miR-148a Transcriptionally Regulated by ZEB1 Suppresses Dermal Papilla Cell Proliferation and Promotes Cell Apoptosis via FGF7/MAPK Axis

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Abstract

Background: Alopecia Areata (AA) is a hair follicle-specifc autoimmune disease. Dermal Papilla (DP) cells play key roles in development and growth of hair follicles. MicroRNAs (miRNAs) are closely related to cell activities of DP cells. Herein, we probe the function of miR-148a in regulating cell activities of DP cells.

Methods: The mRNA and protein expressions were assessed using qRT-PCR and Western blotting. DP cell proliferation was assessed by MTT and EdU assays. In addition, cell apoptosis was evaluated using fow cytometer. Finally, dual luciferase reporter assay was carried out to verify the binding relationship between ZEB1, miR-148a and fbroblast growth factor 7 (FGF7).

Results: Compared with the normal tissues, miR-148a was upregulated in AA skin tissues, while FGF7 was lowly expressed. Function assays displayed that inhibition of miR-148a could promote DP cell proliferation and suppress cell apoptosis, while miR-148a overexpression showed the opposite efects. FGF7 was identifed as a target gene of miR-148a. We subsequently displayed that FGF7 overexpression accelerated DP cell proliferation and suppressed cell apoptosis, which were abolished by RWJ64809 (mitogen-activated protein kinase (MAPK) inhibitor) treatment. Additionally, FGF7 silencing or RWJ64809 treatment abrogated the efects of miR-148a inhibition on cell activities of DP cells. Finally, it was turned out that ZEB1 transcriptionally inhibited miR-148a expression.

Conclusion: MiR-148a might have great potential as therapeutic target for AA, since miR-148a inhibition induced by ZEB1 facilitated DP cell proliferation and suppressed apoptosis during AA progression through activating MAPK signaling pathway by regulating FGF7 expression.

Keywords: Alopecia areata; Dermal papilla cells; miR-148a; FGF7; MAPK signaling pathway

Abbreviations

Alopecia Areata (AA); MicroRNA (miRNA); Dermal Papilla (DP); Zinc Finger E-Box Binding Homeobox 1 (ZEB1); Fibroblast Growth Factor 7 (FGF7); Mitogen-Activated Protein Kinase (MAPK); Extracellular Regulated Protein Kinases (ERK); Immuno uorescence (IF); Ethynyl-2′-deoxyuridine (EdU); 3-(4, 5-Dimethylthiazolyl2)-2, 5-diphenyltetrazolium bromide (MTT); 4',6-diamidino-2-phenylindole (DAPI); Quantitative real-time polymerase chain reaction (qRT-PCR); Standard Deviation (SD); Analysis of Variance (ANOVA).

Introduction

Alopecia Areata (AA) is a disease characterized by autoimmune non-scarring alopecia, with a prevalence of about 2% [1]. AA seriously a ects the patient's appearance, causing great troubles to the lives of patients [2]. However, the current treatment methods for AA, which mainly include local or systemic drug treatment, have serious side e ects [3]. Dermal Papilla (DP) cells act as key roles in hair follicle growth, making it a potential treatment strategy for AA [4]. However, although early-passage DP cells have been shown to induce hair follicle regeneration both in vivo and in vitro, while the agglutinative growth characteristics of DP cells gradually weaken as the number of passages increases [5]. erefore, it is important to nd e ective targets for maintaining the hair inducibility of DP cells.

MicroRNAs (miRNAs) refer to non-coding RNAs with a length of 22-25 nt, which participate in post-transcriptionally regulating gene expressions [6]. As widely reported, miRNAs are involved in AA progression and cell function of DP cells. For instance, Sheng et al. identi ed 36 signi cantly di erentially expressed miRNAs in AA patients, indicating miRNA dysregulation might be one of the main reasons that promoted AA development [7]. In addition, miR-195-5p was reported to suppress hair inducibility of DP cells via inhibiting Wnt/β-catenin pathway [8]. MiR-148a is reported to participate in regulating hair follicle growth and development [9]. Speci cally, miR-148a overexpression could markedly suppress the proliferation of DP cells [9]. Previous study displayed that miR-148a was a factor a ecting cell function of DP cells; however, the potential mechanism by which miR-148a regulated cell phenotypes of DP cells remains unclear. Zinc Finger E-Box Binding Homeobox 1 (ZEB1) is a transcription factor that promotes metastasis and stem cell characterization [10]. As revealed by Kiratipaiboon, ZEB1 inhibition markedly inhibited the stemness of DP cells [11]. Meanwhile, it was previously reported that ZEB1 upregulation enhanced the stemness of DP cells [12]. Herein, by using bioinformatics prediction, it was found that ZEB1 had potential binding sites to miR-148a. Nevertheless, the regulatory relationship between ZEB1 and miR-148a in AA remains unknown, which deserves further research.

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Received: 20-Mar-2023, Manuscript No: CMB-23-92350, **Editor assigned:** 22-Mar-2023, PreQC No: CMB-23-92350(PQ), **Reviewed:** 12-Apr-2023, QC No: CMB-23-92350, **Revised:** 22-Apr-2023, Manuscript No: CMB-23-92350(R), **Published:** 01-May-2023, DOI: 10.4172/1165-158X.1000266

Citation: Wang BH, Wu F, Zhang J, Wang SN, Li YY (2023) miR-148a Transcriptionally Regulated by ZEB1 Suppresses Dermal Papilla Cell Proliferation and Promotes Cell Apoptosis via FGF7/MAPK Axis. Cell Mol Biol, 69: 266.

Fibroblast Growth Factor 7 (FGF7) is a secreted protein mainly located in epithelial cells. It is reported that FGF signal regulates the size of dermal papilla [13]. In addition, as previous described, FGF7 was highly expressed immortalized mouse DP cells [14], and FGF7 overexpression in DP cells could prolong the anagen period [15]. All the above evidence suggested that FGF7 was closely related to cell function of DP cells. However, there's no report about the molecular mechanisms of FGF7 in regulating cell phenotypes of DP cells. As we all known many growth factors are regulated by MAPK pathway [16]. Previous study displayed that MAPK signaling pathway activation contributed to hair growth [17]. More importantly, we observed that FGF7 played an important role in glaucoma optic nerve damage through regulation of the MAPK signaling pathway [18]. However, whether FGF7 is able to regulate cell function of DP cells via MAPK pathway is largely unclear.

Herein, we probed the role of miR-148a in regulating cell function of DP cells. In AA progression, miR-148a inhibition induced by ZEB1 promotes the proliferation of DP cells and inhibited apoptosis through regulation of the FGF7/MAPK axis. Our research provided a new treatment strategy and potential therapeutic targets for AA.

Materials and Method

Clinical samples collection

AA skin tissues were collected from 8 AA patients who treated in the outpatient department of Shandong Provincial Hospital a liated to Shandong First Medical University. e normal skin tissues were collected from 9 individuals selected randomly from the outpatient department with no history of AA dermatological condition. is study was passed the review of Ethics Committee of Shandong Provincial Hospital a liated to Shandong First Medical University and all participants signed informed consent.

Cell culture and treatment

Human DP cells and 293T cells were purchased from Promocell (Heidelberg, Germany) and ATCC (VA, USA), respectively. All cells were cultured in DMEM (Gibco, MD, USA) mixed with 10% FBS (Gibco) at 37°C with 5% CO2. To inhibit MAPK, DP cells were subjected to 10 μmol/L RWJ64809 (MedChemExpress, NJ, USA) for 24 h.

Cell transfection

e overexpression plasmid of FGF7 (oe-FGF7), the short hairpin RNA of FGF7 (sh-FGF7), the short hairpin RNA of ZEB1 (sh-ZEB1), mimics/inhibitor of miR-148a and their negative controls were transfected into cells with Lipofectamine™ 3000 (Invitrogen, CA, USA). e above plasmids were all obtained from GenePharma (Shanghai, China).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cells and tissues with TRIzol (ermo Fisher Scienti c, MA, USA). RNA was reverse transcribed into cDNA with cDNA synthesis kit (Toyobo, Tokyo, Japan). For miRNA, the cDNA was synthesized with the rst-strand cDNA synthesis kits (Sangon, Shanghai, China). en, SYBR (ermo Fisher Scienti c) was employed for qRT-PCR assay. e relative expressions of miRNA and mRNA were respectively normalized by U6 and GAPDH and calculated by $2^{-\Delta\Delta Ct}$ method. e primers used in the study were listed as follows (5'-3'):

miR-148a (F): 5'-CGGCTCAGTGCACTACAGAA-3'

miR-148a (R): 5'-GTCGTATCCAGTGCAGGGTCCGAGG-TATTCGCACTGGATACGACACAAAG-3'

ZEB1 (F): 5'-GATGATGAATGCGAGTCAGATGC -3'

ZEB1 (R): 5'-ACAGCAGTGTCTTGTTGTTGT-3'

FGF7 (F): 5'-CACAGTGGTACCTGAGGATCG-3'

FGF7 (R): 5'-AATTCCAACTGCCACTGTCC-3'

U6 (F): 5'-CTCGCTTCGGCAGCACA-3'

U6 (R): 5'-AACGCTTCACGAATTTGCGT-3'

GAPDH (F): 5'-CTGACTTCAACAGCGACACC-3'

GAPDH (R): 5'-GTGGTCCAGGGGTCTTACTC-3'

Western blot

e proteins were isolated with RIPA, and then BCA kit was used to detect the protein concentration. And proteins were separated by SDS-PAGE, which further transferred to a PVDF membrane (Millipore, MA, USA). e membranes were subsequently blocked with 5% skimmed dry milk for 1 h at room temperature. en, membranes were incubated overnight at 4 with antibodies against Bax (Abcam, 1:1000, ab32503), Bcl-2 (Abcam, 1:1000, ab32124), cleaved caspase 3 (Abcam, 1:1000, ab32042), AKT (Abcam, 1:1000, ab8805), p-AKT (Abcam, 1:1000, ab38449), extracellular regulated protein kinases (ERK) (Abcam, 1:1000, ab32537), p-ERK (Abcam, 1:1000, ab76299), FGF-7 (Abcam, 1:1000, ab131162), ZEB1 (Abcam, 1:1000, ab203829) and GAPDH antibody (Abcam, 1:10000, ab8245). Membranes were then incubated with secondary antibody (Abcam, 1:10000, ab7090, ab97040) for 60 min. e membranes were visualized and imaged by GEL imaging system (Bio-Rad, CA, USA).

Flow cytometry

e Annexin V-FITC apoptosis detection kit (Beyotime) was performed to test cells apoptosis. Cells were re-suspended in 500 μL of 1X Annexin-binding bu er (Beyotime) and then incubated with 10 μL Annexin V-FITC and 5 μL PI stain (Beyotime) for 10 min protect from light. Samples were immediately analyzed using ow cytometry.

3-(4, 5-Dimethylthiazolyl2)-2, 5-diphenyltetrazolium bromide (MTT) assay

Cells were incubated with 5 mg/mL MTT (Sangon) for 4 h at 37 . en DMSO (Sigma-Aldrich, MO, USA) was added and the absorbance at 490 nm was examined using a microplate reader (Bioteke, Beijing, China).

Ethynyl-2′-deoxyuridine (EdU) assay

DP cells were plated in 96-well plates (Corning, NY, USA) and incubated with EdU (Sangon) for 2 h at 37°C. Cells were subsequently xed, permeabilized and incubated with the staining solution for 30 min. Hoechst 33342 was employed to indicate the cells by staining their nucleus. Cells were observed with a uorescence microscope (Olympus, Tokyo, Japan).

Lipofectamine™ 3000 (Invitrogen). Meanwhile, wt and mut reporter plasmids of miR-148a promoter sequences were cloned into pGL3 vector (HonorGene). And 293T cells were co-transfected with wt-miR-148a or mut-miR-148a plasmids and sh-ZEB1 or sh-NC. e luciferase activity was evaluated by a dual-luciferase reporter assay system (Promega, WI, USA).

Data analysis

All the tests conducted in this work were repeated at least three times. GraphPad Prism 8.0 was applied for statistical data analysis and the measurement data were expressed as means ± SD. Between-group di erences and multi-group comparisons were determined using Student's t test and one-way ANOVA, respectively. $P < 0.05$ was considered to represent a signi cant di erence.

Results

miR-148a inhibition promoted DP cell proliferation and suppressed apoptosis

We rstly observed that miR-148a expression markedly increased

proliferation, while suppressed cell apoptosis.

miR-148a knockdown activated MAPK signaling by up-regulating FGF7

Next, we aimed to probe the downstream molecules and signaling pathways of miR-148a in regulating cell function of DP cells. FGF7 was

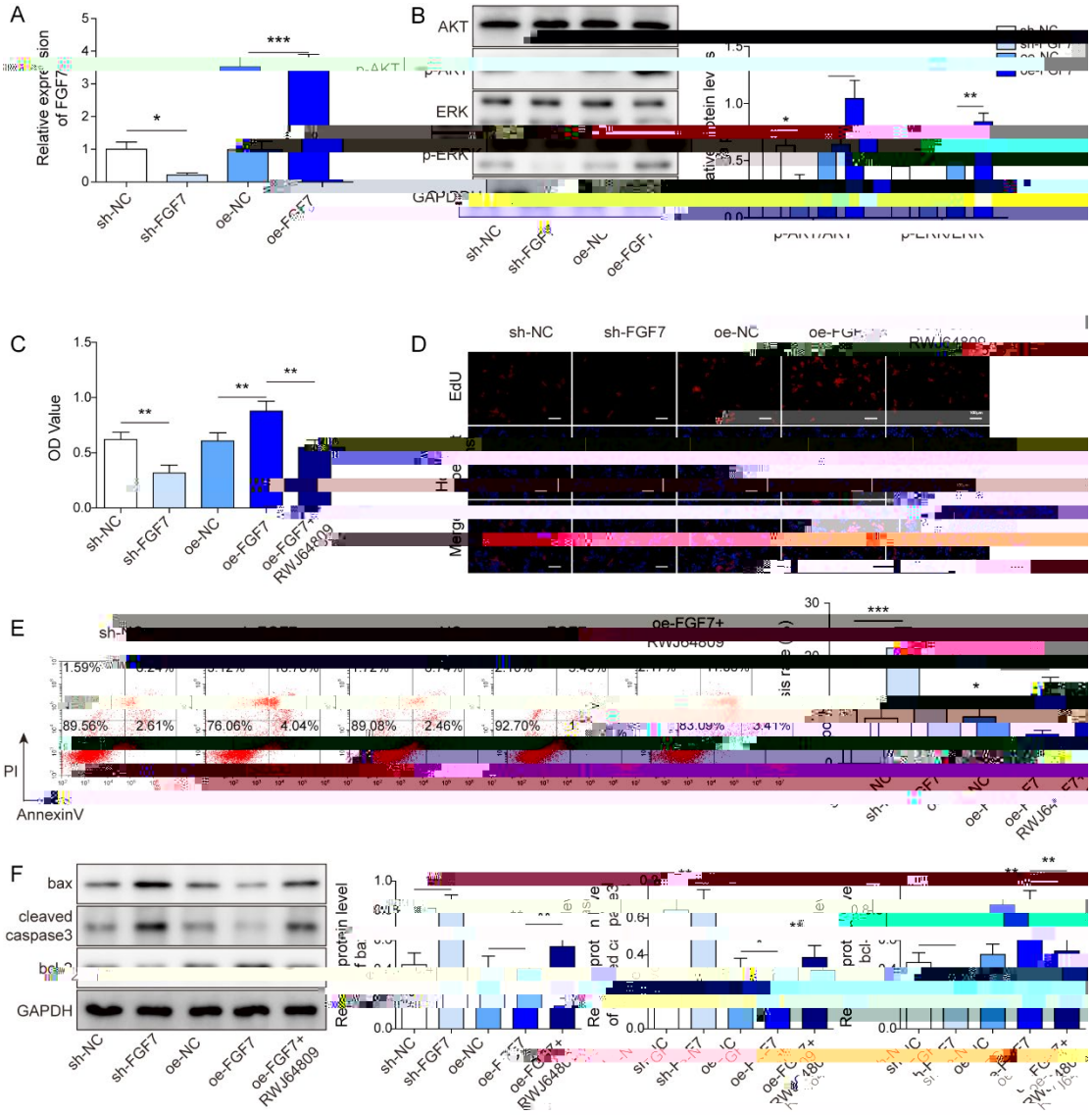

Figure 3: FGF7 overexpression facilitated DP cell proliferation and suppressed cell apoptosis.

(A) FGF7 expression in DP cells following FGF7 overexpression or FGF7 silencing was determined by qRT-PCR. (B) Western blotting was conducted to evaluate the protein levels of AKT, p-AKT, ERK and p-ERK in DP cells following FGF7 overexpression or FGF7 silencing. DP cells were classified into: sh-NC group, sh-FGF7 group, oe-NC group, oe-FGF7 group and oe-FGF7 + RWJ64809 group. (C-D) Cell proliferation was evaluated by MTT assay and EdU assay. (E) Cell apoptosis was detected by fow cytometry assay. (F) Western blotting in l o ḍ ' l } ɪ . ! l Ɣ l ӂ x F ˛ ȝ leve ɔ [. De ⁼ ce

FGF7 knockdown or RWJ64809 treatment abolished the effects of miR-148a knockdown on cell activities of DP cells

To con rm the potential role of miR-148a/FGF7/MAPK axis in regulating cell phenotypes of DP cells, DP cells were co-transfected with miR-148a inhibitor and sh-FGF7 or co-treated with miR-148a inhibitor and RWJ64809. As displayed in Figure 4A-C, miR-148a inhibition resulted in elevated cell proliferation and reduced cell apoptosis of DP cells, while FGF7 knockdown or RWJ64809 treatment attenuated the e ects of miR-148a inhibitor on cell activities of DP cells. In addition, miR-148a inhibitor transfection signi cantly reduced Bax and cleaved-caspase3 levels and elevated Bcl-2 level, while FGF7 knockdown or RWJ64809 treatment abolished the e ects of miR-148a inhibitor (Figure 4D). In summary, miR-148a inhibition promoted DP cell proliferation and suppressed cell apoptosis via activation of FGF7-mediated MAPK signaling.

ZEB1 transcriptionally inhibited miR-148a expression

ZEB1 is a transcription factor which is closely related to the stemness of DP cells [12]. To investigate the function of ZEB1 in regulating AA progression, ZEB1 knockdown was induced in DP cells by transfecting sh-ZEB1 into DP cells. As revealed in Figure 5A, B, ZEB1 expression level in DP cells was markedly reduced by sh-ZEB1 transfection, suggesting the transfection was successful. It was predicted that ZEB1 had potential binding sites to miR-148a promoter by using JASPARE database (Figure 5C). Dual-luciferase reporter gene assay subsequently displayed that ZEB1 knockdown increased the luciferase activity presented by wt-miR-148a (Figure 5D), revealing that ZEB1 directly

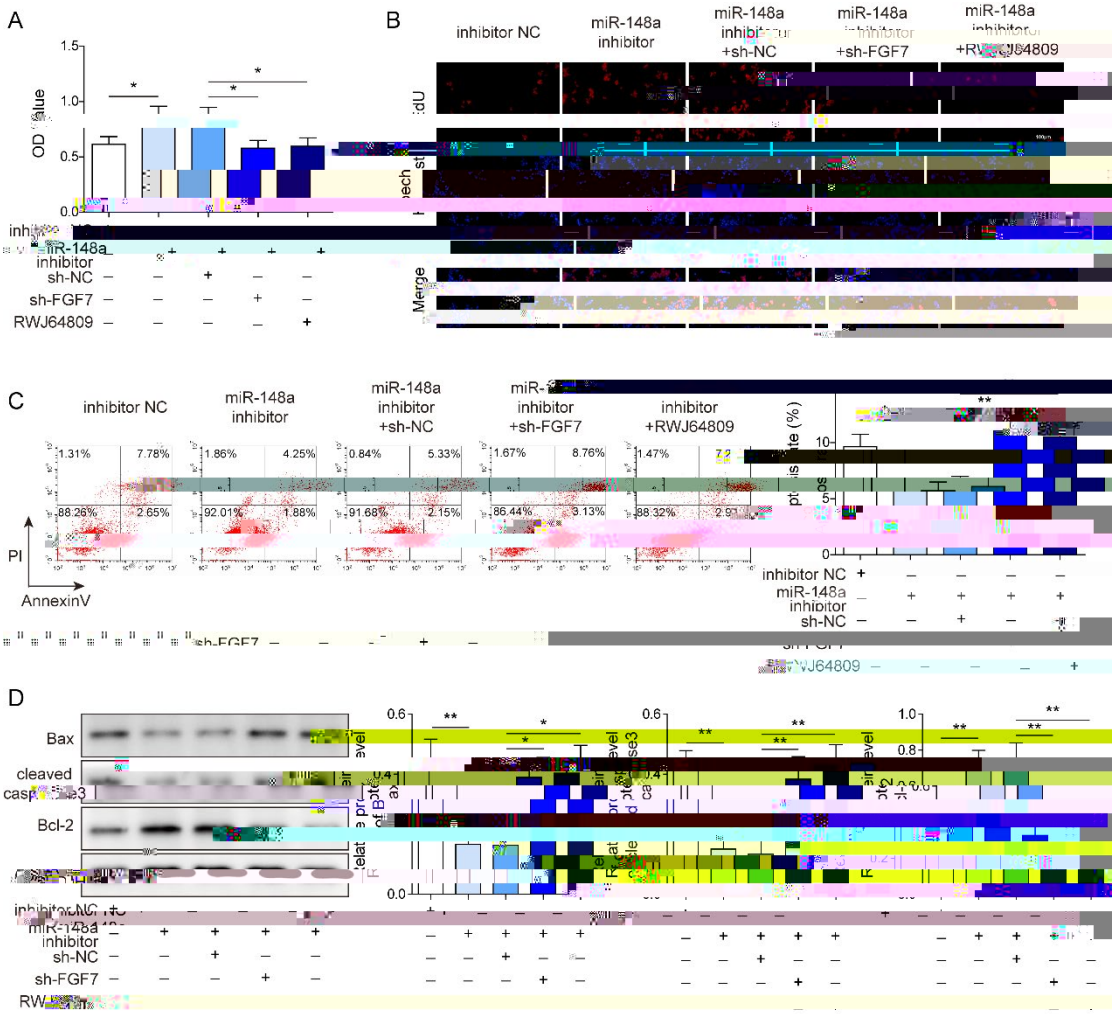

Figure 4: FGF7 knockdown or RWJ64809 treatment abolished the effects of miR-148a knockdown on cell phenotype of DP cells.

DP cells were classified into: inhibitor NC group, miR-148a inhibitor group, miR-148a inhibitor + sh-NC group, miR-148a inhibitor + sh-FGF7 group and miR-148a inhibitor + RWJ64809 group. (A-B) MTT assay and EdU assay were employed to assess cell proliferation. (C) Flow cytometry assay was used to evaluate cell apoptosis. (D) Western blotting was carried out to analyze Bax, cleaved-caspase3 and Bcl-2 levels in DP cells. The measurement data were presented as mean ± SD. All of the tests in this study were conducted for three times. *P < 0.05, ** P < 0.01.

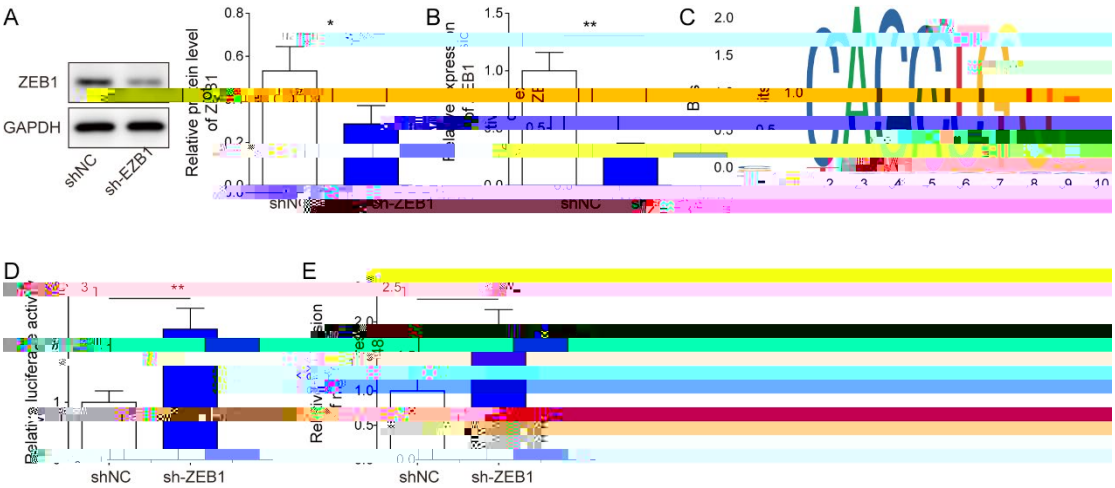

Figure 5: ZEB1 transcriptionally inhibited miR-148a expression.

(A-B) ZEB1 expression level in DP cells after sh-NC or sh-ZEB1 transfection was assessed using qRT-PCR and western blotting. (C) The potential binding sites between ZEB1 and miR-148a promoter was predicted by bioinformatics. (D) Dual-luciferase reporter gene assay was performed to analyze the interaction between ZEB1 and miR-148a promoter. (E) miR-148a expression in DP cells after sh-NC or sh-ZEB1 transfection was assessed using qRT-PCR. The measurement data were presented as mean ± SD. All of the tests in this study were conducted for three times. *P < 0.05, ** P < 0.01.

bound with miR-148a. In addition, it was observed that miR-148a expression in DP cells was markedly increased by ZEB1 knockdown (Figure 5E). In conclusion, ZEB1 transcriptionally inhibited miR-148a expression in DP cells.

Discussion

Much evidence has con rmed that DP cells play key roles in regulating hair induction by a ecting hair follicle circulation [19]. Recently, miRNAs are con rmed to be key regulators for cell activities of DP cells [20, 21]. Herein, we focused on the regulation of miR-148a on DP cell proliferation and apoptosis. miR-148a silencing promoted the proliferation and suppressed cell apoptosis of DP cells through targeting FGF7 to activate MAPK signaling pathway.

It's widely reported that miRNAs are crucial for regulating DP cells mediated hair follicle regeneration [22]. For instance, miR-205 could promote cell apoptosis of DP cells [21]. In addition, miR-133b overexpression was reported to suppress DP cell proliferation [23]. MiR-148a, located on chromosome 7p15.2, is reported to regulate the progress of various human malignancies [24]. In addition to the biological roles of miR-148a in tumors, a recent study revealed that miR-148a suppressed DP cell proliferation [9], indicating the potential e ects of miR-148a in regulating cell activities of DP cells. However, the precise mechanism by which miR-148a regulated cell phenotypes of DP cells is not fully explained. Herein, our results displayed that miR-148a expression markedly increased in AA skin tissues. Besides, functional experiment revealed that miR-148a inhibition accelerated DP cell proliferation and suppressed cell apoptosis, while miR-148a overexpression presented the opposite e ects. Some studies have revealed the interactive regulatory network of transcription factors and miRNAs in diseases [25]. SP1 negatively regulates miR-335 by combining with miR-335 promoter to participate in ovarian cancer metastasis and prognosis [26]. ZEB1 is a transcription factor which is closely related to the stemness of DP cells [11]. In the current research, our results revealed that ZEB1 transcriptionally inhibited miR-148a expression in DP cells. erefore, all our results indicated a key role of ZEB1/miR-148a axis in regulating cell activities of DP cells.

As widely reported, miRNAs regulate post-transcriptional genes expressions by binding to its targets, leading to mRNAs degradation or translational inhibition [27]. Next, we focused on the downstream of miR-148a. Herein, we con rmed that FGF7 was the target of miR-148a. It was also observed that FGF7 expression was enhanced/suppressed by miR-148a inhibition/overexpression. FGF7, a keratinocyte growth factor, is expressed speci cally in mesenchyme [28]. As previously reported, activation of FGF signaling contributes to the increased size of the DP [13]. FGF7 upregulation in DP cells is conducive to DP cell growth [29]. It was also reported that ruxolitinib could directly stimulate anagen-re-entry signals in DP cells through promoting FGF7 expression in DP cells [30]. However, the relationship between miR-148a and FGF7 in regulating cell activities of DP cells is still unclear. In the current study, function assays displayed that FGF7 overexpression accelerated DP cell proliferation and suppressed cell apoptosis, while FGF7 silencing presented the opposite e ects. In addition, FGF7 knockdown eliminated the e ects of miR-148a knockdown on cell activities of DP cells. Taken together, miR-488 inhibition promoted the proliferation and suppressed the apoptosis of DP cells via directly targeting FGF7. ERK1/2 and AKT are related to cell proliferation and apoptosis, and are the main pathways of the MAPK pathway [31]. Recently, accumulated evidence has revealed that MAPK signaling pathway is closely related to hair follicle growth and hair growth. For example, Xiao et al. displayed that MAPK signaling pathway activation participated in the enhancement of fatty acids on hair follicle cell proliferation [32]. Additionally, Kim et al. displayed that quercitrin facilitated DP cell proliferation and suppressed cell apoptosis through activating MAPK pathway [17]. It was also reported that MAPK signaling pathway was the downstream pathway of FGF7 in regulating apoptosis of ganglion cells [18]. However, how MAPK signaling pathway can be regulated by miR-148a in DP cells is unknown. Herein, we found that miR-148a inhibition could markedly elevate p-AKT and p-ERK levels, while miR-148a overexpression showed the opposite e ects. As expected, FGF7 overexpression could markedly elevate p-AKT and p-ERK levels, while FGF7 silencing presented the opposite e ects. In addition, RWJ64809 (MAPK inhibitor) treatment reversed the e ects of FGF7 overexpression or miR-148a inhibition on cell activities of DP cells. erefore, we came to the conclusion that miR-148a inhibition facilitated DP cell proliferation and suppressed cell apoptosis through activating MAPK signaling pathway by interfering with FGF7.

We conclude that ZEB1/miR-148a/FGF7/MAPK is a novel regulatory mechanism for the regulation of DP proliferation and apoptosis. erapeutically inhibition of miR-148a or overexpression of FGF7 may facilitate DP cell proliferation and suppress cell apoptosis. us, our study suggests that miR-148a/FGF7 may be new therapeutic targets for AA treatment.

Declaration

Ethical approval

is study was passed the review of Ethics Committee of Shandong Provincial Hospital a liated to Shandong First Medical University and all participants signed informed consent.

Conflict of Interest

All authors agree with the presented ndings, have contributed to the work, and declare no con ict of interest.

Data Availability Statements

e datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Funding

e authors express their gratitude to for his help for directing our article. is work was supported by grants from Shandong Medical and Health Science and Technology Development Plan (No.2013WS0101) and Natural Science Foundation of Shandong Province (ZR2021QH361).

Authors' Contribution

Bai-He Wang: Guarantor, concepts, design, review

Fan Wu: Experimental studies, data acquisition

Jie Zhang: Experimental studies, preparation, editing

Sheng-Nan Wang: Data acquisition, data analysis, editing

Yuan-Yuan Li: Study concepts, study design, data analysis, preparation, editing

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