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Original Article

Insulin-like growth factor-1 promotes proliferation, migration and osteoblasts differentiation of periodontal ligament stem cells via activating PI3K/AKT signal pathway

Ka Xie, Chun Peng

Department of Stomatology, Yongchuan Hospital of Chongqing Medical University, Chongqing, China

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Abstract: Periodontitis is one kind of the common oral diseases. The periodontal ligament stem cell (PDLSC) is an important cytological basis for the periodontitis treatment. This study was aimed to identify the regulation effect of insulin like growth factor-1 (IGF-1) on PDLSC. PDLSCs were isolated from disease-free impacted rabbit teeth and selected for passage culture. Stable cultured cells were treated with different concentrations of IGF-1 and/or transfected with siRNA for IGF-1 receptor (IGF-1R). Cell proliferation and migration were detected to measure IGF-1 effect on PDLSCs. Activity of alkaline phosphatase (ALP), mineralization and expressions of osteoblastic markers were quantified by using ALP assay kit, Alizarin red staining, qPCR and western blot, respectively. In addition, the related proteins phosphorylation level changes were measured by Western blot to detect activity of PI3K/AKT signal pathway in treated PDLSCs. Results showed that the effect of IGF-1 on PDLSC cell proliferation was concentration-dependent. IGF-1 increased cell proliferation while lose this effect after IGF-1R siRNA transfection. IGF-1 increased cell migration with correlation of IGF-1R, too. Meanwhile, IGF-1 significantly increased ALP activity, mineralization and osteoblasts differentiation marker genes expressions in PDLSCs. The protein phosphorylation levels of PI3K/AKT signal pathway were also significantly increased by IGF-1. To sum up, in PDLSC, IGF-1 increased cell proliferation via binding with its receptor, and promoted migration as well as osteoblasts differentiation, which might be via activating PI3K/AKT signal pathway. The effect of IGF-1 on PDLSCs might provide theoretical basis for clinical application of IGF-1 in periodontal disease.

Keywords: Periodontal ligament stem cell, Insulin like growth factor-1, periodontal inflammation, proliferation, migration, PI3K/AKT signal pathway

Introduction

Periodontal inflammation is a chronic, destructive oral disease which often occurs in the dental support tissues such as gums, periodontal ligament, and alveolar bone. Periodontitis has become the most common disease affecting oral health and it was also the vital reason that caused tooth loose in adults [1]. Normally, periodontal tissue has strong repair ability to achieve tissue regeneration via the undifferentiated mesenchymal cell proliferation and differentiation in periodontal ligament (PDL) [2]. Further studies have shown that these undifferentiated mesenchymal cells could multi-directional differentiate into adult stem cells, and form cementum-periodontal membrane-like complex, which is known as periodontal ligament stem cell (PDLSC) [3]. PDLSCs, as important cytological basis of periodontal defect cell therapy and gene therapy, play important roles in the treatment of periodontal disease, tissue repair around dental implants, and restoration of teeth in orthopedic surgery [4].

Insulin like growth factor-1 (IGF-1) is encoded by human *igf-1* gene and structurally similar to insulin. It plays an important role in the individual growth and development. IGF-1 is widely expressed in human tissues and affect target cells growth and differentiation via autocrine,

paracrine or endocrine modes [5]. The biological function of IGFs is achieved via binding with its receptors or proteins on specific target cell surfaces [6]. PDLSCs as one kind of adult stem cells have potential of high proliferation and multidirectional differentiation. Under appropriate conditions, PDLSCs can differentiate into osteoblasts, osteoblasts-like cells, adipocytes and neuron-like cells [7]. The promotion of PDLSC osteoblast differentiation will be helpful for the treatment of periodontitis [8].

In this study, PDLSCs were treated with different concentrations of IGF-1 and transfected with specific siRNA for IGF-1 receptor (IGF-1R) i to explore the effect of IGF-1 on PDLSC cell proliferation and migration. The effects of IGF-1 on the osteogenic differentiation of PDLSC were determined by assessing the activity of ALP, the degree of mineralization, and expressions of osteogenic makers including collagen I (Col I), osteocalcin (OC) and osteopontin (OPN). In addition, the activity of PI3K/AKT-ERK/-mTOR signaling pathway was assessed by measuring the phosphorylation level of AKT, extracellular signal-regulated kinase (ERK) and ribosomal protein S6 kinase (S6). All of these are aimed to explore the effect of IGF-1 on PDLSC and possible mechanisms. The regulation role of IGF-1 on PDLSC might become a mew research strategy for periodontitis treatment.

Materials and methods

Isolation of PDLSCs and cell culture

New Zealand rabbits (n=3) was provided by our hospital laboratory center and used for isolating PDLSCs. Rabbits were killed after anesthesia by the air embolism method. The experimental protocols were approved by Medical Ethics Committee of the Yongchuan Hospital of Chongqing Medical University. All the surgical operations were conducted by the same person. The disease-free impacted teeth were extracted within 2 h of death, then surfaces of the teeth were cleaned with 75% alcohol and PDL cells were gently scraped from the middle third of the root surface by using forceps, and been digested in a solution of 3 mg/ml collagenase type I (Sigma Chemical, St. Louis, MO, USA) and 4 mg/ml of dispase (Sigma-Aldrich, St. Louis, United States) for 2 h at 37°C. Cells (1×10^{4}/ml) were dispersed into 6-well plate and incubated in the lower sugar Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich) for 3 days, at 37°C with 5% CO_2. The culture medium was changed three times per week. Cells were passaged at a confluency of 70% to 80% by using trypsin-EDTA (0.25% v/v, Sigma-Aldrich) at a ratio of 1:3. To obtain homogeneous populations of PDLSCs, single-cell-derived colony cultures were obtained by using a limiting dilution cloning. PDLSCs were stable cultured and passage. The media was changed every 5 days. The third generation of PDLSCs was used for the following experiments.

Treatment and cell transfection

Stable cultured PDLSCs were cultured in DMEM culture medium supplement with different concentrations of IGF-1 (0, 10, 50, 100, or 150 ng/ml) (Sino Biological Inc., Beijing China). For transfection, cells were grown in serum-free culture medium for 1 h, and then been transfected with IGF-1R specific siRNA (si-IGF-1R) or the siRNA negative control (si-NC) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) by using Lipofectamine 3000 (Invitrogen, USA) according to the manual, After 48 h of transfection, cells were collected for further investigation.

Cell proliferation assay

Cell proliferation was determined by using Cell counting Kit-8 (CCK, Dojindo, Kumamoto Prefecture, Kyushu, Japan) according to the manufacturer's instruction. Briefly, after treatment, PDLSCs were seeded into 96-well plates (2×10^{3}/well) and cultured for 24 h. Then 10 μl of CCK-8 was added into each well and cells were incubated for another 4 h at 37°C. Afterward, the absorbance at 450 nm was measured by microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Migration assay

Cell migration was assessed by using a modified two-chamber with 8.0 μm pore size. In brief, cells after been treated were suspended in 200 μl serum-free culture medium and been seeded on the upper compartment of 24-well Transwell culture chamber, then 600 μl completely culture medium was added into the lower compartment. After 12 h of incubation at

37°C, cell were fixed with 4% methanol (NIST, USA) for 30 min, non-traversed cells were removed from the upper surface of the filter carefully with a cotton swab, traversed cells on the lower side of the filter were stained with 0.1% crystal violet (Sigma-Aldrich) for 20 min and counted under an optical microscope (Leica Microsystems, Wetzlar, Germany).

Induction of osteogenic differentiation and alkaline phosphatase (ALP) activity assay

ALP activity was quantified by using ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions of the manufacturer. Briefly, cells were treated with IGF-1 and/or si-IGF-1R for 48 h. And then been cultured in differentiation medium supplemented with 10^{-8} mol/l dexamethasone (Sigma-Aldrich, St. Louis, United States), 10 mmol/l glycerol phosphate (Sigma-Aldrich), 3.7 g/l sodium bicarbonate (Sigma-Aldrich) and 0.05 g/l ascorbic acid (Sigma-Aldrich) for the induction of osteogenic differentiation for 3, 7 and 9 days [9]. At the indicated time points, cells were harvested and washed with phosphate buffered solution (PBS) and lysed with lysis buffer followed by centrifugation (2500 *g*, 15 min, at 4°C). The supernatants were collected and incubated with SensoLyte-P-Nitrophenylphosphate (NPP) supplied in the kit. The absorbance at 405 nm was measured by a microtiter plate reader (ThermoElectron Corporation, Vantaa, Finland).

Mineralization formation assay

Cells at passage 3 were seed into 6-well plate at 1×10^5/well and been cultured until 80% confluence reached. Then cells were treated with IGF-1 and/or si-IGF-1R transfection for 48 h. The matrix mineralization of treated PDLSCs after osteogenic differentiation for 3, 7, and 9 days, were detected by Alizarin Red S staining. Briefly, the PDLSCs after induction were fixed with 70% ethanol for 1 h. After washing with distilled PBS, cells were stained with 40 mmol Alizarin Red S solution (ScienCell, San Diego, California USA) for 10 min followed by been washed with distilled PBS to remove excessive stain. Alizarin Red S stained mineral deposits were extracted and dissolved in 0.1 N NaOH (Sigma-Aldrich). The absorbance at 540 nm was measured by a microtiter plate reader (Thermo Electron Corporation, Beverly, MA, USA).

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA in treated cells was isolated with TRIzol reagent (Invitrogen, Camarillo, CA, USA) and quantified by spectrophotometry, samples with the ratio of A260/A280 ≥ 1.9 were selected. RNA was reverse transcribed by using a PrimeScript™ RT reagent kit (TaKaRa, Dalian, China) according to the manufacture's protocol. The reverse transcription condition was 10 min at 25°C, 30 min at 48°C, and a final step of 5 min at 95°C. The qRT-PCR was performed on the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). The PCR condition were 95°C for 1 min followed by 35 cycles of amplification consisting of 95°C for 15 s and 72°C for 1 min. All reactions were run in triplicate, each value was normalized to β-actin. The relative expression level of the genes was calculated by using $2^{-\Delta\Delta CT}$ method compare with cells of control group [10]. All primers were synthesized by GenePharma (Shanghai, China). The data were analyzed with Real-Time StatMiner (Integromics).

Western blotting

Specimens were collected at indicated time points and lysed with 1 ml protein extraction reagent (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). All the samples were quantified by using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Western blot system was established by the Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. Equal amounts of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Blots were blocked for 1 h in 5% nonfat milk and then incubated overnight with the primary antibodies to IGF-1R, Col I, OC, OPN, p/t-AKT p/t-ERK, p/t-S6 and GAPDH (at dilution of 1:1,000; Abcam, Cambridge, MA, USA) at 4°C. Then the membranes were incubated with horseradish peroxidase-conjugated (HRP) secondary antibodies (1:5000 diluted, Santa Cruz biotech) at 37°C for 1 h. Blots were visualized by using an enhanced chemiluminescence (ECL) system (Millipore) and the band intensities were determined by the Image Lab™ Software (Bio-Rad, Shanghai, China). The kinase activity was analyzed by band intensity ratio.

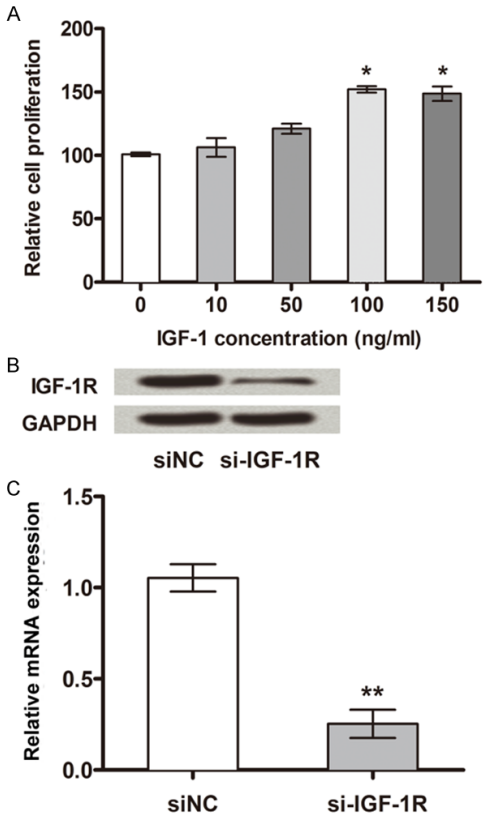

Figure 1. Insulin like growth factor-1 (IGF-1) promoted periodontal ligament stem cell (PDLSC) proliferation. A. Proliferation of PDLSC were measured by Cell Counting Kit-8 (CCK-8) assay after been treated with IGF-1 at different concentrations of 0, 10, 50, 100 and 150 ng/ml. 0 ng/ml was used as control. B. Protein expression level of IGF-1 receptor (IGF-1R) in PDLSCs demonstrating transfection efficiency of IGF-1 specific siRNA (si-IGF-1R) and siRNA negative control (siNC). C. The mRNA expression level of IGF-1R in PDLSC demonstrating transfection efficiency of si-IGF-1R and siNC. *, $P < 0.05$ compared to control without IGF-1 treatment, **, $P < 0.01$ compare to siRNA negative control transfection.

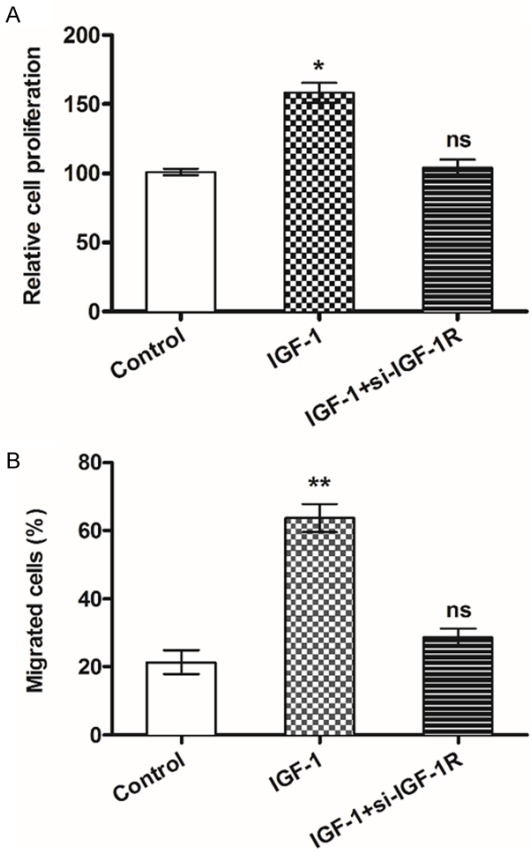

Figure 2. Insulin like growth factor-1 (IGF-1) promoted periodontal ligament stem cell (PDLSC) proliferation and migration via IGF-1 receptor (IGF-1R). PDLSCs were cultured in the medium supplemented with 100 ng/ml IGF-1, and/or been transfected with IGF-1 specific siRNA (si-IGF-1R). Cells without treatment were used as control. A. Proliferation of PDLSCs were measured by Cell Counting Kit-8 (CCK-8) assay. B. Migration of PDLSC were measured by Transwell assay. *, $P < 0.05$ compare with control; **, $P < 0.01$, compare with control; ns, non-significant.

Statistical analysis

All experiments were repeated at least three times. The results were presented as the mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) was used for multiple comparisons. *P*-values of the difference between two groups were estimated by two-tailed unpaired *t*-test. Statistical calculations were performed by Graphpad Prism 6.1 software (GraphPad, San Diego, USA). Statistical significance was defined as $P < 0.05$.

Results

IGF-1 promoted PDLSCs proliferation

PSLSCs were treated with different concentrations of IGF-1 at 0, 10, 50, 100 and 150 ng/ml. The CCK-8 assay results shown in **Figure 1A** suggested that with the increased concentration of IGF-1, cell proliferations were significantly increased compare to 0 ng/ml of IGF-1 treatment as control ($P < 0.05$), and be maximum in the 100 ng/ml concentration test group. It suggested that the effect of IGF-1 on PDLSCs proliferation was concentration-dependent and

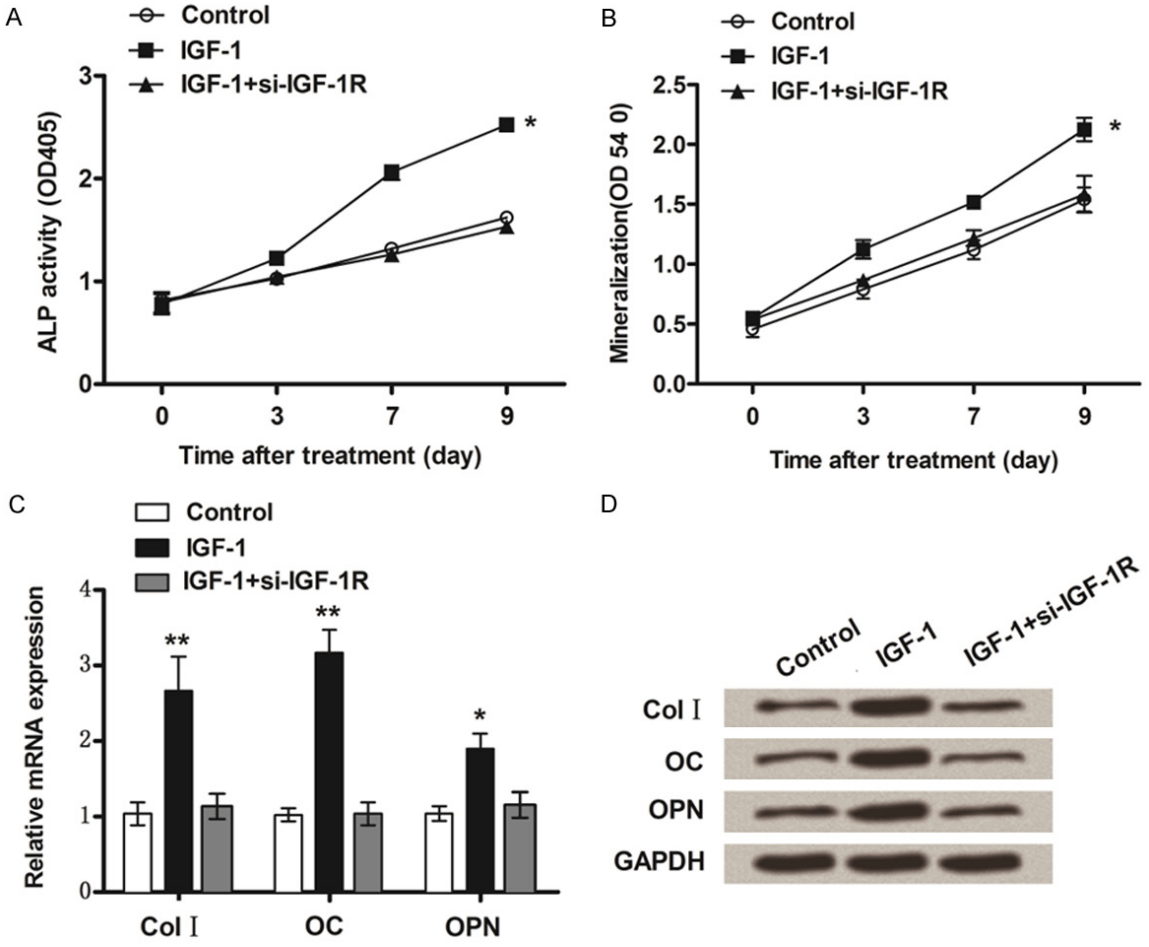

Figure 3. Insulin like growth factor-1 (IGF-1) promoted osteogenic differentiation of periodontal ligament stem cell (PDLSC). PDLSCs cultured in the medium supplement with 100 ng/ml IGF-1, and/or been transfected with IGF-1 specific siRNA (si-IGF-1R). Then cells were induced by been cultured in the osteogenic differentiation medium. The induced cells without treatment were used as control. A. Relative activity of alkaline phosphatase (ALP) in PDLSC was analyzed by using SensoLyte-P-NPP kit. B. Mineralization of PDLSCs after been treated were measured by Alizarin Red S staining. C. Relative mRNA expression level of osteogenic differentiation markers was measured by qRT-PCR. D. Immunoblots of osteogenic differentiation markers protein expression levels were measured by Western blotting assay, GAPDH acted as internal control. *, $P < 0.05$ compare with control; **, $P < 0.01$, compare with control.

100 ng/ml IGF-1 was chosen for the treatment condition of PDLSCs in this study.

Effect of IGF-1 on PDLSC proliferation and migration via IGF-1R

In order to verify whether IGF-1 affect PDLSCs via its receptor, firstly, we detected the efficiecy of si-IGF-1R to suppress IGF-1R expression in PDLSC. The result in **Figure 1B** and **1C** suggested that transfection of si-IGF-1R significantly decreased the mRNA and protein expression levels of IGF-R compared with siRNA negative control ($P < 0.01$).

Then PDLSCs were treated with IGF-1 and/or IGF-1R siRNA transfection to detected effect of IGF-1 on PDLSC proliferation and migration. CCK-8 analysis results (**Figure 2A**) showed that cell proliferation was increased with the presence of IGF-1 compared with control ($P < 0.05$), while after transfected with IGF-1R siRNA with IGF-1 in presence, there was non-significantly increase of cell proliferation. The migration assay results in **Figure 2B** showed the same increasing trend in the group with presence of IGF-1 ($P < 0.01$), and non-significantly increasing of PDLSCs after IGF-1-R siRNA transfection even that the IGF-1 was exist. It suggested that elevation effect of IGF-1 on PDLSCs proliferation and migration might be via its receptor on the cell surface.

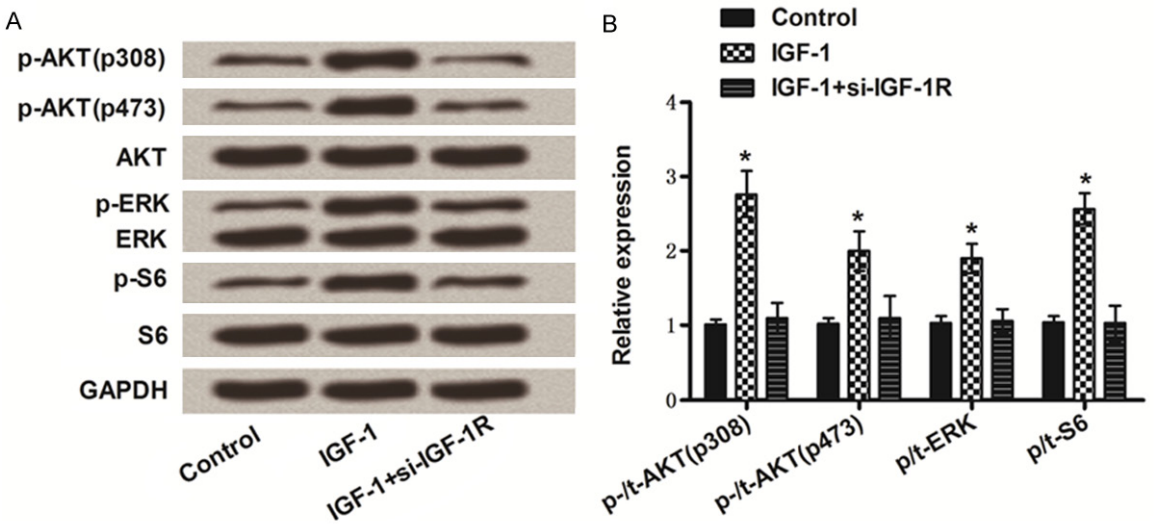

Figure 4. Insulin like growth factor-1 (IGF-1) increased phosphorylation levels of AKT, extracellular signal-regulated kinase (ERK), ribosomal protein S6 kinase (S6) in periodontal ligament stem cell (PDLSC). PDLSCs were cultured in the medium supplement with 100 ng/ml IGF-1, and/or been transfected with IGF-1 specific siRNA (si-IGF-1R). Cells without treatment were used as control. The phosphorylation levels of AKT, ERK and S6 were measured. A. Immunoblots of AKT, ERK and S6 measured by western blot. GAPDH acted as internal control. B. Quantification of phosphorylation levels of AKT, ERK and S6. *, $P < 0.05$ compared with control without treatment.

IGF-1 promoted PDLSCs osteogenic differentiation

We measured osteogenic differentiation of PDLSCs by assessing three aspects including ALP activity, mineralization, and expression of osteogenic markers (Col I, OC and OPN) [11]. The results of ALP activity assay as shown in **Figure 3A** suggested that after osteogenic differentiation induction, the ALP activity in PDLSC were increased until the 9th day and significantly elevated in the group which been treated with IGF-1 alone ($P < 0.05$). While the activity of ALP in PDLSC with si-IGF-1R transfection combine with IGF-1 treatment showed non-significantly difference compare with control without treatment. Meanwhile the results of mineralization assay as showed in **Figure 3B** suggested that IGF-1 increased the mineralization of PDLSC ($P < 0.05$) while knock down of IGF-1R, the effect of IGF-1 was eliminated.

After osteogenic differentiation induction PLDSC cells were treated with IGF-1 and si-IGF-1R. The expression level of Col I, OC and OPN in PLDSCs were measured in both mRNA and protein expression levels compare with control without treatment. In **Figure 3C** and **3D**, both mRNA and protein expression levels of these three factors were significantly increased after IGF-1 treatment, while non-significantly in si-IGF-1R transfected cells ($P < 0.05$ or $P < 0.01$). All these results suggested that IGF-1 promoted osteogenic differentiation of PDLSCs.

IGF-1 regulated PDLSCs via activation of PISK/AKT signal pathway

The phosphorylation levels of AKT, ERK and S6 which related with activation of PI3K/AKT-ERK and -mTOR pathways in PDLSC were determined by Western blot. The results (**Figure 4**) showed that after treated with IGF-1, the expressions of p-AKT, p-ERK and p-S6 were signally increased. While transfected with si-IGF-1R even combine with IGF-1 treatment, the increased trend of p-AKT, p-ERK and p-S6 was reversed as compared to control cells without treatment. It suggested that IGF-1 might affect PDLSCs via activating PI3K/AKT/mTOR signal pathway.

Discussion

Periodontal disease is a common cause of periodontal tissue defects leading to the loss of the teeth [12]. The usage of tissue engineering to promote periodontal regeneration, restoration of teeth physiological function has become a hot research area [13]. There are multi-direc-

tional differentiation stem cells in the periodontal ligament. Periodontal tissues can be repaired via directional migration and differentiation of PDLSCs [4]. PDLSC, directly come from periodontal tissues, has high proliferation and differentiation ability and thus play an important role in the regeneration of periodontal regeneration [14]. The PDLSC has become the seed cell for tissue engineering study, while the effect of PDLSC *in vitro* remains to be further studied. In this study, we investigated the effect of IGF-1 on PDLSCs proliferation and migration. PDLSCs were cultured with different contents of IGF-1, and with the increase concentration of IGF-1, cell proliferation was upregulated, suggesting that the effect of IGF-1 on PDLSCs was in a concentration-dependent manner.

IGF-1 can promote cell differentiation and proliferation and involve in the regulation of multiple organ functions *in vivo* [15]. Most experiments showed that IGF-1 was involved in mediating the proliferation and activity of many adult stem cells including umbilical cord mesenchymal stem cells, embryonic stem cells, neural stem cells, bone-derived stem cells and *etc*. [16-18]. IGF-1 binds to its receptor and affects cell proliferation through receptor-mediated cellular signaling pathways. The activation of IGF-1R by its ligands (IGF-1, IGF-II and insulin at physiological concentrations) plays a major role in the control of proliferation of several types of mammalian cells [19]. In the present study, we knock down the IGF-1R in PDLSC by transfection with IGF-1R specific siRNA, and found that in PDLSCs, the promotion effect of IGF-1 on cell proliferation and migration were eliminated after IGF-1R siRNA transfection, suggesting that effect of IGF-1 on PDLSCs was related with its receptor IGF-1R.

PDLSC is different from other adult stem cells. It can differentiate into both mature periodontal ligament fibroblasts and odontoblasts to achieve a more complete regeneration of periodontal ligament tissue [20]. Studies have shown that IGF-1 was necessary peptide to maintain bone amount, stimulate osteoblast DNA and protein synthesis *in vitro*, as well as promote bone and cartilage growth *in vivo* [21, 22]. In this study, we assessed the effect of IGF-1 on osteogenic differentiation of PDLSCs *in vitro*. By examining ALP activity, the accumulation of mineralization and the expressing of osteogenic related factors in osteogenic differentiation induced PDLSCs after treated with IGF-1 and/or combined with si-IGF-1R were assessed. Results suggested that IGF-1 significantly promoted the increasing trend of three indexes of osteogenic differentiation induced PDLSCs, while si-IGF-1R transfection combined with IGF-1 treatment showed no significant effect on these three indexes. These results suggested that IGF-1 promoted osteogenic differentiation of PDLSCs which might be via IGF-1R as IGF-1 receptor.

IGF-1 plays an important role in reparation of periodontal injury and promotes chemotaxis, proliferation and protein synthesis in the cells *in vitro* [23]. However little was known about the molecular mechanisms underlying effects of IGF-1 on PDLSCs. PI3K/AKT signaling pathway is an evolutionarily conserved signal pathway which plays important roles in cell proliferation, differentiation and apoptosis as well as stem cell differentiation [24, 25]. Alberobell *et al.* have found that IGF-1 signaling selectively via phosphoinositide-dependent kinase-1 prevented the protective effect of IGF-1 on the death of human cancer cells, and IGF-1 binding to its receptor phosphorylates AKT resulting in cascade reaction of downstream target proteins [26]. Therefore, effect of IGF-1 on cell bioactivity is related with PI3K/AKT signal pathway, and AKT phosphorylation level can be used as an indicator to measure PI3K/AKT activity. Many studies showed that effect of IGF-1 on stem cells is achieved through PI3K/AKT signaling pathway. For example, IGF-1 induced neural stem cells to differentiate into neurons through PI3K/AKT [27]. IGF-1/PI3K/AKT was also involved in muscle differentiation [28]. To verify whether IGF-1 acted in activating the PI3K/AKT signal pathway in the PDLSC or not, phosphorylation level of AKT, ERK and S6 were both detected after IGF-1 and/or si-IGF-1R treatment. Among that, ERK as a member of MAPK family is involved in the signal transduction pathway and signal network which regulate cell growth, development and division including stem cells [29]. S6 is substrate for mTOR activation thus the downstream signaling pathway could be measured by S6 phosphorylation measurement. It could promote cell proliferation, as well as. regulation of this pathway also promoted cancer cell apoptosis [30]. The

results in our study showed that the phosphorylation level of these factors were all increased after IGF-1 treatment, while this efficacy was lost after si-IGF-1R transfection, suggesting that IGF-1 might affect PDLSCs via activating PI3K/AKT signaling pathway. In PDLSCs, IGF-1 could binding to its receptor, activating PI3K/AKT signal pathway and might cause a series cascade reactions to affect bioactivity of PDLSCs.

In conclusion, in the present study, IGF-1 binding with its receptor and activating PI3K/AKT signaling pathway to promote the proliferation and migration of PDLSCs. It might also promote osteogenic differentiation of PDLSCs. Although more relevant mechanisms by which IGF-1 effect on PDLSC required deep-going researches, all the findings in combination with the usage of PDLSCs in tissue materials might supply a novel therapeutic strategy in the clinical treatment of periodontitis.

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Disclosure of conflict of interest

None.

Address correspondence to: Chun Peng, Department of Stomatology, Yongchuan Hospital of Chongqing Medical University, 439 Xuanhua Road, Yongchuan District, Chongqing 402160, China. E-mail: pengchun0023@126.com

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