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Huaier Aqueous Extract Mediates Keloid Cell Proliferation and Apoptosis by Regulating miR-128-3p / EPHB2

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ABSTRACT

This study was aimed to investigate the effect of HAE on the proliferation and apoptosis of keloid fibroblasts and its mechanism. Primary HFB cells were isolated and cultured, and treated with different concentrations of HAE for 48 h. Cell viability was measured by MTT. Flow cytometry was used to detect the apoptosis rate. qRT-PCR and Western blot were used to detect the effects of HAE on miR-128-3p and EPHB2. The above method was used to detect cell viability and apoptosis rate after overexpression of miR-128-3p or inhibition of EPHB2. The dual luciferase report experiment verified the targeted binding relationship between miR-128-3p and EPHB2. Western blot was used to detect the expression of Ki-67, PCNA, Bcl-2 and Bax. Our findings showed that after HAE treatment, the cell viability was significantly reduced (P <0.05), the apoptosis rate was significantly increased (P <0.05), the levels of Ki-67, PCNA, Bcl-2 protein were significantly reduced (P <0.05), and the level of Bax protein was significantly increased (P <0.05), the expression level of miR-128-3p was increased significantly (P <0.05), the expression levels of EPHB2 mRNA and protein levels were decreased significantly (P <0.05). After overexpression of miR-128-3p or inhibition of EPHB2, the cell viability was significantly reduced (P <0.05), the apoptosis rate was significantly increased (P <0.05), and the levels of Ki-67, PCNA, and Bcl-2 protein were significantly reduced (P <0.05), the level of Bax protein was increased significantly (P <0.05). Double luciferase reporting experiments confirmed that miR-128-3p could target EPHB2 and negatively regulate its expression and activity. We concluded HAE may inhibit keloid fibroblast proliferation and promote apoptosis by regulating the miR-128-3p / EPHB2 molecular axis.

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Authors' Contribution
XS and YG collected the samples. YW and YM analysed the data. XS and YW conducted the experiments and analysed the results. All authors discussed the results and wrote the manuscript.

Key words
Huaier aqueous extract, miR-128-3p, EPHB2, Keloids, Fibroblasts, Proliferation, Apoptosis.

INTRODUCTION

Keloids, a type of pathological scar tissue with frequent occurrence in the chest and back, is often clinically treated with surgery and radiation therapy, which is however limited in use due to multiple adverse reactions (Zhu *et al*., 2013; Gabriel, 2011). Therefore, searching for keloid's treatment approaches has become a research hotspot. Studies have shown that drugs can play an anti-keloid effect by inducing apoptosis in keloid fibroblast (Hu *et al*., 2009). Huaier is a Chinese herbal medicine with hemostatic and anti-cancer effects. Studies have shown that Huaier aqueous extract (HAE) can promote apoptosis and inhibit cell growth, thereby improving patient immunity (Hu *et al*., 2016). However, the effect of HAE on keloids remains unknown. Imbalance of fibroblast proliferation and apoptosis may be an important reason for keloid formation. Studies have shown that up-regulation of microRNA-128-3p (miR-128-3p) expression can promote tumor cell apoptosis and inhibit cell proliferation (Zhao *et al*., 2019). According to bioinformatics analysis, EPH receptor B2 (EPHB2) may be a target gene of miR-128-3p, which is highly expressed in tumors and can promote cell proliferation and inhibit apoptosis (Inagaki *et al*., 2019). However, it is unknown whether HAE can affect the proliferation and apoptosis of keloid fibroblasts by regulating miR-128-3p/EPHB2 molecular axis. Hence, this study mainly investigates the effect of HAE on the proliferation and apoptosis of keloid fibroblasts, and explores its role in regulating miR-128-3p/EPHB2 molecular axis, thereby providing experimental basis for clinical treatment of keloids by HAE.

MATERIALS AND METHODS

Materials and reagents

Eight patients, 5 males and 3 females with average age of 46.58±9.58 years with keloids in the chest and

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earlobe treated in our hospital from May 2017 to March 2018 were selected as the research subjects. This study was approved by the ethics committee of our hospital. All patients were informed and they signed the consent form.

HAE was purchased from Jiangsu Qidong Gaitianli Pharmaceutical Co., Ltd., with 10 g dissolved in 100 mL serum-free RPMI 1640 culture solution. Human skin fibroblasts (HSF) were purchased from ATCC cell bank, USA. RPMI 1640 medium, fetal bovine serum and trypsin were purchased from Gibco, USA. Penicillin-streptomycin mixed solution was purchased from Beijing Solarbio Science and Technology Co., Ltd. Methylthiazolyl tetrazolium (MTT) was purchased from Shanghai Beyotime Biotechnology. Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit was purchased from Jiangsu KeyGen Biotech Co., Ltd. Trizol reagent and Lipofectamine2000 were purchased from Invitrogen, USA. Reverse transcription kit and real-time quantitative PCR kit were purchased from Tiangen Biochemical Technology (Beijing) Co., Ltd. RIPA lysate was purchased from Shaanxi Beyotime Biological Engineering Co., Ltd. Bicinchoninicacid (BCA) protein quantitative detection kit was purchased from Pierce, USA. EPHB2 small molecule interference RNA (si-EPHB2), disorderly meaningless negative sequence (si-NC), miR-128-3p oligonucleotide mimics (miR-128-3p mimics) and its negative control mimic NC sequence (mimics-NC), miR-128-3p specific oligonucleotide inhibitor (anti-miR-128-3p) and its negative control (anti-miR-NC) were purchased from Guangzhou Ruibo Biotechnology Co., Ltd. Rabbit anti-human EPHB2 antibody was purchased from Cell Signaling Technology, USA. Rabbit anti-human proliferation marker protein proliferating nuclear cell antigen-67 (Antigen identified by monoclonal antibody, Ki-67), proliferating cell nuclear antigen (PCNA), B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax) antibody were purchased from Abcam, USA. Horseradish peroxidase (HRP) labeled goat anti-rabbit IgG secondary antibody was purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.

Isolation and culture of keloid fibroblasts (HFB) and experimental grouping

HFB cells were cultured by adherence of tissue blocks. The resected keloid tissue was placed in a sterilized petri dish and washed with PBS to remove blood stains. The tissue block was cut into 0.5 mm^3 pieces, culture medium was added to prepare particulate suspension, cultured in a flask, and placed in an incubator at 37°C with a volume fraction of 5% CO_2 for 4 h inverted culture. The flask was inverted, allowed to stand still. The culture medium was changed once every 3 days until the primary fibroblasts filled the flask bottom. Cells were then digested with 0.25% trypsin, subcultured, and cells with good growth status in the 4th-6th passage were selected for experiments (Siengdee *et al*., 2018).

After counting, HFB cells were prepared into cell suspension (1×106 cells/mL) and seeded in a 96-well plate (100 μL/well), and treated with HAE at different concentrations (0.05 g/L,0.10 g/L,0.20 g/L,0.40 g/L,0.80 g/L,1.60 g/L) for 48 h (Zhang *et al*., 2010). The appropriate HAE action concentration was selected by MTT experiment. At the same time, normal cultured HFB cells were used as the control group. To investigate the effect of HAE on miR-128-3p and EPHB2 expressions, HSF group (normally cultured human skin fibroblasts), HFB group (normally cultured keloid fibroblasts) and HFB+HAE group (with keloid fibroblasts cultured in a culture medium containing 0.20 g/L HAE) were set in the experiment. Referring to Lipofectamine2000 instructions, si-NC, si-EPHB2, mimics-NC, miR-128-3p mimics were transfected to HFB cells and recorded as si-NC group, si-EPHB2 group, mimics-NC group, miR-128-3p group, respectively. The culture medium was replaced by a serum-containing medium 6 h after the transfection for 48 h further culture.

MTT detection of cell proliferation

HFB cells in logarithmic growth phase were digested with 0.25% trypsin, added with culture solution containing 10% fetal bovine serum to prepare single cell suspension. After cell density adjustment to 3×10^4 cells/mL, cells were seeded to a 96-well plate at a density of 100 μL per well, followed by grouping as described above. Set three replicates in each group. After the treatment, 20 μL MTT solution was added to each well and incubated at room temperature for 4 h. Supernatant was discarded and 150 μL DMSO was added to each well, shaken at a constant speed for 10 min at room temperature and the absorbance (A) of each well was taken at a wavelength of 490 nm using a microplate reader. The experiment was repeated three times.

Apoptosis rate by flow cytometry

HFB cells in logarithmic phase were collected for each group, washed with pre-chilled PBS, centrifuged at 1000 r/min for 6 min at 4°C. Supernatant was discarded, washed with PBS, centrifuged under the same condition before discarding the supernatant. 500 μL binding buffer, 5 μL Annexin V-FITC and 5 μL PI was added and incubated with shaking at room temperature for 10 min. Apoptosis rate of each group was detected using FACS Calibur flow cytometer and Cellauest software.

Detection of miR-128-3p and EPHB2 mRNA expression in cells by quantitative real-time PCR (qRT-PCR)

HFB and HSF cells in logarithmic phase were collected for each group, and total RNA was extracted from the cells by Trizol. RNA concentration and purity were measured by Nanodrop2000c ultra-micro spectrophotometer. Referring to the reverse transcription kit instructions, total RNA was reverse transcribed into cDNA using following primers miR-128-3p F 5'-TCACAGTGAACCGGTCTCTT-3', R 5'-AGCTAGAGAGAGAGAGAGAGAGAGA-3'; EPHB2 F 5'-AAGTACCTGGCGGACATGAA-3', R 5'-CTGGCTGAGGTGAATTTCCG-3'; U6 F 5'-GCTTCGGCAGCACATATACT-3', R 5'-GTGCAGGGTCCGAGGTATTC-3'; β-actin F 5'-TGCTGTCCCTGTATGCCTCT-3', R 5'-TGATGTCACGCACGATTT-3'. The primers were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd. PCR amplification reaction mixture comprised 10×PCR Buffer 2.5 μL, MgSO4 2.5 μL, dNTPs 2.5 μL, 0.5 μL of forward and reverse primers, cDNA 2 μL, RNase-Free ddH_2O complemented to 25 μL. Reaction conditions were as follows: 95°C 2 min, 95°C 30 sec, 60°C 30 sec, 72°C 30 sec, 40 cycles in total. U6 was internal reference for miR-128-3p, while β-actin was internal reference for EPHB2. Relative expressions of miR-128-3p, EPHB2 mRNA were calculated by $2^{-\Delta\Delta Ct}$.

Detection of miR-128-3p target gene by dual luciferase reporter gene

According to StarBase prediction, 3'UTR of EPHB2 contains complementary sequence of miR-128-3p. The binding site was mutated by gene mutation technology, and the sequence containing binding site and mutation site was respectively inserted into the luciferase reporter gene vector to construct wild-type vector WT-EPHB2 and mutant vector MUT-EPHB2. mimics-NC, miR-128-3p mimics were co-transfected with WT-EPHB2, MUT-EPHB2 into HFB cells. Cells were collected after 48 h transfection to detect relative luciferase activity. HFB cells were transfected with mimics-NC, miR-128-3p mimics, anti-miR-NC and anti-miR-128-3p. EPHB2 mRNA and protein levels of the cells were detected by qRT-PCR and Western blot for each group.

Western blot detection of EPHB2, Ki-67, PCNA, Bcl-2, Bax protein expressions

HLB cells in logarithmic phase were collected from each group, to which an appropriate amount of RIPA lysate was added to extract total cell protein. Protein concentration was measured according to the instructions of BCA protein quantitative detection kit. 40 μg protein was isolated by sodium lauryl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The isolated protein gel was transferred to a PVDF membrane, blocked with 5% skim milk powder for 2 h to incubate primary antibody dilution at 4°C for 24 h, washed with TBST. Then, the secondary antibody dilution was incubated at room temperature for 1 h, washed with TBST, exposed and developed in a dark room to analyze gray value of each band using ImageJ software.

Statistical analysis

SPSS 21.0 statistical software was used for data analysis. The measurement data is expressed as ($\bar{x}$±s) and all conform to normal distribution. Independent sample *t* test was used for comparison between two groups, and one-way analysis of variance was taken for comparison between multiple groups. $P<0.05$ indicates statistically significant difference.

RESULTS

Effect of HAE on human keloid cell proliferation and apoptosis

Compared with the control group, all experimental groups have significantly reduced cell viability ($P<0.05$) and increased apoptosis rate ($P<0.05$) (Table I). Since 0.20 g/L HAE acts better, so HAE at a concentration of 0.20 g/L was taken for subsequent experiment. Compared with the control group, HAE group has significantly reduced Ki-67, PCNA and Bcl-2 protein levels ($P<0.05$), significantly increased Bax protein level ($P<0.05$) (Table II).

Table I.- Effect of HAE on human keloid cell proliferation and apoptosis ($\bar{x}$±s, n=9).

Group	Cell viability (A value)	Apoptosis rate (%)
Control	1.00±0.09	6.19±0.61
0.05g/L HAE	0.82±0.08*	12.29±1.23*
0.10 g/L HAE	0.72±0.07*	23.98±2.32*
0.20 g/L HAE	0.56±0.06*	29.68±3.28*
0.40 g/L HAE	0.52±0.05*	32.21±3.23*
0.80 g/LHAE	0.49±0.05*	34.01±3.42*
1.60 g/L HAE	0.46±0.05*	32.86±3.22*
F	83.64	152.0
P	<0.0001	<0.0001

Compared with the control group, *$P<0.05$.

Effect of HAE on miR-128-3p and EPHB2 expressions

Compared with HSF group, HFB group has significantly reduced miR-128-3p expression level ($P<0.05$), significantly increased EPHB2 mRNA and protein levels ($P<0.05$). Compared with HFB group, HFB+HAE

group has significantly increased miR-128-3p expression level (P <0.05) and significantly decreased EPHB2 mRNA and protein levels (P <0.05) (Table III).

Table II.- Effect of HAE on apoptosis-related protein expression in human keloid fibroblasts ($\bar{x}$±s, n=9).

Group	Control	HAE	t	P
Ki-67	0.64±0.06	0.44±0.04*	8.321	0
PCNA	0.68±0.07	0.46±0.04*	8.186	0
Bax	0.35±0.04	0.58±0.06*	9.569	0
Bcl-2	0.65±0.07	0.45±0.05*	7.682	0

Compared with the control group, *P<0.05.

Table III.- Effect of HAE on miR-128-3p and EPHB2 expressions in human keloid cells ($\bar{x}$±s, n=9).

Group	HSF	HFB	HFB+HAE	F	P
miR-128-3p	1.00±0.09	0.42±0.04*	1.88±0.17#	378	0
EPHB2[A]	1.00±0.10	1.58±0.14*	0.86±0.08#	150.759	0
EPHB2[B]	0.52±0.05	0.83±0.09*	0.43±0.04#	97.451	0

Compared with HSF group, *P<0.05; compared with HFB group, #P <0.05. A, EPHB2 mRNA expression; B, EPHB2 protein expression.

EPHB2 knockdown inhibits cell proliferation and promotes apoptosis of human keloid cell

Compared with si-NC group, si-EPHB2 group has significantly reduced cell viability (P <0.05), and significantly reduced Ki-67 and PCNA protein levels (P <0.05) (Table IV). EPHB2 knockdown has significantly increased apoptosis rate (P <0.05), reduced Bcl-2 protein level (P <0.05) and increased Bax protein level (P <0.05) (Table IV).

Table IV.- EPHB2 knockdown inhibits human keloid cell proliferation and promotes its cell apoptosis ($\bar{x}$±s, n=9).

Group	Control	si-NC	si-EPHB2	F	P
Cell proliferation					
EPHB2[A]	1.00±0.09	1.02±0.09	0.48±0.05*	135.33	0
EPHB2[B]	0.53±0.05	0.52±0.05	0.22±0.02*	155.16	0
Cell viability (A value)	1.00±0.09	0.98±0.09	0.45±0.05*	140.48	0
Ki-67	0.63±0.06	0.64±0.06	0.34±0.04*	89.08	0
PCNA	0.66±0.07	0.67±0.06	0.39±0.04*	67.45	0
Cell apoptosis					
Apoptosis rate (%)	6.21±0.60	6.32±0.61	34.89±3.12	704.591	0
Bax	0.36±0.04	0.38±0.04	0.69±0.07	114.111	0
Bcl-2	0.65±0.06	0.64±0.06	0.33±0.04	101.557	0

Compared with si-NC group, *P<0.05. A, EPHB2 mRNA expression; B, EPHB2 protein expression.

miR-128-3p overexpression inhibits proliferation and promotes apoptosis

Compared with mimics-NC group, miR-128-3p group has significantly reduced cell viability (P <0.05), and significantly reduced Ki-67 and PCNA protein levels (P <0.05) (Table V).

Compared with mimics-NC group, miR-128-3p group has significantly increased apoptosis rate (P <0.05), significantly increased Bax protein level (P <0.05), and significantly decreased Bcl-2 protein level (P <0.05) (Table V).

Table V.- miR-128-3p overexpression inhibits proliferation and proliferation-related protein expression and promotes human keloid cell apoptosis (%) and related protein expression in human keloid cell ($\bar{x}$±s, n=9).

Group	Control	mimics-NC	miR-128-3p	F	P
Cell proliferation and proliferation-related protein					
miR-128-3p expression	0.43±0.04	0.42±0.04	1.31±0.11*	460.76	0
Cell viability (A value)	0.58±0.06	0.57±0.06	0.31±0.03*	78.11	0
Ki-67	0.61±0.06	0.64±0.07	0.34±0.03*	78.41	0
PCNA	0.67±0.07	0.66±0.06	0.33±0.03*	107.52	0
Cell apoptosis (%) and related protein					
Cell apoptosis	6.15±0.56	6.11±0.55	38.68±3.62	694.98	0
Bax	0.59±0.06	0.57±0.06	0.72±0.07	14.80	0
Bcl-2	0.66±0.06	0.65±0.06	0.35±0.04	95.23	0

Compared with mimics-NC group, *P<0.05.

Table VI.- Detection of targeting relationship between miR-128-3p and EPHB2 in human keloid cells by dual luciferase activity ($\bar{x}$±s, n=9).

Group	mimics-NC	miR-128-3p	t	P
WT-EPHB2	1.07±0.11	0.51±0.05*	13.904	0
MUT-EPHB2	1.14±0.12	1.20±0.14	0.976	0.344

Compared with miR-con group, *P<0.05.

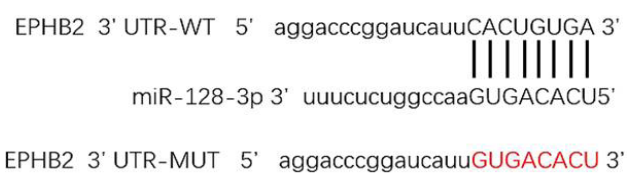

Fig. 1. EPHB2 sequence contains a nucleotide sequence complementary to miR-128-3p.

Table VII.- Effect of knockdown or overexpression of miR-128-3p on EPHB2 protein expression ($\bar{x}$±s, n=9).

Group	Control	mimics-NC	miR-128-3p	anti-miR-NC	anti-miR-128-3p	F	P
miR-128-3p	0.42±0.04	0.43±0.04	1.13±0.12*	0.43±0.04	0.19±0.04#	274.3	0
EPHB2 mRNA expression	1.56±0.16	1.57±0.16	0.92±0.09*	1.55±0.16	2.63±0.25#	115.8	0
EPHB2 protein expression	0.81±0.08	0.82±0.08	0.39±0.04*	0.80±0.08	1.12±0.11#	92.42	0

Compared with mimics-NC group, *P<0.05; compared with anti-miR-NC group, #P <0.05.

miR-128-3p targets negative regulation of EPHB2

According to StarBase prediction, EPHB2 sequence contains a nucleotide sequence complementary to miR-128-3p (Fig. 1). The results of dual luciferase reporter assay show that in the cell experiment on wild-type vector WT-EPHB2 transfection, compared with miR-con group, miR-128-3p group has significantly reduced luciferase activity (P <0.05); In the cell experiment on mutant vector MUT-EPHB2 transfection, no statistically significant difference is found in luciferase activity between miR-128-3p group and miR-con group (P> 0.05) (Table VI). Compared with mimics-NC group, miR-128-3p group has significantly reduced EPHB2 mRNA and protein levels (P <0.05); compared with anti-miR-NC group, anti-miR-128-3p group has significantly increased EPHB2 mRNA and protein levels (P <0.05) (Table VII).

DISCUSSION

The main pathological characteristics in keloid formation are excessive proliferation of fibroblasts and excessive deposition of collagen and fibronectin. The abnormal apoptosis and excessive proliferation of fibroblasts in scars are important reasons for collagen synthesis. Drug induction may promote fibroblast apoptosis, then playing an anti-keloid role. Previous studies have shown that effective drugs screened from natural active ingredients may treat keloids by inhibiting fibroblast proliferation and promoting apoptosis (Lundvig *et al*., 2015; Xu *et al*., 2019). However, therapeutic effect and its mechanism of some Chinese herbal medicines on keloid remain unclear.

In recent years, HAE research mainly focuses on anti-cancer therapy. Previous research has shown that HAE can inhibit the proliferation and growth of tumor cells such as osteosarcoma and promote its apoptosis (Yan *et al*., 2015; Wang *et al*., 2019). However, the effect of HAE on the proliferation and apoptosis of keloid fibroblasts is yet unknown. The results of this study show that HAE can significantly inhibit fibroblast proliferation and promote cell apoptosis, with significant changes shown as concentration increases. It suggests that HAE may regulate keloids occurrence and development by inhibiting keloid fibroblast proliferation and inducing apoptosis. Studies have shown that up-regulation of Ki-67 and PCNA expressions can promote cell cycle progression and thereby promote cell proliferation (Papanastassiou *et al*., 2010). The process of apoptosis is closely related to the abnormal expression of apoptosis-related proteins. Studies have shown that Bcl-2 can inhibit apoptosis while Bax can promote apoptosis (Zhang *et al*., 2014). The results of this study indicate that Ki-67, PCNA and Bcl-2 expression are down-regulated while Bax expression is up-regulated in HAE-treated cells, suggesting that HAE may inhibit keloid fibroblast proliferation and induce its apoptosis by promoting Bax expression and inhibiting Ki-67, PCNA and Bcl-2 expressions.

As our study showed that HAE could down-regulate Ki-67 expression and it was also supported by decreased proliferation. Keloid cells with higher Ki-67 were actively proliferating and cells without Ki67 became senescent.

So, HAE could induce apoptosis in cancer cells or stop the cancer cells proliferation. The study of the Zhang *et al*. (2010) showed that via down-regulation of Bcl-2 and up-regulation of BAX, reduced mitochondrial membrane potential suggested that Huaier mediated mitochondrial pathway apoptosis in breast cancer cells.

Decreased transcription of BCL2 following treatment with HAE happened in the study of Qi *et al*. (2016) on the breast cancer cells. They suggested that other biological intermediaries of HAE's effect on endocrine therapy resistance in breast cancer maight be due to the BCL2 pathways.

To investigate the molecular mechanism by which HAE affects the proliferation and apoptosis of keloid fibroblasts, this study found via detection that HAE can significantly promote miR-128-3p expression and inhibit EPHB2 expression. MiR-128-3p induces apoptosis in glioma cells by targeting pyruvate dehydrogenase kinase 1 (Qu *et al*., 2020). Studies have shown that miR-128-3p inhibits the proliferation of hepatocellular carcinoma by regulating PIK3R1 (Huang *et al*., 2015). Up-regulation of EPHB2 expression can promote cell proliferation and invasion of tumors like cervical cancer (Duan *et al*., 2018; Lian *et al*., 2018). The results of this study suggest that HAE may regulate keloid fibroblast proliferation and apoptosis by up-regulating miR-128-3p expression while down-regulating EPHB2 expression. At the same time,

this study show that after miR-128-3p overexpression or EPHB2 expression inhibition, keloid fibroblasts have significantly reduced viability, significantly increased apoptosis rate, reduced Ki-67, PCNA, Bcl-2 expression levels and increased Bax expression level, suggesting that miR-128-3p overexpression or EPHB2 expression inhibition can inhibit keloid fibroblast proliferation and promote apoptosis. By dual luciferase reporter assay, qRT-PCR experiment and Western blot experiment, this study confirms that miR-128-3p can targetedly bind to EPHB2 and negatively regulate EPHB2 expression, suggesting that HAE may inhibit keloid fibroblast proliferation and promote apoptosis by regulating miR-128-3p/EPHB2 molecular axis.

CONCLUSION

To conclude, HAE can inhibit keloid fibroblast proliferation and induce its apoptosis, and its mechanism of action may concern the regulation of miR-128-3p/EPHB2 molecular axis, which can provide a theoretical basis for keloid treatment by HAE.

Statement of conflict of interest

The authors have declared no conflict of interests.

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