

## Original Article

# MicroRNA-18b mediates the inhibitory effects of nicotine on periodontal ligament-derived stem cell

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**Abstract:** Objective: To investigate the level change of miR-18b in PDLSCs with nicotine exposure and its effect on PDLSCs' proliferation, migration and osteogenic differentiation. Methods: PDLSCs were isolated from periodontal ligament tissues which obtained from smoker patients and age-matched non-smoker. miR-18b levels in PDLSCs from different sources were detected using qRT-PCR. PDLSCs from non-smoker were treated with nicotine and the miR-18b level changes were confirmed at different time point after exposure. miR-18b was downregulated by miR-18b inhibitor transfection in PDLSCs and the influence of miR-18b knockdown on proliferation, apoptosis and migration of nicotine exposed PDLSCs were estimated using Cell Titer-Blue Assay, flow cytometry and time-lapse microscope, respectively. The changes of osteogenic differentiation markers including ALP, RUNX2, OCN and OPN were also analyzed using qRT-PCR and western blot. Furthermore, bioinformatics analysis was performed to predict the target genes of miR-18 followed with gene ontology analysis. Result: Higher miR-18b level was observed in PDLSCs from smoker and nicotine-treated PDLSCs. Restoring of miR-18b level in PDLSCs could relieve the inhibitory effect of nicotine on proliferation, migration and decrease apoptosis; however, it could not change the osteogenic differentiation markers. miR-18b may target the genes involved with protein localization and cellular localization. Conclusion: Nicotine has an inhibitory effect on proliferation, migration and osteogenic differentiation in PDLSCs; miR-18b may be a crucial regulator in these nicotine-associated functional changes.

**Keywords:** Periodontal disease, periodontal ligament-derived stem cells, nicotine, microRNA

## Introduction

Cigarette smoking is a serious health problem around the world, most of the tumors, cardiovascular diseases and respiratory system disease could be attributed to it more or less. Moreover, exposure to cigarette smoking also causes the development of destructive periodontal disease [1, 2]. Periodontal disease is a chronic infectious and inflammation disease usually characterized by the destruction of periodontal tissues including loss or injury of periodontal ligament, alveolar bone, tooth-associated cementum and so on [3]. Cigarette smoking includes 4000 chemicals and most of them are strong oxidant or have cytotoxicity which could induce cells damage and deteriorate the injury of periodontal tissues [4, 5]. Among the chemicals in cigarette smoking, Nicotine is a key component and its relationship with destructive periodontal diseases has

been proven already. One of the potential mechanisms can be considered as that nicotine has an inhibitory effect of nicotine on periodontal ligament-derived stem cells (PDLSCs) proliferation, migration and osteogenic differentiation [6]. PDLSCs are derived from periodontal ligament and participate in periodontal tissue maintenance and regeneration. Due to their potential for self-renewal and multilineage differentiation PDLSCs plays a key role in the periodontal disease healing process and have been considered as excellent cell sources for tissue engineering and cell therapy in periodontal diseases [7, 8]. In the other hand, injury of PDLSCs caused by various factors such as nicotine could prolong the recovery time and hinder the repair of periodontal tissue. Studies have shown that nicotine hinders human PDLSCs' proliferation, migration and differentiation potentials, for instance, Kim et al., found that nicotine decreased the cell viability

ty of PDLSCs and increased percentage of cells in the sub G1 phase which indicated a cell cycle arrest [9]. However, the molecular mechanisms are still unclear. Some of the studies tried to interpret the question through taking microRNAs into account. MicroRNAs (miRNAs) are a kind of 21-24 nucleotides. Non-coding RNA molecules and play a role as post-transcriptional regulators by binding to complementary sequences in the 3'-untranslated region of target mRNAs. miRNAs has a different expression at an extreme dynamic range in different tissues, cells and even in different stages [10, 11].

miRNAs are not only involved in most cells biological processes including proliferation, apoptosis and differentiation, but also implicated in many human diseases. microRNAs could be salivary markers for periodontal Diseases [12]. Na HS et al. found miRNA-128 may be involved in mitigating the inflammatory response in periodontitis [13]. In the case of PDLSCs injury induced by nicotine, Ng et al. reported a miRNA profile changes in nicotine-treated PDLSCs and observed two significantly up-regulated microRNAs including miR-1305 and miR-18b were in PDLSCs from smoker [14]. And then based on the results of Ng et al., Chen et al. confirmed the function of miR-1305 in nicotine-treated PDLSCs and showed the mechanism involved with RUNX2 [15]. But miR-18b is still not be confirmed and further researched. Therefore, the present study was designed to confirm the miR-18b level change in nicotine-treated PDLSCs from both health subject and smoker and reveal the function of miR-18b in here.

### Materials and methods

#### *Isolation and culture of PDLSC*

Periodontal ligament (PLD) tissues were collected from 5 smoker patients with and 5 age-matched non-smoker patients undergoing routine extraction for orthodontic reasons in the department of stomatology, the second hospital of Lanzhou University [16]. The PDLSCs were isolated and cultured according to the protocol mentioned in Gronthos's studies. In brief, obtained PLD tissues were cut into small pieces and digested by collagenase type I and dispase. After digestion, the tissue explants

were seeded into culture dishes with a-MEM medium supplemented with 10% fetal bovine serum (changed every 3 days). The cells were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Then single-cell suspensions of cells were cloned using the limiting-dilution method in order to further isolate. The stem cell properties, including stem cell marker expression and differentiation were detected to confirm the establishment of PDLSC cell line. Passage 3-5 cells were used in further study. This study was approved by the Ethics Committee of the Second Hospital of Lanzhou University and the informed consents were obtained from patients enrolled in.

#### *Nicotine treatment for PDLSC*

PDLSCs from non-smoker patients were seeded into 60 mm dishes (1×10<sup>5</sup> cells/dish) and cultured 24 h. After 24 h, nicotine was added into the medium, the final concentration of nicotine was 0, 0.25, 0.5 and 1.0 μM. Nicotine-treated PDLSCs were cultured for 3 days. The medium was changed every 24 h and fresh nicotine was added after medium changing in order to maintain the constant concentration.

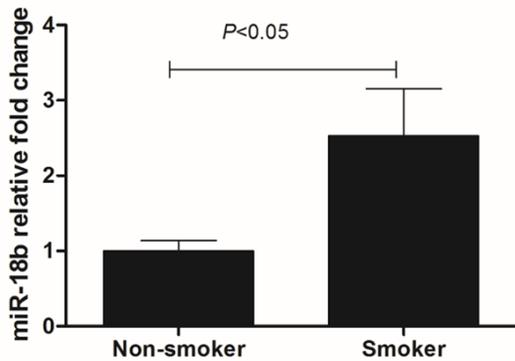
#### *Quantitative real-time PCR assay for microRNA-18b*

Total RNAs of PDLSCs from smoker patients, non-smoker and nicotine-treated PDLSCs from non-smoker were extracted using TRIzol reagent (Thermo Fisher Scientific Inc., USA) in accordance with the manufacturer's protocol. cDNA was synthesized by microRNA reverse transcription KIT: miScript II RT Kit (QIAGEN) following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using SYBR green reagent: miScript SYBR® Green PCR Kit (QIAGEN). The miR-18b level in each sample was calculated by 2-DDCT method. SNORD-48 was served as a reference gene.

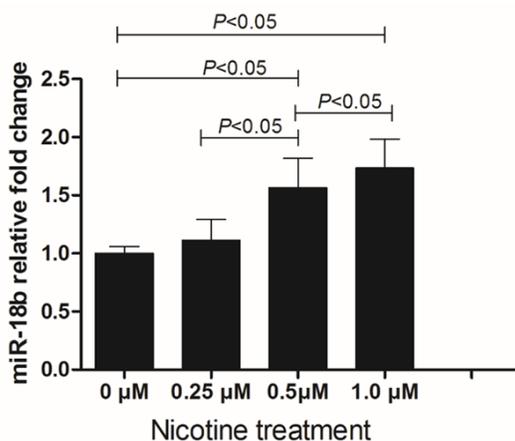
#### *Regulation of miR-18b in PDLSCs by transfection*

PDLSCs were seeded into the 6-wells plate at a 100000 cells/well and cultured until 70% confluency was reached. microRNA-18b inhibitor and all-star negative controls (QIAGEN)

## Effects of microRNA-18b on PDLSCs



**Figure 1.** miR-18b expression analyses of human PDLSC from non-smokers and smokers.



**Figure 2.** The level changes of miR-18b in PDLSCs treated with different nicotine concentrations.

were transfected into PDLSCs at a final concentration of 50 nM using lipofectamin™ RNAiMAX reagent according to the manufacturer's instructions. The effect of microRNA knockdown was confirmed by qRT-PCR after 48 h.

### Cell proliferation analysis

At 48 h after transfection with microRNA-18b inhibitor and all-star negative controls, PDLSCs were seeded into 96-well plates containing medium with 1.0 μM nicotine and cultured for 5 days. Cell Titer-Blue® Assay reagent was used to detect the proliferation of PDLSCs (using PDLSCs without any treatment as untreated control). On each time point medium was discarded and 72 μL Cell Titer-Blue reagent (1:10 diluted) was added to each well. The fluorescence value at 560 nm was measured

by BMG LABTECH microplate reader (Bio-Gene Technology Ltd., Hong Kong).

### Cell apoptosis assay

At 48 h after transfection, PDLSCs were seeded into 6-well plates and treated with 1.0 μM nicotine for 48 h. Cells were collected and then stained with Annexin V-FITC and propidium iodide (Sigma-Aldrich), washed with PBS for three times, and analyzed on a guava Flow Cytometry easy Cyte Systems. PDLSCs without any treatment was used as an untreated control.

### Cell migration assay

Transfected PDLSCs were seeded into 60 mm dishes and scratch wounds were created with pipette tips when cells got more than 90% confluence. Then serum-deprived medium was added and cells treated with 1.0 μM nicotine. After 48 h, 0.5 mg/ml bovine serum albumin was added into dishes for stimulating migration. The 24 h traveling distance of PDLSCs was measured by time-lapse microscope (using PDLSCs without any treatment as untreated control).

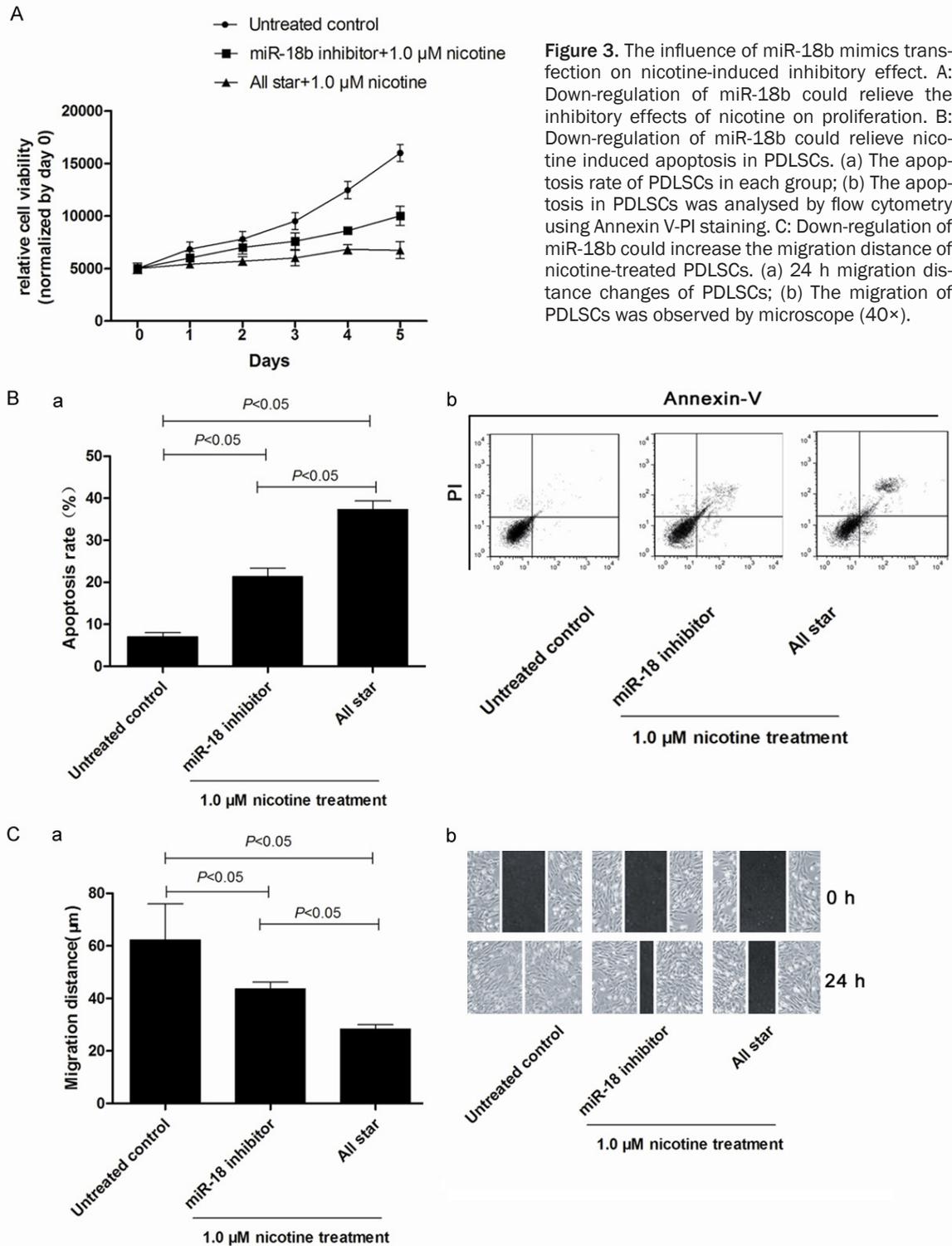
### Detection of osteogenic differentiation markers

Transfected PDLSCs were seeded into 6-well plates at a 100000 cells/well and cultured in osteogenic differentiation medium (DMEM containing 10% fetal bovine serum, 100 nM dexamethasone, 50 μg/ml of ascorbic acid and 10 mM of β-glycerophosphate). After 48 h, 1.0 μM nicotine was supplemented. The medium was changed every 24 h and fresh nicotine was added after medium changing. After 7 days, medium samples were collected. ALP activity was detected by ELISA kit. The mRNA and protein level changes of RUNX2, OCN and OPN were analyzed by qRT-PCR and western blot, respectively.

### Prediction of downstream mRNA targets of the miR-18b and gene ontology analysis

Downstream mRNA targets of the miR-18b were predicted by TargetScan online tool ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)). The predicted potential target gene list was added into DAVID Bioinformatics Resources 6.7 online

## Effects of microRNA-18b on PDLSCs



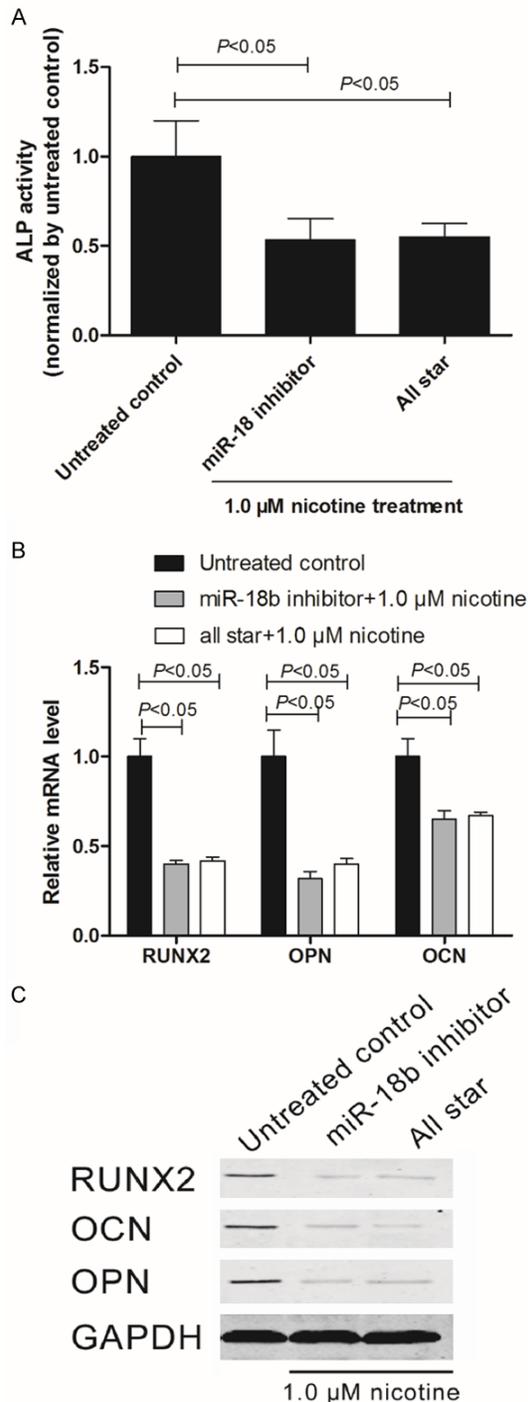
**Figure 3.** The influence of miR-18b mimics transfection on nicotine-induced inhibitory effect. **A:** Down-regulation of miR-18b could relieve the inhibitory effects of nicotine on proliferation. **B:** Down-regulation of miR-18b could relieve nicotine induced apoptosis in PDLSCs. (a) The apoptosis rate of PDLSCs in each group; (b) The apoptosis in PDLSCs was analysed by flow cytometry using Annexin V-PI staining. **C:** Down-regulation of miR-18b could increase the migration distance of nicotine-treated PDLSCs. (a) 24 h migration distance changes of PDLSCs; (b) The migration of PDLSCs was observed by microscope (40 $\times$ ).

tool7 ([HTTP://david.abcc.ncifcrf.gov/](http://david.abcc.ncifcrf.gov/)) then gene enrichment, gene ontology analysis and pathway prediction were performed. An enrichment score greater than 1.3 was considered as significant.

### Statistical analysis

The statistical calculations were performed IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL). Statistical analyses included comparisons

## Effects of microRNA-18b on PDLSCs



**Figure 4.** miR-18b expression regulation have no effect on osteogenic differentiation of nicotine-treated PDLSCs. **A:** The ALP activity was inhibited by nicotine, down-regulation of miR-18b could not relieve this effect; **B:** miR-18b inhibitor transfection has no influence with the mRNA level changes of osteogenic differentiation markers in nicotine-treated PDLSCs; **C:** miR-18b inhibitor transfection has no influence with the protein level changes of osteogenic differentiation markers in nicotine-treated PDLSCs.

with the t-test and one-way ANOVA.  $P < 0.05$  was considered statistically significant.

### Results

#### *miR-18b increased in PDLSCs from smoker and nicotine-treated PDLSCs*

As shown in **Figure 1**, miR-18b level in PDLSCs from smoker was significantly higher than PDLSCs from smoker non-smoker ( $P < 0.05$ ), the relative fold change was  $2.53 \pm 0.09$ . In PDLSCs from non-smokers, nicotine treatment promoted the miR-18b level in a dose-dependent manner, compared with untreated PDLSCs the difference were statistically significant in cells treated with 0.5 and 1.0  $\mu\text{M}$  nicotine ( $P < 0.05$ , **Figure 2**).

#### *Regulating miR-18b in PDLSCs could relieve the inhibitory effect of nicotine on proliferation, migration and apoptosis*

1.0  $\mu\text{M}$  Nicotine suppresses proliferation, the relative cell viability value was lower in nicotine-treated cells, compared with untreated control (**Figure 3A**,  $P < 0.05$ ). Restoration of miR-18b relieves the inhibitory effect of 1.0  $\mu\text{M}$  nicotine on proliferation, miR-18b inhibitor transfected PDLSCs still showed proliferation inhibition compared with untreated control ( $P < 0.05$ ), but it had a higher cell viability than all-star transfected cells at all detection point ( $P < 0.05$ ). Compared with untreated PDLSCs, nicotine exposure for 48 h could induce apoptosis, a  $(37.5 \pm 5.9)\%$  apoptosis rate was detected in all star transfected cells, but the apoptosis rate was  $(21.5 \pm 3.4)\%$  in miR-18b inhibitor transfected cells ( $P < 0.05$ , **Figure 3B**). Cell migration assay indicated that the traveled distance of nicotine-treated PDLSCs was significantly short compared with the PDLSCs without nicotine treatment (**Figure 3C**,  $P < 0.05$ ). After transfected with miR-18b inhibitor, the migration distance of nicotine-treated PDLSCs was increased compared with all-star transfected cells ( $P > 0.05$ ).

#### *Regulating miR-18b in PDLSCs could not change the osteogenic differentiation markers*

To estimate the osteogenic differentiation changes of PDLSCs after nicotine treatment, ALP activity and osteogenic differentiation marker genes (RUNX2, OCN and OPN) were detected.

## Effects of microRNA-18b on PDLSCs

**Table 1.** The gene ontology analysis of the predicted target genes for the miR-18b

Biological process	Count	Enrichment score	P value
Negative regulation of nitrogen compound metabolic process (GO:0051172)	111	1.56	2.73E-02
Protein localization (GO:0008104)	132	1.51	1.74E-02
Cellular localization (GO:0051641)	145	1.49	9.97E-03
Nervous system development (GO:0007399)	156	1.46	1.08E-02
Regulation of cellular component organization (GO:0051128)	162	1.44	1.54E-02
Negative regulation of cellular metabolic process (GO:0031324)	159	1.42	4.44E-02
Nucleobase-containing compound biosynthetic process (GO:0034654)	203	1.38	9.38E-03
RNA biosynthetic process (GO:0032774)	180	1.38	4.87E-02
Heterocycle metabolic process (GO:0046483)	313	1.37	6.60E-06
Organic cyclic compound biosynthetic process (GO:1901362)	215	1.37	5.36E-03

**Table 2.** The top 10 pathways that may be involved with target genes of miR-18b

Pathway name	P value
Thyroid hormone signaling pathway	0.01
cGMP-PKG signaling pathway	0.02
Vitamin digestion and absorption	0.02
Wnt signaling pathway	0.02
SNARE interactions in vesicular transport	0.02
Oxytocin signaling pathway	0.03
Purine metabolism pathway	0.03
MAPK signaling pathway	0.03
Axon guidance	0.03
mTOR signaling pathway	0.04

As shown in **Figure 4A**, nicotine treatment significantly inhibited the ALP activity in PDLSCs, compared with untreated cells ( $P < 0.05$ ). Meanwhile, the mRNA and protein level of RUNX2, OCN and OPN decreasing were confirmed by q-RT-PCR (**Figure 4B**) and western blot (**Figure 4C**) in nicotine-treated PDLSCs. miR-18b inhibitor transfected PDLSCs also showed lower ALP activation and decline of RUNX2, OCN and OPN after nicotine treatment. Most importantly, no significant difference was observed between miR-18b inhibitor transfected PDLSCs and all star transfected cells. It indicated that regulating miR-18b cannot relieve the inhibitory effect of nicotine on osteogenic differentiation.

### *Target prediction of miR-18b and gene ontology analysis of the predicted target genes*

To identify miR-18b-targeted mRNAs, target prediction for all miR-18b was performed using the validated target search engine (TargetScan

online tool). A total of 981 genes were predicted to be targeted by miR-18b. To further understand the biological functions of miR-18b and its potential target genes, a simple gene enrichment and gene ontology analysis was performed by DAVID Bioinformatics Resources. According to the results of gene ontology analysis, miR-18b may target the genes involved with protein localization and cellular localization. The top ten involved biological process were shown in **Table 1**. Moreover, we assessed the predicted target genes of miR-18b with the KEGG database, the top 10 pathways that may be involved with target genes of miR-18b were shown in **Table 2**.

### **Discussion**

Cigarette smoking is firstly exposed to the oral tissues; therefore the consequences to oral tissues induced by cigarette smoking should not be ignored. It has long been proven by epidemiological studies that cigarette smoking is one of the main lifestyle-related risk factors associated with [17, 18]. According to a questionnaire survey conducted by our department, 73.6% of patients with periodontal disease have a smoking history. However, the pathophysiological mechanism of cigarette smoking in periodontal disease has yet to be clarified clearly. Nicotine is a key component of cigarette smoke which constitutes approximately 3% of the dry weight of tobacco and contributes to most of the adverse effects of cigarette smoking. When cigarette smoke is inhaled, the epithelial surface of the oral tissue are exposed to nicotine with a high localized doses and it also exerts systemic effects such as impairing inflammatory and immune responses to periodontal pathogens [19, 20]. Therefore, in the

present study, we focused on the nicotine among thousands of chemical in cigarette smoking. Several *in vitro* studies have already demonstrated that nicotine can inhibit the migration, attachment, proliferation and differentiation potentials of PDLSCs. But the mechanism is still worth being studying due to the import role of in periodontal tissue maintenance, regeneration and the healing process of periodontal disease healing process. Protecting the PDLSCs from nicotine-induced injury may be a prevention or therapy strategy for in periodontal disease in smokers [21].

In the present study, we found that treatment of nicotine had an inhibitory effect on PDLSCs. In brief, the proliferation rate of PDLSCs was decreased by nicotine in a dose-dependent manner. Higher apoptosis rate was detected after nicotine treatment. Meanwhile, the traveling distance of PDLSCs was shorten significantly, compared with nicotine untreated PDLSC. In the other hand, the osteogenic differentiation markers also changed in nicotine-treated PDLSCs which suggested that nicotine suppresses the osteogenic differentiation potential of PDLSCs. These three processes are crucial for the regeneration of PDLSCs. In particular, regular cell proliferation provides an adequate amount of PDLSCs stem exerting a regenerative effect and in contrast, reduced proliferation led to a reduction in available adult stem cells. Cell migration is also necessary; it allows PDLSCs move towards the injury area. In order to exert the repair function, correct osteogenic differentiation of PDLSCs is a key [22, 23].

We also tried to reveal the potential molecular mechanism of nicotine-induced injury on PDLSCs. We analyzed the level difference of miR-18b in PDLSCs from smoker and non-smoker and also compared the expression changes between nicotine-treated and untreated PDLSCs. We selected miR-18b as an interesting target gene based on Ng's report [14]. In the previous study, Ng et al. reported the first global miRNA expression profile of nicotine-treated PDLSCs and identified 16 differentially expressed miRNAs. Among them, hsa-miR-1305 and hsa-miR-18b were up-regulated dramatically. miR-1305 was already confirmed in the other study, it suggested that restoration of miR-1305 relieves the inhibitory effect of nicotine on periodontal ligament-derived stem cell proliferation, migration, and osteogenic

differentiation [15]. Moreover, this studied elucidated the potential mechanism; they proved that miR-1305 may relieve the inhibitory effect of nicotine on PDLSCs depending on its target RUNX2. We found that miR-18b up-regulated in PDLSCs smoker and also increased in nicotine-treated PDLSCs that form non-smoker. After transfected into the nicotine-treated PDLSCs, we observed that inhibited proliferation and migration was recovered, moreover, the apoptosis induced by nicotine also decreased after miR-18b inhibitor transfection. However, there is no influence on differentiation markers in nicotine-treated PDLSCs after transfection. But in Chen's study, they found miR-1305 might target on Runx2 which is associated with bone development.

The limitation of this study is that we only predicted the potential targets of miR-18 using bioinformatics tools but did not confirm any potential target gene of miR-18b and its functions or mechanism in nicotine-treated PDLSCs by experiments such as qRT-PCR. However, we did gene ontology analysis for these potential targets. The gene ontology analysis result revealed that miR-18b may target the genes involved in with protein localization and cellular localization. Moreover, miR-18b may cross with MAPK signaling pathway or Wnt signaling pathway which can regulate proliferation and apoptosis.

In conclusion, this study proved that the proliferation, migration, and osteogenic differentiation human could be inhibited by exposure to nicotine and miR-18b may be a crucial regulator in these nicotine-associated functional changes.

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### Disclosure of conflict of interest

None.

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