



Original Article

# Downregulation of miR-184 facilitates osseous differentiation in periodontal ligament stem cells by modulating nuclear factor I-C



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## KEYWORDS

miR-184;  
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**Abstract** *Background/purpose:* PDLSCs (periodontal ligament stem cells), derived from dental tissues, are candidate cells for regeneration of dental tissues. MiRNAs could regulate osteogenic differentiation and the transformation into osteoblasts. This study was conducted to figure out how miR-184 regulates osteoblastic differentiation in PDLSCs.

*Materials and methods:* PDLSCs were isolated from premolars, and the osteoblastic differentiation was validated via Alizarin red staining and determination of ALP (alkaline phosphatase) activity. Expression of osteogenic specific genes were evaluated by western blot, and the expression pattern of miR-184 was determined by qRT-PCR. Target gene of miR-184 was then verified by dual luciferase reporter assay.

*Results:* Osteogenic-induced PDLSCs were successfully established with increased mineral deposition, ALP activity and protein expression of RUNX2 (runt-related transcription factor 2), osterix and BSP (bone sialoprotein). MiR-184 was reduced during osteoblastic differentiation of PDLSCs, and over-expression of miR-184 suppressed osteoblastic differentiation, as evidenced by reduction in mineral deposition, ALP activity and protein expression of RUNX2, osterix and BSP. MiR-184 could target NFI-C (nuclear factor I-C), and inhibit NFI-C expression in PDLSCs. NFI-C was enhanced during osteoblastic differentiation of PDLSCs, suggesting negative correlation with miR-184. Forced NFI-C expression promoted osteoblastic differentiation, and counteracted with the suppressive effects of miR-184 on osteoblastic differentiation.

*Conclusion:* Downregulation of miR-184 facilitates osteoblastic differentiation in PDLSCs by modulating NFI-C, providing novel therapeutic strategy for regeneration of dental tissues.

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## Introduction

Tooth loss and periodontal tissue defect are related to each other and are considered as the main causes of dental tissue defects, which have adverse effects on the daily life of most people.<sup>1</sup> How to eliminate the negative effects caused by the above factors and achieve tissue function recovery and biological repair is an important problem faced by the researchers of stomatology. In recent years, tissue engineering technology and the in-depth study of various dental stem cells have brought new hope for the biological regeneration of teeth and periodontal tissues.<sup>2</sup>

Since 2004, Seo et al. for the first time successfully isolated and cultured periodontal ligament stem cells (PDLSCs) from human periodontal ligament,<sup>3</sup> and highlighted the strong proliferation ability and multi-directional differentiation potential of PDLSCs to form new periodontal support tissues that play an important role in the process of biological repair and regeneration of periodontal tissue.<sup>4</sup> Under certain culture conditions, PDLSCs could differentiate into osteoblasts, adipocytes and chondroblasts, providing important clinical value for bone tissue maintenance, bone regeneration, repair and orthodontic periodontal reconstruction in the oral cavity.<sup>5,6</sup> The regulation of osteogenic differentiation of PDLSCs should be critical for osteogenic tissue engineering and tooth regeneration.

MicroRNAs (miRNAs), small non-coding RNAs, regulate gene expression by inhibiting post-transcriptional mRNA translation and play an important role in various cellular processes, including cell cycle progression and osteogenesis.<sup>7</sup> Currently, there are numerous studies on the regulation of miRNAs on osteogenic differentiation of stem cells. Abnormal expression of miRNAs have been reported during osteoblasts differentiation,<sup>8,9</sup> and closely associated with bone formation.<sup>10</sup> MiR-124 could inhibit osteogenic differentiation of bone marrow mesenchymal stem cells.<sup>11</sup> MiR-335 inhibited osteogenic differentiation of mesenchymal stem cells.<sup>12</sup> MiR-26 promoted osteogenic differentiation of adipose stem cells.<sup>13</sup> As a tumor suppressor, miR-184 inhibited glioma progression.<sup>14</sup> However, miR-184 was increased in oral squamous cell carcinoma tissues, and functioned as an oncogenic miRNA.<sup>15</sup> Inhibition of miR-184 reduced cell proliferation and induced apoptosis in tongue squamous cell carcinoma cell lines.<sup>16</sup> Recently, miR-184 has been shown to promote epidermal differentiation<sup>17</sup> and retinal pigment epithelium differentiation.<sup>18</sup> Moreover, miR-184 also controlled neural stem cell differentiation.<sup>19</sup> However, the role of miR-184 in osteogenic differentiation is still unknown.

In this study, we first evaluated expressional change of miR-184 during osteogenic differentiation of PDLSCs. The functional role of miR-184 on osteogenic differentiation, as well as the target gene of miR-184, were then investigated. The main result in this study would provide a novel insight

into the mechanism involved in osteogenic differentiation of PDLSCs.

## Materials and methods

### Isolation and differentiation of PDLSCs

Study was approved by the Medical Ethics Committee of Zibo Municipal Hospital with written informed consents from volunteers. Premolars were acquired from ten men (15–32 years old) underwent molar extraction at Zibo Municipal Hospital. Periodontal ligament was then extracted from the root surface and then minced and placed into culture dishes. Periodontal ligament was cultured in  $\alpha$ MEM medium (Gibco; Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco) at 37 °C with 5% CO<sub>2</sub>. Two weeks later, immunomagnetic Dynabeads (Thermo Fisher) were used to isolate Stro-1-positive MSCs. Cells were digested with pronase and collagenase. After centrifugation, PDLSCs were cultured as monolayers in  $\alpha$ MEM medium with change of the medium every 4 days to reach confluence. Three days after confluence, the medium of PDLSCs was exchanged with osteogenic medium. The medium was changed every 3 days during the course of incubation (0–15 days). PDLSCs cultured in  $\alpha$ MEM medium were used as control for undifferentiated cells.

### Alizarin red staining

PDLSCs were seeded with 80% confluence, and cultured in osteogenic medium ( $\alpha$ MEM medium with 10% fetal bovine serum, 50  $\mu$ M ascorbic acid, 100 nM dexamethasone and 5 mM  $\beta$ -glycerophosphate) for 15 days as mentioned before. At days 0, 5, 10, 15, PDLSCs were fixed in 95% ethanol and then stained with Alizarin red (pH 4.1, Sigma Aldrich, St. Louis, MO, USA) and analyzed under an inverted microscope (Nikon, Tokyo, Japan).

### ALP activity

At days 0, 5, 10, 15 during osteogenic differentiation, PDLSCs were lysed with 1% Triton X-100 and the ALP activity was determined via commercialized kit (BioVision, Milpitas, CA, USA) with evaluation of absorbance at 405 nm.

### Cell transfection

Isolated PDLSCs were seeded and transfected with 25 nM miR-184 mimics or negative control mimics (NC mimic), miR-184 inhibitor or NC inh (GenePharma, Shanghai, China). Two days after transfection, cells were harvested for functional assays.

For determination of effect of miR-184 on osteogenic differentiation of PDLSCs, lentiviral plasmid was designed for over-expression of miR-184. The lentiviral plasmid (LV-miR-184: pGC-LV-miR-184-GFP), as well as the negative control (LV-NC: pGC-LV-NC mimic-GFP), were constructed by Genechem (Shanghai, China). The PDLSCs were seeded and then transfected with the lentiviral plasmids (multiplicity of infection of 20). For the over-expression of NFI-C, full length NFI-C was constructed into p3xFLAG-CMV10 vector (Sigma Aldrich). The PDLSCs were seeded and then transfected with vectors (30 nM) or cotransfected with vectors and the lentiviral plasmids. Cells then underwent osteogenic differentiation mentioned before for 15 days.

### Luciferase reporter assay

Wildtype or mutant sequences of 3'-UTR NFI-C were subcloned into pmirGLO luciferase reporter vector (Promega, Madison, Wisconsin, USA). PDLSCs were seeded and co-transfected with miR-184 mimics or NC mimic and the luciferase reporter vector. Luciferase activities were determined 48 h after transfection.

### qRT-PCR

cDNAs were synthesized from the extracted RNAs. qRT-PCR was performed with SYBR Premix Ex Taq™ (Takara, Dalian, China) on ABI 7300 system (Applied Biosystems; Thermo Fisher). Quantitative normalization of miRNA or mRNA were performed on U6 or GAPDH, respectively. The primer sequences were listed as below: miR-184 (F: GACGGA-GAAGTATAAGGG; R: GCGAGCACAGAATTAATACGAC); NFI-C (F: GGACAGGGATGGGCTCTG; R: CGTTCTTCTGAGGCCAGTGC); U6 (F: CTCGCTTCGGCAGCACATA; R: AACGATTCACGAATTTGCGT); GAPDH (F: ACCACAGTCCATGCCATCAC; R: TCCACCACCTGTTGCTGTA).

### Western blot

PDLSCs were lysed and the protein concentration was determined via bicinchoninic acid assay. Proteins (30 µg) were separated by 10% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with 5% milk, and then subsequently incubated overnight with anti-NFI-C (1:2000), anti-RUNX2 (1:2000), anti-Osterix (1:2500), anti-BSP (1:2500), anti-β-actin (1:3000) (Abcam, Cambridge, MA, USA). Secondary antibody with horseradish peroxidase-conjugation was then incubated with the membrane. Signals were determined with enhanced chemiluminescence substrate.

### Statistical analysis

Data were expressed as mean ± standard deviation, and the statistical analysis was performed with SPSS 13.0 (SPSS, Chicago, IL, USA). Difference between groups was evaluated by one-way analysis of variance with Tukey's multiple comparison test.  $P < 0.05$  was considered statistically significant.

## Results

### miR-184 was reduced during differentiation of PDLSCs

*In vitro* osteogenic-induced PDLSCs were visualized with gradually enhancement of mineralized nodule formation by Alizarin red staining (Fig. 1a). Moreover, osteogenic-induced PDLSCs showed unambiguous osteoblastic phenotype compared with the undifferentiated cells, as evidenced by the gradually increased ALP activity (Fig. 1b). Osteogenic-related genes, including RUNX2, Osterix and BSP, were also gradually increased following the osteogenic induction (Fig. 1c), further echoing the observation by Alizarin red staining and ALP activity. These results confirmed that the isolated PDLSCs could differentiate into osteoblasts under the induction condition. Expression of miR-184 was gradually reduced during osteogenic differentiation of PDLSCs in a time-dependent way (Fig. 1d), suggesting that miR-184 might be associated with osteoblastic differentiation in PDLSCs.

### Over-expression of miR-184 suppressed osteoblastic differentiation of PDLSCs

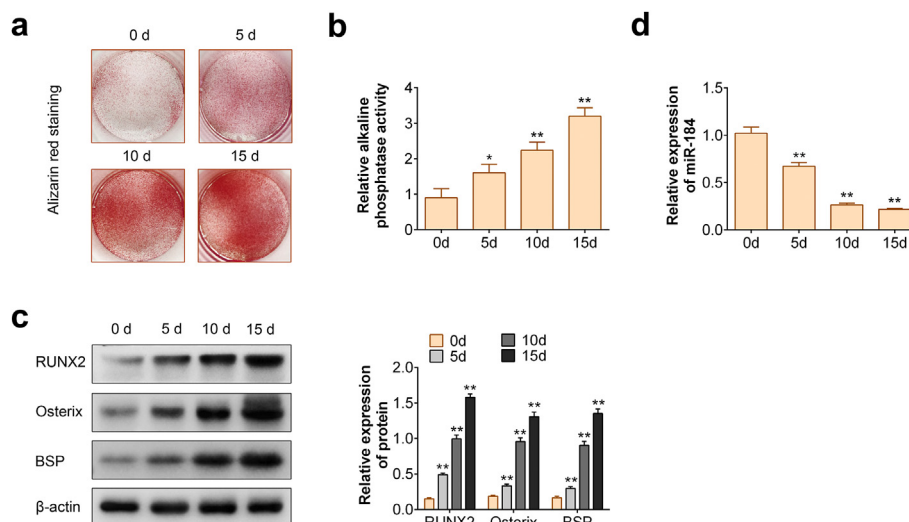
PDLSCs were transfected with lentiviral plasmids (LV-miR-184 or LV-NC mimic) to investigate role of miR-184 on osteoblastic differentiation. A significant up-regulation of miR-184 in cells transfected with LV-miR-184 compared to that transfected with LV-NC mimic was confirmed by qRT-PCR (Fig. 2a). Alizarin red staining (Fig. 2b) and ALP activity assay (Fig. 2c) demonstrated that over-expression of miR-184 significantly suppressed osteogenesis in PDLSCs. Protein expression of RUNX2, Osterix and BSP were dramatically reduced in cells transfected with LV-miR-184 compared to that transfected with LV-NC mimic (Fig. 2d), further confirming the suppressive effect of miR-184 on osteoblastic differentiation of PDLSCs.

### NFI-C was a target of miR-184

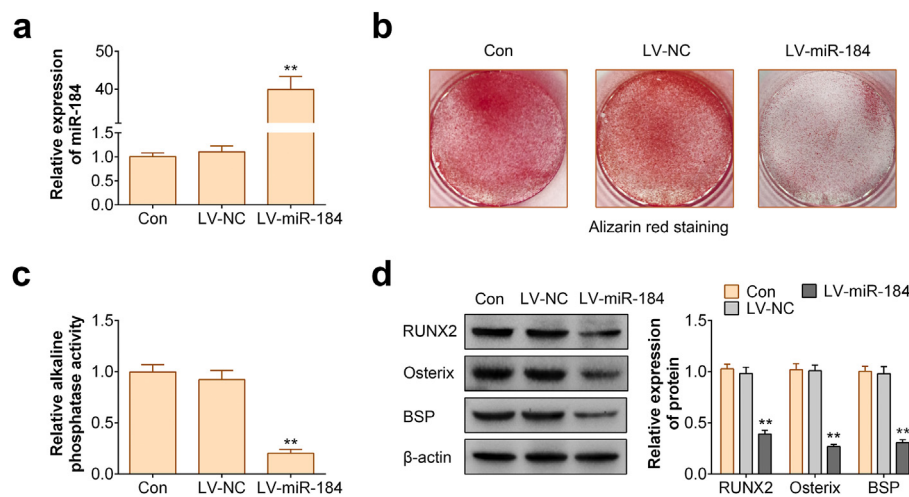
To investigate the mechanism involved in miR-184-mediated osteogenesis, NFI-C was identified as a potential target of miR-184 (Fig. 3a). Luciferase activity of wild-type NFI-C luciferase vector was decreased in PDLSCs transfected with miR-184 mimics (Fig. 3b). However, mutant NFI-C luciferase vector showed no significant change in the luciferase activity (Fig. 3b). PDLSCs were then transfected with miR-184 mimics or inhibitor and verified by qRT-PCR (Fig. 3c). MiR-184 could decrease both mRNA (Fig. 3d) and protein (Fig. 3e) expression of NFI-C, while miR-184 inhibitor enhanced NFI-C expression (Fig. 3d and 3e), suggesting that miR-184 bind to NFI-C and inhibit its expression in PDLSCs.

### NFI-C expression was enhanced during differentiation of PDLSCs

mRNA (Fig. 4a) and protein (Fig. 4b) expression of NFI-C were gradually enhanced during osteogenic differentiation of



**Figure 1** miR-184 was reduced during differentiation of PDLSCs. (a) Alizarin red staining of osteogenic-induced PDLSCs. (b) ALP activity was measured in osteogenic-induced PDLSCs via ELISA. (c) Protein expression of RUNX2, osterix, BSP in osteogenic-induced PDLSCs via western blot. (d) Expression of miR-184 in osteogenic-induced PDLSCs via qRT-PCR. \*, \*\* $P < 0.05$ ,  $P < 0.01$ .



**Figure 2** Over-expression of miR-184 suppressed osteoblastic differentiation of PDLSCs. (a) Transfection efficiency of LV-miR-184 in PDLSCs via qRT-PCR. (b) Effect of miR-184 on osteoblastic differentiation of PDLSCs by Alizarin red staining. (c) Effect of miR-184 on osteoblastic differentiation of PDLSCs by determination of ALP activity. (d) Effect of miR-184 on osteoblastic differentiation of PDLSCs by determination of protein expression of RUNX2, osterix, BSP. \*\* $P < 0.01$ .

PDLSCs in a time-dependent way, showing a negative correlation with miR-184 during osteogenesis in PDLSCs. These results suggested that miR-184/NFI-C axis might participate in the regulation of PDLSCs osteogenesis.

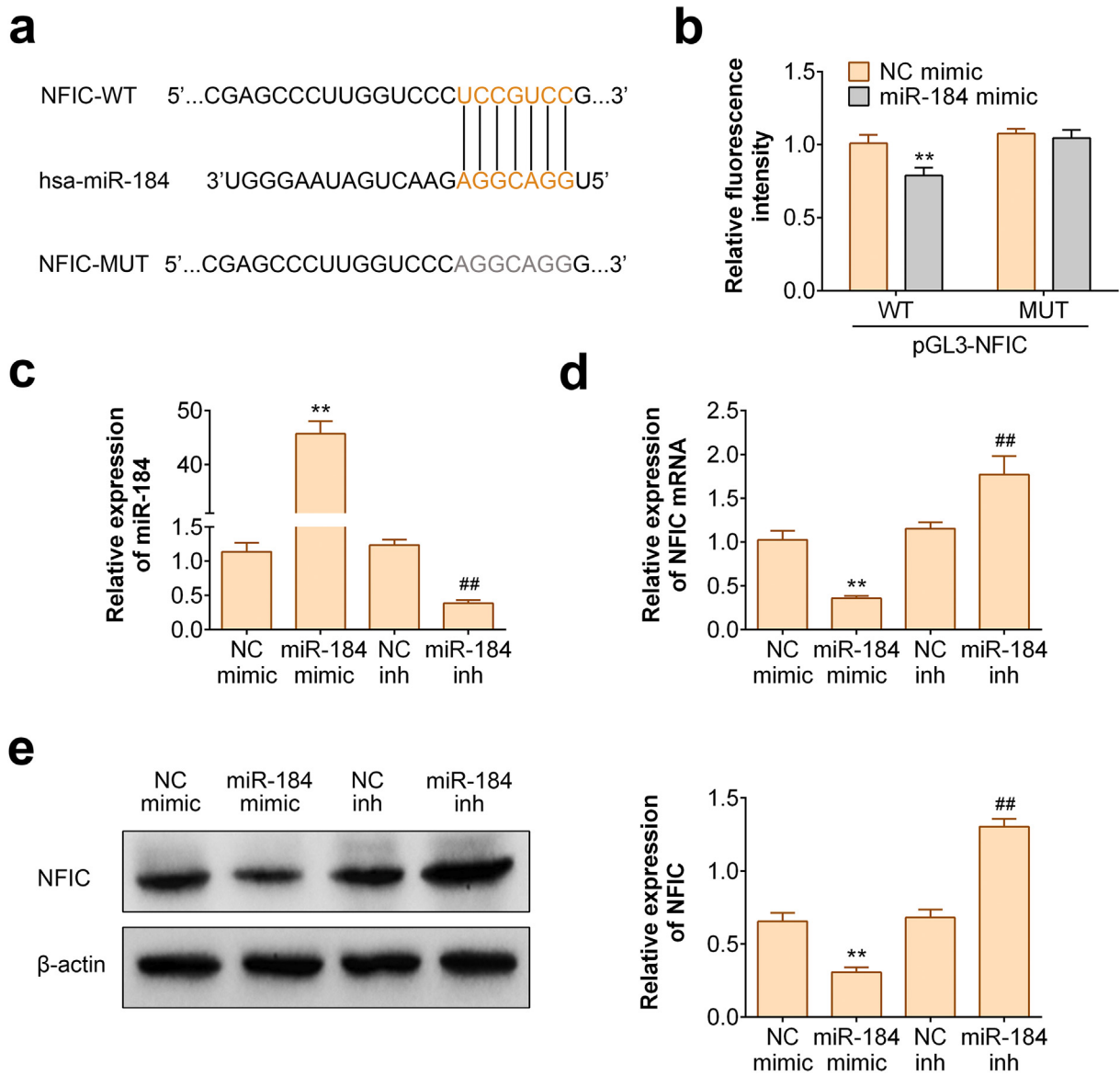
### Forced NFI-C expression counteracted the suppressive effects of miR-184 on osteoblastic differentiation

To investigate the role of miR-184/NFI-C axis on PDLSCs osteogenesis, PDLSCs were cotransfected with p3xFLAG-CMV10-NIFC and LV-miR-184. A significant up-regulation of NFI-C was found in PDLSCs transfected with p3xFLAG-CMV10-NIFC compared to the empty vector (Fig. 5a).

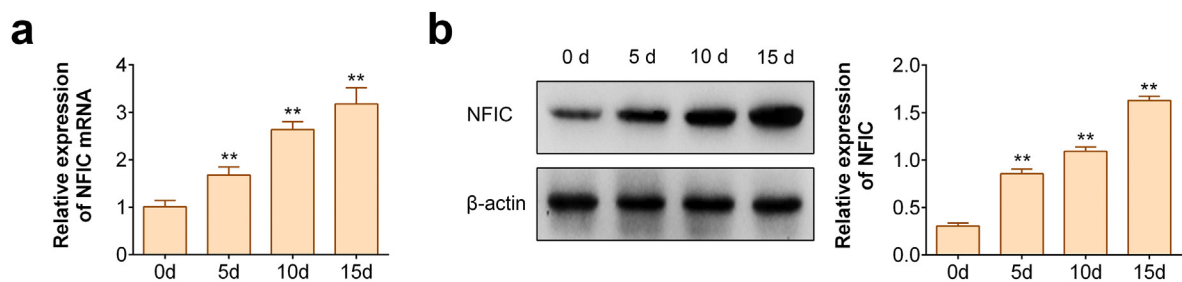
Forced NFI-C expression promoted osteoblastic differentiation with increased mineral deposition (Fig. 5b), ALP activity (Fig. 5c) and protein expression of RUNX2, osterix and BSP (Fig. 5d). Moreover, miR-184-induced decrease of mineral deposition (Fig. 5b), ALP activity (Fig. 5c) and protein expression of RUNX2, osterix and BSP (Fig. 5d) were reversed by over-expression of NFI-C, suggesting that forced NIFC counteracted the suppressive effects of miR-184 on osteoblastic differentiation.

### Discussion

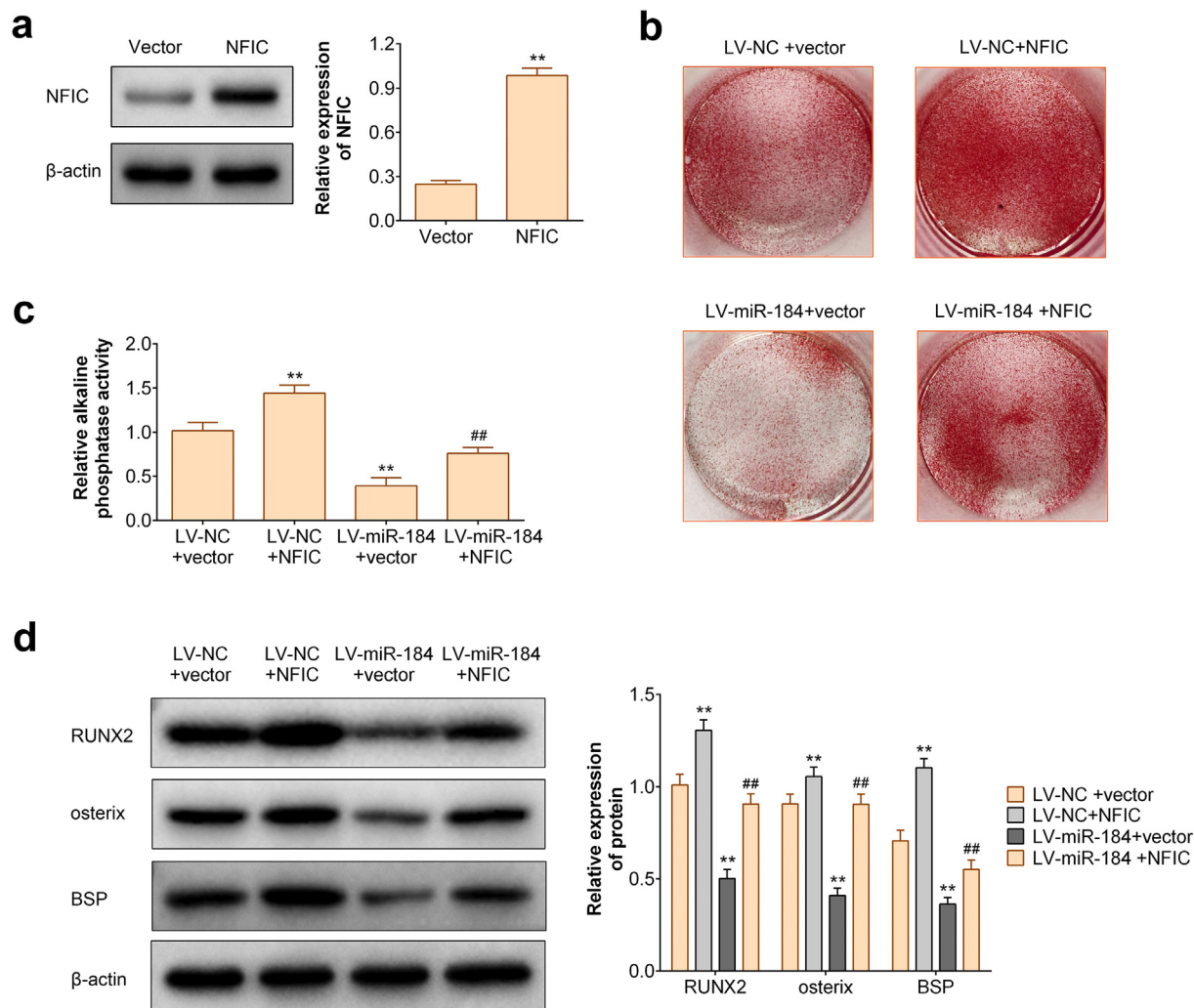
Recently, oral stem cells, with the pluripotency of osteogenic differentiation, have been regarded as promising



**Figure 3** NFI-C was target of miR-184 (a) Potential binding site of miR-184 in 3'UTR of NFI-C. (b) Effect of miR-184 mimics on luciferase activity of wild-type or mutant NFI-C luciferase vector. (c) Transfection efficiency of miR-184 mimics or inhibitor in PDLSCs were verified by qRT-PCR. (d) Effect of miR-184 on mRNA expression of NFI-C in PDLSCs. (e) Effect of miR-184 on protein expression of NFI-C in PDLSCs. \*\*, ## $P < 0.01$ .



**Figure 4** NFI-C expression was enhanced during differentiation of PDLSCs. (a) mRNA expression of NFI-C in osteogenic-induced PDLSCs via qRT-PCR. (b) Protein expression of NFI-C in osteogenic-induced PDLSCs via western blot. \*\* $P < 0.01$ .



**Figure 5** Forced NIF-C expression counteracted the suppressive effects of miR-184 on osteoblastic differentiation. (a) Transfection efficiency of p3xFLAG-CMV10-NFIC in PDLSCs via qRT-PCR. (b) Effect of miR-184 and NFI-C on osteoblastic differentiation of PDLSCs by Alizarin red staining. (c) Effect of miR-184 and NFI-C on osteoblastic differentiation of PDLSCs by determination of ALP activity. (d) Effect of miR-184 and NFI-C on osteoblastic differentiation of PDLSCs by determination of protein expression of RUNX2, osterix, BSP. \*\*, ### $P < 0.01$ .

therapeutic strategy for dental tissue defects.<sup>20</sup> PDLSCs, as postnatal stem cells, could differentiate into osteoblasts and have been widely used in periodontal tissue regeneration.<sup>21</sup> For example, transplantation of PDLSCs into porcine model has been reported to regenerate the root-periodontal complex and support normal dental function.<sup>22</sup> *In vivo* injection with PDLSCs could regenerate periodontal tissues in swine.<sup>23</sup> MiRNAs, with the ability to regulate pathways involved in osteogenic differentiation of PDLSCs, have been considered as key regulators in bone tissue engineering.<sup>24</sup> This study was then conducted to investigate the role of miR-184 on osteogenesis in PDLSCs.

First, osteogenic-induced PDLSCs was established in this study. The important osteogenic transcription factor, RUNX2, was elevated in osteogenic-induced PDLSCs.<sup>25</sup> RUNX2 would then regulate osterix-mediated osteoblast-specific genes, such as ALP.<sup>26</sup> Here, both ALP activity and protein expression of RUNX2 and osterix were enhanced in osteogenic-induced PDLSCs. Moreover, mineralization of

osteogenic-induced PDLSCs were validated by Alizarin red staining and increase of BSP.<sup>27</sup>

Previous study has shown that miR-184, conserved from fly to human, was up-regulated during retinal pigment epithelium differentiation.<sup>18</sup> Data from this study showed that miR-184 was gradually reduced during osteogenic differentiation of PDLSCs in a time-dependent way. Over-expression of miR-184 suppressed osteoblastic differentiation of PDLSCs with decreased RUNX2, osterix, BSP and ALP activity. MiR-184 could promote differentiation of retinal pigment epithelium via inhibition of AKT2/mTOR pathway,<sup>18</sup> and induce epidermal differentiation via activation of Notch pathway.<sup>17</sup> AKT/mTOR<sup>28</sup> and Notch<sup>29</sup> pathways play crucial roles in osteogenic differentiation of PDLSCs, whether these pathways participate in miR-184-mediated osteogenesis in PDLSCs should be investigated in the further research.

MiR-184 could inhibit differentiation of adult neural stem/progenitor cells via targeting Numbl-like,<sup>19</sup> the target

gene of miR-184 involved in osteoblastic differentiation of PDLSCs was then verified. Results indicated that miR-184 was able to specifically target 3'UTR of NFI-C, and NFI-C was gradually enhanced during osteogenic differentiation of PDLSCs in a time-dependent way, showing a negative correlation with miR-184 during osteogenesis in PDLSCs. NFI-C has been shown to be a critical regulator during tooth development via influencing dental follicle cells, stem cells in the dental germ.<sup>30</sup> Destruction of NFI-C leads to the differentiation of odontogenic cells<sup>31</sup> and the imbalance of the formation of dentine in mice, resulting in the deformity of the root of tooth.<sup>32</sup> Recently, NFI-C has also been found to play an important role in bone formation and bone homeostasis.<sup>33</sup> Loss of NFI-C damaged osteoblast differentiation and bone formation.<sup>34</sup> NFI-C, as a downstream gene of RUNX2, regulated osterix expression to control osteoblast differentiation.<sup>34</sup> Results in this study showed that gain-of NFI-C promoted osteoblast differentiation, and forced NFI-C expression counteracted the suppressive effects of miR-184 on osteoblastic differentiation. Therefore, miR-184/NFI-C axis might be involved in RUNX2/osterix/ALP network during osteoblast differentiation of PDLSCs. However, the clinical application of miR-184/NFI-C axis on osteoblast differentiation of PDLSCs should be further investigated in mice model.

In summary, a new layer of miRNA regulation in osteoblast differentiation of PDLSCs was discovered in this study. The results showed that miR-184 could inhibit osteoblast differentiation of PDLSCs through targeting NFI-C. This study suggested that miR-184 could serve as a novel therapeutic strategy for the regeneration of dental tissues.

## Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

## Acknowledgements

Not applicable.

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