

# DNMT1 mediated methylation silences miR-214-3p gene and promotes hair follicle stem cell differentiate into adipogenic lineages

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## Research

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# Abstract

## Background

Dysfunction of the DNA methylation was associated with stem cell reprogramming. Moreover, DNA methyltransferase 1 (DNMT1) deficiency was involved in the differentiation of hair follicle stem cell (HFSc), but the molecular mechanisms remain unknown.

## Methods

HFSc from human scalp tissues were isolated and cultured. The oil red O staining was used to detect the adipogenesis. The interaction relationship between microRNA (miR)-214-3p and mitogen-activated protein kinase 1 (MAPK1) was accessed by dual-luciferase reporter gene assay. The methylation level of miR-214-3p promoter was detected by methylation-specific PCR, and the enrichment of DNMT1 in miR-214-3p promoter was detected by chromatin immunoprecipitation assay (ChIP). The mouse model of trauma was established to observe the skin regeneration at 0, 6 and 14 days.

## Results

Expression of DNMT1 and MAPK1 was increased in the HFSc, while the expression of miR-214-3p was reduced. Moreover, DNMT1 inhibited the expression of miR-214-3p by promoting the promoter methylation of miR-214-3p. Overexpression of DNMT1 could reduce the expression of miR-214-3p but increased the expression of MAPK1 and the extent of extracellular signal regulated kinase (ERK)1/2 phosphorylation. DNMT1 also significantly enhanced the adipogenic differentiation, and increased the proportion and markers of adipogenic cells. Importantly, DNMT1 promoted skin regeneration *in vivo*. Conversely, overexpression of miR-214-3p could reverse the effects of DNMT1 on adipogenesis of HFSc.

## Conclusion

DNMT1 promotes adipogenesis of HFSc by mediating miR-214-3p/MAPK1/phosphorylated-ERK1/2 axis. This study may provide more biomarkers for the potential application in stem cell therapy.

## Background

In the early stage of skin trauma, the basal cells at the edge of the wound begin to proliferate and form a single layer of epithelium, which cover the surface of the granulation tissue of the wound and then differentiate into squamous epithelium [1, 2]. Differentiation of epithelial cells is the key process of epidermal wound repair. Many complicated diseases, such as venous insufficiency [venous ulcer (VU)] and diabetes [diabetic foot ulcer (DFU)], could slow down the differentiation of epithelial cells [3]. However, the molecular mechanisms of regulation process of epithelial differentiation are still not fully

understood. Accumulating evidence suggests that epigenetic regulation of gene expression affects a variety of stem cell phenotypes [4]. The dynamic epithelial-mesenchymal crosstalk characteristic of hair follicle stem cells (HFSc) from embryonic development to adulthood can be found during normal hair follicle growth and cyclic regeneration [5]. Interestingly, emerging studies have revealed important functions of DNA methylation in skin biology such as can regulate HFSc differentiation [6, 7]. DNA methylation is an epigenetic modification to change the epigenome that was mediated by DNA methyltransferase. The function of DNA methyltransferases (DNMT)-1 plays a central role in DNA methylation and could silence several miRNA genes and accumulates in the promoter regions of miRNA genes [8]. However, the role of DNMT-1 in wound healing and skin regeneration remains poorly understood.

Recent study found that about 70 miRNAs have been playing a role in controlling the development and differentiation of skin stem cells [9]. Some studies found that over 200 miRNA were aberrantly expressed during the regeneration process of HFSc in the skin of mice [10]. Induction of dermal deletion in *Dicer* or *Drosha* in the skin of mice after birth also demonstrated the important role of miRNA in maintaining the normal process of growth cycle and differentiation of HFSc [11]. More importantly, emerging evidence now supports the idea that DNA methylation is crucially involved in the dysregulation of miRNAs in many diseases, such as cancer, metabolic disorder and atherosclerosis [12–14]. In the present study, we aim to detect the relationship between DNMT1 and miR-214-3p in the process of HFSc differentiation into adipogenic lineages.

## Materials And Methods

### Ethics statement

This study was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University and the informed consent of the patients was obtained. We performed this study following the principles recommended by *Declaration of Helsinki*. All experimental procedures involving animals were performed in accordance with animal protocols approved by the Institutional Animal Use and Care Committee.

### Hair follicle stem cell isolation

Scalp tissues were obtained from 9 patients (6 males and 3 females) with a scalp laceration and contusion at the First Affiliated Hospital of Zhengzhou University. HFSc were isolated as described previously [15] with minor changes to the protocol. The scalp tissues were rinsed with penicillin-containing Hank's solution for 3 times and penicillin-free Hank's solution for 2 times. The scalp tissues were cut into 2mm × 2mm size and digested with 0.48U/mL neutral protease for overnight at 4 °C. The intact hair follicles were gently extracted from hair follicles with surgical tweezers. Add 0.05% trypsin and 0.02% ethylene diamine tetraacetic acid (EDTA) mixture into the cut hair follicles tissue and digest at 37 °C for 30 min. Terminate the digestion then filter with 100 mesh steel mesh, and centrifuge the supernatant with 1000 rpm for 5 min. Added the Dulbecco's modified eagle medium (DMEM) F12 (3: 1), insulin (5 mg/L), transferrin (5 mg/L), hydrocortisone (0.4 mg/L), EGF (20 µg/L), amphotericin B (2.5

mg/L), penicillin (1051 U/ L), streptomycin (100 mg/L) and 20% fetal bovine serum (FBS) into centrifugal. The cells were cultured at 5% CO<sub>2</sub> and 37 °C saturated humidity. Finally, flow cytometry was used to detect the surface markers including CK14, CD200, Integrin α 6, p63 of HFSc to identify the successful isolation.

### **Differentiation and transfection of HFSc**

HFSc were differentiated into adipocytes according to previously described methods [16]. Briefly, primary HFSc were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin (growth medium) in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. Forty-eight hours after confluence, differentiation was induced with DMEM supplemented with 10% FBS, 1 μM dexamethasone, 10 μg/mL insulin, 0.2 mmol/L indomethacin, 0.1 mmol/L ascorbic acid and 0.5 mM 3-isobutyl-1-methylxan-thine (IBMX) (MDI medium). Two days later, the medium was replaced with DMEM (10% FBS) and was changed every 2 d.

To construct lentiviral vectors overexpressing DNMT1 and MAPK1 and miR-214-3p gene, the human DNMT1 and MAPK1 and miR-214-3p gene DNA fragment was PCR amplified from human SGC7901 cell genomic DNA. The PCR-amplified fragments were inserted into a lentiviral vector pLV-EF1α-MCSIRES-Puro (pLV-ctrl) to generate pLV-DNMT1 and pLV-MAPK1 and pLV- miR-214-3p. Viral vector pLV-DNMT1 and pLV-MAPK1 and pLV- miR-214-3p and pLV-ctrl were transfected into HEK293T cells. Media containing lentiviruses (pLV-DNMT1 and pLV-MAPK1 and pLV- miR-214-3p and pLV-ctrl) were collected every 24 h for three times and the lentiviruses were purified by ultra-speed centrifugation. Full-length cDNA encoding human DNMT1 and MAPK1 and miR-214-3p were amplified by PCR, and the PCR product was sub-cloned into pBOBI and pCMV-HA vectors to obtain DNMT1 and MAPK1 and miR-214-3p gene overexpressing plasmids.

For cell transient transfection, the HFSc were seeded and grown in 6-well plates overnight. The short interfering RNA (siRNA) was used for inhibiting endogenous RNA expression [17]. The HFSc were transfected with negative control siRNA oligonucleotides, positive control siRNA oligonucleotides, siRNA against DNMT1 and miR-214-3p, using by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Besides, the HFSc were transfected with DNMT1 and MAPK1 and miR-214-3p gene overexpressing plasmids. The PD98059 (19-143, Sigma, St. Louis, MO, USA), a MAPK1 inhibitor, was also administrated in HFSc. Subsequent experiments were carried out 48 h after transfection.

### **Reverse transcription quantitative polymerase chain reaction (RT-qPCR)**

TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from cells, and the total RNA concentration and purity were detected by nanodrop2000 microultraviolet spectrophotometer (1011U, Nanodrop, USA). The RNA was reversely-transcribed to complementary (cDNA) according to the instructions of TaqMan MicroRNA Assays Reverse Transcription primer (4427975, Applied Biosystems, USA), and the primer of miR-214-3p was designed and synthesized by TaKaRa (Table 1) with GAPDH as a

control. For miRNAs, qPCR was performed with the stem-loop primers as reported previously [18]. U6 RNA served as an internal control. Each experiment was repeated three times.

## Western blotting

Total protein was extracted from radio immunoprecipitation assay (RIPA) lysis buffer containing phenylmethanesulfonyl fluoride (PMSF) (P0013C, Beyotime Biotechnology, Shanghai, China) on ice for 30 min, centrifuged at 8,000g for 10 min at 4 °C. The 50 ug protein was dissolved in 2 × SDS loading buffer and boiled at 100°C for 5 min. Proteins were separated by electrophoresis on 8% to 12% SDS-polyacrylamide gels and transferred moist to polyvinylidene difluoride membranes. Membranes were blocked by 5% nonfat dry milk in PBS and incubated with antibodies for DNMT1 (1:1000, ab188453, Abcam, Cambridge, UK), MAPK1 (1:200, R&D, MAB1230-SP), ERK1/2 (1:10000, ab184699, Abcam), phosphorylated (p)-ERK1/2 (1:1000, ab214362, Abcam), PPAR-γ2 (1:500, ab23673, Abcam), perilipin (1:1500, ab3526, Abcam), Adipoq (1:1000, ab62551, Abcam), aP2 (1:1000, ab218107, Abcam) and GAPDH (1:2500, ab9485, Abcam) overnight at 4°C. The membrane was incubated with HRP-labeled goat anti-rabbit IgG (1:2000, ab97051, Abcam) for 1 h. The ECL fluorescence detection kit (BB-3501, Ameshame, UK) was added on the membrane, then using Bio-Rad image analysis system (Bio-Rad, USA) with Quantity One v4.6.2 analysis software to analysis and GAPDH was used as internal control. The experiment was repeated three times. **Oil-red-O staining**

The HFSc were cultured in DMEM/F12 medium and collected on 7d and 14d, respectively. The cells were fixed with 10% formalin, washed with 60% isopropanol, and stained with oil red O working fluid. Being fixed by glycerine gelatin, cells were observed under a microscope (Olympus optics, Tokyo, Japan). The number of positive cells stained with oil red O was counted under the microscope.

## Dual-luciferase reporter assay

MAPK1 was identified as a miR-214-3p target in TargetScan7.1 ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)). Human HEK293T cells were cultured in DMEM medium containing 10% FBS. The cDNA fragment of MAPK1 3' -untranslated region (UTR), MAPK1-wild type (Wt) containing the miR-214-3p binding site was inserted into the pmiRGLO vector. The cDNA fragment of MAPK1 3' -UTR, MAPK1-mutant type (Mut) was synthesized by point mutation and inserted into pmiRGLO vector. The inserted sequence was verified to be correct by sequencing performed by RiboBio Co., LTD (Shanghai, China). The recombinant vector pmiRGLO-MAPK1-Wt or pmiRGLO-MAPK1-Mut was co-transfected with a miR-214-3p mimic (miR-214-3p overexpression sequence) or an NC mimic (negative control sequence) into HEK293T cells by liposome transfection, and the cells were incubated and cultured for 48 h before being collected and lysed. Take 100 μL lysate supernatant of was taken and 100 μL Renilla luciferase assay solution was added to detect the activity. In addition, take 100 μL lysate supernatant and 100 μL firefly luciferase to detect the luciferase activity. After 48 hours, the cells were collected, and the luciferase and Renilla luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to Renilla luciferase activity. The SpectraMax M5 (Molecular devices instruments CO., LTD., Shanghai, China, Origin: USA) with an interval of 2 s and a

determination time design of 10 s was used to detect the luciferase activity of Renilla luciferase and Firefly luciferase, respectively.

### **Real-time quantitative methylation-specific PCR (MSP)**

The methylation of gene promoter was detected by MSP. Genomic DNA was extracted by Genomic DNA extraction kit (Tiangen Biochemical technology CO., LTD, Beijing, China) according to the instructions. The DNA concentration and purity were determined by ultraviolet spectrophotometry. DNA was treated with sodium sulfite using the EZ DNA Methylation Kit (Zymo Research, USA), and the reaction column was used for desulfurization and purification. The purified DNA could be used for subsequent PCR reaction. The methylation and non-methylation primers (Table 2) were designed for the miR-214-3p gene promoter by CpG island enrichment area. The reaction products were subjected to agarose gel electrophoresis, gel electrophoresis imaging and image analysis system. If the CpG island in the promoter region is completely methylated, only the methylated primer can amplify the target band. If there is no methylation, only non-methylated primers can amplify the target band. If partial methylation occurs, the target bands can be amplified from both primers. Partial methylation is classified as methylation. Serial dilutions of plasmid DNA was used as standards for quantification [19]. Each experiment was repeated three times.

### **Chromatin immunoprecipitation (ChIP)**

Differentiated and cultured HFSc were taken, the cell fusion degree reached 70-80%, 1% formaldehyde was added and fixed at room temperature for 10 min to make the DNA and protein in the cell fixed and cross-linked. Then, the protein was broken randomly by ultrasonic treatment for 10 s, and interval for 10 s and 15 cycles and to make fragments of appropriate size. The supernatant was collected by centrifugation at 13000 rpm and divided into two tubes, and added with antibody for negative control and rabbit anti-IgG (1: 100, ab172730, Abcam, UK) and mouse anti-DNMT1 (1: 100, ab13537, Abcam, UK) at 4°C for overnight, respectively. The endogenous DNA-Protein complex was precipitated by Protein Agarose/Sepharose, and the non-specific complex was washed, the cross-linking was performed overnight at 65°C, and the DNA fragments were extracted and purified by phenol and chloroform to detect the binding of miR-214-3p gene promoter fragment to DNMT1.

### **Mouse model of trauma**

A total of 24 mice (weight  $20 \pm 2$  g) were purchased from Hunan SJA Laboratory Animal Co., Ltd. The mice were anesthetized by intraperitoneal injection with 3% barbiturate. Two wounds at a position of 1.0 cm on both sides of the back spinal column were created to form a circular skin incision and not touch the muscles. After the wound was formed, it was not bandaged and treated with medicine. The mice was kept separately in a sterile laboratory and sterilized every day. Lentivirus vectors ( $5 \times 10^8$  pfu/100 L) were introduced into the wound surface of mice beside the wound surface. The mice were randomly divided into oe-NC+sh-NC group (n=8), and oe-DNMT1+sh-NC group (n=8) and oe-DNMT1+sh-MAPK1 group (n=8). The overexpressing lentivirus vectors were used by LV5-GFP and silencing lentivirus vectors were used by pSIH1-H1-copGFP. The wound area of each group was photographed and recorded on day 0, 6,

10 and 14, respectively. The mice were sacrificed on day 18, and skin tissue was extracted from the wound. The tissue sections were embedded by paraffin or used to extract proteins for detection.

### **Immunofluorescence staining**

Mouse skin tissues were fixed with 4% paraformaldehyde for overnight. Then, the tissue was washed and sealed with normal saline of 0.01 M phosphate buffer for three times, and then sealed with 10% goat serum at room temperature for 30 min. After that, TGF- $\beta$ 1 (1:200, ab92486, abcam, Cambridge, UK), vascular endothelial growth factor (VEGF) (1:200, ab2350, abcam, Cambridge, UK) and platelet derived growth factor (PDGF-BB) (1:200, ab9704, abcam, Cambridge, UK) were incubated at 4°C for overnight. The second antibody and DAPI were incubated at room temperature in the dark for 1 hour, and glycerol was fixed. The confocal laser scanning microscope was used to analyze (LSM, FV1000; Olympus corp., Tokyo, Japan).

### **Statistical analysis**

All statistical tests were performed using SPSS 21.0 (IBM SPSS Statistics, Armonk, NY, USA). The data were presented as mean  $\pm$  standard deviation. The data in the two groups were compared by unpaired Student's *t* test, and the data in multiple groups were compared by one-way analysis of variance (ANOVA) and Tukey's post-test.  $p < 0.05$  was considered statistically significant.

## **Results**

### **HFSc are successfully isolated**

The markers for HFSc at passage 3, CK14, CD200, Integrin  $\alpha$ 6 and p63, were tested, to confirm the isolation of HFSc. There was an increase expression of CK14, CD200, Integrin  $\alpha$ 6 and p63 in HFSc (Fig. 1), suggesting that HFSc were successfully isolated.

### **DNMT1 promotes differentiation of HFSc into adipogenic lineages**

In order to demonstrate the effect of DNMT1 in the differentiation of HFSc, the HFSc were induced differentiation into adipogenic lineages. The Oil red O staining detected lipid droplets in the cytoplasm at the 7d after induction and progressively increased to 14d (Fig. 2A), suggesting that HFSc have successfully undergone differentiation of adipogenic lineages. The expression of DNMT1 was increased at 7d and peaked at 14d by successful induction (Fig. 2B). After transfection with overexpressing DNMT1 vectors, the expression of DNMT1 was increased (Fig. 2C), and the proportion of differentiated cells was significantly up-regulated by Oil red O staining (Fig. 2D). Moreover, the expression of adipose formation markers, PPAR- $\gamma$ 2, perilipin, Adipoq and aP2, were significantly increased by overexpressing of DNMT1 (Fig. 2E). These data indicated that DNMT1 promotes HFSc differentiate into adipogenic lineages.

# DNMT1 promotes differentiation of HFSc into adipogenic lineages by silencing miR-214-3p expression

Subsequently, we found the expression of miR-214-3p was reduced at 7d and lowest at 14d by differentiation (Fig. 3A). The MSP showed that the methylation level of miR-214-3p promoter was significantly increased at 7d and 14d by differentiation (Fig. 3B). We further investigated whether DNMT1 can directly influence differentiation process of HFSc by methylation effects in the promoter region of the miR-214-3p gene. We found that the enrichment of DNMT1 on miR-214-3p promoter was significantly increased at 7d and 14d by differentiation (Fig. 3C).

To detect the role of miR-214-3p in the process of DNMT1 promoting HFSc differentiation, we test the mRNA and protein level of DNMT1 in different HFSc groups. We found that overexpression of DNMT1 could significantly reduce the level of miR-214-3p, and which could be reversed by administration of miR-214-3p mimic (Fig. 3D-E). The Oil red O staining showed the proportion of differentiated cells was significantly increased in oe-DNMT1 + NC mimic group than that in oe-NC + NC mimic group, and which was restored in oe-DNMT1 + miR-214-3p mimic group (Fig. 3F). Moreover, the expression level adipose formation markers, PPAR- $\gamma$ 2, perilipin, Adipoq and aP2, were higher in oe-DNMT1 + NC mimic group than that in oe-NC + NC mimic group, and which was restored in oe-DNMT1 + miR-214-3p mimic group (Fig. 3G).

## The miR-214-3p inhibits adipogenic differentiation of HFSc by inhibiting MAPK1

To detect the regulation mechanism of miR-214-3p, we predicted through the Starbase2 (<http://starbase.sysu.edu.cn/starbase2/>) and found MAPK1 might be the downstream regulatory gene of miR-214-3p (Fig. 4A). More importantly, Cai *et al.* showed that MAPK1 promotes HFSc proliferation, wound contraction and epidermal regeneration in mouse model [20]. Here, we found the expression of MAPK1 was significantly increased in HFSc by differentiation at 7d and 14d (Fig. 4B). To investigate the potential targeting of MAPK1 by miR-214-3p, a luciferase activity assay was designed. Expression of miR-214-3p in 293T and SGC7901 cells obviously inhibited the luciferase activity of the MAPK1-3UTR reporter. Additionally, our qPCR analyses indicated that, compared to the control transfected group, transfection of 293T and SGC7901 cells with miR-214-3p significantly increased miR-214-3p expression (Fig. 4C), suggest that MAPK1 mRNA was subjected to post-transcriptional control of miR-214-3p by targeting the MAPK1-3' UTR. We further transfection HFSc with miR-214-3p inhibitor to decrease the expression of miR-214-3p (Fig. 4D), but the expression of MAPK1 was significantly increased by miR-214-3p inhibitor (Fig. 4E). These data indicated that miR-214-3p can target to inhibit MAPK1.

To explore whether miR-214-3p affect adipogenic differentiation of HFSc by regulating MAPK1, the HFSc were incubated with inhibitor of miR-214-3p and MAPK1. We found the gene expression level of miR-214-3p was reduced by silence of miR-214-3p, while the gene expression level of MAPK1 was increased (Fig. 4F). Similar results were confirmed by western blotting test (Fig. 4G). The Oil red O staining showed

the proportion of differentiated cells was significantly increased in miR-214-3p inhibitor + sh-NC group than that in NC inhibitor + sh-NC group, while the proportion of differentiated cells was significantly reduced in miR-214-3p inhibitor + sh-MAPK1 group (Fig. 4H). The similar tendency was observed on detection the expression levels of adipose formation markers protein including PPAR- $\gamma$ 2, perilipin, Adipoq and aP2 (Fig. 4I).

## **The miR-214-3p inhibits adipogenic differentiation of HFSc by reducing MAPK1-mediated expression of p-ERK1/2**

Previous studies showed that MAPK1 promotes the expression of p-ERK1/2 [21], While the epidermal growth factor (EGF) that promotes the expression of p-ERK1/2 [22] could induce the differentiation of HFSc, suggesting that p-ERK1/2 may play an important role in the differentiation process of HFSc. Here, we found the expression of phosphorylation of ERK1/2 was significantly increased in HFSc by differentiation at 7d and 14d (Fig. 5A). We also found the expression of phosphorylation of ERK1/2 was significantly increased by miR-214-3p inhibitor, while it was reversed by miR-214-3p inhibitor and sh-MAPK1 (Fig. 5B) suggesting that miR-214-3p could promote the expression of p-ERK1/2 by regulating MAPK1.

To test whether miR-214-3p promotes MAPK1 by regulating p-ERK1/2, the PD 98059, an inhibitor of p-ERK1/2, was administrated in HFSc. Here, we found overexpression of MAPK1 significantly enhanced the expression level of p-ERK1/2, while PD 98059 reversed the effect (Fig. 5C). The Oil red O staining showed the proportion of differentiated cells was significantly reduced in miR-214-3p inhibitor + PD 98059 group, while it was significantly increased by overexpression of MAPK1. In addition, the effect of MAPK1 was restored by PD 98059 (Fig. 5D). Moreover, the similar results of adipose formation markers protein including PPAR- $\gamma$ 2, perilipin, Adipoq and aP2 were observed by western blotting test (Fig. 5E). Taken together, these data indicated that miR-214-3p inhibits adipogenic differentiation of HFSc by reducing MAPK1-mediated expression of p-ERK1/2.

## **DNMT1 increased the expression MAPK1/p-ERK1/2 axis by silences miR-214-3p, thereby promotes wound healing and skin regeneration in mice**

The mouse model of trauma was established to test the effect of DNMT1 on the adipogenic differentiation of HFSc. The expression of DNMT1, MAPK1 and phosphorylated-ERK1/2 gene was increased and miR-214-3p expression reduced in oe-DNMT1 + sh-NC group. While the expression levels of MAPK1 and p-ERK1/2 gene were reduced in oe-DNMT1 + sh-MAPK1 group compare with oe-DNMT1 + sh-NC group (Fig. 6A). The similar expression tendency of protein was found by western blotting test (Fig. 6B). Here, we found the epidermis of mice in each group showed different degrees of growth and recovery, and the skin regeneration was peaked by overexpression of DNMT1 that reserved by sh-MAPK1 (Fig. 6C). Moreover, the wound healing growth factors including TGF- $\beta$ 1 and VEGF and PDGF-BB in all groups were detected by immunofluorescence. Compare with the oe-NC + sh-NC group, the expression of TGF- $\beta$ 1 and VEGF and PDGF-BB were significantly increased in oe-DNMT1 + sh-NC group, while it was restored in oe-DNMT1 + sh-MAPK1 group (Fig. 6D). These data indicated that DNMT1 increased the

expression MAPK1/p-ERK1/2 axis by silences miR-214-3p, thereby promotes wound healing and skin regeneration in mice.

## Discussion

In the present study, we found that DNMT1 gene was upregulated in adipogenic differentiation of HFSc. In addition, our results showed that miR-214-3p expression was reduced when its gene promoter was hypermethylated in adipogenic differentiation of HFSc. Moreover, treatment with MAPK1 could restore the proportion of differentiated cells in cultured adipogenic differentiation of HFSc by miR-214-3p. Altogether, hypermethylation of miR-214-3p gene promoter accounts as a primary source for the loss of miR-214-3p expression in adipogenic differentiation of HFSc. As a result, the skin regeneration was enhanced by overexpression of DNMT1 in the mouse model of trauma. Thus, our findings may help to clarify the mechanism of adipogenic differentiation of HFSc, and address the possible relationship between DNMT1 and miR-214-3p in the differentiation process of HFSc.

DNA methylation is an epigenetic modification method in which is mediated by DNA methyltransferase. DNA methyltransferase consists of three subtypes, including DNMT1, DNMT2 and DNMT3, which catalyze the methylation of cytosine bases and cause changes in apparent modification. DNMT1 is a kind of semi-methylated DNA methyltransferase, which can accumulate in the promoter region of many genes, leading to gene silencing. Previous study has found that DNMT1 gene deficiency can reduce the activation rate of stem cells in the aging process of mice, suggesting that DNMT1 is closely related to stem cell differentiation. [23]. Moreover, the expression of DNMT1 is reduced in de-differentiation of HFSc [24], suggesting that DNMT1 may play an important role in the differentiation process of HFSc. In the present study, we found that inducing epidermal stem cell differentiation can significantly increase the expression of DNMT1 (Figure 2B), while overexpression of DNMT1 can significantly increase the proportion of epidermal stem cell differentiation (Figure 2C), suggesting that DNMT1 can promote epidermal stem cell differentiation by inducing DNA methylation. Numerous studies have shown that DNA methylation and miRNA influence each other and that maintain the stability of the body in a balanced relationship. For example, the promoters of miR-122, miR-129 and miR-191 are affected by DNA methylation and participate in the occurrence of a variety of tumors. Chen et al found that inhibition of DNMT1 activity could up-regulate the expression by inducing miR-214-3p DNA demethylation [25], and miR-214-3p was reduced in the in the differentiation process of HFSc [15]. Therefore, we speculated that DNMT1 may affect the differentiation of HFSc by inhibiting the expression of miR-214-3p. In the present study, we found that the expression of miR-214-3p was reduced in lipogenic HFSc, and the methylation level of miR-214-3p promoter was significantly increased, suggesting that the adipogenic differentiation of HFSc may be caused by miR-214-3pDNA methylation. In addition, we found that miR-214-3p could reverse the differentiation of HFSc induced by overexpression of DNMT1. In order to further clarify the molecular mechanism of the regulation of miR-214-3p by DNMT1, we used ChIP and dual-luciferase reporter gene detection to confirm the directly effect of DNMT1 and miR-214-3p.

Although it has been found that inhibition of miR-214-3p could induce HFSc differentiation, the specific molecular mechanism has not been elucidated. Through the prediction of the website, we found the downstream binding target MAPK1 of miR-214-3p. In the present study, we found that miR-214-3p can directly inhibit the expression of MAPK1, and the dual-luciferase reporter genes also confirmed the direct binding between miR-214-3p and MAPK1 gene. Cai et al. showed that MAPK1 could promote HFSc proliferation, wound contraction and epidermal regeneration of mouse model [20]. Consistent with the previous study, we found that inhibition of miR-214-3p could induce HFSc differentiation, while inhibition of MAPK1 can reverse this effect, suggesting that MAPK1, as a downstream effector molecule of miR-214-3p, plays a role in promoting HFSc differentiation. ERK1/2 is a classical effector molecule downstream of MAPK1. Here, we found that miR-214-3p inhibits the adipogenic differentiation of HFSc by inhibiting the MAPK1-mediated up-regulated expression of p-ERK1/2. Finally, we constructed a wound healing mouse model and found that overexpression of DNMT1 could promote the skin regeneration, while inhibition of MAPK1 could reverse the above effects.

In conclusion, the present study confirmed for the first time that DNMT1 can inhibit miR-214-3p-mediated MAPK1 expression and promote HFSc differentiation by increasing the methylation of miR-214-3p promoter. In injured human epithelial cells, the gradual loss of DNMT1 protein or enzyme activity has been reported, indicating that the loss of DNMT1 in epithelial cells is part of the process of skin regeneration. Future work will identify more DNMT molecular targets in stem cells and further clarify the relationship between epidermal DNMT defects and human skin lesion repair.

## Abbreviations

DNMT1 (DNA methyltransferase 1); HFSc (hair follicle stem cell); miR (microRNA); MAPK1 (mitogen-activated protein kinase 1); VU (venous ulcer); DFU (diabetic foot ulcer); EDTA (ethylene diamine tetraacetic acid); DMEM (Dulbecco's modified eagle medium); FBS (fetal bovine serum); siRNA (short interfering RNA); PMSF (phenylmethanesulfonyl fluoride).

## Declarations

### Acknowledgements

We would like to acknowledge the colleagues for their helpful assistance on this study.

### Author contributions

Fangcao Jin and Min Li conceived and designed the study, Xuyang Li and Yunpeng Zheng collected the data, Kun Zhang and Xiaojun Liu analyzed the data, Bingjie Cai and Guangwen Yin wrote the manuscript. All authors read and approved the final manuscript.

## Funding

None.

## Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article.

## Ethics approval and consent to participate

This study was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University and the informed consent of the patients was obtained. We performed this study following the principles recommended by *Declaration of Helsinki*. All experimental procedures involving animals were performed in accordance with animal protocols approved by the Institutional Animal Use and Care Committee.

## Consent for publication

Not applicable.

## Competing interests

The authors declare no conflicts of interest.

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## Tables

**Table 1** Primer sequences for RT-qPCR

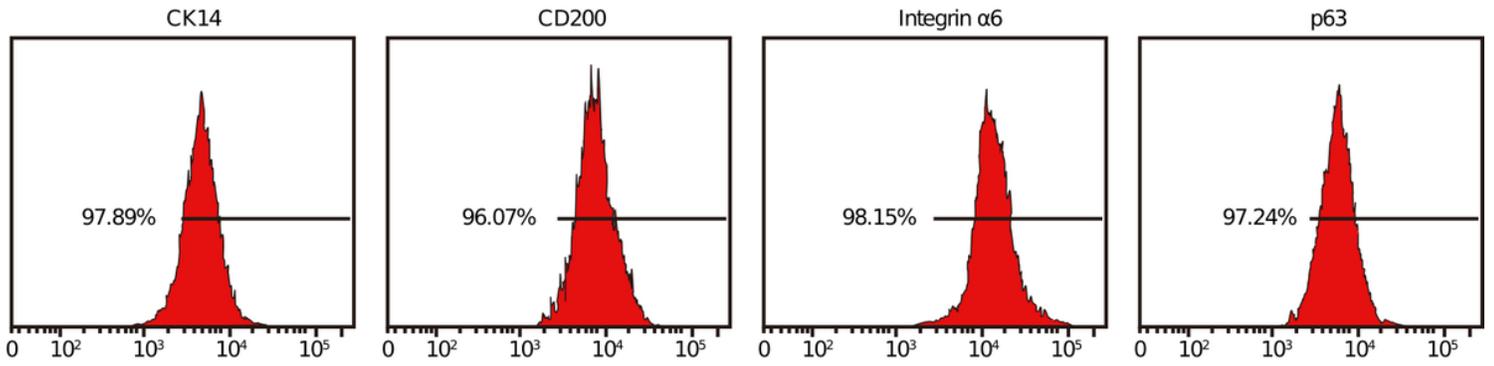
Primer sequences	
miR-214-3p	F: 5'-ACACTCCAGCTGGGACAG-3' R: 5'-CTCGCTTCG GCAGCACA-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3'

Notes: F, forward; R, reverse

**Table 2** Primer sequences for MS-PCR

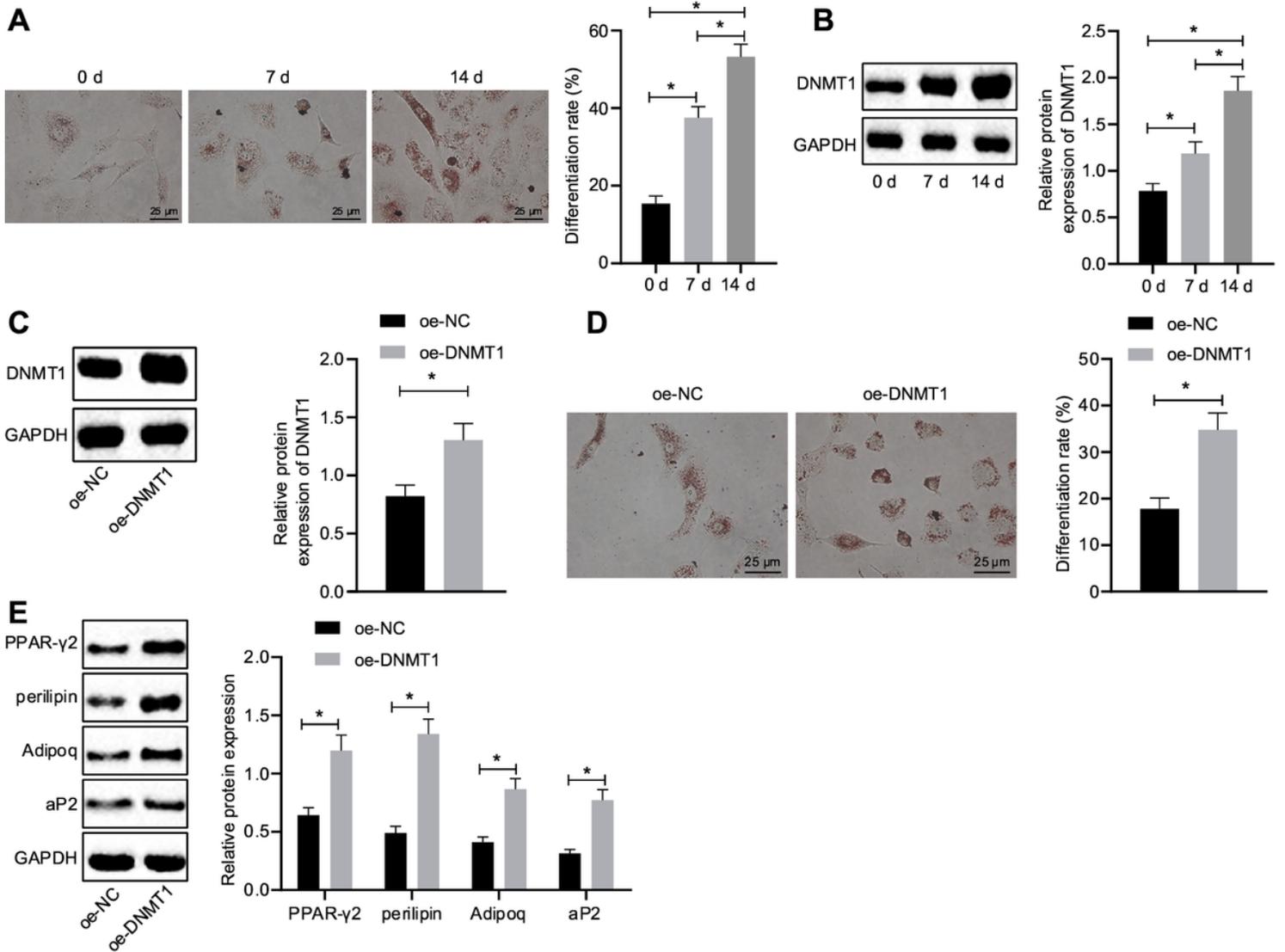
Primer sequences	
miR-214-3p-M	F: 5'-TTGTTTGGTATTCGGGTTTTTC-3' R: 5'-AAAAAATAAAAAAATTCTTTCGTT-3'
miR-214-3p-U	F: 5'-TTTTTGTGGTATTTGGGTTTTT-3' R: 5'-AAAAAATAAAAAAATTCTTTCATT-3'

# Figures



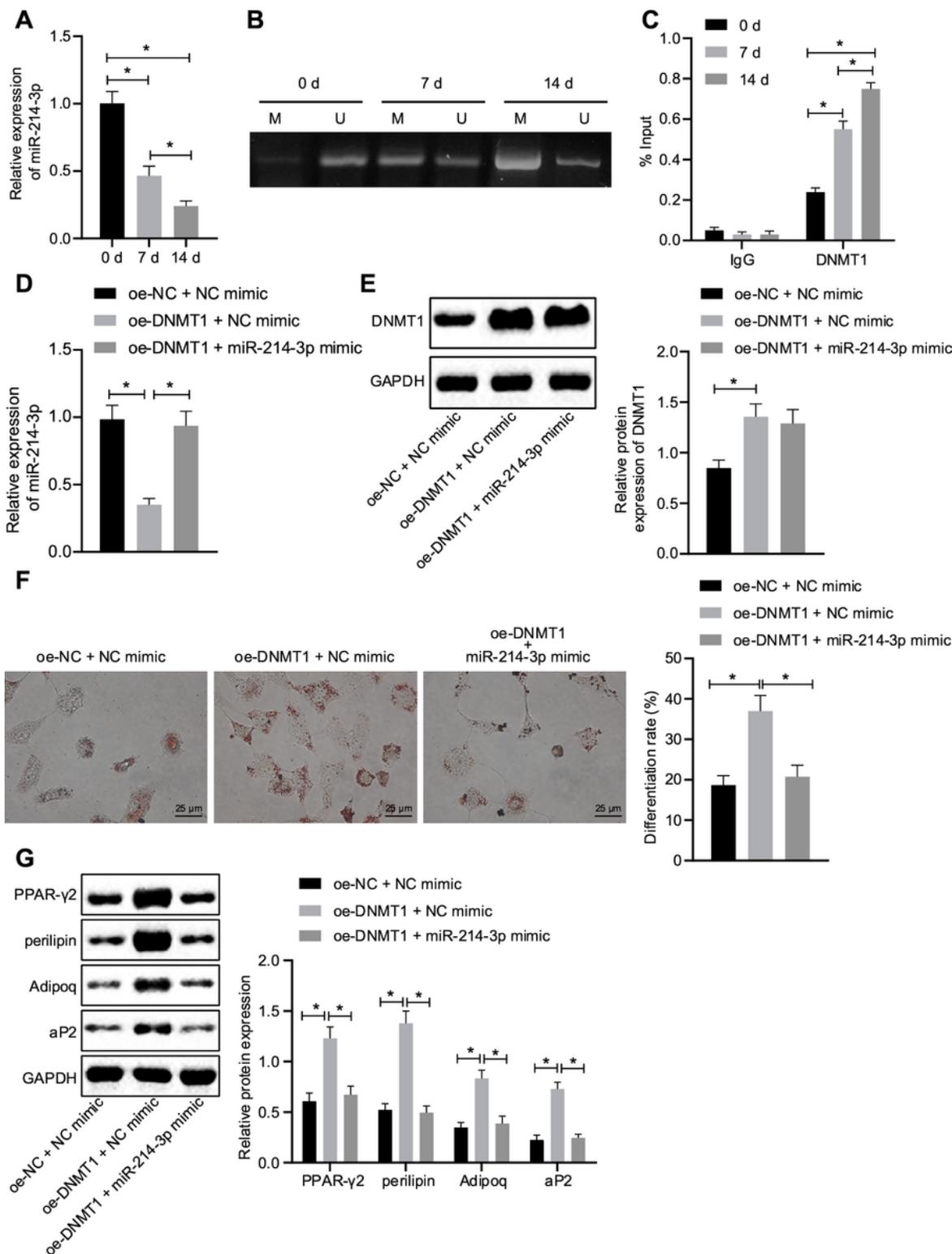
**Figure 1**

Surface markers of HFSc were detected by flow cytometry.



## Figure 2

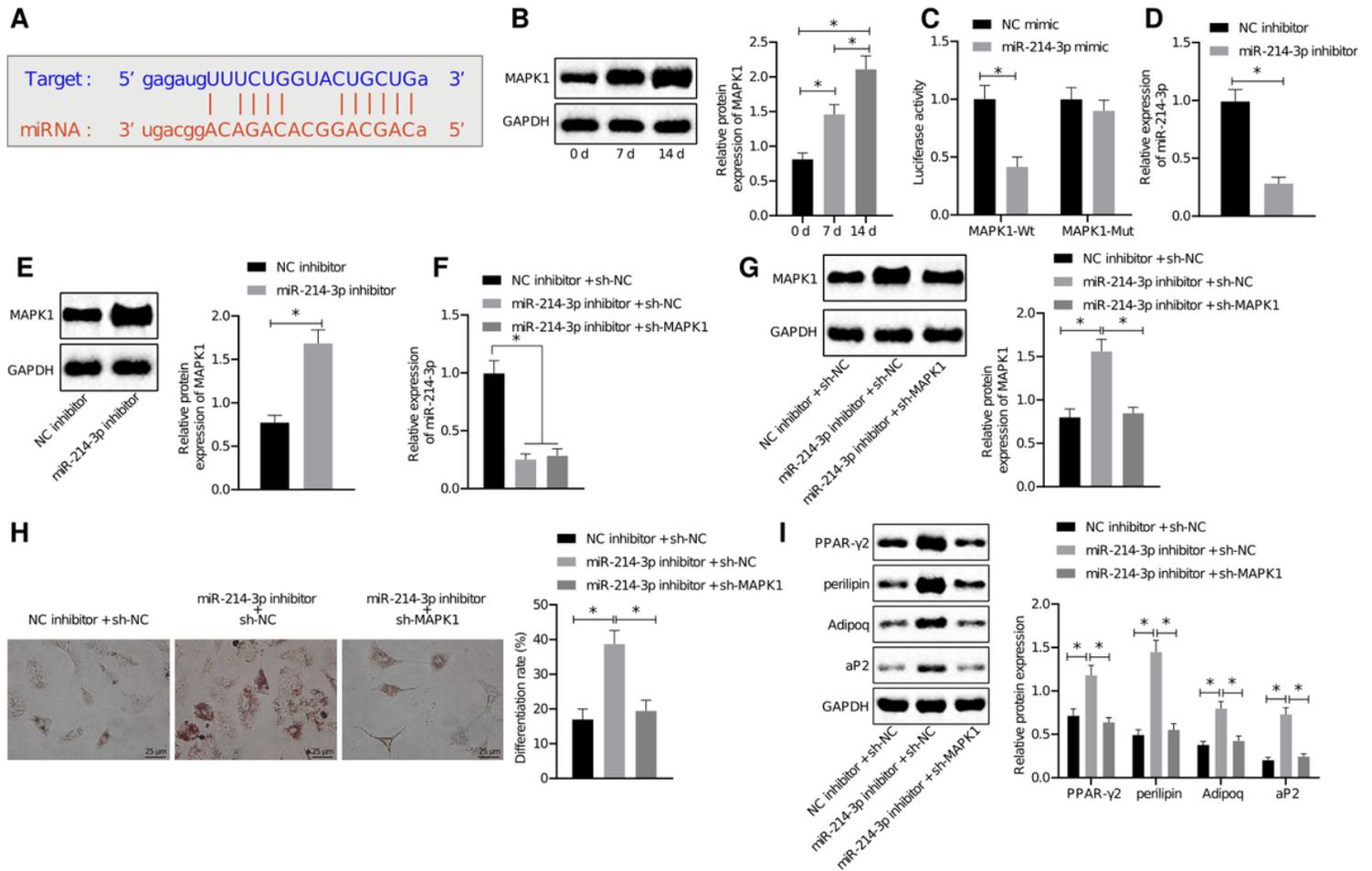
DNMT1 promoted adipogenic differentiation of HFSc. (A) Oil red o staining was used to detect the adipogenic differentiation of HFSc. (B) WB detected the expression of DNMT1 in HFSc cultured in differentiation medium for 0d, 7d and 14d. (C) WB detects the expression level of DNMT1 by overexpression of DNMT1. (D) The degree of adipogenic differentiation of HFSc after overexpression of DNMT1 was detected by Oil red O staining. (E) WB was used to detect the expression level of adipose formation markers. Data were expressed as mean  $\pm$  standard deviation. (A, B) by one-way ANOVA and Tukey's post hoc test. (C-E) by unpaired Student's t test, n=3 per group, \*P < 0.05.



**Figure 3**

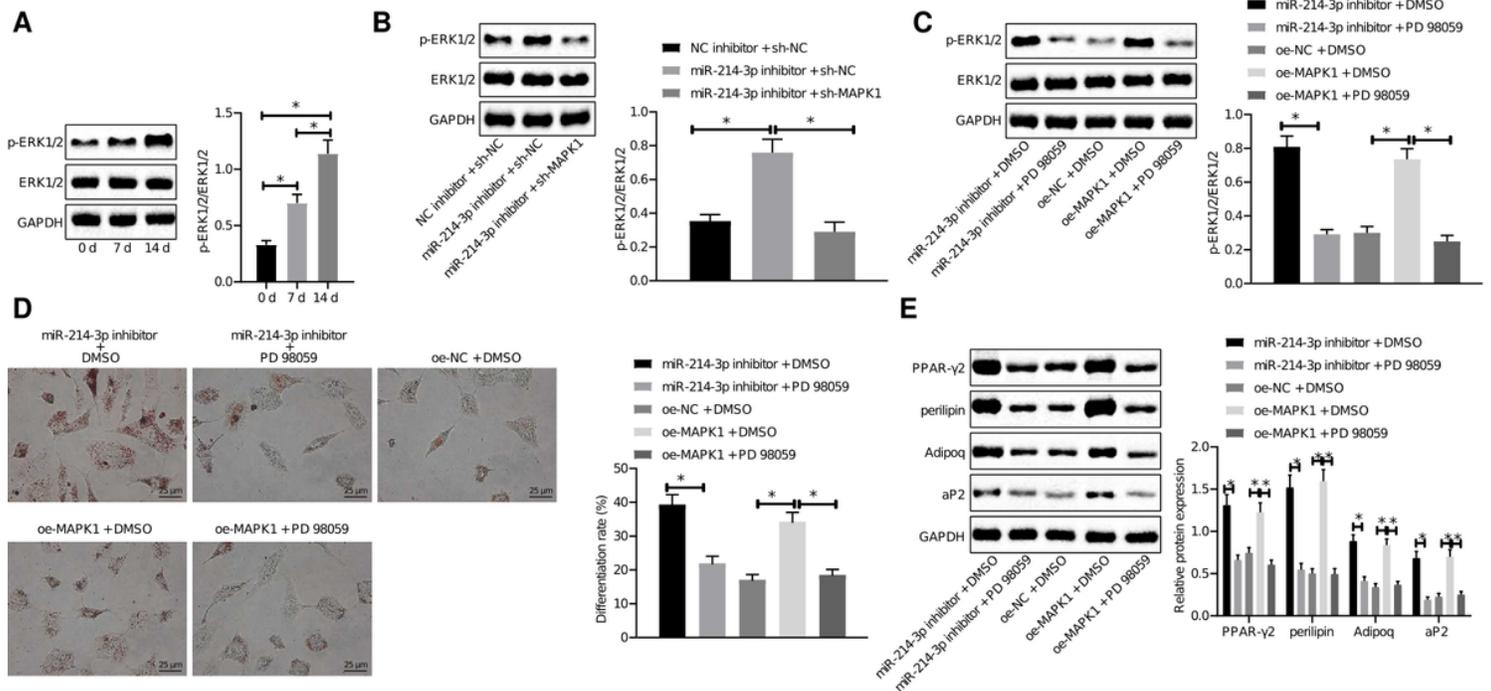
DNMT1 promotes Hair Follicle Stem Cell Differentiate into Adipogenic Lineages by inhibition of miR-214-3p. (A) The expression level of miR-214-3p was detected by RT-qPCR after the adipogenic differentiation of HFSc. (B) MSP detected the methylation level of miR-214-3p promoter after the adipogenic differentiation of HFSc. (C) ChIP detected the enrichment of DNMT1 on the miR-214-3p promoter after the HFSc were cultured in differentiation medium for 0d, 7d and 14d. (D) The expression level of miR-214-3p

in each group was detected by RT-qPCR. (E) WB was used to detect the expression level of DNMT1 in each group. (F) The degree of adipogenic differentiation of HFSc was detected by oil red o staining. (G) WB was used to detect the expression level of adipose formation markers. The data were expressed as mean  $\pm$  standard deviation. n=3 per group. \*P < 0.05.



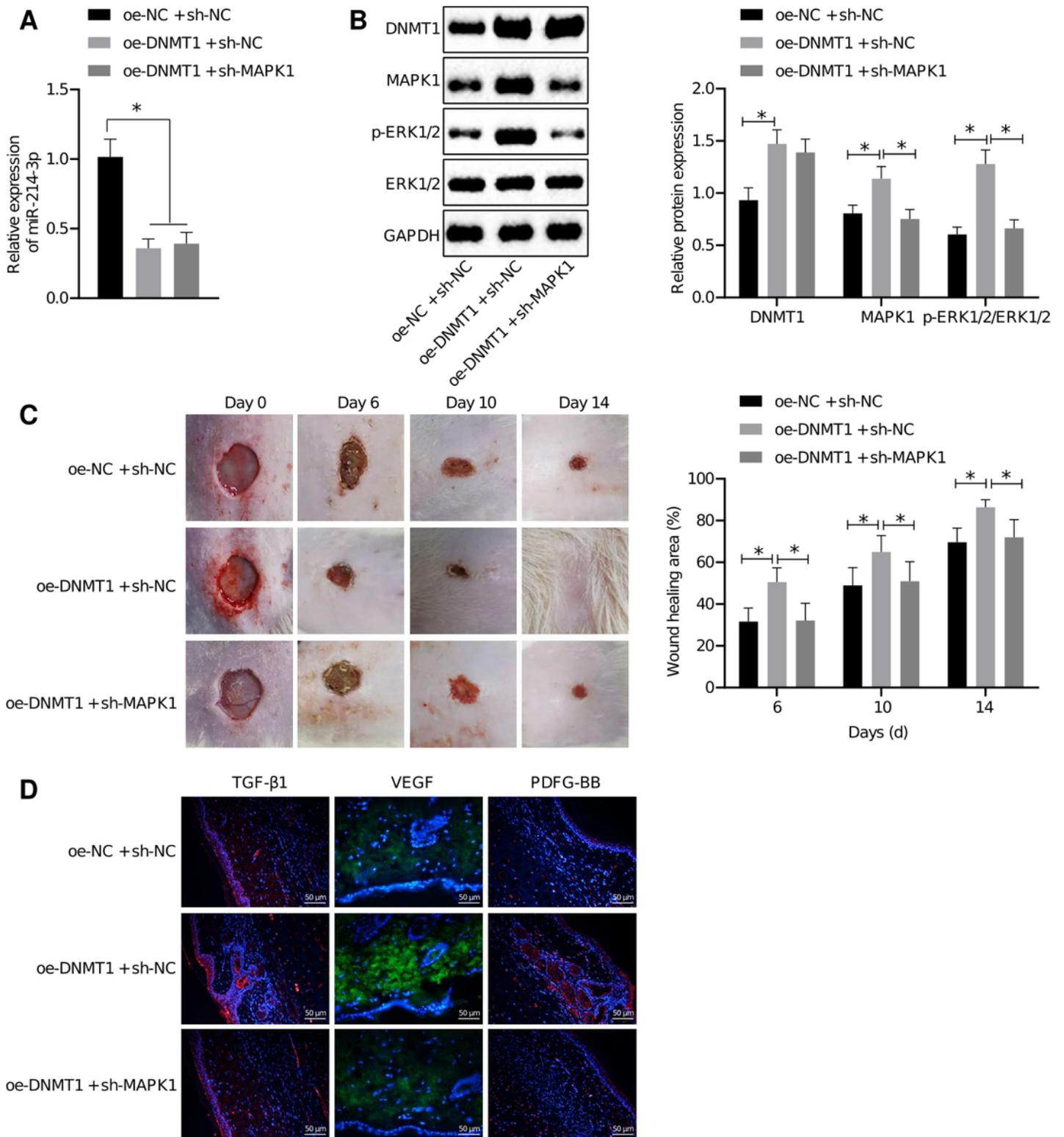
**Figure 4**

MiR-214-3p inhibits adipogenic differentiation of HFSc by silences MAPK1. (A) The binding sites of miR-214-3p and MAPK1 mRNA in 3'UTR were predicted. (B) WB was used to detect the expression of MAPK1 in HFSc after adipogenic differentiation. (C) Dual-luciferase reporting experiment verified the targeting relationship between miR-214-3p and MAPK1. (D) Transfection efficiency of miR-214-3p after silencing of miR-214-3p by RT-qPCR. (E) MAPK1 expression level after silence of miR-214-3p was detected by Western Blot. (F) The expression levels of miR-214-3p in each group were detected by RT-qPCR. (G) Western Blot was used to detect the expression level of MAPK1 in each group. (H) The degree of adipogenic differentiation of HFSc was detected by oil red o staining. (I) WB was used to detect the expression level of adipose formation markers. The data were expressed as mean  $\pm$  standard deviation. n=3 per group. \*P < 0.05.



**Figure 5**

miR-214-3p inhibits adipogenic differentiation of HFSc by reducing MAPK1-mediated expression of p-ERK1/2. (A) WB was used to detect the phosphorylation level of ERK1/2 in HFSc after adipogenic differentiation of HFSc. (B) WB detected the phosphorylation level of ERK1/2 in each group. (C) WB detected the phosphorylation level of ERK1/2 in each group. (D) Oil red O staining was used to detect the degree of adipogenic differentiation of HFSc. (E) WB was used to detect the expression level of adipose formation markers. n=3 per group. \*P < 0.05.



**Figure 6**

DNMT1 increased the expression MAPK1/p-ERK1/2 axis by silences miR-214-3p, thereby promotes wound healing and skin regeneration in mice. (A) The expression levels of miR-214-3p in each group were detected by RT-qPCR. (B) WB detected the phosphorylation levels of DNMT1 and ERK1/2 in each group. (C) Epidermal regeneration and quantitative analysis of epidermal tissues in each group. (D)

Immunofluorescence staining was used to detect wound healing growth factors in wound tissues of each group. n=8 per group. \*P < 0.05.