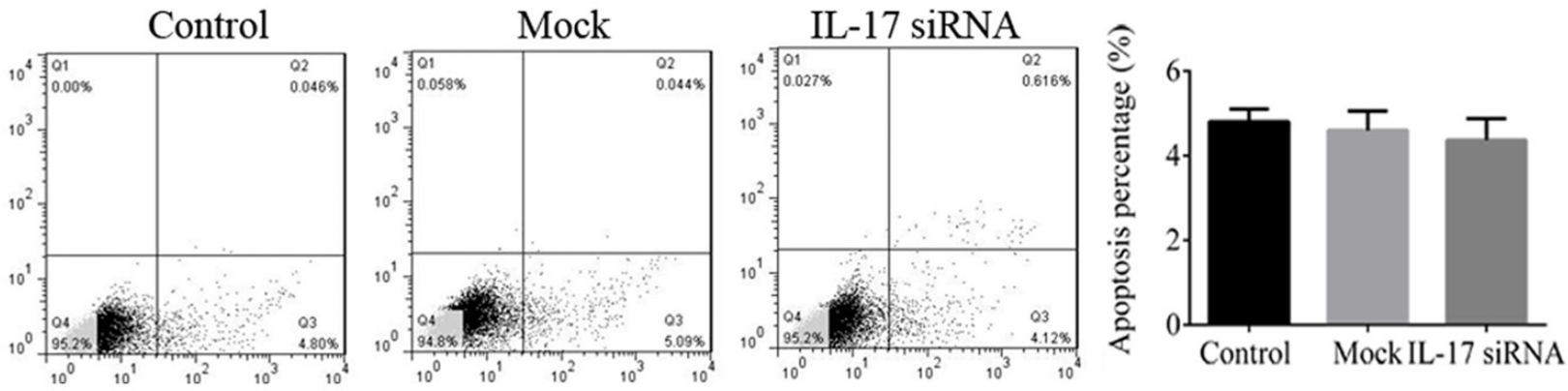

Figure 3. Cell apoptosis percentage of hPDLSCs. Cell apoptosis ratio was determined using flow cytometry with Annexin V apoptosis detection kit.

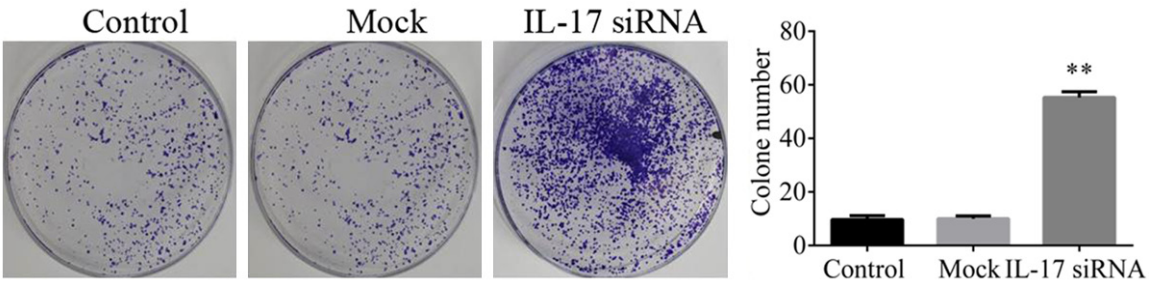

Figure 4. Soft agar colony formation assay for hPDLSCs. Colony formation ability of hPDLSCs cells were evaluated using the soft agar colony formation assay. Colony formation determined by crystal violet stained cell colonies with more than 50 cells counted. **Indicates significant level at $P < 0.01$, vs. Control and Mock, respectively.

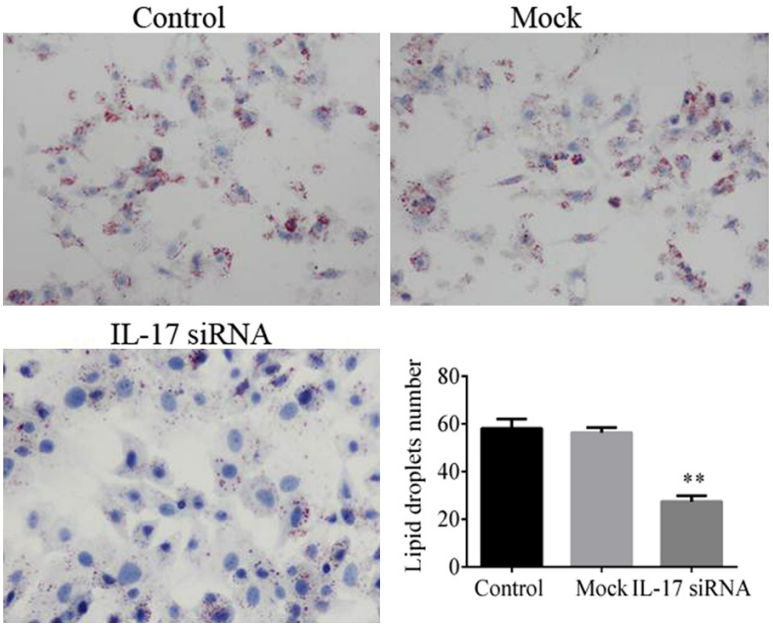

Figure 5. Adipogenesis induction of hPDLSCs. Transfected hPDLSCs were induced in the hMSC adipogenic differentiation medium for 28 days, and adipogenic differentiation was determined using Oil Red O staining. Averaged numbers of dyed red lipid droplets from 5 separated view field was calculated (100 ×). **Indicates significant level at $P < 0.01$, vs. Control and Mock, respectively.

Red O staining assay on hPDLSCs which were pretreated with adipogenic differentiation medium for 28 days, transfected with IL-17 siRNA plasmid or not. As shown in **Figure 5**, hPDLSCs transfected with IL-17 siRNA showed less dyed red lipid droplets out of cells, as compared with the Control and Mock ($P < 0.01$, **Figure 5**). More lipid droplets, or lipid-drop accumulation, were found in control cells and mock transfected cells, with no difference between them ($P > 0.05$). These showed IL-17 siRNA attenuated the adipogenesis of hPDLSCs.

IL-17 siRNA promotes osteogenesis of hPDLSCs

The osteogenesis ability of hPDLSCs was Alizarin red S staining. The mineralization nodules formed by hPDLSCs were significantly enhanced by IL-17 siRNA plasmids transfection in comparison with those of control and mock transfected hPDLSCs ($P < 0.01$, **Figure 6**). This fact revealed that IL-17 siRNA promoted hPDLSCs mineralization.

IL-17 siRNA activates ERK, JNK, and p38 MAPK signaling

ERK, JNK, and p18 MAPK signaling had been reported to associate with osteogenic differentiation of hPDLSCs [5, 6]. In order to investigate the influence of IL-17 siRNA on activation of ERK, JNK, and p38 MAPK signaling, we detected the expression of the phosphorylated (p)-ERK, p-JNK, p-p38 MAPK, and total proteins of them in hPDLSCs. We confirmed that the expression of p-ERK, p-JNK, and p-p38 MAPK were significantly upregulated by IL-17 siRNA transfection, while no differences were observed in expression of total proteins, or between control and mock groups (**Figure 7**). These might suggested that IL-17 promoted cell proliferation and osteogenesis of hPDLSCs via activation of ERK, JNK, and p38 MAPK signaling.

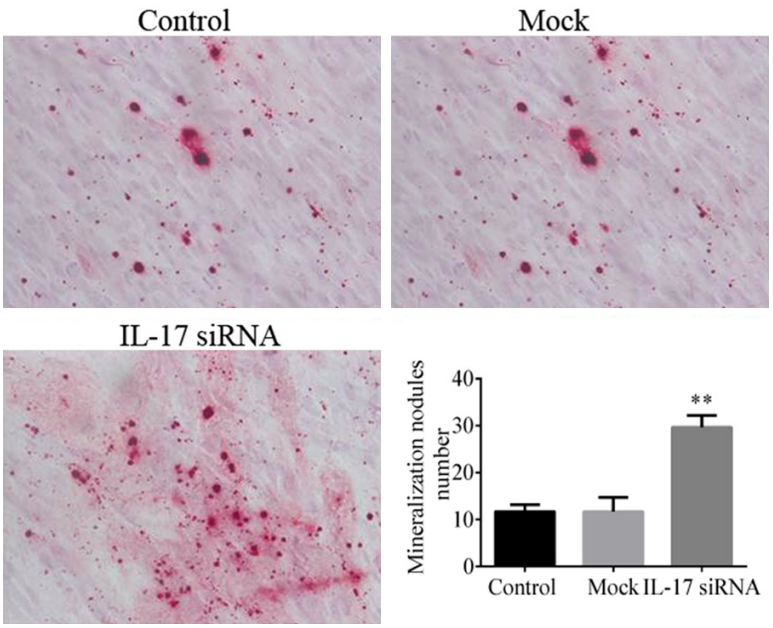

Figure 6. Osteogenesis induction of hPDLSCs. Transfected hPDLSCs were induced in the chondro-inductive medium for 28 days, and chondrogenic differentiation was determined using alizarin red S staining. Averaged numbers of mineralization nodules from 5 separated view field was calculated (100 ×). **Indicates significant level at $P < 0.01$, vs. Control and Mock, respectively.

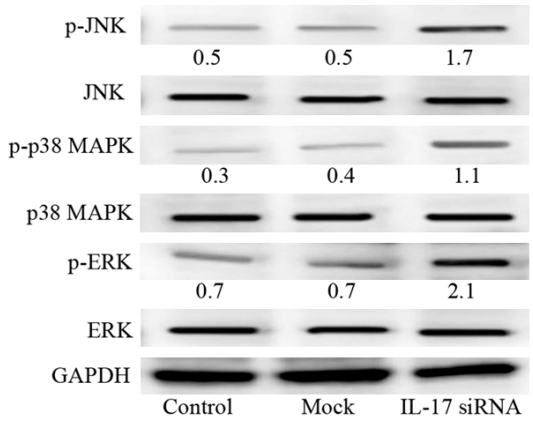

Figure 7. Immunoblotting analysis for ERK, p38 MAPK and JNK signaling. Cellular protein from transfected hPDLSCs were detected using western blot analysis to analyze the activation of the key signaling factors related to chondrogenic differentiation, proliferation of hPDLSCs. Numbers below the immunoblots indicate the fold change normalized to GAPDH and controls.

Discussion

The dual role of IL-17 in cell proliferation and osteogenic differentiation had been studied in previous studies [6, 17-20]. In this present study we confirmed that inhibition of IL-17 with siRNA technology significantly promoted cell proliferation, colony formation ability, and osteogenic differentiation, with reduced adipogenesis. All these changes were companied with activated JNK, p38, and ERK signaling.

As reported that IL-17 administration inhibited the proliferation and osteogenic differentiation of PDLSCs and MSCs [6, 15, 20]. As interestingly as reported, however, IL-17 not only accelerated the hMSCs proliferation, but also induced hMSCs osteoblastic differentiation [20], which was in contrary to the results in our study. In this present study we determined that IL-17 silencing in hPDLSCs significantly enhanced cell proliferation and colony formation ability. Moreover, the osteogenic differentiation of hPDLSCs transfected with IL-17 siRNA plasmid was significantly enhanced. However, no effect was observed on cell apoptosis of hPDLSCs. These demonstrated that IL-17 siRNA promoted proliferation, colony formation, and osteogenic differentiation of PDLSCs, while had no impact on cell apoptosis.

Effect of IL-17 on adipogenesis had been reported. Evidence had shown that IL-17 inhibited adipogenesis and glucose metabolism in mouse-derived 3T3-L1 preadipocytes, and thus impedes obesity in mice [25]. Additionally, IL-17 signaling negatively regulates adipogenesis and leptin production, which is mainly secreted by adipocyte [26, 27]. In this present study, we suggested that the adipogenic differentiation of hPDLSCs was significantly inhibited by IL-17 siRNA. We revealed that expression of IL-17 in hPDLSCs might promote hPDLSCs adipogenesis.

As shown that MEK-ERK pathway activation was important for IL-17-dependent hMSC proliferation [20]. Moreover, the ERK1/2, JNK MAPKs, and p38 MAPK involve in IL-17-mediated

osteogenic differentiation of PDLSCs [5, 6, 12, 13]. As reported, the inhibition of PDLSCs proliferation, migration, and osteogenic differentiation by IL-17 was mediated by ERK1/2 and MAPKs activation [6]. On the contrary, we demonstrated that the expression of p-ERK1/2, p-JNK MAPK, and p-p38 MAPK, as well as cell proliferation and osteogenic differentiation were significantly elevated by IL-17 siRNA. These results were accordant with previous results that the activation of ERK1/2, Akt, JNK MAPK, and p38 MAPK signaling in enhanced osteogenesis of hMSCs, bone marrow stromal cells (BMSCs), or hPDLSCs [28-32]. Thus, we confirmed that IL-17 siRNA promoted cell proliferation, colony formation and osteogenic differentiation of hPDLSCs via activated ERK, JNK, and p38 MAPK pathways.

Conclusion

In summary, we concluded that IL-17 siRNA contributed to enhanced cell proliferation and osteogenic differentiation, and reduced adipogenic differentiation of hPDLSCs. In hPDLSCs transfected with IL-17 siRNA, activation of ERK, JNK, and p38 MAPK signaling were detected. These showed that IL-17 siRNA promoted osteogenesis or hPDLSCs through ERK, JNK, and p38 MAPK signaling. However, more experiments should be performed to dig the mechanism in IL-17 mediated osteogenic differentiation.

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Disclosure of conflict of interest

None.

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