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Long non-coding RNA H19/SAHH axis epigenetically regulates odontogenic differentiation of human dental pulp stem cells

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ABSTRACT

Long noncoding RNAs (lncRNAs) are emerging as important regulators in molecular processes and may play vital roles in odontogenic differentiation of human dental pulp stem cells (hDPSCs). However, their functions remain to be elucidated. As lncRNA H19 is one of the most classical lncRNA, which plays essential roles in cellular differentiation, thus we explored the effects and mechanisms of H19 in odontogenic differentiation of hDPSCs. Stable overexpression and knockdown of H19 in hDPSCs were constructed using recombinant lentiviruses containing H19 and short hairpin-H19 expression cassettes, respectively. Alkaline phosphatase (ALP) assay, Alizarin red staining assay, von kossa staining, quantitative polymerase chain reaction (qPCR), Western blot analysis, and immunofluorescent staining results indicated that overexpression of H19 in hDPSCs positively regulates the odontogenic differentiation of hDPSCs, while knockdown of H19 in hDPSCs inhibits odontogenic differentiation of hDPSCs. Further, we found that H19 promotes the odontogenic differentiation of hDPSCs through S-adenosylhomocysteine hydrolase (SAHH) epigenetically regulates the methylation and expression of distal-less homeobox (DLX3) gene. Herein, for the first time, we determined that H19/SAHH axis epigentically regulates odontogenic differentiaiton of hDPSCs by inhibiting the DNA methyltransferase 3B (DNMT3B)-mediated methylation of DLX3. Our findings provide a new insight into how H19/SAHH axis play its role in odontogenic differentiation of hDPSCs, and would be helpful in developing therapeutic approaches for dentin regeneration based on stem cells.

1. Introduction

Human dental pulp stem cells (hDPSCs) are derived from neural crest mesenchymal stem cells, which have the capacity of pluripotent differntiation and self-renewal.[21,28] Those abilities allow hDPSCs to play an important role in dentin repair and regeneration, therefore, they may be commonly used in clinical applications.[14,22] Despite a great deal of research has been performed to exlpore the mechanisms involved in odontogenic differentiation process of hDPSCs,[9,23] the precise molecular mechanisms remain unclear.

Many factors and molecular processes are invovled in regulating differentiation of hDPSCs.[8,13,18] Emerging evidence suggests that long non-coding RNAs (lncRNAs) play important roles in the process of odontogenic differentiation of hDPSCs.[6,11,16,26] LncRNAs are long non-coding RNAs > 200 nucleotides in length that serve important regulatory roles in cell differentiation and biological control,[1,20] which indicates vital roles of lncRNAs in regulating odontogenic differentiation of hDPSCs.

Notably, lncRNA H19, one of the best known lncRNA, is a highly conserved imprinted gene and encodes a ~2.6 kb polyadenylated lncRNA and exerts a variety of functional activities both in the nucleus and in the cytoplasm.[7] H19 has been identified to exert a crucial role in embryonic stem cell differentiation, myogenic differentiation, skeletal muscle differentiation, and mesenchymal stem cell osteogenic differentiation.[3,4,10,19] These findings suggest that H19 has an important role in cellular differentiation. It has been shown that H19. Further, H19 can bind to and inhibit the activity of S-adenosylhomocysteine hydrolase (SAHH), and results in altering gene methylation and expression via DNA methyltransferases 3B (DNMT3B), indicative of an important epigenetic regulation function.[29,30] Thus, considering the complex function of H19 in cellular differentiation and the epigenetic regulation function of H19, we wondered whether H19 can invovle in the odontogenic differentiation of hDPSCs in an epigenetic regulation manner.

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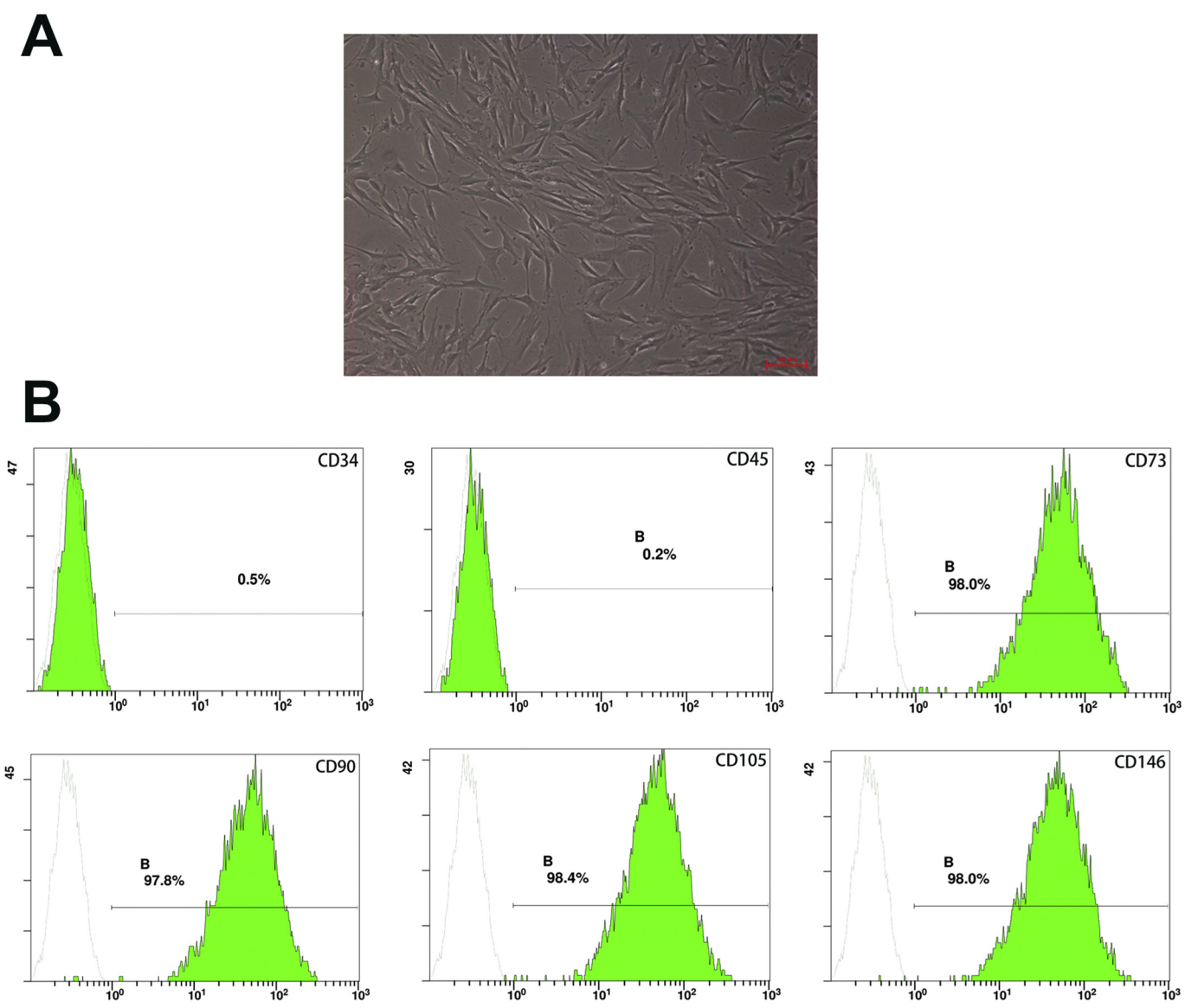

Fig. 1. Cells morphology and flow cytometry analysis of hDPSCs. A. Morphological characteristics. B. Flow cytometry analysis of various cell-surface markers on the isolated hDPSCs.

In this report, we firstly identified the dynamic expression profile of H19 in odontogenic differentiation of hDPSCs. Then, stably over-expression and knockdown of H19 in hDPSCs were used to explore the function of H19 on odontogenic differentiation of hDSPCs. Finally, we verified that H19/SAHH axis epigenetically promotes odontogenic differentiation of hDPSCs through distal-less homeobox 3 (DLX3) gene. This study unveils the role of H19 in odontogenic differentiation of hDPSCs through epigenetic regulation of DLX3, and provide an explanation for epigenetic regulatory mechanism in odontogenic differentiation of hDPSCs from a new insight.

2. Materials and methods

2.1. Cell culture, identification and treatment

This study was approved by the Ethics Committee of Peking University School and Hospital of Stomatology. All individuals participated in this study with informed consent which followed the instructions of Helsinki compliance. Dental pulp tissues were collected from impacted premolars and isolated hDPSCs were maintained in a humidified atmosphere with 95% air and 5% CO_2 at 37 °C in α-MEM growth medium (GM) supplemented with 10% fetal bovine serum (Gibco, Paisley, UK). Cells at passage 3 were analyzed by using stem cell associated markers through flow cytometry. hDPSCs were suspended in ice-cold phosphate buffer solution (PBS) with 2% fetal bovine serum and incubated with antibodies for 30 min on ice. Then the following antibodies were used: anti-phycoerythrin (PE), anti-fluorescein isothiocyanate (FITC), anti-CD34-FITC, anti-CD45-FITC, anti-CD73-PE, anti-CD90-FITC, anti-CD105-FITC, and anti-CD146-PE (BD Pharmingen, Franklin Lakes, NJ, USA). Mouse IgG1-PE and IgG1-FITC were used as isotype controls. All procedures were performed in the dark at 4 °C. The expression profiles were performed by using a flow cytometer (Beckman Coulter. Inc., Brea, CA, USA). For odontogenic differentiation, cells were cultured in odontogenic medium (OM), which contains 50 mg/ml ascorbic acid, 100 nmol/L dexamethasone, and 10 mmol/L β-glycerolphosphate (Sigma, St Louis, MO, USA).

2.2. Lentivirus infection and establishment of stably infected hDPSCs

Recombinant lentiviruses carrying H19 expression cassettte (H19) and its control (H19-NC), recombinant lentiviruses targeting H19 (shH19) and the control (shH19-NC) were obtained from GeneChem

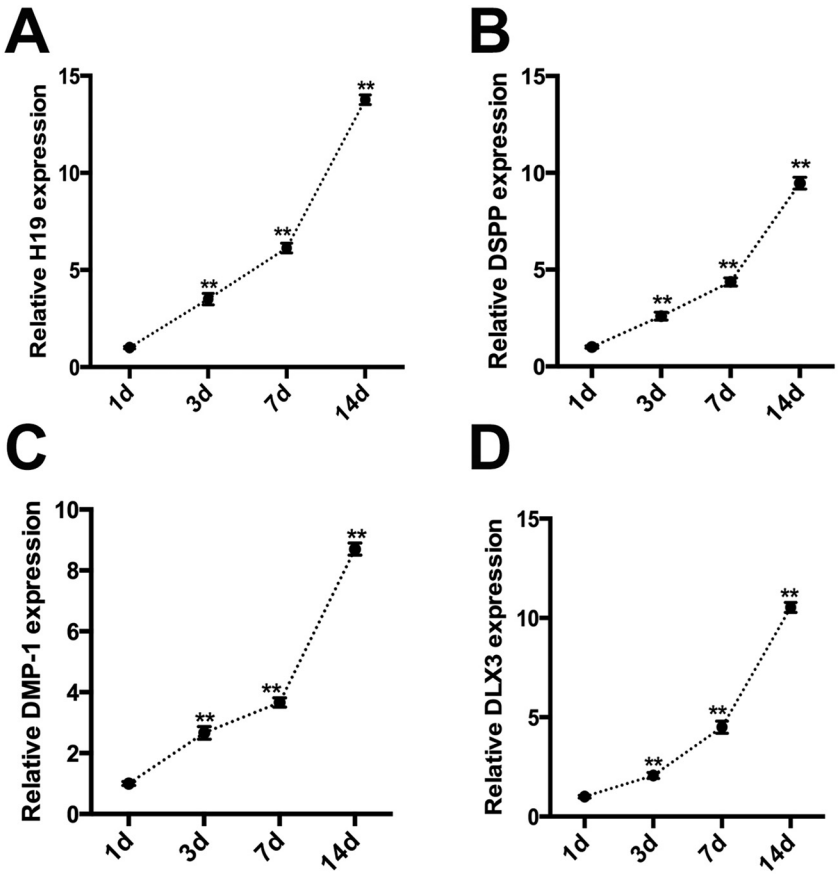

Fig. 2. Dynamic expression profile of H19 and odontogenic markers during odontogenic differentiation of hDPSCs. A-D mRNA expression levels of H19, DSPP, DMP-1 and DLX3 on days 1, 3, 7 and 14. GAPDH was used as an internal control. Significant differences versus with the control hDPSCs at the indicated days, *$p < 0.05$, **$p < 0.01$.

company (GeneChem, Co.,Ltd. Shanghai, China). The lentiviruses were used to infect hDPSCs and establish stably infected hDPSCs, which are stably expressing exogenous gene for overexpression and knockdown of H19. Infection of the hDPSCs was performed by exposing hDPSCs to varies dilutions of the virus in the presence of polybrene (5 μg/ml) for 12 h. Then the medium was changed after 12 h and the cells were incubated for another 48 h. Next, the infected cells were cultured in the presence of 1 μg/ml puromycin. The screened cells were used as the stably expressing model in the following experiments.

2.3. Alkaline phosphatase (ALP) staining and quantification of ALP activity

hDPSCs were cultured in 12-well plates and induced by OM. At the indicated time, ALP staining was performed based on the protocol provided in the NBT/BCIP staining kit (Cwbiotech, Beijing, China). ALP activity was measured using an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.4. Alizarin red staining and quantification of matrix mineralization

Cells were cultured in 6-well plates and induced by OM for 28 days. At the indicated time, Alizarin red staining and quantification were performed according to the manufacturer's instructions. Specifically, cells were rinsed with PBS for 3 times and fixed in 4% formaldehyde for 30 min, then washed with distilled water for 3 times. The cells were stained with 2% Alizarin red pH 4.2 (Sigma) for 10 min at room temperature, then the stained samples were eluted with 100 mM cetylpyridinium chloride (Sigma) for 1 h, and the released Alizarin red was analyzed using a spectrophotometric instrument at 562 nm.

2.5. Von kossa staining

Cells were cultured in odontogenic induction media for 28 days. The calcified nodules in the cell culture were detected by von Kossa staining. Briefly, 6-well cells were washed in PBS for 3 times and fixed with 4% paraformaldehyde for 30 min, and then incubated with 5% silver nitrate solution for 30 min in the dark, then under ultraviolet light for 30 min. Unreacted silver was removed with 5% sodium thiosulfate for 5 min, followed by several washes with water.

2.6. Quantitative polymerase chain reaction (qPCR)

Total RNA was isolated with Trizol reagent (Life Technologies, Carlsbad, CA, USA) and 2 μg of RNA was reverse-transcribed into cDNA. qPCR reactions were conducted with the ABI Prism 7500 Real-time PCR System (Life Technologies) by using SYBR green master mix (Roche Diagnostics, Indianapolis, IN, USA). The relative mRNA expression was normalized to glyceraldehyde phosphate dehydrogenase (GAPDH), and calculated using the $2^{-\triangle\triangle Ct}$ method. The sequences of each primer are listed in Supplementary Table 1.

2.7. Western blot assay

Cells were collected and lysed in RIPA buffer with protease inhibitors. Thirty micrograms of total protein were loaded into a 10% sodium dodecyl sulfate polyacrylamide gel for electrophoresis and subsequently transferred to a polyvinylidene difluoride membrane. The membranes were blocked in 6% skim milk for 1 h and incubated with antibodies against DLX3, DMP-1, GAPDH (Abcam, Cambridge, UK), DSPP (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) separately at 4 °C overnight, then followed by peroxidase-linked secondary antibodies (1:10,000) for 1 h. The immunoreactive bands were visualized on an Odyssey infrared imaging system (Odyssey LI-COR Biosciences,

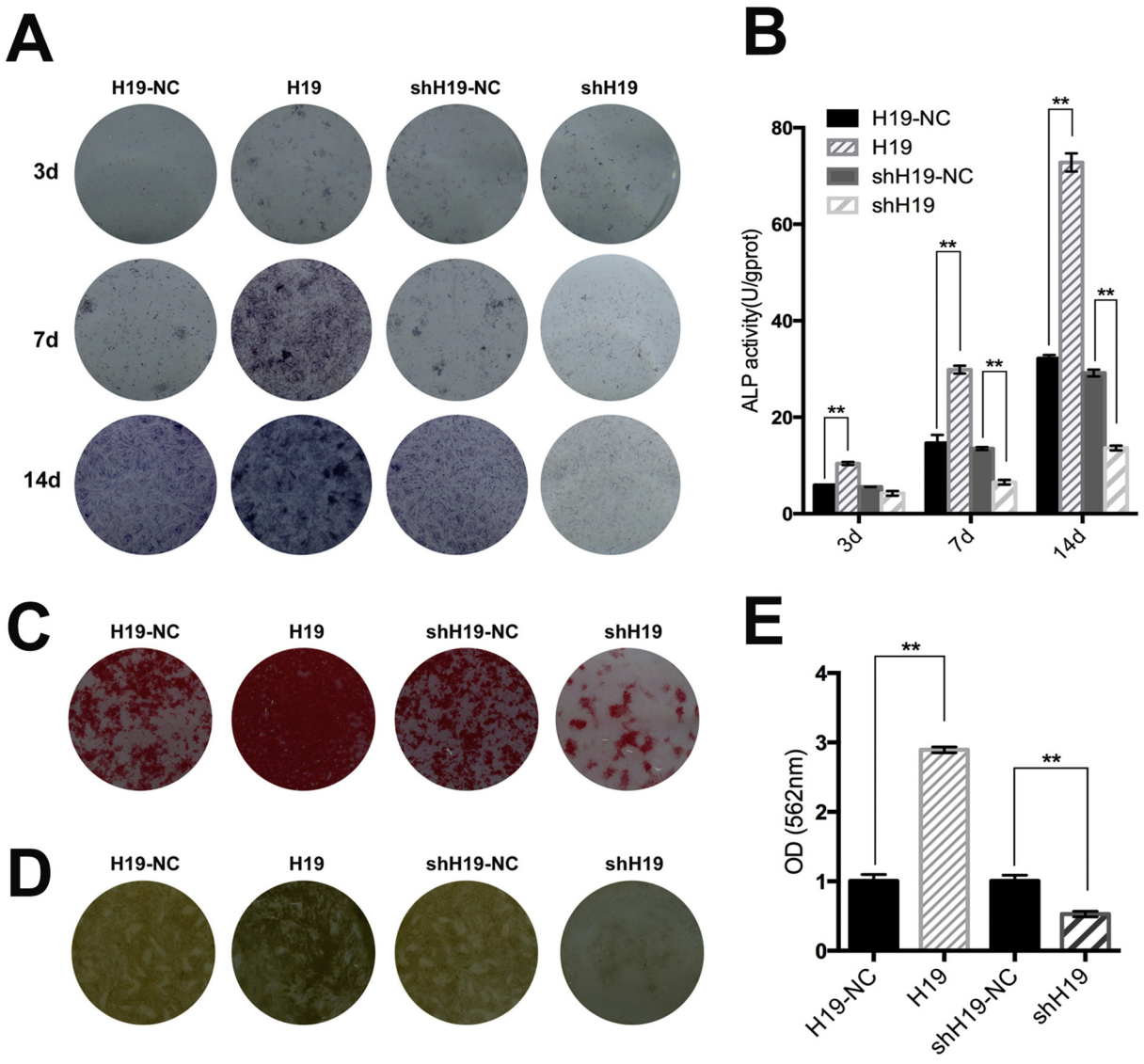

Fig. 3. Effects of H19 on ALP expression, activity, and mineralized tissue formation of hDPSCs. A.B. ALP staining and activity assays in the H19-NC, H19, shH19-NC, shH19 groups on days 3, 7 and 14 after odontogenic induction. C–E. Mineralized tissue formation of hDPSCs was measured by Alizarin red staining assays, von kossa staining assays and quantification of alizarin red staining on days 28 post-odontoinduction. Significant differences versus the NC group, *$p < 0.05$, **$p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Lincoln, NE, USA).

2.8. Immunofluorescent staining

At the indicated time, cells grown on sterile glass coverslips were fixed with 4% formaldehyde for 30 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min, and followed by the incubation in blocking buffer for 30 min. After that, cells were incubated with primary antibody diluted in bovine serum albumin at 4 °C overnight, and then incubated in the specified second antibody for 1 h at room temperature in the dark. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and the coverslips were mounted on a glass slide. Images were captured under a Leica TCS SP8 X confocal imaging system (Leica, Wetzlar, Germany).

2.9. S-adenosylhomocysteine hydrolase (SAHH) activity assay

The experiment was performed using a SAHH activity assay kit (Abcam, Cambridge, UK), following the manufacturer's instructions. Briefly, cells were washed with cold PBS and resuspended in 300 μl cold homogenization buffer, followed by agitation on a rotary shaker at 4 °C for at least 15 min and centrifugation at 16,000*g* at 4 °C for 15 min. After centrifugation, the supernatant was collected and used for SAHH activity measurement. The absorbance of the samples was determined using a spectrophotometric instrument (PerkinElmer, Waltham Mass, MA, USA). A serial dilution of adenosine was prepared to obtain a standard curve.

2.10. DNA methyltransferases 3B (DNMT3B) activity assay

Quantification of DNMT3B activity was performed and analyzed using DNMT3B Activity Assay Core kit (Epigentek, Farmingdale, NY, USA). Briefly, cells were washed with cold PBS and lysed on plate in RIPA buffer with protease inhibitors. The lysate was ultra-sounded and centrifuged at 12,000 *g* at 4 °C for 30 min immediately following the centrifugation, 5 μlof the supernatant was collected and used for DNMT3B activity measurement according to the manufacture's instructions. The absorbance of the samples was determined using a spectrophotometric device (PerkinElmer). The assay was performed in triplicate and the DNMT3B activity was calculated by following the

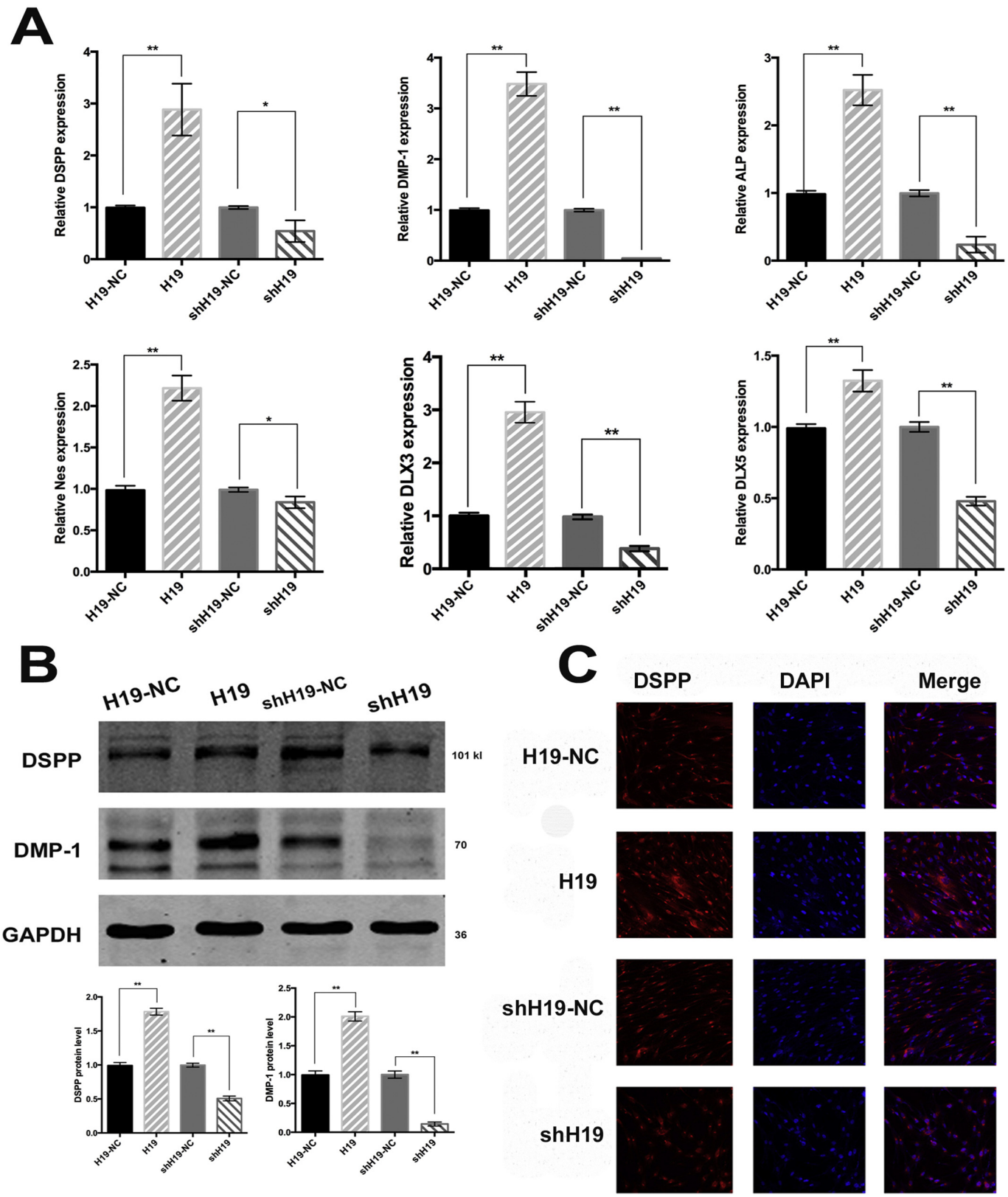

Fig. 4. H19 promotes odontogenic differentiation of hDPSCs. A. Odontogenesis-related genes (DSPP, DMP-1, ALP, Nes, DLX3 and DLX5) mRNA expression of H19-NC, H19, shH19-NC, shH19 groups after odontogenic induction. B. Western blots of DSPP, DMP-1 and GAPDH of H19-NC, H19, shH19-NC, shH19 groups under odontogenic stimulation. C. Confocal microscopy of DSPP with DAPI counterstaining in H19-NC, H19, shH19-NC and shH19 groups after induction to the odontogenic lineage. GAPDH served as an internal control. Significant differences versus the NC group, *$p < 0.05$; **$p < 0.01$.

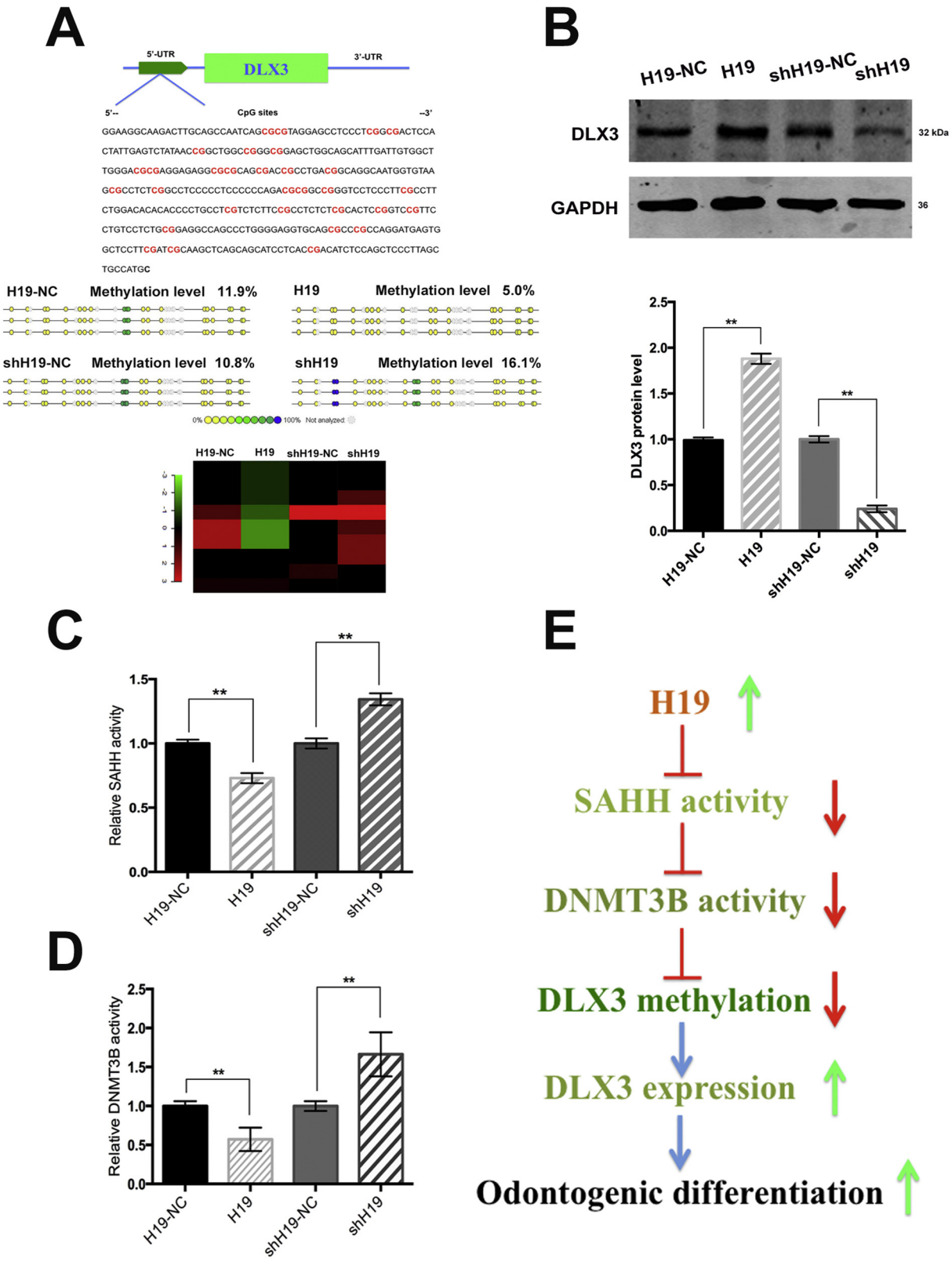

Fig. 5. H19/SAHH axis epigenetically regulates odontogenic differentiation of hDPSCs via DLX3. A. Quantitative methylation level and heat-map of DLX3 gene promoter region in H19-NC, H19, shH19-NC and shH19 groups. B. Western blots of DLX3 in H19-NC, H19, shH19-NC and shH19 groups under odontogenic stimulation. C. SAHH activity in H19-NC, H19, shH19-NC and shH19 groups. D. DNMT3B activity in H19-NC, H19, shH19-NC and shH19 groups. E. A model for H19/SAHH axis epigenetically regulates odontogenic differentiation of hDPSCs via DNMT3B by regulation of DLX3. Green arrow means promoting, red T and red arrow mean inhibiting. Significant differences versus the NC group, *$p < 0.05$; **$p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

manufacture's instructions.

2.11. Quantitative methylation analysis

Quantitative methylation analysis of the gene promoter was performed using the Sequenom MassARRAY platform (CapitalBio, Beijing, China). Genomic DNA was extracted using a Universal Genomic DNA kit (Cwbiotech) according to the manufacturer's instructions. The isolated DNA sample was converted with sodium bisulfite and the modified DNA was amplified by PCR. The target regions were amplified using the primers listed in Supplementary Table 2. For each reverse primer, an additional T7 promoter tag for in vitro transcription was added, and a 10-mer tag on the forward primer to balance PCR. After amplification, the procedures were started by patterning samples on a 384-pad Spectro-CHIP (Sequenom, San Diego, CA, USA) using a MassARRAY nanodispenser (Samsung, Irvine, CA, USA), followed by spectral acquisition on a MassARRAY analyzer compact MALDI-TOF MS (Sequenom). The spectra methylation levels were analyzed by Epityper software version 1.0 (Sequenom) to produce quantitative results for each CpG site or a cluster of multiple CpG sites.

2.12. Statistical analysis

All data was representative of each assay repeated independently at least three times. Statistical analyses were performed by SPSS version 13.0 software package (SPSS Inc., Chicago, IL, USA). Statistical significance was determined by one-way analysis of variance and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Cells characterization

Mesenchymal stem cells from dental pulp tissues were successfully isolated. Cells reached 70% confluence after 10 days culture and showed a typical spindle-shaped fibroblastic morphology (Fig. 1A). The flow cytometry assay showed that the isolated cells were negative for the hematopoietic marker CD34 and endothelial cells marker CD45, and positive for the mesenchymal stem cell-associated markers CD73, CD90, CD105, and CD146 (Fig. 1B), indicating that the isolated cells derived from dental pulp tissues were hDPSCs.

3.2. Expression pattern of H19 during the odontogenic differentiation of hDPSCs

To explore the function of H19 in odontogenic differentiation of hDPSCs, we firstly detected the dynamic expression profile of H19 in hDPSCs after induction to the odontogenic lineage. H19 expression was gradually up-regulated during odontogenic differentiation with more than tenfold on day 14 (Fig. 2A). Further, qPCR results showed that the mRNA expression of odontogenic markers DSPP, DMP-1, and DLX3 were significantly up-regulated during odontogenesis (Fig. 2B–D). These results indicated that the expression profiles of H19 and the odontogenic markers are identical, and suggested that H19 can involve in the odontogenic differentiation of hDPSCs.

3.3. H19 promotes ALP activity and accelerates mineralized tissue formation of hDPSCs

Considering that hDPSCs express H19 gene and its level increased during odontogenic differentiation, we next hypothesized that H19 may contribute to reprogram hDPSCs towards to odontogenic phenotype. Lentiviruses were used to overexpress or knock down H19 in hDPSCs. The infection efficiency was > 90% (Supplementary S1A), and qPCR analysis showed more than tenfold expression in the H19-overexpression group and a 60% decrease in the H19-knockdown group compared to NC group (Supplementary S1B). After odontogenic induction for 3, 7 and 14 days, the expression and activity of ALP were increased in H19-overexpression group and decreased in H19-knockdown group (Fig. 3A, B). Following odontogenic induction for 28 days, Alizarin red staining and von kossa staining of calcified deposition with more staining intensity in H19 overexpression group and less staining intensity in H19 knockdown group, and quantification of mineralized deposition further confirmed these results (Fig. 3C–E).

3.4. H19 induces the expression of odontogenic markers of hDPSCs

To identify the effect of H19 on odontogenic differentiation of hDPSCs, the expression of odontogenic markers were detected. qPCR results showed that mRNA levels of DSPP, DMP-1, ALP, Nes, DLX3 and DLX5 were increased in hDPSCs after H19 overexpression, while the opposite effects were observed in H19 knockdown hDPSCs (Fig. 4A). Western blot analysis and immunofluorescence staining further indicated that the protein levels of DSPP and DMP-1were up-regulated in Lentivirus-H19 infected hDPSCs, while the protein levels of DSPP and DMP-1 were dramatically down-regulated in Lentivirus-shH19 infected hDPSCs (Fig. 4B, C). Those data suggested that H19 exerts as a positive factor in odontogenic differentiation of hDPSCs.

3.5. H19/SAHH axis epigenetically regulates the methylation and expression of DLX3

Since H19 could alter DNMT3B-mediated DNA methylation genome wide by regulating SAHH,[30] we wondered whether H19 can regulate the methylation of odontogenesis-related genes in hDPSCs to elaborate the role of H19 in odontogenic differentiation. To test this possibility, hDPSCs stably overexpression and knockdown of H19 were used to explore the methylation level of odontogenesis-related genes. Quantitative methylation analysis revealed that odontogenesis-related gene DLX3 promoter is hypo-methylated in H19 group compared with shH19 group (Fig. 5A). Western blot analysis corroborated the findings from quantitative methylation analysis and showed that the protein level of DLX3 in H19 group was significantly up-regulated, whereas the opposite effect was observed in shH19 group (Fig. 5B). Those results strongly suggested that H19 can regulate the expression of DLX3 through methylation at DLX3 promoter region.

To further address whether SAHH might contribute to the decreased methylation of DLX3 in H19 group via DNMT3B activity, SAHH activity assay and DNMT3B activity assay were carried out. Activity assays results showed that SAHH activity was significantly down-regulated in H19 group, which results in a decrease of DNMT3B activity in H19 group (Fig. 5C, D). Therefore, we proposed that H19 overexpression promotes the expression of DLX3 via hypo-methylation of DLX3 by decreasing the SAHH activity and DNMT3B activity, finally leading to the promotion of odontogenic differentiation of hDPSCs (Fig. 5E).

4. Discussion

Herein this is the first work to report on the epigenetic regulation of H19/SAHH axis in odontogenic differentiation of hDPSCs. Our studies provide an explanation for the mechanism role of lncRNA H19 in dentin development. Therefore, the in-depth functional investigations extended our knowledge of H19 function in odontogenic differentiation of hDPSCs.

hDPSCs is a heterogenic cell population that has multi-differentiation potential ability, which is capable of differentiation from hDPSCs into functional odontoblasts, and produces reparative dentin in response to different stimulations.[12,17,25] Therefore, exploration of the regulatory mechanism in odontogenic differentiation of hDPSCs has optimistic therapeutic implications in the repair of injured pulp and dentin tissues.

Works performed in the last several years have demonstrated that

DLX3 is a key regulator in odontogenic differentiation of hDPSCs. In vitro function studies have revealed that DLX3 has the power to promote odontogenic differentiation, in other words, overexpression of DLX3 in odontoprogenitors promotes odontogenic differentiation. [15,27] Those results are confirmed by experiment in vivo. Neural crest deletion of DLX3 in Wnt1-cre mice presented major dentin defects throughout the lifetime due to directly weaken odontoblast differentiation and down-regulation of odontogenic essential marker DSPP. [5] Undoubtedly, these results showed that DLX3 is a positive regulator in odontogenic differentiation.

Given the fact that differentiation is an intricate process directed by hundreds of factors, an interesting concept emerges is that lncRNAs H19 performs its role in differentiation by coordinating with signal pathways. Dey et al.[4] found that lncRNA H19 promotes skeletal muscle differentiation and regeneration by interacting with BMP pathway. Huang et al.[10] reported that H19 promotes the osteogenic differentiation of human bone marrow mesenchymal stem cells and bone formation via TGF-β1/Smad3/HDAC pathway. Further, dentinogenesis is a sophisticated process that many lncRNAs are involved in odontogenic differentiation,[2] while the function of lncRNA H19 in odontogenic differentiation of hDPSCs is still unknown. Therefore, we explored the function of the most classical lncRNA H19 in odontogenic differentiation of hDPSCs. In the present study, we found that H19 significantly up-regulated the odontogenesis-related genes expression and the formation of mineralized nodules in hDPSCs, while knockdown of H19 in hDPSCs weakened the odontogenic differentiation of hDPSCs, suggesting that H19 has a positive effect on the odontogenic differentiation of hDPSCs.

To explore how H19 plays its role in odontogenic differentiation, we focused on epigenetic regulation axis H19/SAHH. Previous studies have shown that H19 can regulate DNA methylation at many genomic sites via SAHH. Further detailed investigations showed that H19 overexpression leads to a decreased DNMT3B-mediated methylation by decreasing the activity of SAHH.[29,30] SAHH is the only enzyme to catalyze S-adenosylhomocysteine (SAH) into homocysteine in human body. Moreover, SAH serves as a potent inhibitor of S-adenosylmethionine (SAM) methyltransferases. As DNMTs belong to SAM methyltransferases, thus, SAH can block the methylation activity of DNMTs.[24] Previous studies showed that H19 inhibits the SAHH activity, and results in a increase in SAH level and a decrease in DMNT3B activity, finally altering global DNA methylation [29,30]. Our findings offers a novel insight into H19/SAHH axis epigenetically regulates DLX3, a positive regulator in odontogenic differentiation, methylation via DNMT3B, which is in line with previous studies.[29,30] In addition, other DNMTs, including DNMT1 and DNMT3A, also belong to SAM methyltransferase. Accumulation of SAH can inhibit DNMTs activity and cause methylation change. Therefore, other DNMTs may contribute to the alteration of DLX3 methylation. However, there is no direct evidence yet, which warrants further investigations.

5. Conclusions

Taken together, our data highlight that H19/SAHH axis epigenetically regulates odontogenic differentiation of hDPSCs via epigenetic regulation of DLX3. Those findings broaden our understanding of H19 effect on odontogenic differentiation of hDPSCs, and extend the role of H19/SAHH axis in dentin development.

Conflict of interest

The authors deny any conflict of interest.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2018.08.015.

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