

Nuclear Factor I-C promotes proliferation and differentiation of apical papilla-derived human stem cells in vitro

Zhang, Jing; Wang, Zhihua; Jiang, Yong; Niu, Zhongying; Fu, Lei; Luo, Zhirong; Cooper, Paul; Smith, Anthony; He, Wenxi

DOI:

[10.1016/j.yexcr.2015.01.020](https://doi.org/10.1016/j.yexcr.2015.01.020)

License:

Other (please specify with Rights Statement)

Document Version

Peer reviewed version

Citation for published version (Harvard):

Zhang, J, Wang, Z, Jiang, Y, Niu, Z, Fu, L, Luo, Z, Cooper, P, Smith, A & He, W 2015, 'Nuclear Factor I-C promotes proliferation and differentiation of apical papilla-derived human stem cells in vitro', *Experimental Cell Research*, vol. 332, no. 2, pp. 259-266. <https://doi.org/10.1016/j.yexcr.2015.01.020>

[Link to publication on Research at Birmingham portal](#)

Publisher Rights Statement:

NOTICE: this is the author's version of a work that was accepted for publication. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published as Jing Zhang, Zhihua Wang, Jiang Yong, Zhongying Niu, Lei Fu, Zhirong Luo, Paul R. Cooper, Anthony J. Smith, Wenxi He, Nuclear factor I-C promotes proliferation and differentiation of apical papilla-derived Human stem cells in vitro, *Experimental Cell Research*, <http://dx.doi.org/10.1016/j.yexcr.2015.01.020>

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Author's Accepted Manuscript

Nuclear factor I-C promotes proliferation and differentiation of apical papilla-derived Human stem cells in vitro

Jing Zhang, Zhihua Wang, Jiang Yong, Zhongying Niu, Lei Fu, Zhirong Luo, Paul R. Cooper, Anthony J. Smith, Wenxi He



www.elsevier.com/locate/yexcr

PII: S0014-4827(15)00040-3
DOI: <http://dx.doi.org/10.1016/j.yexcr.2015.01.020>
Reference: YEXCR9867

To appear in: *Experimental Cell Research*

Received date: 21 October 2014
Revised date: 26 January 2015
Accepted date: 31 January 2015

Cite this article as: Jing Zhang, Zhihua Wang, Jiang Yong, Zhongying Niu, Lei Fu, Zhirong Luo, Paul R. Cooper, Anthony J. Smith, Wenxi He, Nuclear factor I-C promotes proliferation and differentiation of apical papilla-derived Human stem cells in vitro, *Experimental Cell Research*, <http://dx.doi.org/10.1016/j.yexcr.2015.01.020>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting galley proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Nuclear Factor I-C promotes proliferation and differentiation of apical papilla-derived human stem cells in vitro

Jing Zhang, DDS, PhD^{1a,2}, Zihua Wang, DDS, MS^{1a}, Jiang Yong, DDS, MS^{1a}, Zhongying Niu DDS, PhD³, Lei Fu DDS, MS¹, Zhirong Luo, DDS, MS¹, Paul R. Cooper PhD⁴, Anthony J. Smith PhD⁴ and Wenxi He DDS, PhD^{1*}

1. State Key Laboratory of Military Stomatology, Department of Operative Dentistry & Endodontics, School of Stomatology, The Fourth Military Medical University, Xi'an, China
2. Stomatologic Hospital & College, Anhui Medical University, Key Lab of Oral Diseases Research of Anhui Province, Hefei, China
3. Treatment center of oral diseases, The 306th Hospital of People's Liberation Army, Beijing, China
4. Oral Biology, School of Dentistry, University of Birmingham, B4 6NN, UK

^aThese authors contributed equally to this work.

* Corresponding Author: Wenxi He, 145 Chang-le Xi Road, Xian 710032, PR China. Tel.: +86-29-84776476 (o), Fax: +86-29-84776476 (o); E-mail: hewenxi@fmmu.edu.cn;

Introduction:

Tooth formation is a complex developmental process mediated through a series of epithelial-mesenchymal interactions, the regulatory mechanisms for which have been extensively studied in the crown of the tooth. However, the mechanisms involved in the regulation of tooth root development are relatively poorly understood. Stem cells from the apical papilla (SCAPs) residing in the root apex of immature permanent teeth have been implicated in the development of the radicular pulp and primary odontoblasts, which contribute to the formation of root dentin [1]. Removal of the apical papilla from molars at an early age of root development in minipigs abrogated root development despite the remaining pulp tissue being intact [2]. *Ex vivo* expanded SCAPs combined with biological scaffolds can regenerate dentin-pulp-like tissues and bioengineered root tissue *in vivo* and these cells have been suggested to be a superior source for dental tissue engineering applications [3].

During tooth morphogenesis, Nuclear Factor I-C (NFIC) has been reported to be a critical regulator of root formation [4]. The Nuclear Factor I (NFI) family of transcription factors includes four members in vertebrates (NFIA, NFIB, NFIC and NFIX), which share a highly conserved N-terminal DNA-binding domain [5]. Involvement of NFI subtypes in organ development has been proposed based on the specific disruption of distinct subtypes of NFI in mice and the resultant phenotypes [6-8]. Interestingly, disruption of NFIC most prominently interferes with the differentiation of preodontoblasts into odontoblasts resulting in defects in root development in mice [4, 9]. The affected odontoblasts show loss of intercellular junctions, decreased expression of ZO-1 and occludin, morphological disruption and exhibit abnormal dentin formation [10]. These data highlight the critical role that NFIC plays in the terminal differentiation and function of odontoblasts in root formation.

Despite the importance of NFIC in physiological root development, there is a lack of information on the influence of this transcription factor on the biologic functions of SCAPs. Thus, the aim of this study was to investigate the role of NFIC in the proliferation and differentiation

regulation of SCAPs and importantly, to explore whether it may be an effective therapeutic target for pulp regeneration and root dentin tissue engineering.

Materials and Methods

Isolation of human SCAPs and culture

The apical papilla of human third molar teeth still undergoing root development were collected from patients aged 16-18 years with informed consent using a protocol approved by the Institutional Review Board of the Fourth Military Medical University. Briefly, root apical papilla tissue was dissected from the extracted teeth, minced and digested in a solution of 3mg/ml collagenase type I and 4mg/ml dispase (both from Invitrogen, Carlsbad, USA) for 45 minutes at 37 °C. Single cell suspensions of SCAPs were obtained by passing the digest through a 70 µm strainer (Falcon, BD Labware, Franklin Lakes, NJ), followed by seeding of 1×10^4 cells in 10-cm culture dishes, and cultured in alpha-modification of Eagle's Medium (α -MEM; Hyclone, Logan, USA) supplemented with 15% fetal bovine serum (Hyclone), 100 units/mL penicillin streptomycin (Roche, Basel, Switzerland) and 100 mM/L ascorbic acid (Roche) at 37 °C in 5% CO₂ in air. Subculturing was at a ratio of 1:3 when cultures reached 80-90% confluence. Culture medium was changed every 2–3 days. Cultured SCAPs were used in the study at passages 2-6 and all experiments were performed at least in triplicate.

Immunocytochemical staining

To identify the mesenchymal origin of the putative SCAPs, cells were seeded in a six-well plate at 2×10^4 cells/well. After 24 hours of culture, the cells were washed in phosphate-buffered saline (PBS, pH 7.4) and fixed in 4% phosphate-buffered paraformaldehyde at room temperature (RT) for 30 min and then immersed in 3% H₂O₂/methanol for 15 minutes to quench the endogenous peroxidase activity. After blocking with 5% bovine serum albumin for 20 min, the samples were incubated with primary anti-human vimentin antibody (1:200 dilution) (Boster, Wuhan, China) overnight at 4°C. Following washing in PBS three times, the cells were stained using the broadspectrum immunoperoxidase ABC kit (Boster) and the chromogen 3, 3-Diaminobenzidine tetrahydrochloride (Zsgb-Bio, Beijing, China) prior to counterstaining with hematoxylin.

Multi-lineage differentiation in vitro

Cells were cultured in 6-well plates in osteo/odontogenic differentiation medium containing 50 mg/ml ascorbic acid, 10mM β -glycerophosphate, and 10nM dexamethasone (all from Sigma-Aldrich, St. Louis, USA) with 10% FBS for 4weeks. Cultures were rinsed twice in PBS and fixed with 4% polyoxymethylene for 15 minutes. The cells were washed with ddH₂O and stained with alizarin red S (Sigma-Aldrich) (pH 4.2) at the end of the culture period time. Photomicrographs of mineralized nodules were captured and then 500µl 10% cetylpyridinium chloride was added to each well to dissolve the nodules. Aliquots (100µL) of the supernatant were then measured spectrophotometrically at 562 nm. For adipogenic induction, the cells were incubated in adipogenic medium containing 1µM dexamethasone, 0.2 mM indomethacin, 1µg/ml insulin, 0.5 mM isobutyl-methylxanthine (IBMX), and 50 mM indomethacin (all from Sigma-Aldrich), and 10% FBS for 5 weeks. The presence of lipid droplets was identified by staining with 0.3% (w/v) Oil-Red O (Sigma-Aldrich)/60% isopropanol reagent for 60 min

followed by washing with water.

Flow cytometric analysis of stem cell surface markers

For flow cytometric analysis, cells were harvested by trypsinization and washed followed by fixation with 4% paraformaldehyde for 30 min at room temperature. Subsequently, cells were stained with the fluorochrome-conjugated mouse anti-human antibodies CD90, CD105, CD34 and CD45 (Becton & Dickinson, CA, USA) in PBS containing 3% FBS for 1h in the dark at room temperature. After staining, cells were washed twice with ice-cold PBS and centrifuged at 1000×g for 5 min. Samples were analyzed with a BD LSR II Flow Cytometer (BD Biosciences) and the data were analyzed with a Mod-Fit 2.0 cell cycle analysis program (Becton & Dickinson).

Lentivirus production and virus infection

A pCMV6-XL4 plasmid expression vector for human NFIC (GenBank accession No: NG_030333.1) was purchased from Origene (Rockville, USA). After digestion with EcoRI and NotI (Takara, Otsu, Japan), the fragment was subcloned into the Lentiviral expression vector pLenti6.3/V5-DEST (Invitrogen) to give pLenti6.3-NFIC (P-N). The empty vector (pLenti-Cherry) (P-C) (Invitrogen) was used as a control. All vector constructs were confirmed by PCR and DNA sequencing (data not shown).

HEK293T cells were seeded at a density of 7×10^5 per 6 cm tissue culture plate and cultured overnight without antibiotics. pLenti6.3-NFIC (P-N) or pLenti-Cherry (P-C) together with the packaging plasmid and envelope plasmid were added to the medium using Lipofectamine 2000 (Invitrogen). After 48h, viral supernatants were harvested and filtered through a 0.45 μ m filter. The viruses were concentrated with the Lenti-X concentrator (Clontech, Mountain View, USA) and stored at -80 °C. SCAPs from the 3rd passage at 90% confluence in six-well plates were transduced with the concentrated virus at 1ml/well in the presence of 6 μ g/mL Polybrene (Sigma-Aldrich). Transduced cells were selected with 4 μ g/ml blasticidin (Invitrogen) to obtain stable transfectants expressing the NFIC protein.

MTT assay

SCAPs transduced with NFIC were seeded into 96-well plates at a density of 3×10^3 cells/well. The cells transduced with pLenti-Cherry (P-C) or normal untreated cells both acted as control groups. After incubation in medium containing 2% FBS at the different times indicated, the cultures were supplemented with 20 μ L 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (5g/L; Sigma-Aldrich) and incubated for 4 hours. The supernatants were then removed and 150 μ L dimethyl sulfoxide was added to each culture and incubated for 10 minutes with gently agitation. The MTT assay was quantified spectrophotometrically at a wavelength of 490 nm (Powerwave 340; Bio-tek, Winooski, USA) and the assay was performed in triplicate.

ALP activity assay

The ALP (alkaline phosphatase) activity of SCAPs transduced with plenti-NFIC (P-N) or plenti-Cherry (P-C) was assayed. Cells were seeded at 2×10^4 cells/well in 96-well plates with standard medium. After an attachment period of 24 hours, the medium was changed to osteo/odontogenic differentiation medium. After culture for 1, 4, 7 and 14 days, cells were rinsed in PBS and treated with 1% Triton X-100 at 37 °C. The ALP activity of the cell lysate was assayed

with the ALP Activity Assay Kit (Jiancheng, Nanjing, China) according to the manufacturer's protocol. After incubation, the absorbance of each sample was measured at 520 nm with an automatic microplate reader (Bio-tek, Winooski, USA).

Quantitative Reverse Transcriptase Polymerase Chain Reaction

SCAPs were transduced with pLenti6.3-NFIC (P-N) or pLenti-Cherry (P-C), respectively. After cells had been cultured in osteo/odontogenic media for the times indicated, total RNA was isolated from SCAPs using the RNeasy Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. cDNA was synthesized from 1 μ g RNA using the SuperScript III System (Invitrogen) followed by DNase treatment using the Qiagen RNAeasy Mini kit. Quantitative real-time PCR (RT-PCR) gene expression analyses were performed on triplicate samples with SYBR Green (Takara) using the 7900 RT-PCR System (Applied Biosystems, Foster City, USA) over 40 cycles (95 °C/5s, 60 °C/34s) after an initial degradation step of 95 °C for 30s. Specific primers were purchased from Sangon (Shanghai, China), as human ALP (alkaline phosphatase) (Forward: CCACGTCTTCACATTTGGTG Reverse: AGACTGCGCCTGGTAGTTGT); OCN (osteocalcin) (Forward: GGCAGCGAGGTAGTGAAGAG Reverse: CTGGAGAGGAGCAGAAGCTGG); Col I (collagen type I) (Forward: AAGGACAAGAGGCACGTCTG Reverse: CGCTGTTCTTGCAGTGGTAG); NFIC (Forward: GGACAGGGATGGGCTCTG Reverse: CGTTCTTCTGAGGCCAGTGC). Data acquisition and analyses were performed with the Sequence Detection System Version 2.3 software (Applied Biosystems). The expression level of GAPDH (Forward: GCACCGTCAAGGCTGAGAAC Reverse: TGGTGAAGACGCCAGTGGA) was used as an internal control.

Western blot analysis

SCAPs transduced with pLenti6.3-NFIC (P-N) or pLenti-Cherry (P-C) were collected after 1, 2 and 3 weeks, then cells were washed three times with ice-cold phosphate-buffered saline and subjected to lysis with ice-cold radioimmunoprecipitation (RIPA) lysis buffer (Santa Cruz Biotechnology, CA, USA) containing protease inhibitors. The proteins (30 μ g) were separated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking in Tris-Buffered Saline with Tween (TBST) containing 5% non-fat dry milk, the membrane was incubated overnight at 4 °C with a primary antibody against NFIC or DSP (dentin sialoprotein) (1:400 dilution) (Santa Cruz Biotechnology, CA, USA). After washing, the membrane was reacted with a horseradish peroxidase-conjugated secondary antibody at 1:5000 (Santa Cruz) at room temperature for 1 hour. Protein bands were finally visualized on X-ray film using the ECL system (GE Healthcare, Buckinghamshire, UK). β -actin (1:1000; Abgent, Flanders Count, USA) served as an internal control in these experiments.

siRNA transfection

Cells were transfected with specific-labelled siRNA (100 nM final concentration) (RiboBio, Guangzhou, China) targeting NFIC mRNA (si-NFIC), while control cells were transfected with non-specific siRNA (si-CON) to provide a negative control. The sequences of the sense and antisense strands of the human si-NFIC were as follows: forward:

5'-GAGCGAGAUGCAGAGCAAA-3' (sense) and 5'-UUUGCUCUGCAUCUCGCUC-3' (antisense). The transfection was performed according to the manufacturer's instructions. Briefly, 8×10^4 cells were seeded in six-well culture plates without antibiotics and incubated with siRNA diluted with Opti-Mem (Invitrogen) for 6h according to the manufacturer's instructions. After transfection, cells were cultured in 6-well plates at 37 °C until required.

Statistical Analysis

Data obtained from this study are expressed as the mean \pm standard deviation (SD) from at least three independent experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) or Student's t-test with a significance level of $p < 0.05$.

Results

Characterization of isolated SCAPs

The apical papilla tissue of extracted immature teeth was easily identified (Fig. 1A) prior to its dissection and subsequent isolation of SCAPs. Immunocytochemical staining revealed that the *in vivo*-expanded cells were positive for the mesenchymal cell marker vimentin (Fig. 1B and C). The isolated cells demonstrated pluripotential capacity and were able to differentiate along several cell-restricted lineages. Under appropriate inductive conditions, the isolated cells formed extensive mineralized nodules stained by Alizarin red S after 4 weeks of culture in odontogenic/osteogenic medium (Fig. 1D) and stained positively for lipid droplets with Oil-Red O after 5 weeks culture in adipogenic medium (Fig. 1E). Flow cytometric analysis further corroborated the characteristics of the cells as those resembling SCAPs with strong expression of the putative MSC associated markers CD90 and CD105 (Fig. 1F and G) and the absence of expression of the hematopoietic markers CD45 and CD34 (Figs. 1H and I).

NFIC promotes the proliferation of SCAPs *in vitro*

The MTT assay was performed to investigate the effect of NFIC on the proliferation of SCAPs. Cells were transduced with pLenti6.3-NFIC (P-N) to overexpress NFIC protein, and the cells transduced with empty vector pLenti-Cherry (P-C) were treated as control. NFIC protein levels were analyzed by Western blot. The results showed overexpression of NFIC (P-N) significantly increased the expression of NFIC protein (Figs. 1J and K). Cell proliferation was markedly increased in the P-N group at both 5 days and 7 days compared with normal untreated cells or the P-C group (Figure 1L).

NFIC promotes osteo/odontogenic differentiation of SCAPs *in vitro*

To further evaluate the functional role of NFIC, the formation of mineralization nodules was analyzed at times of up to 21 days in culture. Mineralized nodules were clearly formed in a time-dependent manner in cells cultured with osteo/odontogenic differentiation medium and were more extensive in the P-N group than the control group (Figs. 2A and B). The calcium concentrations were consistent with the Alizarin red staining and were markedly higher in the P-N group at 14 days and 21 days compared with the control (Fig. 2C). After osteo/odontogenic induction for 14 days, the ALP activity was much greater in the P-N group compared with the P-C group (Fig. 2D). To verify that NFIC had stimulated odontoblastic differentiation in SCAPs, we examined the expression of appropriate markers by RT-PCR and Western blot analysis. The

mRNA expression of Col I (collagen type I) was significantly increased in the P-N group after osteo/odontogenic induction for 1 week and 2 weeks (Fig. 3A and B), although differences were not statistically significant at 3 weeks (Fig. 3C). OCN (osteocalcin) was significantly up-regulated in the P-N group at each time interval, especially at 3 weeks (Fig. 3A-C). Consistent with the ALP (alkaline phosphatase) activity assay (Fig. 2D), the ALP mRNA peaked in the P-N group at week 2 and continued at an elevated level to week 3 (Fig. 3A-C). In addition, the protein expression of DSP (dentin sialoprotein) was also enhanced in the P-N group over the culture period after osteo/odontogenic induction (Fig. 3D), although the increase at week 3 relative to the control was relatively minimal.

Knockdown of NFIC inhibits odontogenic differentiation in SCAPs

To determine the effect of NFIC on the differentiation of SCAPs, we initially compared the odontoblastic differentiation of SCAPs knocked down for NFIC expression by transfection with si-RNA (si-NFIC) or control siRNA vector (si-CON). Mineralized nodules formation was decreased in knockdown NFIC cells compared with the control (Fig. 4A-D) and this was associated with lower calcium concentrations in the knockdown NFIC cells (Fig. 4E) after cultured in osteo/odontogenic differentiation medium for 2 weeks. Expression levels of osteo/odontogenic markers, including ALP, OCN and Col I, assessed by RT-PCR showed that treatment with si-NFIC blocked the mRNA expression of these markers for ALP, OCN and Col I at 1 week and 2 weeks (Fig. 4F and G).

Discussion

Growing evidence has highlighted the role of the transcription factor NFIC in tooth root development [4, 11]. Disruption of NFIC in mice causes development of short molar roots with aberrant odontoblasts and abnormal dentin formation [10]. SCAPs isolated from the root tip have significant potential to differentiate into odontoblast-like cells and form ectopic dentin emphasizing their potential clinical therapeutic application in “bio-root” tissue engineering [12]. Cell-based gene therapy can overcome some of the limitations of direct administration of stimulatory factors for tissue engineering and regenerative strategies, providing specific and continued transgene expression [13]. In view of the importance of NFIC in root formation, we hypothesized that NFIC has specific effects on SCAPs behaviour and may be a valuable target for regenerative strategies.

It was recently demonstrated that knockout of NFIC increased the expression of cell cycle inhibitors such as p21 and p16, but decreased expression of cyclin D1 and cyclin B1 in pulp cells, leading to the suppression of odontogenic cell proliferation and differentiation [11]. These effects were considered responsible for the apoptosis of aberrant odontoblasts during root formation, thereby contributing to the formation of short roots [11]. Similarly, lack of NFIC resulted in a delay in progenitor cell proliferation and hair follicle regeneration in mice, indicating impaired activation of the hair follicle cycle [14]. In the present study, a plasmid over-expressing NFIC was constructed and transduced into SCAPs to obtain cells with over-expressed NFIC protein. Over-expression of NFIC increased the proliferation of SCAPs, while transduction of empty vector had minimal effect on the proliferation of SCAPs, indicating that the presence of the virus had little impact on cell growth.

To investigate whether NFIC was involved in odontoblastic differentiation of SCAPs, we

first assessed mineralization by alizarin red staining after NFIC activation. Results demonstrated that NFIC markedly promoted mineralized nodule formation, and this was confirmed by quantification of calcium following the extraction of the alizarin red stain. Moreover, ALP activity is also recognized as a characteristic marker of the early stages of odontoblastic and mineralized cell differentiation. Consistently, increases in ALP activity paralleled the increased calcium levels induced by NFIC. In addition, NFIC up-regulated the mRNA expression of several osteo/odontogenic-related markers (ALP, OCN and Col I). Indeed both ALP and Col I are considered as early markers for osteoblast or odontoblast differentiation and are expressed by cells undergoing mineralization, while OCN was used as a marker of late stage differentiation [15, 16]. In our study, ALP and Col I mRNA were significantly enhanced from week 1 to 2 while OCN was consistently up-regulated in the P-N group. While mineralized matrix proteins, such as ALP, OCN and Col I, are expressed by both odontoblasts and osteoblasts, dentin sialoprotein (DSP) is considered as a representative marker for odontogenic differentiation and is derived from dentin sialophosphoprotein (DSPP) [17]. Although DSP is not exclusively expressed in odontoblasts, there is considerable evidence supporting its involvement in the nucleation and control of hydroxylapatite mineral formation during dentin calcification [18]. Thus, the up-regulation of DSP in the P-N group suggested that NFIC can enhance the odontogenic differentiation of SCAPs. Indeed our previous findings have demonstrated that transforming growth factor- β (TGF- β) down-regulated DSP in a Smad3-dependent manner in odontoblasts [19]. However, NFIC induces dephosphorylation of p-Smad2/3 to prevent TGF- β signaling from translocating to the nucleus [20]. Furthermore, NFIC, as a transcription factor, is known to directly binds the DSPP promoter and activate the transcription of the DSPP gene in a mouse odontoblast cell line [21]. Therefore, we anticipated that NFIC may promote the differentiation of SCAPs to odontoblasts through mechanisms partly involving both direct and indirect interactions with DSP. Indeed NFIC enhanced DSP protein expression in both normal and mineralization medium compared with control cells (P-C group) respectively, suggesting that NFIC is an up-stream regulator of DSP.

To further examine the effect of NFIC on differentiation of SCAPs, NFIC gene silencing was also undertaken. Knockdown cells formed fewer mineralized nodules and exhibited inhibited ALP and Col. I expression at 1 week and 2 weeks suggesting that odontoblastic differentiation of SCAPs was suppressed by NFIC inhibition.

In the present study, we have demonstrated that the proliferation and multi-lineage differentiation of SCAPs are enhanced by NFIC activation, while suppressed by NFIC gene silencing, indicating that NFIC gene activity in SCAPs may be a potential therapeutic target for dental tissue regeneration, which need to be further confirmed by additional *in vivo* experiment in future.

Acknowledgments

This work was supported by grant 81070831, 81470733, 81271125, 81400497 from the Chinese National Natural Science Foundation and Changjiang Scholars and Innovative Research Team in University (No. IRT13051).

References

- [1] Huang GT, Gronthos S and S. S. (2009). Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. *J Dent Res.* **88**: 792-806.
- [2] Huang GT, Sonoyama W, Liu Y, Liu H, Wang S and S. S. (2008). The hidden treasure in apical papilla: the potential role in pulp/dentin regeneration and bioroot engineering. *J Endod.* **34**: 645-651.
- [3] Huang GT, Yamaza T, Shea LD, Diouad F, Kunhn NZ, Tuan RS and S S. (2010). Stem/progenitor cell-mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model. *Tissue Eng Part A.* **16**: 605-615.
- [4] Steele-Perkins G, Butz KG, Lyons GE, Zeichner-David M, Kim HJ, Cho MI and G RM. (2003). Essential role for NFI-C/CTF transcription-replication factor in tooth root development. *Mol Cell Biol.* **23**: 1075-1084.
- [5] Gronostajski RM. (2000). Roles of the NFI/CTF gene family in transcription and development. *Gene.* 249:31-45.
- [6] Das Neves L, Duchala CS, Tolentino-Silva F, Haxhiu MA, Colmenares C, Macklin WB, Campbell CE, Butz KG and G RM. (1999). Disruption of the murine nuclear factor I-A gene (Nfia) results in perinatal lethality, hydrocephalus, and agenesis of the corpus callosum. *Proc Natl Acad Sci U S A.* **96**:11946-11951.
- [7] Steele-Perkins G, Plachez C, Y. Butz KG, Yang G, Bachurski CJ, Kinsman SL, Litwack ED, Richards LJ and G RM. (2005). The transcription factor gene Nfib is essential for both lung maturation and brain development. *Mol Cell Biol.* **25**: 685-698.
- [8] Driller K, Pagenstecher A, Uhl M, Omran H, Berlis A, Grunder A and S AE. (2007). Nuclear factor I X deficiency causes brain malformation and severe skeletal defects. *Mol Cell Biol.* **27**:3855-3867.
- [9] Lee TY, Lee DS, Kim HM, Ko JS, Gronostajski RM, Cho MI, Son HH and P JC. (2009). Disruption of Nfic causes dissociation of odontoblasts by interfering with the formation of intercellular junctions and aberrant odontoblast differentiation. *J Histochem Cytochem.* **57**: 469-476.
- [10] Park JC, Herr Y, Kim HJ, Gronostajski RM and C. MI. (2007). Nfic gene disruption inhibits differentiation of odontoblasts responsible for root formation and results in formation of short and abnormal roots in mice. *J Periodontol.* **78** :1795-1802.
- [11] Lee DS, Park JT, Kim HM, Ko JS, Son HH, Gronostajski RM, Cho MI, Choung PH and P JC. (2009). Nuclear Factor I-C Is essential for odontogenic cell proliferation and odontoblast differentiation during tooth root development. *J Biol Chem.* **284**:17293-17303.
- [12] Sonoyama W, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, Liu H, Gronthos S, Wang CY, Wang S and S S. (2006). Mesenchymal stem cell-mediated functional tooth regeneration in swine. *PLoS One.* **1**: e79.
- [13] Kim JJ, Kim SJ, Kim YS, Kim SY, Park SH and K. EC. (2012). The role of SIRT1 on angiogenic and odontogenic potential in human dental pulp cells. *J Endod.* **38**:899-906.
- [14] Plasari G, Edelmann S, Högger F, Dusserre Y, Mermod N and C. A. (2010). Nuclear factor I-C regulates TGF- β -dependent hair follicle cycling. *J Biol Chem.* **285**: 34115-34125. .
- [15] Rickard DJ, Sullivan TA, Shenker BJ, Leboy PS and K. I. (1994). Induction of rapid osteoblast differentiation in rat bone marrow stromal cell cultures by dexamethasone and BMP-2.

Dev Biol. **161**: 218-228.

[16] Lesot H, Osman M and R. JV. (1981). Immunofluorescent localization of collagens, fibronectin, and laminin during terminal differentiation of odontoblasts. Dev Biol. **82**: 371-381.

[17] B. WT., Dentin specific proteins. (1987). Methods Enzymol. **145**: 290-303.

[18] Qin C, Brunn JC, Cadena E, Ridall A, Tsujigjwa H, Nagatsuka H, Nagai N and B WT. (2002). The expression of dentin sialophosphoprotein gene in bone. J Dent Res. **81**: 392-394.

[19] He WX, Niu ZY, Zhao SL, Jin WL, Gao J and S. AJ. (2004). TGF-beta activated Smad signaling leads to a Smad3-mediated down-regulation of DSPP in an odontoblast cell line. Arch Oral Biol. **49**: 911-918.

[20] Lee DS, Yoon WJ, Cho ES, Kim HJ, Gronostajski RM, Cho MI and P JC. (2011). Crosstalk between nuclear factor I-C and transforming growth factor- β 1 signaling regulates odontoblast differentiation and homeostasis. PLoS One. **6**: e29160.

[21] Oh HJ, Lee HK, Park SJ, Cho YS, Bae HS, Cho MI and P JC. (2012). Zinc balance is critical for NFI-C mediated regulation of odontoblast differentiation. J Cell Biochem. **113**: 877-887.

[22] Wang S, Mu J, Fan Z, Yu Y, Yan M, Lei G, Tang C, Wang Z, Zheng Y, Yu J and Z G. (2012). Insulin-like growth factor 1 can promote the osteogenic differentiation and osteogenesis of stem cells from apical papilla. Stem Cell Res. **8**: 346-356.

[23] Wu J, Huang GT, He W, Wang P, Tong Z, Jia Q, Dong L, Niu Z and N L. (2012). Basic fibroblast growth factor enhances stemness of human stem cells from the apical papilla. J Endod. **38**: 614-622.

[24] Liu Y, Chen C, He H, Wang D, E L, Liu Z and L H. (2012). Lentiviral-mediated gene transfer into human adipose-derived stem cells: role of NELL1 versus BMP2 in osteogenesis and adipogenesis in vitro. Acta Biochim Biophys Sin. **44**: 856-865.

Figure Legends

Fig. 1. Characterization of SCAPs and effects of NFIC on the proliferation of SCAPs. A: Root apical papilla from an extracted immature third molar (arrow). B-C: SCAPs stained positive for vimentin by immunocytochemistry. D: SCAPs formed mineralized nodules stained by Alizarin red S after culture in osteo/odontogenic medium for 4 weeks. E: Oil red O staining after adipogenic induction for 5 weeks. F-I: Flow cytometric analysis of SCAPs for molecular surface antigen markers. (P2 refers to the positive percentage for the antigen; F:CD90; G:CD105; H:CD45; I:CD34); J-K: NFIC levels were analyzed and quantified in P-N or P-C cells L: Cell proliferation was assessed for proliferation by MTT assay (* $P < 0.05$, ** $P < 0.01$). Scale bar for upper and lower panel represents 500 μ m, 100 μ m and 50 μ m respectively. P-N, SCAPs over-expressing NFIC protein; P-C, SCAPs transduced with empty vector pLenti-Cherry; Ctrl, normal untreated cells. P-C and Ctrl were both used as control groups.

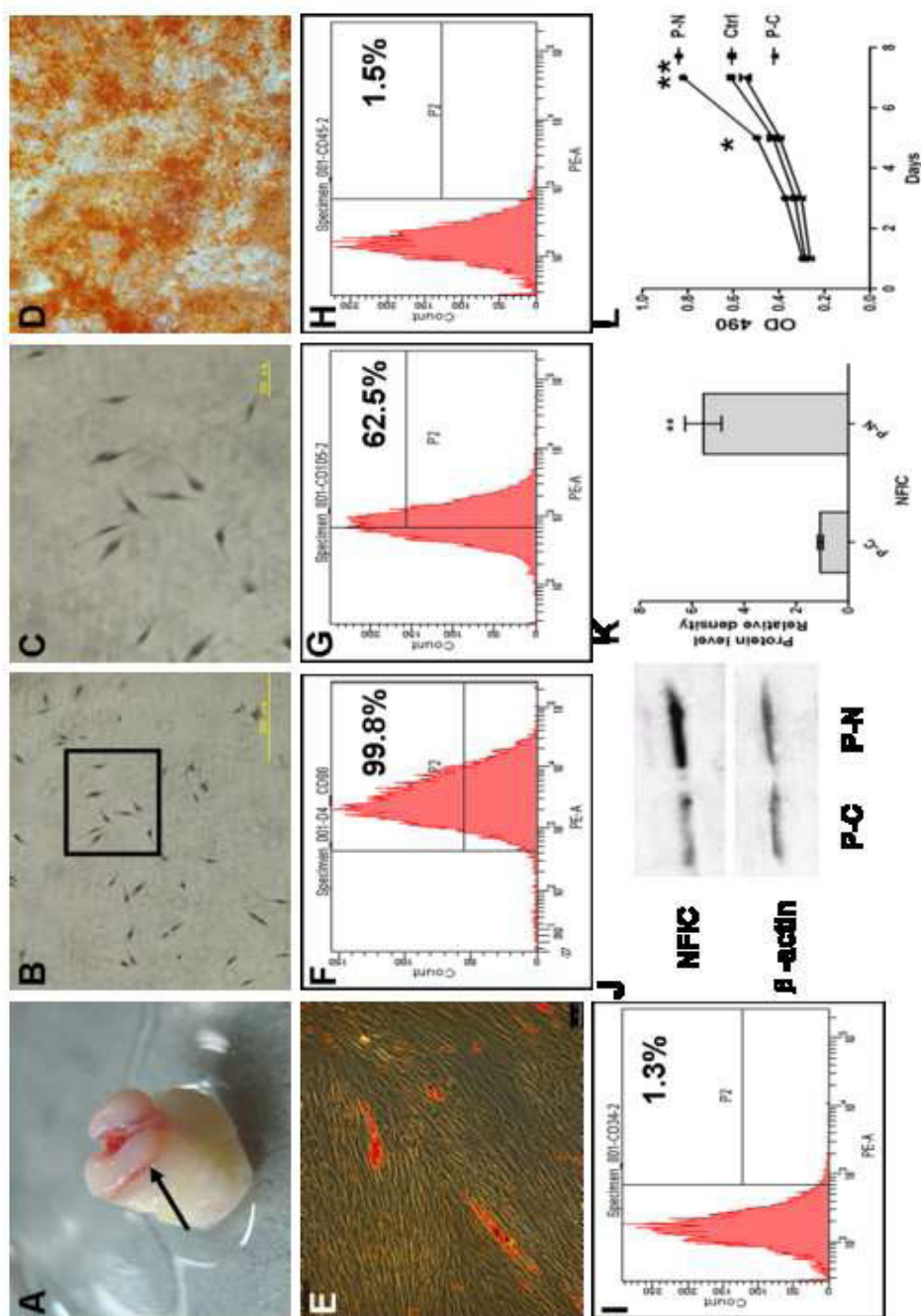
Fig. 2. NFIC enhanced mineralization and ALP activity of SCAPs in vitro. Cells transduced with NFIC or transduced with empty vector pLenti-Cherry as control were incubated in osteo/odontogenic medium or normal medium for 4, 7, 14 and 21 days. A: Mineralized nodules were formed in the P-N groups and P-C groups with or without osteo/odontogenic differentiation medium in each of the indicated times. B: Microscopic images of Alizarin red S (ARS)-stained mineralized nodules in P-N groups and P-C groups cultured in osteo/odontogenic differentiation medium in the indicated times. C: Quantitative measurements of mineralized nodules in ARS

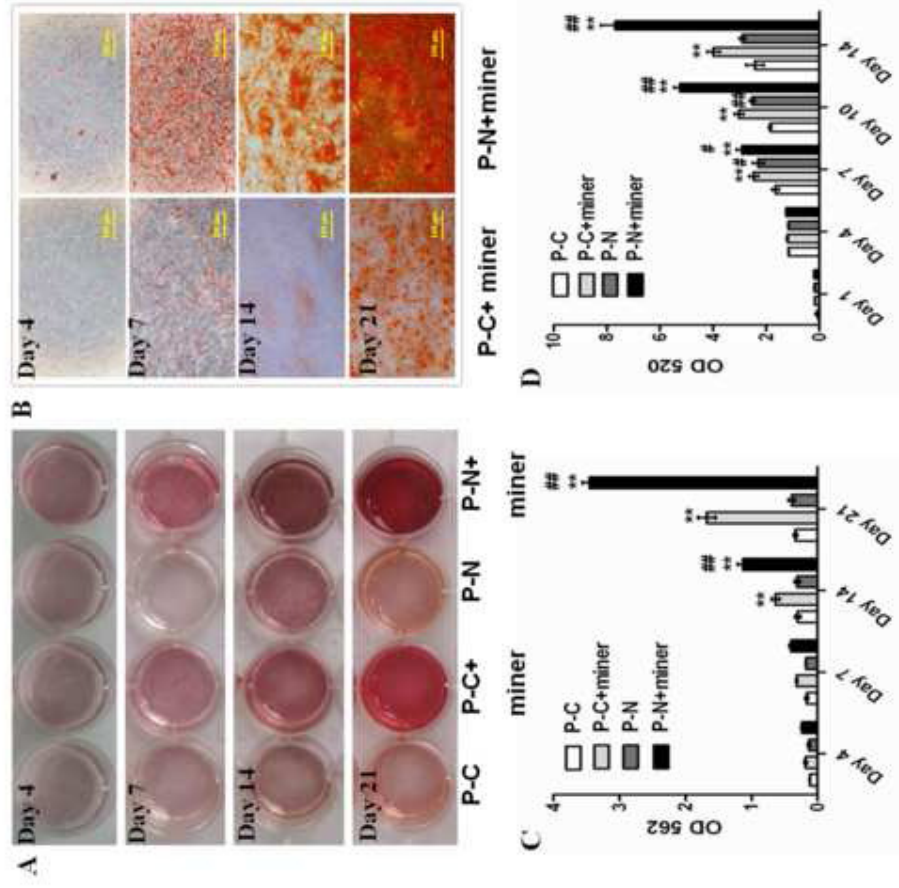
staining in the P-N groups and P-C groups with or without osteo/odontogenic differentiation medium in the indicated times. D: ALP activity in the P-N groups and P-C groups with or without osteo/odontogenic differentiation medium in the indicated times. * $p < 0.05$ or ** $p < 0.01$ when compared with the cells in normal medium. # $p < 0.05$ or ## $p < 0.01$ when compared with the P-C group. Scale bars: 100 μm . P-N, SCAPs over-expressing NFIC protein; P-C: SCAPs transduced with empty vector pLenti-Cherry; Miner: cultured in osteo/odontogenic differentiation medium.

Fig. 3. Effect of NFIC on osteo/odontogenic gene expression of SCAPs after induction of differentiation. Cells over-expressing NFIC protein or transduced with empty vector pLenti-Cherry as control were cultured in osteo/odontogenic medium or normal medium for 1-3 weeks. A-C: RT-PCR was used to assess the effects of NFIC regulation on mRNA transcriptional levels for Col I, ALP and OCN. GAPDH was used as an internal control. * $p < 0.05$ or ** $p < 0.01$ when compared with the cells in normal medium. # $p < 0.05$ or ## $p < 0.01$ when compared with the P-C group. D: Levels of DSP protein were assessed by Western blot analysis. β -actin was used as a loading control. P-N, SCAPs over-expressing NFIC protein; P-C, SCAPs transduced with empty vector pLenti-Cherry; miner, cultured in osteo/odontogenic differentiation medium; W, week.

Fig. 4. Knockdown of NFIC inhibited the osteo/odontogenic differentiation of SCAPs. SCAPs transfected with the NFIC expression vector or control empty vector were cultured in osteo/odontogenic medium for 1-2 weeks. A-D: Decreased formation of mineralized nodules stained by Alizarin red S after knockdown of NFIC and culture in the same osteo/odontogenic inductive conditions for 2 weeks. E: The calcium concentration in the si-NFIC group was significantly lower than in the si-CON group (** $p < 0.01$). F-G: Knockdown of NFIC gene expression in SCAPs. Expression of ALP, Col I mRNA and OCN were reduced significantly in si-NFIC group (* $p < 0.05$, ** $p < 0.01$). Scale bars: 100 μm . si-NFIC, SCAPs transfected with siRNA NFIC expression vector; si-CON, SCAPs transfected with control empty vector as control.

1. NFIC promotes the proliferation of SCAPs *in vitro*.
2. NFIC promotes osteo/odontogenic differentiation of SCAPs *in vitro*.
3. Knockdown of NFIC inhibits odontogenic differentiation in SCAPs.





Accepted

