MiR-146a-5p Promotes Dental Stem Cells Osteo/odontogenic Differentiation through NF-Kappa B Signaling Pathway by Targeting *TRAF6*

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Objective: *To screen miRNAs that could simultaneously regulate osteo/odontogenic differentiation of multiple stem cells, including dental pulp stem cells (DPSCs), stem cells from the apical papilla (SCAPs) and periodontal ligament stem cells (PDLSCs).*
Methods: *Differentially expressed miRNAs analysis on three miRNA microarrays data of dental stem cells undergoing osteo/odontogenic differentiation (GSE138180, GSE154466 and GSE159508) was performed, and miR-146a-5p were identified by bioinformatic prediction, dual-luciferase reporter assay and quantitative real-time polymerase chain reaction (PCR). In addition, differentially expressed genes between miR-146a-5p overexpressed group and control group (GSE79341) were applied for KEGG pathways enrichment analysis.*
Results: *MiR-146a-5p expression increased in the osteo/odontogenic differentiation of DPSCs, SCAPs and PDLSCs. Tumour necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) was identified as the target gene of miR-146a-5p. Furthermore, miR-146a-5p could influence the NF-Kappa B signalling pathway.*
Conclusion: *This study suggests that miR-146a-5p could promote differentiation in multiple dental stem cells through the NF-Kappa B signalling pathway by targeting TRAF6.*

Key words: *dental stem cells, miR-146a-5p, NF-Kappa B signalling pathway, osteo/odontogenic differentiation, TRAF6*
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Osteo/odontogenic differentiation of dental stem cells, including dental pulp stem cells (DPSCs), stem cells from the apical papilla (SCAPs) and periodontal ligament stem cells (PDLSCs), is a key process in tooth development[1]. DPSCs are mesenchymal stem cells that originate from the embryonic cranial neural crest and that are capable of self-renewal and pluripotent differentiation[2,3]. DPSCs can differentiate into osteoblasts and odontoblasts, and play an important role in dentine repair and regeneration[4]. SCAPs are derived from the apical papilla of immature teeth and can promote the formation of root dentine during root maturation[5-7]. PDLSCs are isolated from the periodontal ligament (PDL), which connects the alveolar bone to the cementum. They are also pluripotent stem cells and show great potential in the formation of new bone and other periodontal tissues[8].

To date, several studies have suggested that a variety of miRNAs are involved in the process of osteo/odontogenic differentiation of dental stem cells. For example, *miR-497-5p* influences the Smad signalling pathway by regulating *Smurf2* to promote osteo/odontogenic differentiation of SCAPs[1]. Yao et al[9] found the *miR-214-ATF4* axis to be a novel pathway for regulating the osteogenic differentiation of PDLSCs. Besides, *miR-143* inhibited DPSCs osteogenic differentiation by targeting TNF-α to block NF-Kappa B signaling path-

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way10. *MiR-146a-5p* is a frequently studied miRNA and is simply called *miR-146a* in previous nomenclature, whereas *miR-146a-3p* was named *miR-146a**[11]. *MiR-146a-5p* has been widely reported to be associated with cancers, such as liver[12], lung[13], breast[14] and oral cancers[15], as a tumour-suppressor miRNA in some cancers and an oncogenic miRNA in others.

Currently, there are few studies on shared miRNAs that regulate osteo/odontogenic differentiation of multiple dental stem cells, and the molecular mechanism remains unclear. In the present study, we explored miRNAs that could simultaneously regulate differentiation of multiple stem cells (DPSCs, SCAPs and PDLSCs), which might become a new target for promoting tooth tissue regeneration.

Materials and methods

Analysis of microarray datasets and bioinformation

The Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) is a public database[16] that contains a large amount of gene/miRNAs expression microarray and sequencing data. We retrieved and downloaded four datasets from the database, including three miRNA microarrays datasets of dental stem cells osteo/odontogenic differentiation (GSE138180, GSE154466, GSE159508) and one gene expression datasets (GSE79341).

GSE138180 explored the roles of miRNAs in the odontogenic differentiation of DPSCs extracted from healthy premolars of 19 healthy patients aged from 15 to 25 years, whose premolars were extracted for orthodontic treatment at the Department of Stomatology, Nanfang Hospital, Southern Medical University, Guangzhou[17]. DPSCs from the differentiated group were cultured with an odontogenic differentiation medium containing 50 mg/ml ascorbic acid, 100 nmol/L dexamethasone and 10 mmol/L β-glycerolphosphate (Sigma, St Louis, MO, USA) for 14 days, while DPSCs from the undifferentiated group were cultured in basal medium with 10% foetal bovine serum (FBS).

GSE154466 applied miRNA microarrays to analyse differentially expressed miRNAs in osteo/odontogenic differentiation of SCAPs obtained from immature human third molars of patients aged 16 to 20 years for orthodontic reasons at the School and Hospital of Stomatology, Shandong University[1]. The researchers cultured SCAPs in a differentiation medium and selected two stages to obtain the stem cells: pre-osteo/odontogenic differentiation and 7 days after osteo/odontogenic differentiation, followed by RNA extraction and Affymetrix (Santa Clara, CA, USA) microarray detection[1].

GSE159508 analysed miRNA expression profile during osteogenic differentiation of PDLSCs provided by the Department of Stomatology, Nanfang Hospital, Southern Medical University, Guangzhou. PDLSCs from the differentiated group were cultured in an odontogenic differentiation medium for 14 days. Both groups had three replicates and were investigated by microarray profiling[18].

GSE79341 contained gene expression data of *miR-146a-5p* overexpressed cells using the Affymetrix GeneChip[19]. Hepatocyte-like Huh7.5.1 cells were transfected with *miR-146a-5p* mimics and control mimics, followed by total RNA extraction and purification, cDNAs hybridisation and scanning of gene chips.

The website named *Gene expression in tooth* that presented gene expression in developing dental tissues (http://bite-it.Helsinki.fi/) contains schematic illustrations of gene expression in different stages of development. These data were derived from previous studies describing the expression of genes at one or more stages of tooth development, mainly obtained by in situ hybridisation analysis to reveal the location of mRNA or by immunochemical methods to detect proteins.

Predicting the potential binding targets of miRNAs

The potential target genes of *miR-146a-5p* were predicted and identified by four databases, namely Targetscan (http://www.targetscan.org/vert_71/)[20], miRDB (http://mirdb.org/)[21], Starbase (http://starbase.sysu.edu.cn/)[22] and miRWalk (http://mirwalk.umm.uni-heidelberg.de/)[23] according to the following criteria: consistently predicted by all four databases, and had the highest target rank.

Cell culture

The HEK-293 (human embryonic kidney 293 cells), a common cell to investigate cell function and molecular regulation, was cultured in Eagle's minimum essential medium (EMEM) comprised of 10% FBS, 100 U/ml penicillin and 100 mg/ml of streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C.

Transfection and quantitative real-time PCR

HEK-293 cells, seeded into12-well culture plates, were instantly transfected with *miR-146a-5p* mimics by EndoFectin MAX (GeneCopoeia, Germantown, MD,

USA). After 48 hours, the cells were collected for RNA extraction and reverse transcribed into single stranded cDNA (Takara, Shiga, Japan). The primer sequence information of tumour necrosis factor receptor (TNFR)-associated factor 6 (*TRAF6*) is shown in Table S1 (provided on request). We performed real-time quantitative PCR by Power SYBR Green on a CFX96 PCR System (Bio-Rad, Hercules, CA, USA). Data were collected and analysed using the $2^{-\Delta\Delta Ct}$ method for qualification of the relative expression levels of *TRAF6*, with *GAPDH* as corresponding internal controls.

Construction of reporter plasmids and dual-luciferase reporter assay

The *TRAF6* 3'-UTR wild-type fragment was inserted at the NheI-SalI restriction site downstream of the luciferase gene in the pmirGLO vector. HEK-293 cells were transfected with the reporter plasmids and *miR-146a-5p* mimic. After transfection for 24 hours, the luciferase activity in the lysates was quantified by a dual-luciferase reporter assay system (Promega, Madison, WI, USA). The binding activity of *miR-146a-5p* to *TRAF6* 3'-UTR was characterised by measuring the ratio of firefly luciferase to renilla luciferase activity.

Pathway analyses and PPI network

Differentially expressed genes identified in GSE79341 were applied for Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways enrichment analysis, which was performed using clusterProfiler R software package (R Core Team, Vienna, Austria) to explore the downstream pathways affected by *miR-146a-5p* overexpression. The Search Tool for the Retrieval of Interacting Genes (STRING, https://string-db.org/) website was used to construct a protein–protein interaction[24] network of genes in target pathways, with an interaction score of 0.4 as the cutoff criterion[25].

Statistical analysis

Differentially expressed miRNAs (DEMs) and genes (DEGs) were identified using a linear model with the GEO2R online analysis tool based on GEOquery, Limma and Umap R software packages[26]. $P < 0.05$ and $|\log2(FC)| > 1$ was taken as the criterion for screening DEMs and $P < 0.05$ for DEGs. We conducted KEGG pathway enrichment analyses of DEGs using clusterProfiler R software[27]. The volcano plots, heatmaps and Venn diagrams were created using OmicStudio tools (https://www.omicstudio.cn/tool). Luciferase activity analysis and qRT-PCR results were calculated using a two-tailed Student *t* test, with the level of significance set at $P < 0.05$.

Results

Identification of DEMs associated with osteo/odontogenic differentiation

In this study, we first separately identified differentially expressed miRNAs in three miRNA microarray datasets (GSE138180, GSE154466 and GSE159508) using GEO2R software. As a result, 185 miRNAs in DPSCs were screened out from GSE138180, 20 miRNAs in SCAPs from GSE154466 and 174 miRNAs in PDLSCs from GSE159508. The volcano plots, as well as the heatmaps, showed upregulation or downregulation of miRNAs between the two groups (Fig 1). Furthermore, we took the intersection of the results for the three datasets and finally found that *miR-146a-5p* expression increased in the differentiated groups (Fig 2), suggesting that it played an important role in dental stem cell osteo/odontogenic differentiation.

TRAF6 was identified as the target gene of miR-146a-5p

TRAF6 was consistently predicted to be the target gene of *miR-146a-5p* in the four databases, and its target rank was the first according to target scores (Table S2, provided on request). In addition, there were six possible binding sites for *miR-146a-5p* in the 3'-UTR of *TRAF6* (Fig 3a). In addition, a luciferase reporter plasmid containing *TRAF6* 3'-UTR was cloned and co-transfected with *miR-146a-5p* mimics in HEK-293 cells. Luciferase reporter gene results showed that *miR-146a-5p* reduced the activity of 3'-UTR of *TRAF6* significantly (Fig 3b). To further verify whether *TRAF6* was the target gene, we transfected *miR-146a-5p* mimics in HEK-293 cells and extracted total RNA 48 hours later to detect changes in *TRAF6* expression, which showed that increased expression of *miR-146a-5p* inhibited the expression of *TRAF6* significantly (Fig 3c). The above results suggested *TRAF6* was the target gene of *miR-146a-5p*.

Traf6 was expressed during multiple stages of mouse tooth germs

Traf6 was expressed continuously in multiple stages of mouse teeth, including the initiation, bud, cap and bell stages. In addition, *Traf6* expression signal could be

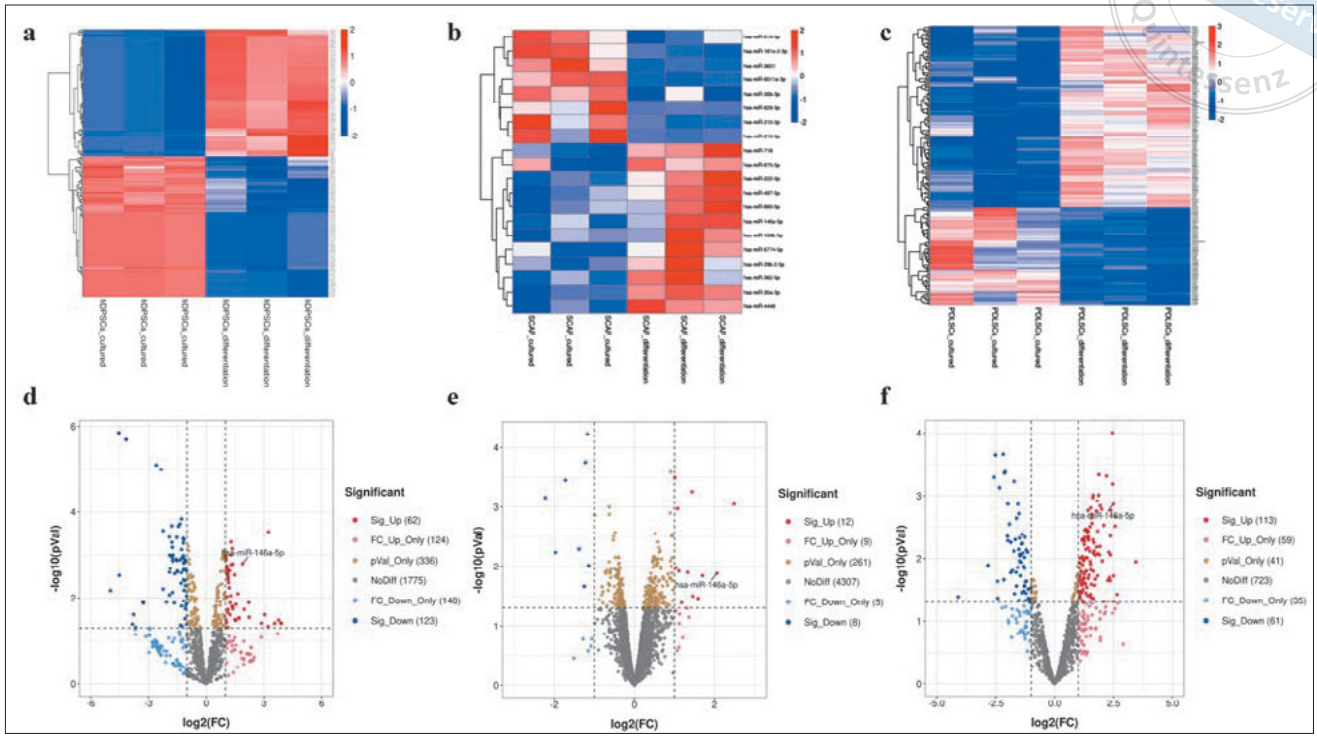

Fig 1 Heatmaps of differentially expressed miRNAs in **(a)** GSE138180, **(b)** GSE154466 and **(c)** GSE159508. The screening criteria were as follows: |log2(FC)| > 1, $P < 0.05$ for miRNAs. The expression value is described by a colour scale. The intensity increased from red to blue. Each column represents one sample, and each row represents one transcript. Volcano plots of miRNAs in **(d)** GSE138180, **(e)** GSE154466 and **(f)** GSE159508. Red, blue and grey colours indicate relatively high, low and equal expression of miRNAs, respectively.

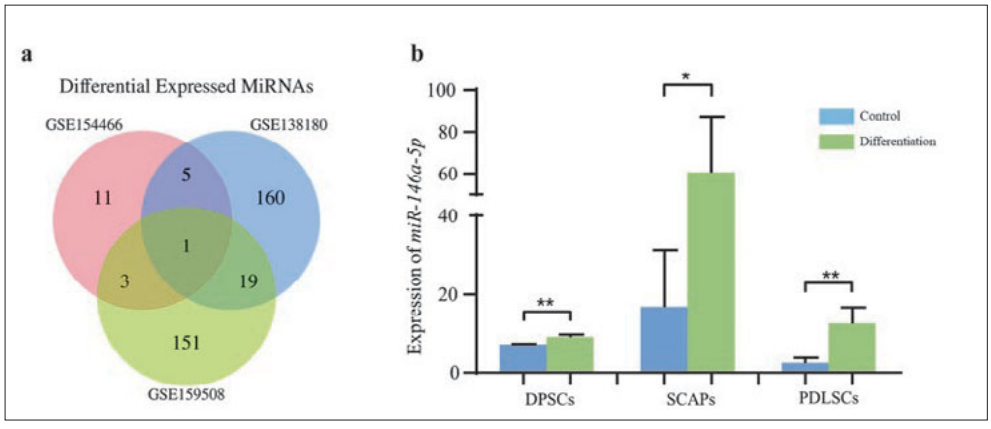

Fig 2 **(a)** A Venn diagram shows the overlapping differentially expressed miRNAs in GSE138180, GSE154466 and GSE159508. **(b)** Expression of *miR-146a-5p* was increased in the dental stem cells osteo/odontogenic differentiation groups.

detected in various tissues at each stage, especially in the dental epithelium, stellate reticulum and dental papilla (Fig 3d). The expression pattern of *Traf6* was mapped by in situ hybridisation in mouse embryonic mandibular first molar tooth germs between E11.5 and E15.5 from the study by Ohazama et al[28].

MiR-146a-5p affected the NF-Kappa B signalling pathway

As shown in the volcano plot and heatmap, we identified 2229 differentially expressed genes (duplicate and invalid gene names were removed) between *miR-146a-5p* overexpressed groups and control groups. KEGG

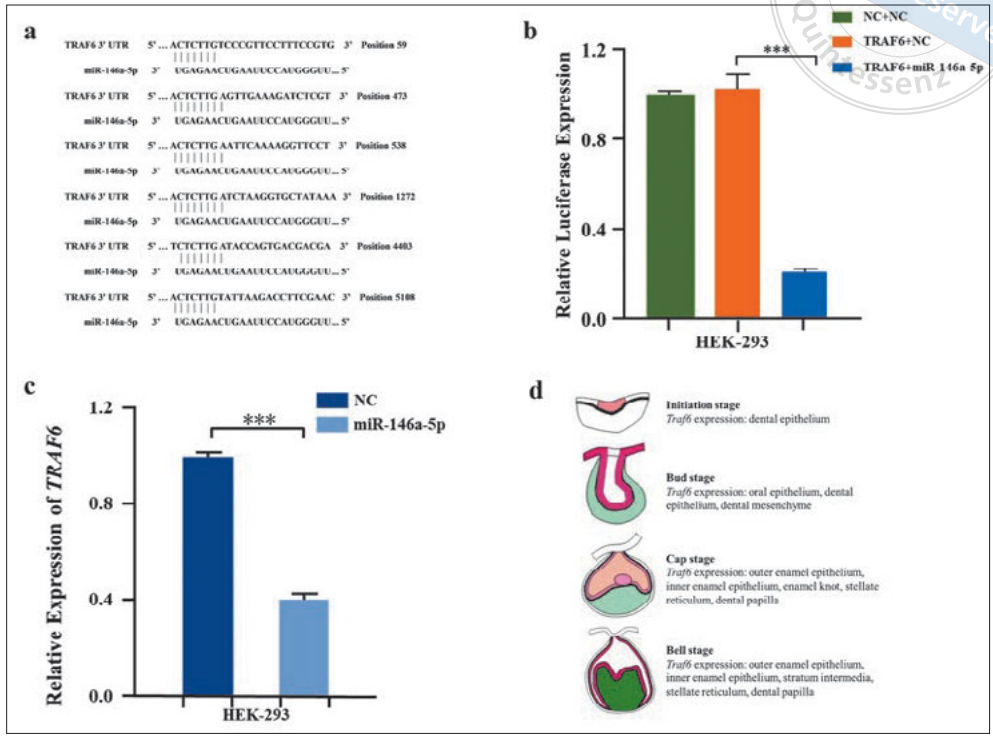

Fig 3 **(a)** The predicted binding sites for *miR-146a-5p* in the 3'-UTR of *TRAF6*. **(b)** Dual luciferase reporter assays in HEK-293 cells demonstrated that *TRAF6* was the direct target of *miR-146-5p*. **(c)** *TRAF6* mRNA expression in HEK-293 cells after transfection with *miR-146a-5p* mimics. Transcript levels were normalised to GAPDH levels (***$P < 0.001$). **(d)** *TRAF6* was expressed continuously at multiple stages in various tissues of mouse teeth.

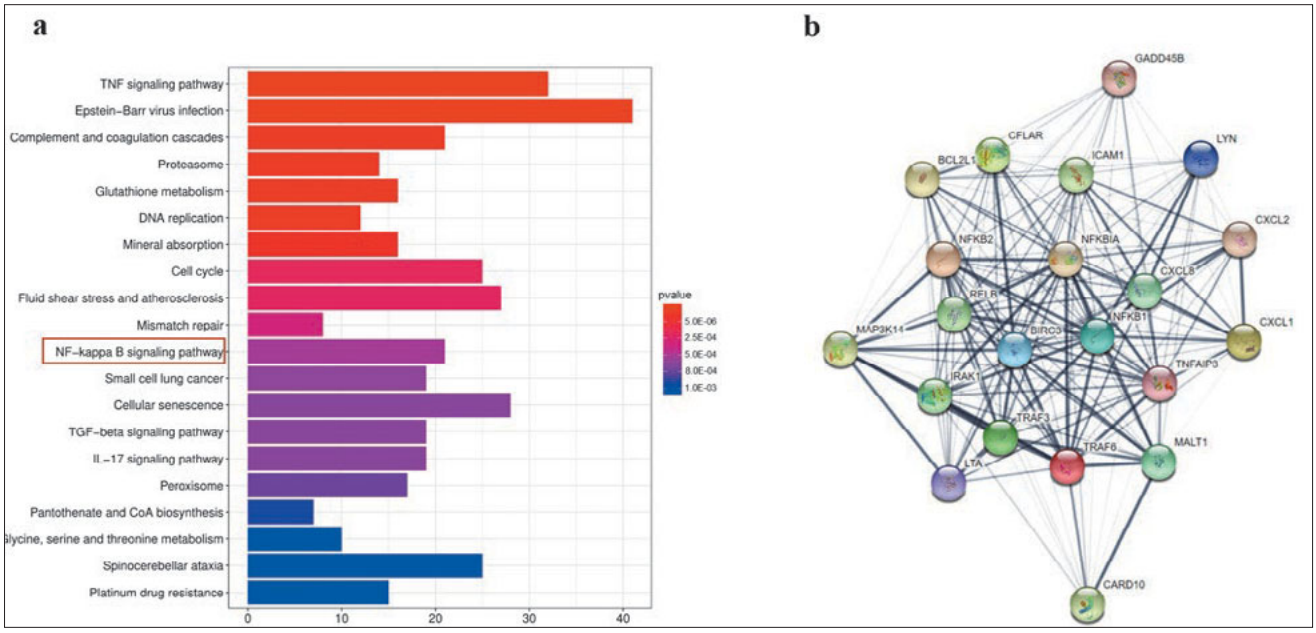

Fig 4 KEGG pathway analyses of differentially expressed mRNAs in GSE79341. **(a)** Pathways marked in the red box were reported to be associated with dental stem cell osteo/odontogenic differentiation. **(b)** The PPI network of the NF-Kappa B signalling pathway suggested *TRAF6* was the core gene of the pathway.

pathway analysis detected 48 significant pathways ($P < 0.05$), and the top 20 pathways were selected for presentation (Fig 4a). Notably, we found that the TNF, NF-Kappa B and TGF−beta signalling pathways were reported to be associated with tooth development[29-31]. In particular, it was reported that the NF-Kappa B signalling pathway significantly influenced the osteo/odontogenic differentiation ability of dental stem cells[32-35]. Meanwhile, *TRAF6* was the core gene of the NF-Kappa B signalling pathway, as shown in the protein–protein interaction (PPI) network (Fig 4b).

Discussion

In this study, we identified *miR-146a-5p* as a potential target for promoting dental stem cell osteo/odontogenic differentiation, which has been widely reported to be associated with various cancers. Interestingly, tooth development and cancer development share common molecular pathways, and almost all types of tumours are more common in families with tooth agenesis[36]. Furthermore, Pan et al[37] suggested that *miR-146a* was susceptible to non-syndromic orofacial cleft (NSOC). NSOC patients always present with dental abnormalities, especially tooth agenesis and maxillary defects[38].

Here, we further identified *TRAF6* as the target gene of *miR-146a-5p*. *TRAF6* was expressed strongly in the signal centre of the epithelial enamel knot, which was believed to regulate the morphogenesis of tooth tips in the process of mouse tooth development[38]. Besides, *TRAF6* gene silencing could enhance the proliferation of murine odontoblast-like cells (MDPC-23), which would affect the ability of odontoblast cells to form and repair dentine, suggesting that *TRAF6* was an important signalling molecule regulating the process of tooth development and dentine repair[39]. This was essentially consistent with our conclusion that the decreased expression of *TRAF6* could promote osteo/odontogenic differentiation and tooth eruption.

The classical NF-Kappa B signalling pathway was commonly believed to play an important part in the tooth organogenesis and osteo/odontogenic differentiation process. Wang et al[33] reported that inhibition of NF-Kappa B signalling pathway can reduce osteo/odontogenic differentiation of SCAPs. They also provided evidence that the 17β-oestradiol promoted osteo/odontogenic differentiation of DPSCs through activation of the NF-Kappa B signalling pathway[34]. In addition, a study found that the osteo/odontogenic capacity of PDLSCs could be improved by mineral trioxide aggregate by activating the NF-Kappa B signalling pathway[40].

Conclusion

We discovered that *miR-146a-5p* could promote osteo/odontogenic differentiation in multiple dental stem cells (DPSCs, SCAPs and PDLSCs) through the NF-Kappa B signalling pathway by targeting *TRAF6*. Our findings tended to enhance the current understanding of the molecular events regulating osteo/odontogenic differentiation and could serve as a basis for researching functional miRNAs in the differentiation of multiple dental stem cells.

Acknowledgments

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Author contribution

Dr Xin YU performed the experiments, analysed the data and drafted the article; Dr Jian Feng LU contributed to the analysis and interpretation of the data; Drs Mei Qin GAO, Bin XIONG and Wen Qian XIA designed the study, analysed the data and critically revised the manuscript.

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