Hsa-miRNA-143-3p Regulates Differentiation of Human Stem Cells From The Apical Papilla Through Targeting Nuclear Factor I-C

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Research

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Hsa-miRNA-143-3p regulates differentiation of human stem cells from the apical papilla through targeting Nuclear factor I-C

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Abstract

Background: Dental root development is independent and time-space-specific. Nuclear factor I-C (NFIC) plays a key role in human root development through regulating the differentiation of stem cells from the apical papillary (SCAPs). The function of microRNAs during the differentiation of SCAP and post-transcriptional regulation of NFIC remain unclear.

Methods: We examined the microRNA expression profiles in human immature permanent teeth and SCAPs differentiation. hSCAPs were treated with miR-143-3p over/low-expression viruses, then the odonto/osteogenic differentiation of these stem cells and the involvement of NFIC pathway were investigated. Next, luciferase reporter and its mutant plasmids were used to confirm direct target gene of miR-143-3p. Mineralization induction assays *ex vivo* and *in vitro* were used to investigate the functional significance of miR-143-3p.

Results: MiR-143-3p was screened by microarray expression profiling and bioinformatics technology, which decreased during hSCAPs differentiation. Overexpression of miR-143-3p inhibited the odontogenic differentiation of hSCAPs and downregulated the related genes, whereas the functional inhibition of miR-143-3p yielded the opposite effect. The luciferase reporter gene detection and bioinformatics approach identified NFIC as a potential target of miR-143-3p. Furthermore, NFIC overexpression reversed the inhibitory effect of miR-143-3p on the odontogenic differentiation of hSCAP.

Conclusions: MiR-143-3p maintains the stemness of hSCAPs and negatively modulates their differentiation and mineralization by directly targeting transcription factor NFIC, which serves as an contribution towards a better understanding of the developmental mechanisms of root formation.

Key words: Dental root development, SCAPs, microRNA, NFIC, odontogenic differentiation

Background

Tooth root development is a complex process and requires exact temporal and spatial regulation of cell proliferation and differentiation. Many signaling molecules are involved in the regulation of growth and eventually apical closure[1]. Stem cells from apical papilla (SCAPs) are multipotent progenitor cells residing in the root apex of immature teeth. SCAPs can differentiate into radicular pulp as well as the odontoblasts that are responsible for root dentinogenesis and undergo morphological and functional changes[2]. These postnatal stem cells play a crucial role in pulp-dentin complex healing and regeneration when the roots of young permanent teeth are damaged. A greater understanding of the cellular and molecular mechanisms that regulate SCAPs activity is likely to improve the tooth tissue engineering.

MicroRNAs (miRNAs) are small RNA molecules that negatively regulate gene expression at the post-transcriptional level by binding to their target mRNAs through base pairing to the 3'-untranslated region (UTR), causing translational repression of the mRNA[3]. Evidence has been accumulating that, as fine-tuners of gene expression, miRNAs play essential roles in tooth development and homeostasis[4, 5]. Conditional inactivation of DICER, which is essential for miRNA maturation, leaded to abnormal tooth development in mice, indicating participation of miRNAs during tooth formation[6]. However, certain specific miRNAs operating through transcription factors in dentin formation and SCAP differentiation remain largely unknown.

Nuclear factor I-C (NFIC) plays a paramount role in tooth root development. The most striking defect caused by *Nfic* disruption in mice is the loss of molar roots formation[7]. NFIC has no effect on tooth germ and crown dentin, which indicates that the potential mechanism in root formation may be different from that in the tooth germ/crown formation. NFIC belongs to the Nuclear Factor-I (NF-I) family, which preferentially bind DNA target sites near transcription initiation sites as transcriptional activators[8]. Previous studies have revealed that NFIC has a close association with human root development and can significantly enhance SCAPs cytodifferentiation[9], and is important for the early epithelial-mesenchymal interactions and may be involved in regulation of both dentin and enamelextracellular matrix production[10]. Reports suggest that NFIC is related to both TGF-β/BMP and SHH signaling pathways and comprises a complex signaling cycle[11, 12]. However, the functional significance of NFIC signaling and its interaction with other signaling molecules in regulating the fate of human SCAPs (hSCAPs) are still elusive.

To date, few study has been conducted on the miRNAs expression profiles during human tooth root formation. To fully understand the biological roles of miRNAs in SCAP differentiation, we performed miRNA microarray profiling to reveal the expression patterns of miRNAs involved in dental root development, and investigated the influence of specific miRNA on the proliferation and differentiation of hSCAPs. Our findings first revealed that miR-143-3p can regulate differentiation of hSCAPs through targeting NFIC. We aimed to unveil the function of miR-143-3p and the exact molecular mechanism, especially in its operation through transcription factors and participation in signaling pathways and regulatory networks controlling tooth root formation.

Methods

This study was approved by the Ethical Committee of the Institute of Stomatological Research, Sun Yat-sen University (Guangzhou, China). Patients provided written informed consent. Mice were obtained from the Experimental Animal Department of Peking University Health Science Center. Care and the handling of animals was in accordance with Institutional and National guidelines.

Cell Culture and Cell-proliferation assays

Apical papilla tissues separated gently from the end of normal human impacted third molars with open apical foramina were digested in a solution of 3 mg/ml collagenase type I (Sigma-Aldrich, Basel, Switzerland) and 4mg/ml dispase (Sigma-Aldrich) for 1 h at 37℃. Cultures were maintained in α-modified Eagle' s minimum essential medium (α-MEM; Gibco, Grand Island, NY, USA). The basic concentration of fetal bovine serum (FBS) was 10%, and it was decreased to 5% when the cells were cultured in mineralization medium containing 10nM dexamethasone, 50 mg/ml ascorbate phosphate and 10 nM 1,25-di-hydroxyvitamin D3. The proliferation of hSCAPs after transfection was assessed using a Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan) according to the manufacturer's instructions.

MiRNA Microarray and Bioinformatics Analysis

miRNA microarray was performed to assess miRNA expression patterns of apical papilla

tissues and dental root pulp tissues from human immature teeth. Total RNA was collected with TRIzol (TaKaRa) and small RNAs of 18–30 nt were obtained using 15% denaturing polyacrylamide gel electrophoresis (PAGE). PCR products were purified and submitted for sequencing via an IlluminaHi-Seq 2000 platform. The threshold of differentially expressed miRNA was at least 2-fold change. Databases (containing TargetScan, miRTarBase, miRDB, and miRWalk) were used to perform bioinformatics analysis (Ribobio, Guangzhou, China).

Virus transfection

miR-143-3p over/low-expression lentiviruses were purchased from Genechem (Shanghai, China). The appropriated multiplicity of infection (MOI) was screened in the preliminary experiment. At the confluence of 60-70%, hSCAPs were transfected with the miR-143-3p over/low-expression lentiviral vectors in α-MEM medium (6% FBS) containing 8 μg/mL polybrene (POL). Thus four groups were applied, i.e., Con-over group, miR-143-3p-over group, Con-low group and miR-143-low group.

Alizarin red staining and Alkaline phosphatase activity

The mineralization ability of transfected hSCAPs in mineralization-inducing medium was investigated. The Alkaline phosphatase (ALP) activity was detected by using an ALP activity assay kit (Jiancheng, Nanjing, China) according to the manufacturer's instructions. Calcium deposition of the extracellular matrix was evaluated by staining with 1% alizarin red-S (Sigma-Aldrich).

Transplantation and Immunohistochemistry

Approximately 2.0×10^6 *in vitro*-expanded hSCAPs mixed with 40 mg hydroxyapatite ceramic particles (Bio Osteon, Beijing, China) were transplanted subcutaneously into the dorsal surfaces of 10-wk-old immunodeficient mice (CB-17/SCID; Vitalriver, Beijing, China). Transplants were harvested 8 wk after transplantation. Immunostaining was performed with 7-μm paraffin samples with the antibody against NFIC (Abcam, Cambridge, UK).

Luciferase Reporter Assay

Plasmids encoding wild-type (WT) or mutant (MUT) 3’ untranslated regions (UTR) of NFIC (encoding NFIC receptor tyrosine kinase) were synthesized by Genechem (Shanghai, China). MiR-143-3p mimic and miR-143-3p mimic-control were produced by GenePharma (Shanghai, China). The luciferase vector (150 ng) was cotransfected into cells transfected with either miR-143-3p mimic or mimic-control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After incubation for 36-48h, the cells were collected and lysed, and the luciferase activities were detected by the Dual-Luciferase Reporter Assay Kit (Beyotime Biotechnology, Shanghai, China).

Quantitative Real-Time PCR and Western blotting

The real-time PCR primer sequences for gene expression analysis were obtained from PrimerBank. Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the

manufacturer's instructions. cDNA was synthesized using an AMV Reverse Transcriptase kit (Fermentas, St. Leon-Rot, Germany). The qRT-PCR was performed on a LightCycler 480 (Roche, Indianapolis, USA) with the Fast Start Universal SYBR GreenMasterMix (Roche) according to themanufacturer's instructions. The relative mRNA expression was calculated using the comparative cycle threshold ($^{\triangle\triangle}$Ct) method. Protein extracts were resolved by 10-12% SDS-PAGE, transferred to PVDF membranes, and probed with antibodies against NFIC, KLF4, DMP1 (Abcam, Cambridge, UK) and DSP (Santa cruz, USA).

Statistical analyses

Statistical analysis was performed with SPSS software, version 16.0 (SPSS, Chicago, IL, USA). p <0.05 was considered statistically significant. Student's t-test and chi-square test was used to analyze differences between groups. All experiments were independently performed at least three times.

Results

MiR-143-3p expression is inversely associated with hSCAPs differentiation

Apical papilla tissues located in the root apex of immature teeth are reliable cell source for pulp-dentine complex formation. MiRNA microarray assay was performed to analyze the miRNA profiles of mesenchymal tissues isolated from root pulp (RP) and apical papilla (AP) of young permanent teeth via Ion Torrent/MiSeq sequencing. Among the differentially expressed miRNAs, miR-143-3p was significantly downregulated in the root pulp (Fig. 1A, B). Additionally, real-time

PCR was conducted to detect miR-143-3p expression in five RPs and matched APs, showing the same trends that the levels of miR-143-3p decreased significantly in RPs compared with that in APs (Fig. 1C). MiRNAs related to NFIC were predicted using bioinformatics database, showing that miR-143-3p is one of the miRNAs retrieved from all three databases (Table 1). Therefore, miR-143-3p was selected for further study.

To investigate the miR-143-3p expression patterns *in vitro*, hSCAPs were cultured in mineralization medium. Alizarin red-S staining revealed the presence of red mineralized nodules from day 7 after the differentiation induction (Fig. 1D). RT-PCR analysis indicated that the expression level of miR-143-3p were gradually decreased during the mineralization of hSCAPs, while the expression of odontogenic marker genes in dental root (NFIC and DSPP) had rising and fluctuating trends before day 12 and then decreased (Fig. 1E). This indicates that miR-143-3p expressions is down-regulated during hSCAPs differentiation *in vitro* and *in vivo*.

Figure 1. miR-143-3p is associated with hSCAPs differentiation. (A) Heatmap diagram of differential miRNA expression profiles between root pulp (RT) and apical papilla (AP) tissues of young permanent teeth. Red= miRNAs with higher expression, blue= miRNAs with lower expression, and white= miRNAs with equal expression. (B) Differential miRNAs expression profiles between RPs and APs. Red= miRNAs with higher expression, green= miRNAs with lower expression, and gray= miRNAs with equal expression. (C) Real-time PCR analysis of miR-143-3p expression in RPs and APs (n=5). (D) Representative images of calcium nodules in different groups under the inverted microscope. Scale bars= 500um. (E) Expression of miR-143-3p, *NFIC* and *DSPP* was evaluated by RT-PCR during hSCAP differentiation. **p* <0.05, ***p* <0.01.

Table 1 Bioinformatic analyses of miRNAs related to NFIC

Targetsean	MicroRNA.org	miRandaSites	miRNApath
hsa-miR-4770	hsa-miR-365	hsa-miR-210-3p	hsa-miR-519B
hsa-miR-143-3p	**hsa-miR-143**	hsa-miR-193b-3p	hsa-miR-519C
hsa-miR-6088	hsa-miR-210	hsa-miR-365a-3p	hsa-miR-519D
		hsa-miR-193a-3p	hsa-miR-365
		hsa-miR-143-3p	hsa-miR-95
			hsa-miR-519A
			hsa-miR-488

MiR-143-3p influences odontogenic differentiation of hSCAP *in vitro* and *in vivo*

To determine the effect of miR-143-3p on cellular behavior of hSCAPs, we stably overexpressed and silenced miR-143-3p expression in the cells with lentiviral vectors. MOI refers to the average number of viral particles in a cell with an active viral infection, and the cytotoxicity of the virus increase with the MOI values. According to the cell vitality and fluorescence expression, the best MOI value equals to 5 in this study (Fig. 2A). RT-PCR results demonstrated that the expression level of miR-143-3p increased sharply in miR-143-3p over-expression group (miR-143-3p-over), but decreased in miR-143-3p low-expression group (miR-143-3p-low, Fig. 2B, $^{**}p<0.01$) compared with the control group.

To explore whether miR-143-3p could act on hSCAPs proliferation, we performed the CCK-8 cell proliferation assay. The results presented no distinct difference of the cell proliferation rate in miR-143-3p-over group and miR-143-3p-low group compared with that in the miR-control transfected group (Fig. 2C), indicating that miR-143-3p had no significant effect on the proliferative capability of hSCAPs.

The effect of miR-143-3p on the odontoblast differentiation potency of hSCAPs was studied. RT-PCR and western blotting results confirmed that the overexpression of miR-143-3p could

191 significantly downregulate the expression levels of NFIC, Krüpple-like factor 4 (KLF4), dentin
192 sialophosphoprotein (DSPP) and upregulate dentin matrix protein 1 (DMP1), and levels of
193 Alkaline phosphatase (ALP), osteocalcin (OCN) and collagen type I (COLLA I) was not
194 significantly changed (Fig. 2D, E). Alizarin red staining (Fig. 2F) and quantitative calcium
195 measurement (Fig. G) demonstrated that hSCAPs with the miR-143-3p over-expression generated
196 less calcium nodules after 15 days of mineralized induction than the control group.
197 Correspondingly, the downregulated miR-143-3p exhibited the opposite effects. To study the
198 effect of miR-143-3p on odontoblast differentiation *ex vivo*, hSCAPs with hydroxyapatite carriers
199 were transplanted into immunocompromised mice. Eight weeks after transplantation, the hSCAPs
200 with the over-expression of miR-143-3p hardly generated shape structures, but dentin-like
201 structures formed in the control-over group with odontoblast-like cells aligned in a layer along the
202 surface and positive to NFIC immunohistochemical straining (Fig. 2H). These results
203 demonstrated that enforced expression of miR-143-3p significantly retarded odonto/osteogenic
204 differentiation of hSCAPs, and the down-regulation could promote this differentiation, and NFIC,
205 KLF4, DSPP and miR-143-3p probably act as a whole especially in the process of hSCAP
206 differentiation.

207 **Figure 2.** The effects of miR-143-3p on the proliferation and differentiation of hSCAPs. (A) Representative
208 photographs of cell fluorescence expression under an inverted microscope with different virus titers. (B) RT-PCR
209 analysis for the expression of miR-143-3p in con-over group, miR-143-3p-over group, con-low group and
210 miR-143-low group, respectively. $^{**}p < 0.01$. (C) Results for the viability of hSCAPs obtained by the colorimetric
211 CCK-8 method, expressed as absorbance values, showed no significant difference in hSCAPs viability between

con-over group and miR-143-3p-over group, or con-low group and miR-143-low group. *p>0.05. (D) RT-PCR analysis for the expression of *NFIC, KLF4, DSPP, DMP1, ALP, OCN* and *COLLAI*. (E) Protein expression levels of NFIC, KLF4, DSP and DMP1 were evaluated by western blotting. (F) Alizarin red staining showed formation of mineralized nodules at day 15. Scale bars= 500um. (G) Quantitative analysis for calcium contents. *p< 0.05. (H) Representative histology of tissue mass generated by miR-143-3p-over-expression hSCAPs and dentin-pulp-like complex with NFIC immunostaining positive odontoblast-like cells produced by con-over hSCAPs after transplantation into the dorsum of immunocomprized mice. (*arrows*, dentin-like structures surrounding pulp-like tissue; HA, hydroxyapatite).

MiR-143-3p directly targets NFIC in hSCAPs differentiation

Four publicly available bioinformatics tools (miRDB, miRWalk, TargetScan, miRTarBase) were used to analyze genes targeted by miR-143-3p. NFIC ranks among the predicted genes and was retrieved in all four databases (Fig. 3A). The expression of NFIC was significantly lower in miR-143-3p overexpressed hSCAPs and increased in miR-143-3p knockdown cells (Fig. 2D, E). Then we obtained the sequence of miR-143-3p (UGAGAUGAAGCACUGUAGCUC) and found the predicted consequential pairing of target region (top) and miRNA (bottom) between NFIC and miR-143-3p on the TargetScanHuman website (Fig. 3B). The luciferase reporter assay was applied to verify whether miR-143-3p could target the 3′UTR of NFIC directly. We cloned the 3′UTR fragment (WT-NFIC) of NFIC containing a miR-143-3p binding site and mutant fragments (MUT-NFIC) into luciferase reporter vectors and found that miR-143-3p could significantly reduce WT-NFIC luciferase activity in hSCAPs but had no effect on that of MUT-NFIC group (Fig. 3C). Next, to further investigate whether miR-143-3p exerted its effects through targeting

NFIC in hSCAPs, a plasmid containing full-length NFIC was transfected into the miR-143-3p overexpressing hSCAPs, then we found that the cells showed the upregulated level of NFIC, DSPP and KLF4, the downregulated DMP1 (Fig. 3D, E) and generated more calcium nodules (Fig. 3F) and higher ALPase activity (Fig. 3G) compared with the miR-143-3p overexpression group and the control group, indicating that the over-expression of NFIC antogonized the effect of miR-143-3p on the differentiation capacity of hSCAP. Taken together, these results identified NFIC as miR-143-3p downstream target genes during hSCAP differentiation.

Figure 3. Identification of NFIC as miR-143-3p target gene. (A) The target genes of miR-143-3p were predicted using publicly available bioinformatics tools (miRDB, miRWalk, TargetScan, miRTarBase). (B) Predicted miR-143-3p binding sites in the 3´ UTR of wild-type (NFIC-3´ UTR-WT) and mutant (NFIC-3´ UTR-MUT) NFIC sequences. (C) Luciferase reporter assays were performed 48h after co-transfection of hSCAPs with control or miR-143-3p mimics and a luciferase vector encoding the wild-type or mutant NFIC 3´ UTR region. *$p < 0.05$. (D) RT-PCR analysis for the expression of *NFIC*, *KLF4*, *DSPP* and *DMP1* in miR-143-3p overexpressing hSCAPs transfected with the plasmid containing full-length NFIC and control plasmid. **$p< 0.01$. (E) NFIC and DSP expression of hSCAPs transfected was examined by western blotting. (F) Mineralization of hSCAPs assessed by alizarin red staining. Calcium nodules in different groups under the inverted microscope. Scale bars = 500um. (G) ALPase activity for hSCAPs after mineralized induction for 2 weeks. **$p < 0.01$.

Discussion

Stem cells from apical papilla (SCAPs) are a population of stem/progenitor cells residing in the apical papilla. As a key event during tooth root development, the differentiation of SCAPs is regulated by various genes and signaling molecules. SCAPs share many features with dental pulp stem cells but also feature their own specific mechanisms at cellular and molecular levels in the dental root formation[13]. Ample evidence suggests that microRNAs are necessary for maintaining homeostasis and proper functionality in many organs and are also implicated in tooth germ development, differentiation and regeneration of pulp-dentin complex and periodontal tissue[14, 15].

In this study, to screen specific miRNAs that function in human tooth root formation, we first performed miRNA microarray assay in roots of human young permanent teeth and found that the expression of miR-143-3p decreased significantly in root pulp compared with that in apical dental mesenchyme. Mineralization induction experiment showed that, miR-143-3p expression was downregulated gradually during hSCAPs differentiation *in vitro*, which showed the opposite trend with NFIC and DSPP, that are regarded as dentin-specific markers of odontoblasts differentiation. In addition, the bioinformatic analysis was performed to suggest NFIC as the possible target gene of miR-143-3p. These results implied that miR-143-3p may act as an essential factor in human tooth root development and hSCAPs differentiation, and is related to the regulation of NFIC. The gene of miR-143-3p is located on human chromosome 5q32 and can be transcribed and processed into two isoforms: miR-143-3p and miR-143-5p. MiR-143-3p is common isoform in normal tissues and highly expressed in mesenchymal cells[16]. MiR-143-3p has been shown to suppress the differentiation of mouse pre-odontoblast cell line through *Klf4* transcription factor signaling pathways[17], but the functions in root dentin development have rarely been reported. As a

DNA-binding transcription factor, NFIC has been considered as a crucial regulator of tooth root formation. *Nfic* knockout mice cannot develop molar roots but crowns are normal, which may result from the interference to the differentiation of odontoblasts due to disruption of NFIC[7, 18]. Our previous studies also demonstrated that NFIC expression was restricted within odontoblasts and preodontoblasts and weakly within the pulp and apical papilla, and was involved in the development of human root dentin and the regulation of odontoblastic differentiation of hSCAPs[9]. Our findings, together with previous reports, suggest that miR-143-3p is likely to be a participant in the regulation of tooth root development through interactions with NFIC. These findings promoted us to hypothesize that miR-143-3p could have a negative effect on hSCAPs differentiation and be related with NFIC signaling pathway. On the other hand, former studies on the regulatory mechanism of *Nfic* mainly focused on its downstream signals[19], for example, *Nfic* activates hedgehog (Hh) attenuator Hhip in the mesenchyme, so that mesenchymal cells respond correctly to the sonic hedgehog (Shh) signal from the epithelium to maintain growth patterns of the apical papilla[20]. Our study provided new insights into understanding the developmental mechanisms of root formation.

miR-143-3p has been found to act as a key regulator in various biological processes and diseases, and become one of the best known of the tumor suppressor miRNA[21] by targeting several oncogenes in various human cancers, such as breast cancer[22], gastric cancer[23], renal cell cancer[24]. miR-143 serum levels were recently suggested as a biomarker for critical illness and sepsis[25]. However, we have not found definite reports on cellular behavior of hSCAPs influenced by miR-143-3p. In the present study, we describe the direct effects of miR-143-3p overexpression and knockdown on the proliferation and differentiation of hSCAPs. The cell proliferation assay

294 based on the CCK-8 marker showed that miR-143-3p is not required for the hSCAPs proliferation.
295 On the other side, we observed that overexpression of miR-143-3p resulted in decreased
296 expression levels of odontogenic markers (NFIC and DSPP) and weakened mineralization ability
297 *in vitro*, manifested by less mineral nodules and weaker ALP staining in mineralized induction.
298 The findings above were consistent with the effects of knockdown NFIC in hSCAPs[9].
299 Furthrtmore, downregulation of miR-143-3p showed the opposite effects on hSCAPs, that is,
300 promoted the odontogenic differentiation and mineralization abilities. When hSCAPs of
301 enforced-expression miR-143-3p were transplanted into the dorsum of immunocompromized mice,
302 dentin-like structure and odontoblast-like cells were not generated and no or weak expression of
303 NFIC were observed. These results demonstrated that miR-143-3p negatively regulated
304 odontogenic differentiation of hSCAPs.

305 Odontoblasts synthesize and secrete dentin extracellular matrix proteins, among which DSPP
306 and DMP1 are considered as typical marker for the odontogenic differentiation of mesenchyma
307 stem cells[26], and DMP1 might regulate the expression of DSPP[27]. Our results showed that the
308 expression of NFIC, DSPP and KLF4 were inhibited and DMP1 was promoted by miR-143-3p.
309 The reason might be the synergistic effect of miR-143-3p on DSPP and DMP1 by directly acting
310 on NFIC. The function of KLF4 as a transcription factor is relative complex, and it is responsible
311 for promoting the differentiation of mouse pre-odontoblast and the expression of Dmp1 and Dspp,
312 but shows inhibitory effect on some embryonic stem cells to maintain their stemness[28]. ALP and
313 OCN expression was not affected by miR-143-3p, which may be because they are more related to
314 the dentin mineralization in the late-stage of root development[29], or associated with regulation by

other post-transcriptional mechanisms. The interaction of these molecules in regulating root development needs further investigation.

MiRNAs lead to translational inhibition of target genes through gene silencing mechanisms. To explore the underlying mechanisms of regulation between miR-143-3p and NFIC, the luciferase reporter assay was used and found that miR-143-3p directly represses the gene expression through binding to 3'-UTRs of NFIC, furthermore, NFIC overexpression reversed the effect of miR-143-3p on the differentiation capacity of hSCAP, indicating that miR-143-3p could inhibit hSCAP differentiation by targeting NFIC. Although some signaling pathways for crown and root development may be similar, NFIC pathway reflects the independence of root development. NFIC knockout may disrupt the nexus of critical signaling pathway specific to tooth root, leading to the absent root phenotype in *Nfic* null mouse[30]. So this study considers that the regulation of miR-143-3p is involved in specific signaling events that are unique to root formation, and reduced miR-143-3p may activate the NFIC signalling pathway during initiation of SCAP differentiation. MiR-143-3p was identified miRNA markers of the human naive, that are specifcally expressed in naive and primed pluripotent states and are downregulated upon diferentiation[31], which is in line with our observation of downregulation during root development, that is, miR-143-3p can maintain the stemness of SCAPs and degrade its expression in the process of differentiation into odontoblasts or pulp cells.

Although previous works have reported miR-143 up-regulation during cell differentiation as promoters of differentiation, such as embryonic stem[32] and smooth muscle cells[33], our data showed that miR-143-3p served as the maintainer of the undifferentiated state of SCAP, which is consistent with the reports where it suppressed the differentiation of mouse pre-odontoblast[17, 34].

Accordingly, miR-143-3p-dependent transcription may modulate in different ways depending on the cell types. For example, miR-143-3p is upregulated with stimulation with TGF-β and may be a mediator of glomerulonephropathy[35], but TGF-β1 down-regulated miR-143 leading to the progression of nasopharyngeal carcinoma[36]. These findings illustrate the dual effects of these multifunctional miRNAs in different biological roles, which may be tissue- and cell-specific. It awaits further investigation to understand the complete mechanisms of root complex formation.

Conclusions

Altogether, our study demonstrated that miR-143-3p maintained the stemness of hSCAPs and negatively modulated their differentiation and mineralization by directly targeting transcription factor NFIC, which serves as an contribution towards a better understanding of the developmental mechanisms of root formation. SCAPs are considered as a more potential cell source for tooth root regeneration, showing to form more uniform dentin-like tissue and possess much higher dentinogenic capacity, an example of which is that SCAPs recombined with biological scaffolds in the empty root canal space could generate bioengineered roots that can provide anchorage for a porcelain crown[37]. It is hoped that the results of this study could promote regenerative medicine, concentrating on guiding differentiation of SCAPs into functional cells to repair damaged tissues.

Ethical approval and consent to participate All procedures were in accordance with the ethical standards of the institutional research committee. Informed written consent was taken from each patient.

Consent for publication Written informed consent for publication was obtained from all participants.

Availability of data and material The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests None declared.

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Authors' contributors SG contributed to implementation of the experiment, the data analysis and manuscript preparation. PL and YL contributed to the material preparation and data collection. WZ, LG and YZ supervised the data collection, data analysis and critical revisions. All authors contributed to the study conception and design and approved the final manuscript.

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Abbreviations

BMP: Bone morphogenetic protein

SHH: Sonic hedgehog

MOI: Multiplicity of infection

WT: Wild-type

MUT: Mutant

KLF4: Krüpple-like factor 4

DSPP: Dentin sialophosphoprotein

DMP1: Dentin matrix protein 1

ALP: Alkaline phosphatase

OCN: Osteocalcin

COLLA I: Collagen type I

HH: Hedgehog

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Figures

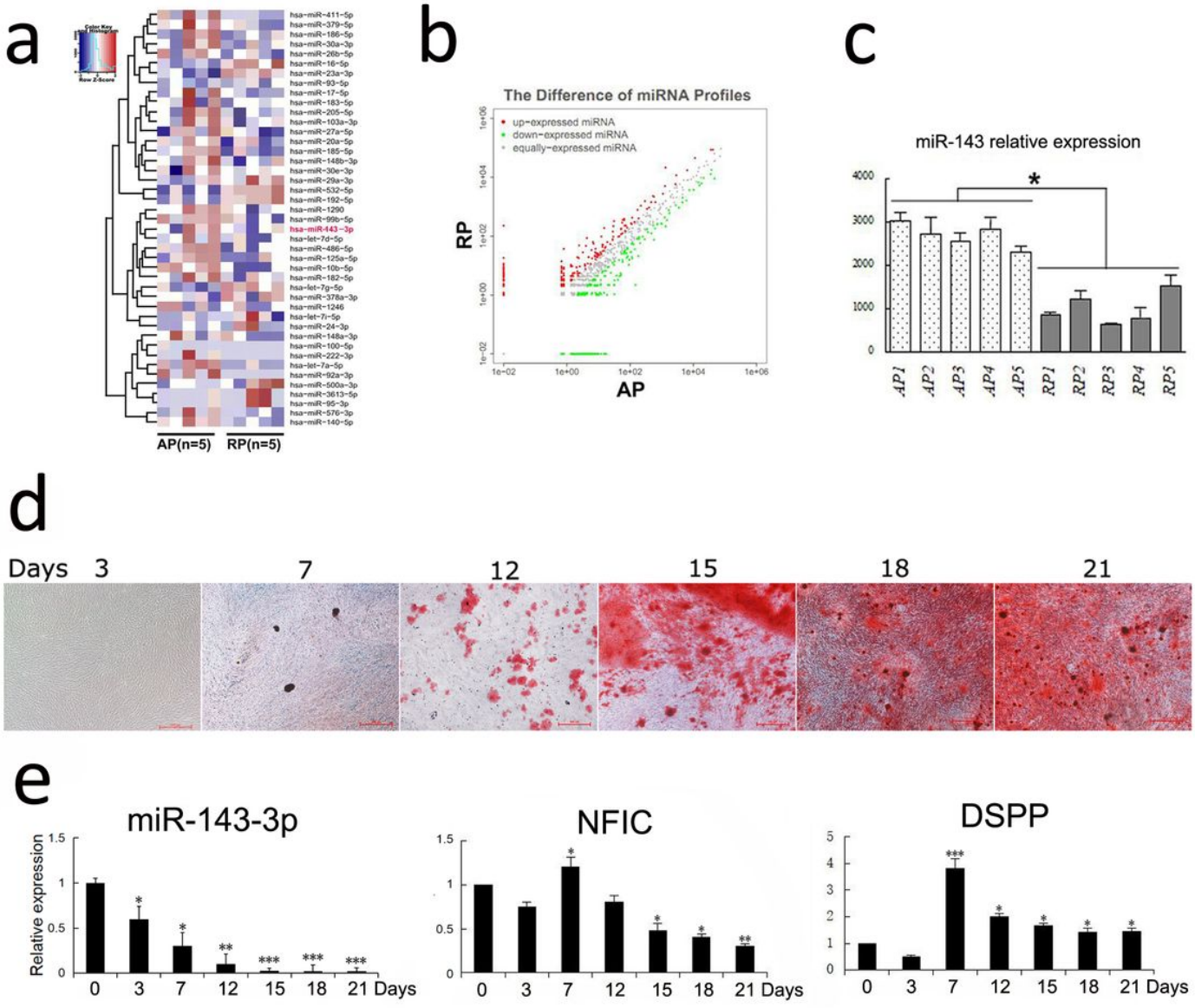

Figure 1

miR-143-3p is associated with hSCAPs differentiation. (A) Heatmap diagram of differential miRNA expression profiles between root pulp (RT) and apical papilla (AP) tissues of young permanent teeth. Red= miRNAs with higher expression, blue= miRNAs with lower expression, and white= miRNAs with equal expression. (B) Differential miRNAs expression profiles between RPs and APs. Red= miRNAs with higher expression, green= miRNAs with lower expression, and gray= miRNAs with equal expression. (C) Real-time PCR analysis of miR-143-3p expression in RPs and APs (n=5). (D) Representative images of calcium nodules in different groups under the inverted microscope. Scale bars= 500um. (E) Expression of miR-143-3p, NFIC and DSPP was evaluated by RT-PCR during hSCAP differentiation. *p <0.05, **p <0.01.

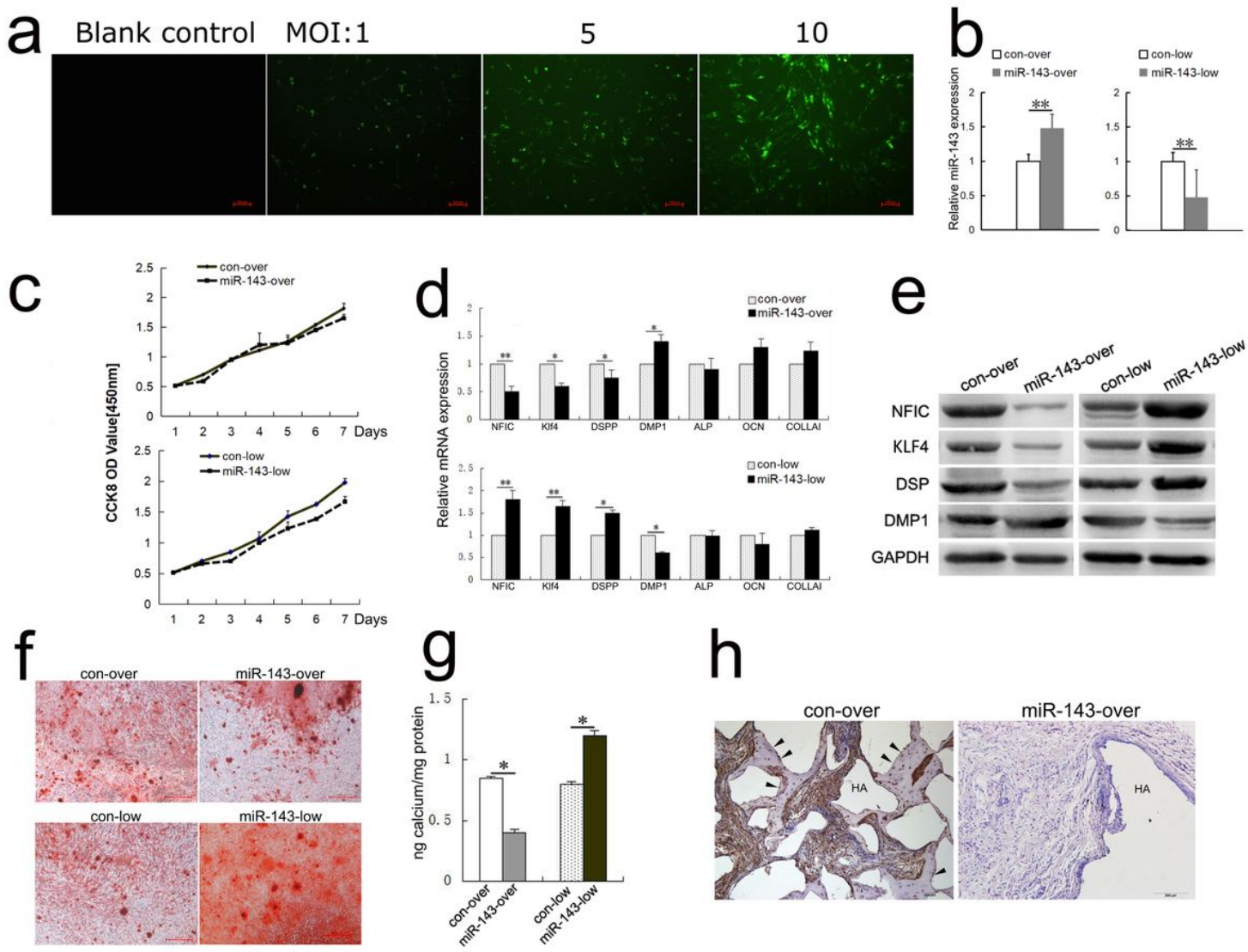

Figure 2

The effects of miR-143-3p on the proliferation and differentiation of hSCAPs. (A) Representative photographs of cell fluorescence expression under an inverted microscope with different virus titers. (B) RT-PCR analysis for the expression of miR-143-3p in con-over group, miR-143-3p-over group, con-low group and miR-143-low group, respectively. **p< 0.01. (C) Results for the viability of hSCAPs obtained by the colorimetric CCK-8 method, expressed as absorbance values, showed no significant difference in hSCAPs viability between con-over group and miR-143-3p-over group, or con-low group and miR-143-low group. 212 *p>0.05. (D) RT-PCR analysis for the expression of NFIC, KLF4, DSPP, DMP1, ALP, OCN and COLLAI. (E) Protein expression levels of NFIC, KLF4, DSP and DMP1 were evaluated by western blotting. (F) Alizarin red staining showed formation of mineralized nodules at day 15. Scale bars= 500um. (G) Quantitative analysis for calcium contents. *p< 0.05. (H) Representative histology of tissue mass generated by miR-143-3p-over-expression hSCAPs and dentin-pulp-like complex with NFIC immunostaining positive odontoblast-like cells produced by con-over hSCAPs after transplantation into

the dorsum of immunocomprized mice. (arrows, dentin-like structures surrounding pulp-like tissue; HA, hydroxyapatite).

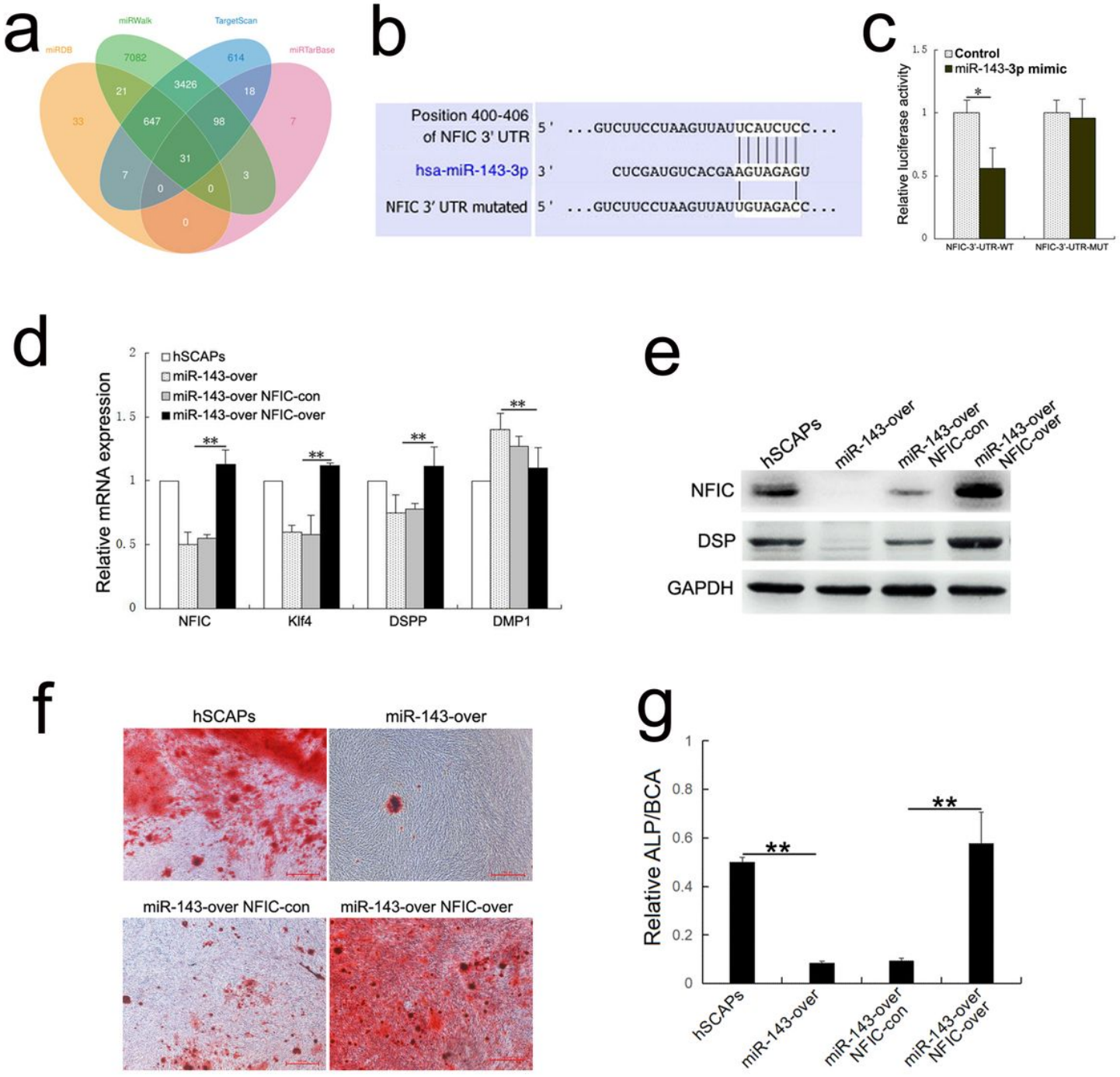

Figure 3

Identification of NFIC as miR-143-3p target gene. (A) The target genes of miR-143-3p were predicted using publicly available bioinformatics tools (miRDB, miRWalk, TargetScan, miRTarBase). (B) Predicted miR-143-3p binding sites in the 3´UTR of wild-type (NFIC-3´UTR-WT) and mutant (NFIC-3´UTR-MUT) NFIC sequences. (C) Luciferase reporter assays were performed 48h after co-transfection of hSCAPs with control or miR-143-3p mimics and a luciferase vector encoding the wild-type or mutant NFIC 3´UTR

region. *p < 0.05. (D) RT-PCR analysis for the expression of NFIC, KLF4, DSPP and DMP1 in miR-143-3p overexpressing hSCAPs transfected with the plasmid containing full-length NFIC and control plasmid. **p< 0.01. (E) NFIC and DSP expression of hSCAPs transfected was examined by western blotting. (F) Mineralization of hSCAPs assessed by alizarin red staining. Calcium nodules in different groups under the inverted microscope. Scale bars = 500um. (G) ALPase activity for hSCAPs after mineralized induction for 2 weeks. **p < 0.01.