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FAM20C could be targeted by TET1 to promote odontoblastic differentiation potential of human dental pulp cells

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Abstract

Objectives: Ten-eleven translocation 1 (TET1) is a DNA methylcytosine (mC) dioxygenase discovered recently that can convert 5-mC into 5-hydroxymethylcytosine (5hmC). We previously reported that TET1 promotes odontoblastic differentiation of human dental pulp cells (hDPCs). The gene encoding the family with sequence similarity 20, member C (FAM20C) protein, is a potential TET1 target and showed demethylation during odontoblastic differentiation of hDPCs in our previous study. This study aimed to explore whether TET1-mediated hydroxymethylation could activate the FAM20C gene, thereby regulating hDPC differentiation.

Materials and methods: The expression pattern of FAM20C and its potential changes during odontoblastic induction of hDPCs were assessed by Western blotting. Lentivirus-mediated transduction with short hairpin RNA (shRNA) was used to knock down FAM20C and TET1 expression in hDPCs. The mineralization potential of hDPCs was evaluated with an ALPase activity assay and by observing the mineralized matrix deposition and the expression of odontoblast-related markers DSPP and DMP1. Recombinant human FAM20C protein (rh-FAM20C) was reintroduced into shTET1 cells in a rescue experiment. The dynamic hydroxymethylation status of the FAM20C gene promoter was examined using hydroxymethylated DNA immunoprecipitation (IP)-PCR. Chromatin IP-PCR and agarose gel electrophoresis were utilized to validate the recruitment of TET1 to its target loci in the FAM20C promoter.

Results: FAM20C protein level was upregulated after the odontoblastic induction of hDPCs. shRNA-mediated FAM20C suppression reduced the expression of DSPP and DMP1 after odontoblastic induction for 7 and 14 days. ALPase activity was reduced on day 7, and the formation of mineralized nodules was attenuated on day 14 after odontoblastic induction in FAM20C-inhibited hDPCs. Genomic 5hmC levels significantly decreased, and total 5mC levels increased in TET1-deficient hDPCs. In addition, a significant reduction in FAM20C also emerged. The rhFAM20C treatment of shTET1 cells attenuated the mineralization abnormalities caused by TET1 depletion. TET1 depletion prompted a decline in 5hmC levels in several regions on the FAM20C promoter. Enhanced TET1 recruitment was detected at the corresponding loci in the FAM20C promoter during odontoblastic induction.

Conclusion: TET1 knockdown suppressed odontoblastic differentiation by restraining its direct binding to FAM20C promoter, and hence inhibiting FAM20C hydroxymethylation and subsequent transcription. These results suggest that TET1 potentially

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promotes the cytodifferentiation potential of hDPCs through its DNA demethylation machinery and upregulation of FAM20C protein expression.

1 | INTRODUCTION

DNA methylation is an essential epigenetic modification associated with transposable element repression, genomic imprinting, cellular differentiation and development.[1,2] DNA methylation is quite dynamic and conducted by an accurate molecular network of regulators.[3,4] Compared with acquisition of DNA methylation, the methylation removal process, namely DNA demethylation, has not been well delineated. Ten-eleven translocation (TET) proteins are recently discovered DNA methylcytosine (mC) dioxygenases which can convert 5-mC into 5-hydroxymethyl-cytosine (5hmC), 5-formylcytosines and 5-carboxylcyosines.[5,6] The sequential DNA repair mechanism is directed by activation-induced cytidine deaminase or thymine DNA glycosylase, which can deaminate 5hmC and replace the modified base with an unmodified cytosine and thus represents a pathway of active demethylation in vivo.[7,8] TET proteins, designated TET1, TET2 and TET3, are demonstrated to be imperative for embryonic development, meiosis and neurogenesis, and they are implicated in various cancers.[9-11] TET1 recruited by NANOG can accelerate the expression of certain pivotal reprogramming target genes, such as OCT4, by increasing their 5hmC levels in mouse embryonic stem cells.[12] TET2 mutations are frequently noted in myeloid malignancies, and TET2 inactivation in haematopoietic progenitor cells blocks myeloid differentiation.[13] TET3 knockout impaired the maintenance and terminal differentiation of neural progenitor cells.[11] Thus, the TET family of proteins can impact particular genes and plays multiple roles in various cell populations.

Human dental pulp cells (hDPCs) are of mesenchymal origin and exhibit high proliferative and self-renewal capacity, and they possess the ability to differentiate into odontoblast-like cells, which are required for reparative dentinogenesis when caries or trauma occurs.[14,15] Numerous studies have confirmed that signalling pathways, growth factors and epigenetic regulators are involved in the odontoblastic differentiation process of hDPCs.[16-18] Our previous studies indicated for the first time that all TETs were expressed in the nucleus and cytoplasm of hDPCs, and only TET1 expression was elevated during both early spontaneous differentiation and odontoblastic induction.[19] Moreover, TET1 knockdown suppresses the odontoblastic differentiation potential of hDPCs.[20] Herein, to investigate the specific mechanisms by which TET1 hydroxylase modulates hDPC cytodifferentiation, we aimed to identify its downstream targets.

Previous studies have generated a considerable number of chromatin immunoprecipitation (IP) coupled with high-throughput sequencing (ChIP-seq) data sets of TET1 from mouse embryonic stem cells, which are available in Gene Expression Omnibus databases.[10,21,22] We retrieved the available ChIP-seq data sets and performed gene ontology (GO) analysis with the Database for Annotation, Visualization and Integrated Discovery online analysis tool (david.abcc. ncifcrf.gov/) to investigate the TET1-occupancy genes that may be involved in odontogenesis, osteoblast differentiation, dentinogenesis or other related GO biological processes both in Mus musculus and in Homo sapiens. We found that the gene encoding the family with sequence similarity 20, member C (FAM20C) protein, is a potential target of TET1. FAM20C, also named "dentin matrix protein 4 (DMP4)," is a Golgi-enriched kinase that phosphorylates the Ser-x-Glu/pSer motif of secretory pathway proteins. Its 350-amino acid C-terminal region (corresponding to 218-569 in the mouse FAM20C sequence), known as the "conserved C-terminal domain," contains a highly conserved casein kinase domain.[23] FAM20C is highly expressed in mineralized tissues, such as bone and teeth.[24,25] FAM20C expression has also been observed in other cell types such as chondrocytes, osteoblasts/osteocytes, cementoblasts and ameloblasts.[26] Notably, FAM20C was one of the significantly hypomethylated genes in hDPCs identified from our previous study on odonto/osteoblastic differentiation (Li QM, Zhang DQ, Li JL, et al. unpublished data). However, the role of FAM20C as a differentiation factor-like protein in this process remains unknown. In this study, we found that TET1 modulates the odontoblastic differentiation potential of hDPCs by directly targeting FAM20C. We examined the expression pattern of FAM20C and the consequences of FAM20C knockdown during odontoblastic differentiation in hDPCs. Furthermore, we investigated whether TET1 promotes the odontoblastic differentiation potential of hDPCs by directly targeting FAM20C.

2 | MATERIALS AND METHODS

2.1 | Cell culture and odontoblastic differentiation

The donors in this research were 18-25 years old and provided informed consent according to the guidelines of Ethical Review Board of the affiliated stomatological hospital of Sun Yat-sen University. Primary hDPCs were obtained from freshly extracted third molars and cultured as described previously.[27] Concisely, minced pulp tissues were digested for 20 minutes at 37°C in the Dulbecco's minimum essential medium (DMEM) with 3 g/L collagenase type I (Gibco, Carlsbad, NM, USA). The pulp fragments were then cultivated in complete medium containing DMEM with 10% foetal bovine serum, 100 g/L streptomycin and 100 000 U/L penicillin (Gibco). When the cells reached 80% confluence, they were trypsinized using 0.25% trypsin (Gibco), harvested and serially passaged. The cells from the 1st to the 4th passage were utilized for further experiments.

For odontoblastic differentiation, hDPCs were cultured for 7 and 14 days in odonto/osteoblastic induction medium,[28,29] which was supplemented with 100 nmol/L dexamethasone, 50 g/L ascorbic acid and 10 mmol/L β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA).

2.2 | shRNA, transfection and transduction

Short hairpin RNA (shRNA) against FAM20C (shRNA_1: 5′-GGAATAGTTTGCAATGTCATA-3′ and shRNA_2: 5′-CCGTCGT GAATTCAGTGAATT-3′), shRNA against TET1 (shRNA_1: 5′-CCA CTTTCTAAGGGTTTAGAA-3′ and shRNA_2: 5′-CCAATTG CTACC TTTAATGCT-3′), and a non-specific shRNA construct were designed and cloned into a psi-LVRU6GP vector. The recombinant lentiviral vector was transfected into 293FT cells with pLP1, pLP2 and pLP/VSVG packaging vector mix using Lipofectamine™ 3000 Transfection Reagent (Invitrogen, Carlsbad, NM, USA) as per the manufacturer's guidelines. The packaged lentiviruses were obtained after 48 hours and transduced into hDPCs, which were then cultured for 2 weeks in complete medium with 1 μg/mL puromycin (Sigma-Aldrich). The stable clones were then maintained in 0.5 μg/mL puromycin to ensure that the transfection rate was over 90%, and the knockdown effect was confirmed using a Western blotting assay.

2.3 | Western blotting analysis

The hDPCs were harvested using RIPA lysis buffer (Cell Signaling Technology, Boston, MA, USA) containing protease inhibitor cocktail (Cwbiotech, Beijing, China). Totally, 30-70 μg of protein was electrophoresed in 7%-8% sodium dodecyl sulphate polyacrylamide gel electrophoresis and then blotted to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) in transfer buffer with 10% methanol. The membranes were blocked in Tris-buffered saline with Tween-20 containing 2%-5% bovine serum albumin for 1 hour at room temperature and then incubated with primary antibody overnight at 4°C as follows: anti-FAM20C (1:500; Abcam, Cambridge, UK), anti-DMP1 (1:500; Abcam), anti-DSPP (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TET1 (1:1000; GeneTex, Irvine, CA, USA), anti-TET2 (1:500; Abcam), anti-TET3 (1:1000; Abcam), anti-GAPDH (1:1000; Abcam) and anti-VINCULIN (1:1000; Cell Signaling Technology). After washing for 30 minutes, the membrane was then steeped in blocking buffer with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000; Abcam) at room temperature for 1 hour. The immune-reactive blots were visualized using Immobilon Western HRP substrate (Millipore) and captured via an ImageQuant™ Las 4000 mini system (GE Healthcare Life Sciences, Piscataway, NJ, USA). ImageJ 1.47v software (National Institutes of Health, Bethesda, MD, USA) was applied to quantify the band blots.

2.4 | Alizarin red S staining

To test the mineralized matrix deposition, the hDPCs seeded in six-well plates were allowed to undergo odonto/osteoblastic induction for 0, 7 and 14 days. After culturing, the cells were rinsed with PBS thrice, fixed in 4% paraformaldehyde solution for 20 minutes, stained using 1% alizarin red S (GL Biochem, Shanghai, China) solution at room temperature for 10 minutes and rinsed several times again. Mineralized nodules were then detected under an inverted phase contrast microscope (Axiovert 40; Zeiss, Jena, Germany).

2.5 | Alkaline phosphatase activity assay

Human dental pulp cells seeded in 24-well plates underwent 7 days of cultivation for odonto/osteoblastic induction and were rinsed twice with ice-cold PBS. After lysing the cells with 1% Triton X-100 at 4°C for 30 minutes, alkaline phosphatase activity was assessed by an ALPase assay kit (Beyotime Biotechnology, Haimen, China) in accordance with the manufacturer's protocol. The optical density (OD) value of the solution was examined at 520 nm by a microplate reader (Tecan, Hombrechtikon, Switzerland). The protein concentration was quantitated by a bicinchoninic acid protein assay (Beyotime Biotechnology), and ALPase activity was standardized to total protein concentration.

2.6 | Global 5mC and 5hmC quantification

Genomic DNA was obtained using a tissue DNA isolation kit (Omega Bio-Tek, Norcross, GA, USA), and 100 ng (for 5mC detection) or 400 ng (for 5hmC detection) of genomic DNA per sample was prepared for the next step. A MethylFlash Methylated and a MethylFlash Hydroxymethylated DNA Quantification Kit (Epigentek, New York, NY, USA) were used for colorimetric detection of 5mC and 5hmC according to the manufacturer's guidelines. Reference DNA fragments containing 5mC, 5hmC and cytosine were used as positive and negative standards. OD values were detected at 450 nm with a microplate reader (Tecan). The amount of 5mC and 5hmC is proportional to OD values and was calculated based on the relative quantification generated using the kit standards.

2.7 | Immunofluorescence analysis

Human dental pulp cells at passage 3, including the cells before and after odontoblastic induction and the transduced cells, were seeded in coverglass-bottomed dishes for 2 days. The cells were fixed in 4% paraformaldehyde solution, permeabilized with 0.2% Triton X-100 for 5 minutes and blocked in PBS with 5% bovine serum albumin at room temperature for 1 hour. The dishes were steeped in blocking buffer with primary antibody against FAM20C (1:200; Abcam) overnight at 4°C followed by a diluted fluorescein-conjugated secondary antibody (1:300; EarthOx, San Francisco, CA, USA) incubation at room temperature for 1 hour. The cells were then stained using DAPI Dyeing Kit (KeyGEN Biotech, Nanjing, China) at room temperature for 5 minutes. The dishes were mounted with antifade solution (Applygen, Beijing, China) and visualized under an inverted fluorescence microscope (Zeiss).

2.8 | Recombination human FAM20C protein treatment

For the rescue experiment, primary hDPCs and the shTET1 group were treated with recombinant human FAM20C protein (rhFAM20C; R&D Systems, Minneapolis, MN, USA) at the concentrations of 0, 100, 300 or 500 ng/mL for 14 days during odontoblastic induction as described previously.[30-32]

2.9 | Hydroxymethylated DNA immunoprecipitation and real-time quantitative polymerase chain reaction

The FAM20C gene promoter, which is defined as 2000 bp upstream and 1000 bp downstream of the transcription start site (TSS), was retrieved through the UCSC Genome Browser (genome.ucsc.edu/cgi-bin) initially, and its CpG content was predicted using the website analysis tool (www.urogene.org). A long CpG island (CGI) from 1205 bp upstream and 808 bp downstream of TSS was identified in its promoter region, (Figure 5A), and multiple scattered CpG sites were identified around the CGI. The specific primers were designed using Primer Express v3.0 software (Table 1) and were synthesized by BGI technology (BGI, Shenzhen, China). Total DNA harvested from 10^7 cells from the TET1-shRNA groups and the control group was sonicated into random fragments with a size range of 200-1000 bp using an ultrasonic processor (Sonics, Newtown, CT, USA). Approximately 1 μg of fragmented DNA was denatured at 94°C for 10 minutes and then incubated with a 5hmC antibody at 4°C overnight (Diagenode, Liege, Belgium). As a negative control, non-specific human IgG IP was performed in parallel to the methyl DNA IP. The immunoprecipitated DNA was eluted and assessed by subsequent real-time quantitative polymerase chain reaction (qRT-PCR) analyses using LightCycler 480 SYBR Green I Master (Roche, Basel, Switzerland).

TABLE 1 Sequences of FAM20C promoter region-specific primers

Primer	Sequences of FAM20C promoter region-specific primers
1	Forward: 5′-GTGAAGCTGTCTCCTGAGGG-3′
	Reverse: 5′-CGTCTTGGGCCATTTGAAGT-3′
2	Forward: 5′-CATCTCATCTGTCACCCACAA-3′
	Reverse: 5′-ACATCTTAGGCTGACATCCAG-3′
3	Forward: 5′-CTACAGGGAGCTGCACGG-3′
	Reverse: 5′-TCTGTGTGCATGTGTGTGTG-3′
4	Forward: 5′-GCACATCCACACACACACAT-3′
	Reverse: 5′-GCTGAGAGGGGCTGGTGT-3′
5	Forward: 5′-CCTGCACTCACACACCCC-3′
	Reverse: 5′-CCCGCCTTAACCCTCCAG-3′
6	Forward: 5′-GCTCCTTGGGCCTCTCTC-3′
	Reverse: 5′-TCTCGGCTCCTCCCCAGA-3′
7	Forward: 5′-GCTTCCCTCTGCAAACCG-3′
	Reverse: 5′-CTCCCGCTCTCCTTCCTC-3′
8	Forward: 5′-CTCCTCCAACCTCTCGTCC-3′
	Reverse: 5′-GGTCGTGGGGTCTTAGGG-3′
9	Forward: 5′-CAATGTGAACAGCGACACCA-3′
	Reverse: 5′-CCCCTGAACCTCTCTACACC-3′

2.10 | Chromatin immunoprecipitation (ChIP) and qRT-PCR analysis

For the ChIP assay, EZ-Magna ChIP™ G Chromatin Immunoprecipitation Kits (Millipore) were used following the manufacturer's instructions. Approximately 5×10^7 hDPCs cultivated in odonto/osteoblastic induction medium for 0 and 14 days were crosslinked with 1% formaldehyde (Sigma-Aldrich). Cells were lysed and shattered using a Dounce homogenizer and sheared with an ultrasonic processor (Sonics). The chromatin solution was then subjected to IP by adding 5 μg of TET1 antibody (GeneTex) at 4°C with rotation overnight. After washing, the DNA was unwound from the protein-DNA cross-links and purified with the ChIP™ kit. qRT-PCR was performed to validate the sequences of interest with the same primers used for hydroxymethylated DNA immunoprecipitation (hMeDIP)-qPCR. Normal rabbit IgG served as a negative control IP. The PCR product was loaded onto 1.5% agarose gel (Biowest, Nuaille, France) in Tris/boric acid/EDTA buffer (TBE; Cwbiotech) with 0.5% EtBr (Zomanbio, Beijing, China) and run for 25 minutes. Images were captured using a FluorChem™ Q system (Alpha Innotech, San Jose, CA, USA).

2.11 | Statistical analyses

All experiments were replicated at least in triplicate, and the technical repetition was likewise 3 times at least. The data were displayed as the mean ± standard deviation and calculated using the SPSS 20.0 software program (SPSS, Chicago, IL, USA). One-way analysis of variance was applied to compare the experimental groups, and statistically significant levels of difference were indicated with $P < .05$.

3 | RESULTS

3.1 | Odontoblastic differentiation and FAM20C expression in hDPCs

During odontoblastic induction of hDPCs, odontoblastic marker (DSPP and DMP1) expression was upregulated (Figure 1A, $P < .05$), as shown by Western blotting analysis. Mineralized matrix deposition increased after 14 days of odontoblastic induction compared with deposition 0 and 7 days after induction (Figure 1B), further confirming the odontoblastic differentiation of hDPCs.

To investigate the role of FAM20C protein in hDPC differentiation, FAM20C expression was examined during odontoblastic induction. FAM20C protein level increased after 7 and 14 days of odontoblastic induction, as observed by Western blotting (Figure 1C, $P < .05$). Accordingly, the expression pattern of FAM20C during odontoblastic differentiation was consistent with those of the mineralization-related markers and TET1 protein described in our previous study.[33]

3.2 | FAM20C knockdown suppressed odontoblastic marker expression and inhibited the mineralization capacity of hDPCs

To ascertain the function of FAM20C in the odontoblastic differentiation process of hDPCs, specific shRNAs were utilized to knock down its expression. In the FAM20C-sh1 and FAM20C-sh2 groups, FAM20C protein level exhibited an approximate 60% decrement

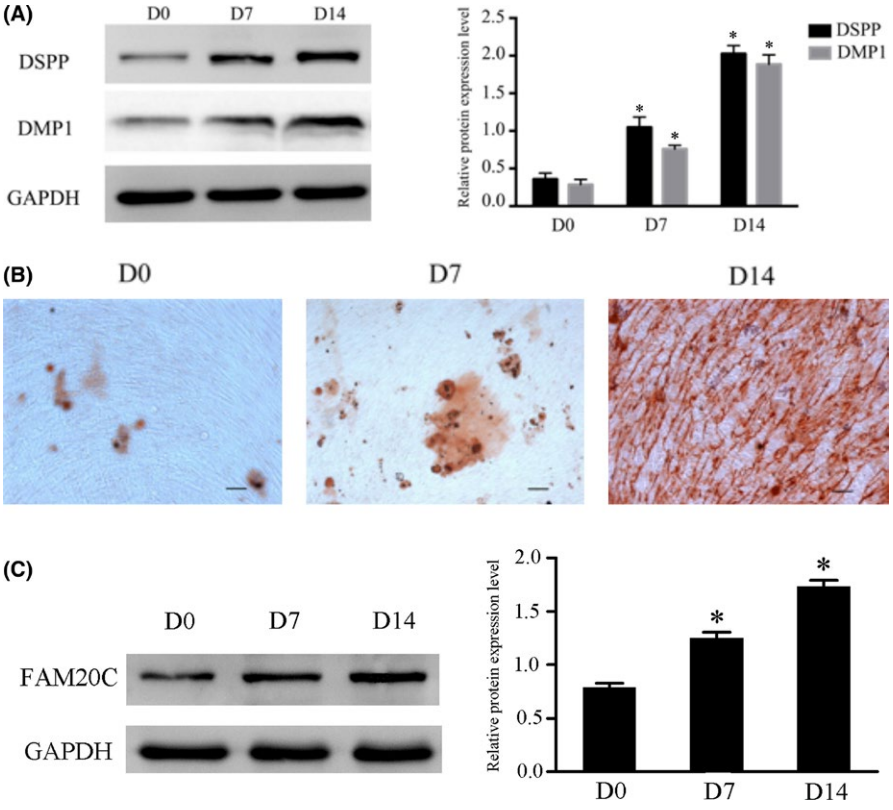

FIGURE 1 Odontoblastic differentiation of human dental pulp cells (hDPCs). A, The expression of the odontoblastic markers DSPP and DMP1 was assessed using Western blotting; the band intensities were analysed using ImageJ software; GAPDH was used as an internal control. B, Alizarin red S staining of hDPCs on day 0, 7 and 14 of culture in odontoblastic induction medium. The "black" scale bars represent 100 μm (original magnification ×100). C, The FAM20C protein was detected using Western blot analysis, and the band intensities were analysed using ImageJ software; GAPDH was used as an internal control. All of the results represent the mean ± standard deviation of 3 independent experiments (n = 3). *Significant difference compared with the control ($P < .05$)

compared with the FAM20C-shCtrl group, suggesting that FAM20C was significantly silenced (Figure 2A, $P < .05$).

The expression of DSPP and DMP1 was then evaluated by Western blotting after FAM20C knockdown. The results revealed that shRNA-mediated depletion of FAM20C reduced DSPP and DMP1 protein levels in hDPCs after odontoblastic induction for 7 and 14 days (Figure 2C, $P < .05$).

Both ALPase activity and mineralized nodule formation are considered to be essential in mineralization of hDPCs. To detect the effect of FAM20C depletion on the hDPC mineralization potential, ALPase activity and mineralized matrix deposition were estimated. In the FAM20C-shRNA groups, ALPase activity decreased on day 7 (Figure 2B, $P < .05$), and the mineralized nodule formation diminished on day 14 after odontoblastic induction (Figure 2D).

3.3 | TET1 knockdown downregulated global 5hmC level and FAM20C expression

Because FAM20C is a TET1-occupancy gene in *Mus musculus*, and both are supposed to be involved in regulating odontoblastic differentiation of hDPCs, we hypothesized that FAM20C may be a downstream effector of TET1 demethylase. As expected, TET1 expression was significantly reduced by shRNAs (Figure 3A, $P < .05$). To exclude the possible involvement of TET2 and TET3 in TET1 knockdown hDPCs, their expression levels were detected and were found to be unaltered. As a result of TET1 knockdown, genomic 5hmC levels significantly decreased, whereas total 5mC levels increased in the TET1-shRNA groups (Figure 3B, $P < .05$). In good agreement with our previous report, DSPP and DMP1 expression levels were attenuated after 7 and 14 days of odontoblastic induction. Notably, TET1-deficient groups exhibited a significant reduction in FAM20C protein (Figure 3C, $P < .05$). Immunofluorescence staining of FAM20C also confirmed the decreased expression upon TET1 depletion (Figure 3D). These findings indicate that the FAM20C gene might be a potential target of TET1 in hDPCs.

3.4 | Recombinant FAM20C rescued the mineralization abnormalities in TET1-deficient hDPCs

To confirm that TET1 mediates odontoblastic differentiation of hDPCs via regulation of FAM20C expression, a rescue experiment was designed to reintroduce FAM20C into TET1-knockdown cells. First, we investigated the mineralization-related markers of hDPCs undergoing rhFAM20C treatment with 4 different concentrations ranging from 0 to 500 ng/mL. After odontoblastic induction for 14 days, 300 or 500 ng/mL exogenous rhFAM20C significantly increased FAM20C protein expression. DSPP and DMP1 were also stimulated by 500 ng/mL rhFAM20C treatment for 14 days (Figure 4A). Next, 500 ng/mL rhFAM20C was administered to shTET1 hDPCs to elucidate whether it could rescue the aberrant cytodifferentiation in TET1-knockdown cells. As shown by Western blotting and alizarin red S staining, rhFAM20C not only enhanced FAM20C expression but also partially rescued the expression of DSPP and DMP1 and mineralized nodules formation (Figure 4B, C). These findings indicate that FAM20C could attenuate the reduction in odontoblastic differentiation caused by TET1 knockdown.

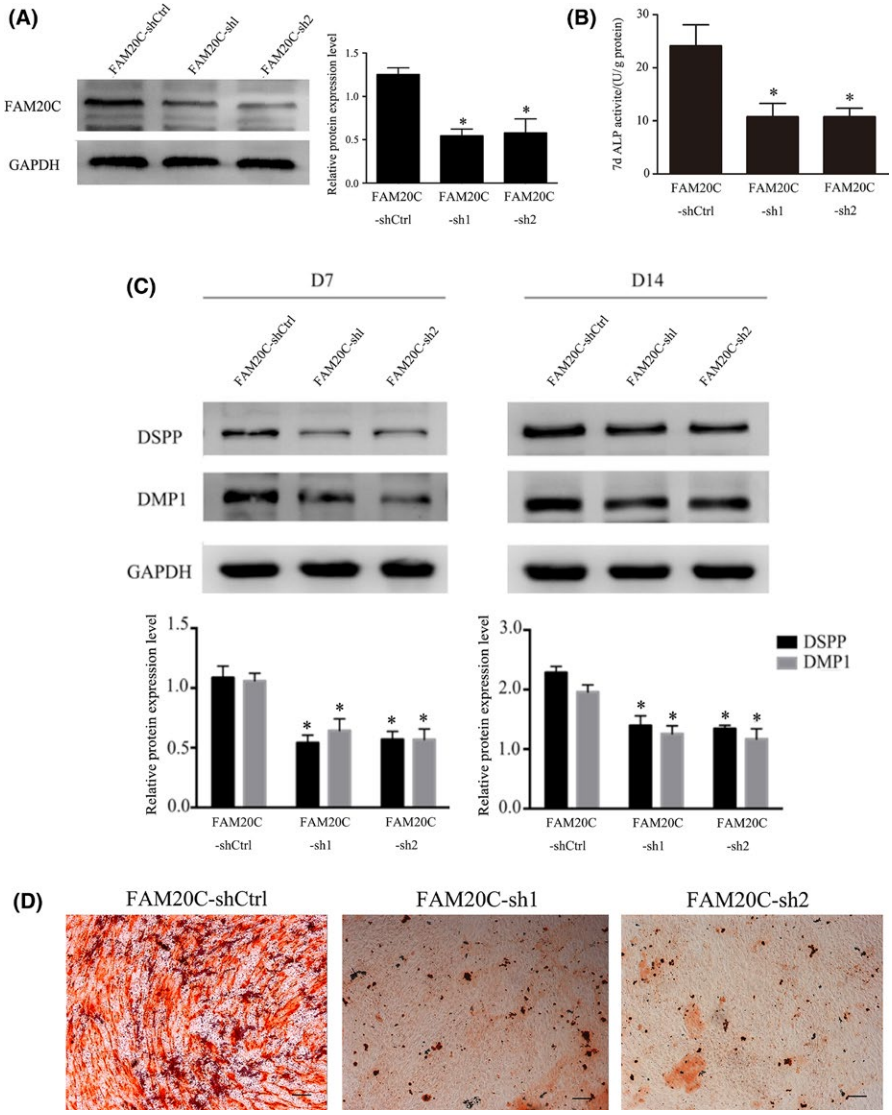

FIGURE 2 Effect of FAM20C knockdown on the odontoblastic potential of human dental pulp cells. A, The expression level of FAM20C in FAM20C-shCtrl and FAM20C-shRNA groups was assessed using Western blotting. The band intensities were analysed using ImageJ software; GAPDH was used as an internal control. B, ALP activity in the odontoblastic differentiation medium at day 7 was determined in FAM20C-shCtrl and FAM20C-shRNA groups. C, The protein levels of odontoblastic markers in FAM20C-shCtrl and FAM20C-shRNA groups after 7 and 14 days of odontoblastic induction were assessed using Western blotting. The band intensities were analysed using ImageJ software; GAPDH was used as an internal control. D, Mineralization was analysed using alizarin red S staining. The formation of mineralized nodules was analysed at day 14 in FAM20C-shCtrl and FAM20C-shRNA groups undergoing odontoblastic induction. The "black" scale bars represent 100 μm (original magnification ×100). All of the results represent the mean ± standard deviation of 3 independent experiments (n = 3). *Significant difference compared with the control ($P < .05$)

3.5 | Dynamic epigenetic status of the FAM20C gene mediated by TET1 binding to DNA

To assess the dynamic DNA hydroxymethylation status of the FAM20C gene in hDPCs, relative hMeDIP-DNA levels were quantified by each input DNA in the TET1-shRNA and TET1-shCtrl transduced cells. TET1 depletion prompted a significant decline in 5hmC levels in several regions around the predicted long CGI on the FAM20C promoter (Figure 5B), which is −2000 to 1000 bp around the TSS (Figure 5A). To further verify that TET1 determines FAM20C epigenetic status by directly locating to the gene promoter of FAM20C, ChIP analysis was implemented with a specific antibody against TET1. The results revealed that TET1 can directly bind particular regions on adjacent sites of the FAM20C promoter where the 5hmC levels of corresponding loci were analysed as described above. Moreover, during odontoblastic induction, significant enrichment of TET1 was detected among these regions on the FAM20C promoter (Figure 5C). Agarose gel electrophoresis was performed to confirm the significant results of ChIP-qPCR (Figure 5D), which verified enhanced recruitment of TET1 at target loci.

4 | DISCUSSION

Human dental pulp cells are a heterogeneous cell population that possess self-renewal ability and can terminally differentiate into odontoblast-like cells for dental pulp regeneration.[34] Elucidating the long-sought mechanisms that regulate hDPC odontoblastic differentiation would contribute significantly to providing diverse routes in the field of reparative dentinogenesis. Our recent reports implied that DNA methylation/demethylation offers an epigenetic mechanism to illustrate this topic.[20,33,35]

In 2009, Rao's group first indicated that the TET1 protein can hydroxylate 5mC to 5hmC and initiate the active DNA demethylation pathway.[5] TET1 has been detected subsequently in the foetal heart, brain, adult skeletal muscle and other organs.[36] Our previous study

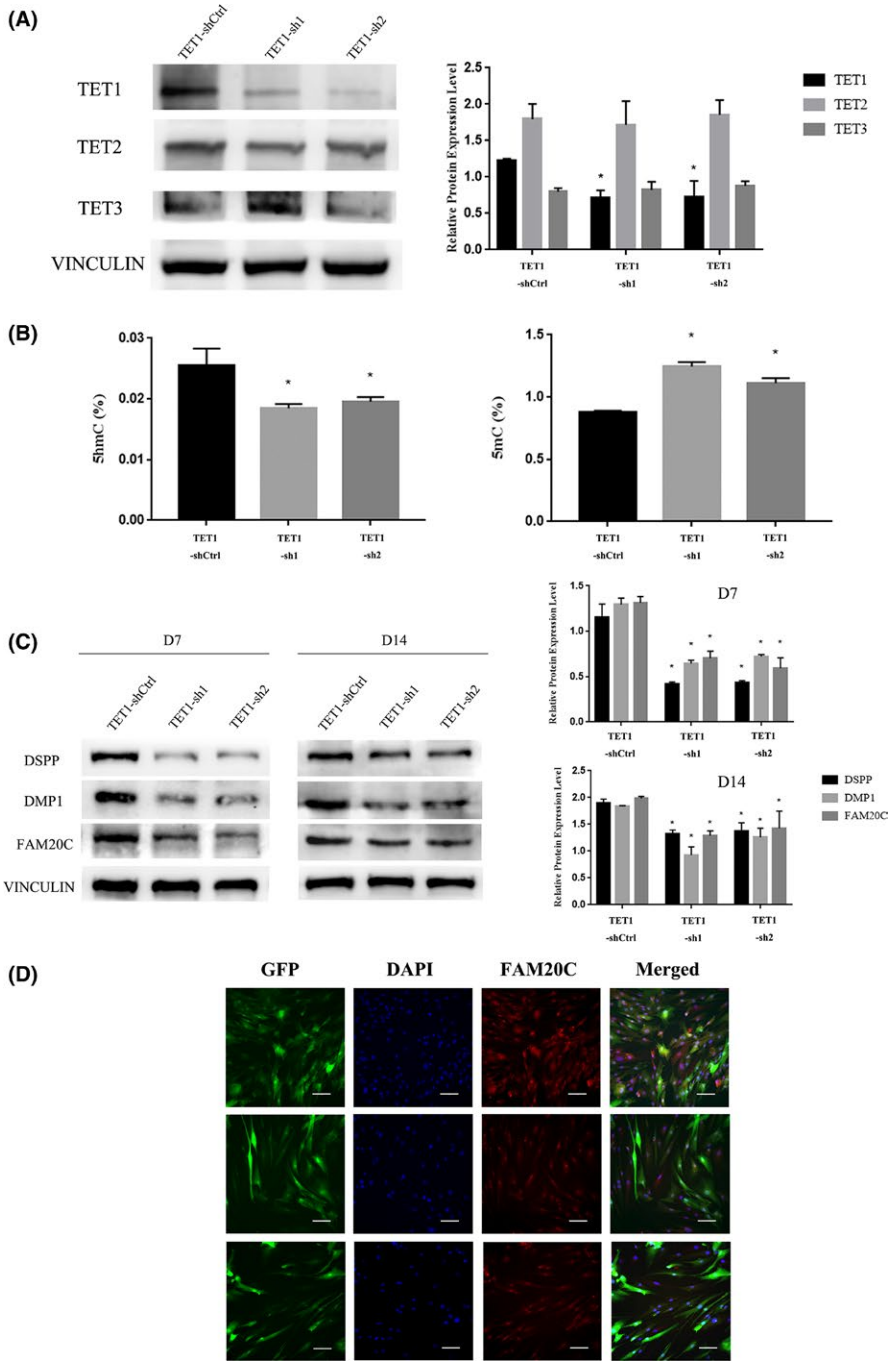

FIGURE 3 Effect of ten-eleven translocation (TET)1 knockdown on global epigenetic status, odontoblastic markers and FAM20C expression in human dental pulp cells (hDPCs). A, TETs expression was assessed using Western blotting. The band intensities were analysed using ImageJ software; VINCULIN was used as an internal control. B, Global 5hmC and 5mC levels were detected using a DNA Quantification Kit. C, The protein levels of DSPP, DMP1 and FAM20C of shCtrl, TET1-sh1 and sh2 groups were detected after 7 and 14 days of odontoblastic induction. The band intensities were analysed using ImageJ software; VINCULIN was used as an internal control. All of the results represent the mean ± standard deviation of 3 independent experiments (n = 3). *Statistically significant difference compared with the control; $P < .05$. D, Immunofluorescence staining for FAM20C in hDPCs. GFP marks the plasmid of the lentivirus vector psi-LVRU6GP(FITC), red colour indicates positive staining for FAM20C (RHOD), and blue colour shows the nucleus (DAPI). The "white" scale bars represent 100 μm (original magnification ×100)

demonstrated that this protein also exists in hDPCs.[33] Recently, TET1 has been speculated to have an effect on the expression of key genes involved in cellular pluripotency and differentiation.[37] TET1 knockdown causes a self-renewal defect and a bias towards trophectoderm differentiation in preimplantation embryos.[38] Female mice with TET1 deficiency exhibit meiotic aberrations due to reduced expression of specific meiotic genes associated with promoter hypermethylation.[39] TET1 hypoactivity in the adult brain can result in 5mC accumulation on the promoters of neurogenesis-related genes and the impairment of cell proliferation.[9] We previously manifested that TET1 depletion may restrain the odontoblastic differentiation of hDPCs by repressing the expression of DMP1 and DSPP, ALPase activity and mineralized matrix deposition.[20] Here, we attempt to further clarify the epigenetic mechanisms by which TET1-mediated demethylation adjusts the physiological features of hDPCs. According to the current literature and our previous high-throughput information (Li QM, Zhang DQ, Li JL, et al. unpublished data), we reasoned that FAM20C may be a candidate target of TET1 in hDPCs.

FAM20C is an extracellular serine/threonine protein kinase. Hao et al[23] found that FAM20C overexpression in C3H10T1/2 (a mouse multipotential mesenchymal cell line) and MC3T3-E1 (a pre-osteoblast cell line) cells increased the expression of DMP1 and DSPP and accelerated mineralized nodule formation. Loss of function mutations in the

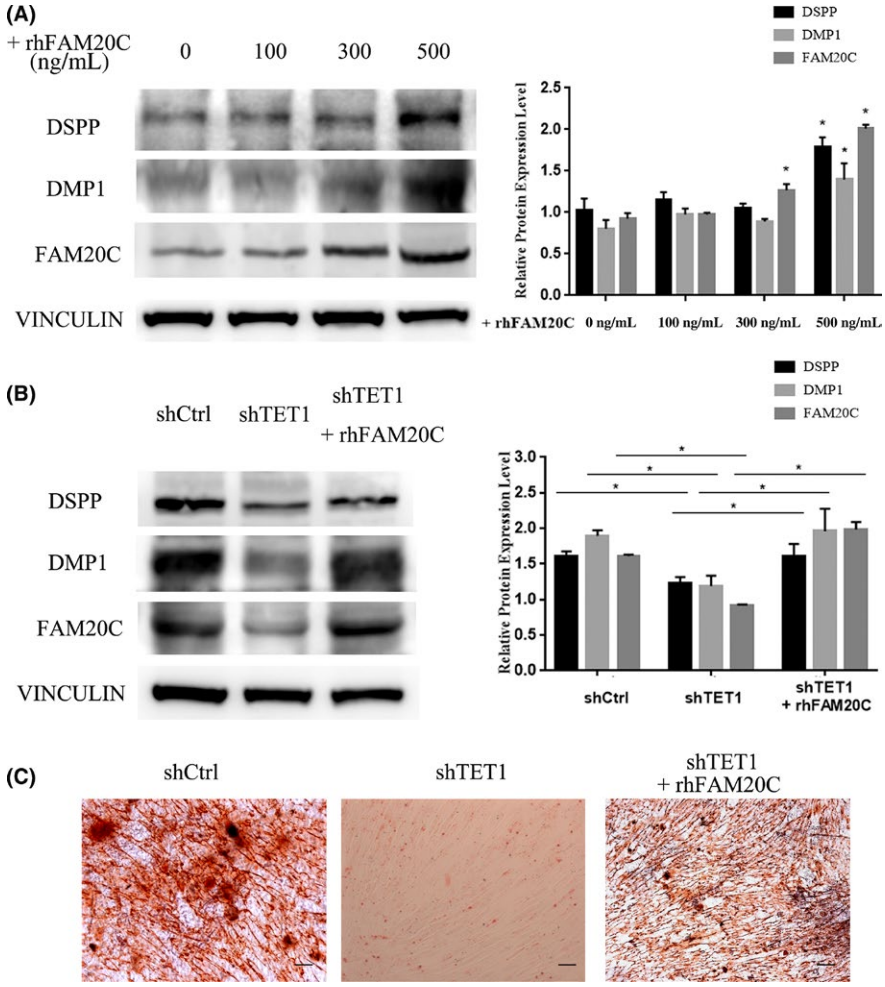

FIGURE 4 Recombinant FAM20C rescued the mineralization defects in ten-eleven translocation (TET)1-knockdown human dental pulp cells (hDPCs). A, FAM20C, DSPP and DMP1 expression levels were assessed using Western blotting on day 14 of culture in odontoblastic induction medium containing different concentrations of rhFAM20C. The band intensities were analysed using ImageJ software; VINCULIN was used as an internal control. B, FAM20C, DSPP and DMP1 protein levels of shCtrl and shTET1 groups with or without rhFAM20C were detected after 14 days of odontoblastic induction. The band intensities were analysed using ImageJ software; VINCULIN was used as an internal control. All of the results represent the mean ± standard deviation of 3 independent experiments (n = 3). *Statistically significant difference compared with the control; $P < .05$. C, The formation of mineralized nodules was assessed in shCtrl and shTET1 groups on day 14 of culture in odontoblastic induction medium with or without rhFAM20C treatment. The "black" scale bars represent 100 μm (original magnification ×100)

FAM20C gene leads to bone and craniofacial/dental abnormalities.[40] Conditional FAM20C gene knockout mice exhibit hypophosphatemic rickets and alveolar bone defects.[30,41] These studies confirm an essential role of FAM20C in the maintenance and development of mineralized tissues. However, its role in odontoblastic differentiation of hDPCs is still unclear. This study showed that FAM20C was elevated during odontoblastic differentiation of hDPCs in a time-dependent manner. FAM20C knockdown repressed the expression of DSPP and DMP1, ALPase activity and the mineralized matrix deposition, indicating that FAM20C was positively involved in regulating hDPC odontoblastic differentiation. The promoting effect of the FAM20C protein on hDPC differentiation was in conformity with previous studies regarding MC3T3-E1 cells and mouse odontoblasts.

TET1 tends to locate in CG rich promoters and CGIs genomewide.[42] In addition, TET1 knockdown cells lost 5hmC at CGI promoters of genes with high expression on average.[43] Louisa et al[44] reported that TET1 directly binds to the promoter of insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1) and influences the DNA hydroxymethylation status of its promoter, which can contribute to the stemness of human mesenchymal stem cells. Knocking down the rat spinal TET1 decreased 5hmC enrichment and further increased 5mC enrichment on the promoter CpG sites of the brain-derived neurotrophic factor gene, attenuating its expression.[45] Given that the expression pattern of FAM20C and the effect of FAM20C depletion on the odontoblastic differentiation of hDPCs were quite similar to those of TET1 and that FAM20C with a putative long CGI in its promoter is thought to be one of the potential TET1-occupancy genes in Mus musculus, we were prompted to explore the possibility that TET1 might regulate FAM20C transcription through its hydroxymethylation status in hDPCs. Our results revealed that TET1 deficiency with no compensatory upregulation of TET2 or TET3 led to significantly reduced odontoblast marker expression and a concomitant reduction in FAM20C levels. Moreover, administration of rhFAM20C in TET1-knockdown cells could rescue their mineralization defects. To test the hypothesis that TET1 might directly bind the FAM20C promoter to increase its 5hmC signal, we analysed dynamic 5hmC deposition and TET1 occupation of FAM20C. The results indicated that TET1 knockdown downregulated hydroxymethylcytosine (hmC) both globally and specifically at the FAM20C gene promoter locus. ChIP analysis combined with PCR further demonstrated that FAM20C was under epigenetic control of the TET1 enzyme through promoter binding. These findings revealed that enhanced recruitment of TET1 at target loci in the FAM20C promoter might hydroxylate nearby 5mC and alter gene transcription.

In general, the present study illustrated that TET1 knockdown restrained its promoter binding to the FAM20C gene and hence inhibited FAM20C hydroxymethylation and subsequent transcription. Thus, the

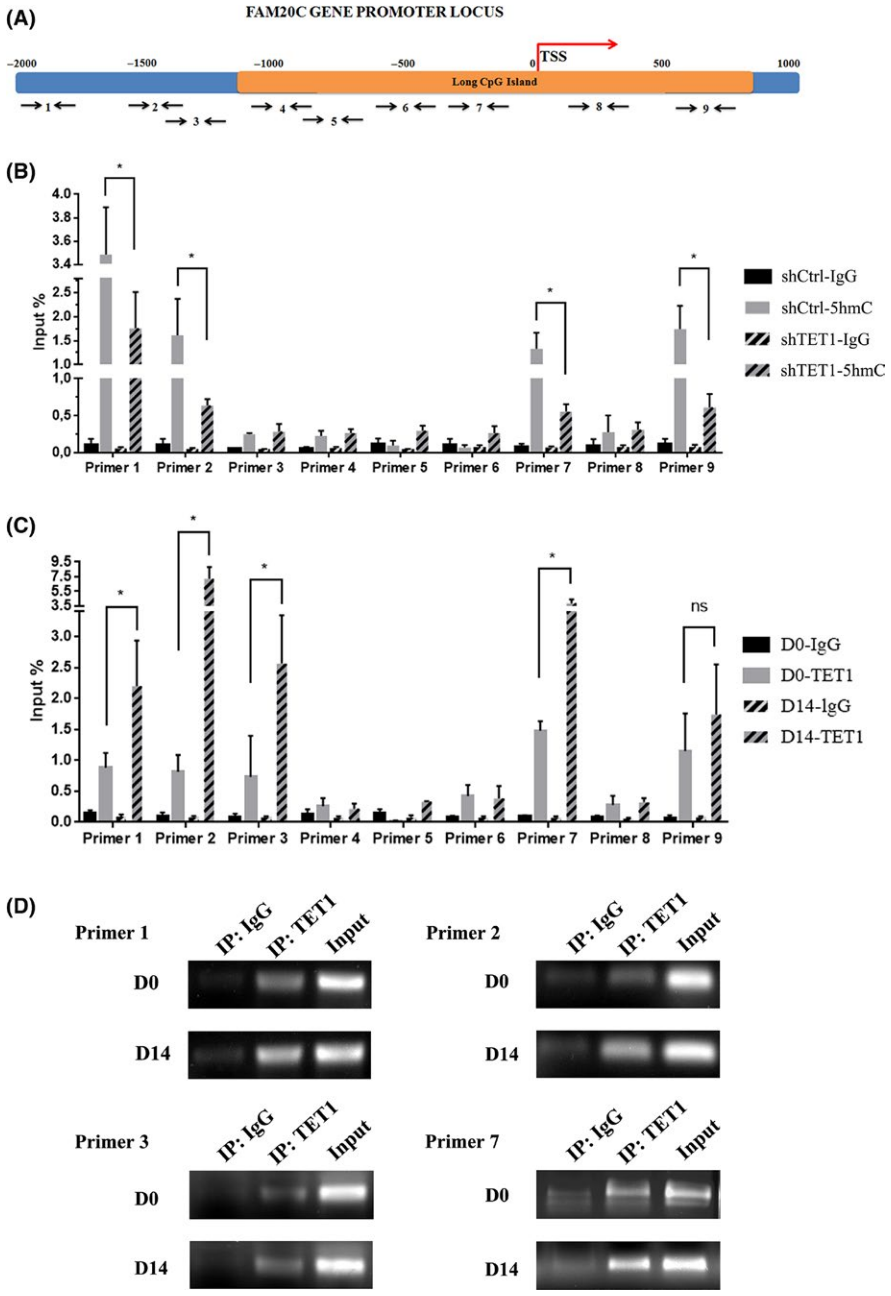

FIGURE 5 TET1 directly binds to FAM20C and engages its hydroxymethylation. A, Promoter region of the FAM20C gene. Arrows and numbers show the location of primers used in these analyses. The long CpG island is shown in orange. B, Dynamic hydroxymethylation levels of IP-DNA were evaluated with qPCR using the corresponding primer pairs indicated above. Bars represent shCtrl (no stripes) and shTET1 (stripes) groups. C, Signal of TET1 IP-DNA relative to the total amount of input DNA in human dental pulp cells extracted before (no stripes) and after (stripes) odontoblastic induction, as analysed with qPCR using the specific primer pairs indicated in (A). D, The significant results of (C) were verified via agarose gel electrophoresis. All of the results represent the mean ± standard deviation of 3 independent experiments (n = 3). *Statistically significant difference compared with the control; $P < .05$

resulting FAM20C deficiency led to decreased mineralization ability of hDPCs. These findings indicated that TET1 might promote odontoblastic differentiation of hDPCs through its DNA demethylation machinery and upregulation of FAM20C expression. This report might shed new light on the cytodifferentiation mechanisms of hDPCs. More studies are necessary to further illuminate the exact mechanisms of TET1-dependent FAM20C activation in hDPC proliferation and differentiation.

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