

Retraction

Retraction Notice

Experimental Biology and Medicine 2022; X: 1. DOI: 10.1177/15353702221144939

The following article has been retracted at the request of the Editor and Publisher at SAGE Publishing:

An G, Liang S, Sheng C, Liu Y, Yao W. Upregulation of microRNA-205 suppresses vascular endothelial growth factor expression-mediated PI3K/Akt signaling transduction in human keloid fibroblasts. *Experimental Biology and Medicine*. 2017;242(3):275-285. doi:10.1177/1535370216669839

Concerns were raised on multiple figures on Pubpeer:

1. Figure 3a NC Invasion panel and 4b siVEGF panel lower image appear to be duplicated.
2. The immunoblot images in figure 4F appear to be similar to immunoblots in Figure 4A rotated 180°.

The corresponding author provided new figures, but original images were not provided upon request.

Due to the lack of uncropped, unedited images underlying figure panels, the image concerns remain unresolved. In accordance with COPE guidelines, the *Experimental Biology and Medicine* Editor retracts the article.

The corresponding author did not respond to requests for comments on this retraction. The co-authors of this publication were unreachable via email.

RETRACTED: Upregulation of microRNA-205 suppresses vascular endothelial growth factor expression-mediated PI3K/Akt signaling transduction in human keloid fibroblasts

Gang An, Shuzeng Liang, Chunhong Sheng, Yan Liu and Wei Yao

Shandong Provincial Qianfoshan Hospital, Jinan, Shandong 250014, China

Corresponding author: Gang An. Email: cloudseasky@126.com

Abstract

Keloid is one of the most frustrating problems related to wounding healing and presents a great challenge in clinic. MicroRNAs (miRs) have shown their potential as a novel therapy for the prevention and treatment of keloid. Vascular endothelial growth factor (VEGF) plays a critical role in the regulation of scar development. In the current study, it was hypothesized that miR-205-5p was capable of suppressing keloid formation by inhibiting the VEGF-mediated wound healing cascade. The expression statuses of miR-205-5p and VEGF in clinical keloid tissues and keloid cell line human keloid fibroblasts (HKF) were detected. Then the direct action of miR-205-5p on *VEGF* gene was assessed using dual-luciferase assay. Thereafter, orchestrated administrations on HKF with miR-205-5p mimic, specific VEGF siRNA, PI3K agonist (740 Y-P), and PI3K inhibitor (LY294002) were performed to reveal the roles of miR-205-5p and VEGF in keloid formation and further explain the mechanism through which miR-205-5p affected the VEGF-mediated signaling transductions. Our results showed that there was significant low expression of miR-205-5p in keloid tissue specimens and the cell line while the expression of VEGF in keloid tissues was augmented. Moreover, miR-205-5p over-expression dramatically impaired the cell viability, induced the cell apoptosis, and inhibited the cell invasion and migration ability in HKF. Based on the detection of dual luciferase assay and detection at protein level, miR-205-5p antagonized the keloids by directly targeting VEGF expression and subsequently inhibiting PI3K/Akt pathway. The current study is the first one demonstrating that miR-205-5p inhibits the pathogenesis of keloids, indicating the potential of miR-205-5p in the development of therapies for prevention and treatment of keloids.

Keywords: Keloid, microRNA-205, vascular endothelial growth factor, Akt

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Introduction

Skin is the largest organ in human bodies and protects fragile intrinsic systems against surrounding effects. Due to its constant contact with the environment, skin is frequently subjected to wounds. After being wounded, a complete restoration of cutaneous skin architecture is the optimal outcome of skin repair,¹ but only fetal skin is capable of healing in this manner. Generally, scars are the endpoint of the normal continuum of skin repair. However, once the delicate balance of the reparative process is disrupted, wound healing can turn into drastic impairments and result in two pathological extremes: chronic wounds or excess scar formation range from hypertrophic scars to keloids.²

Among the disorders related to wound healing, keloids are one of the most frustrating problems. Keloid scarring, also known as keloid disease, is defined as locally aggressive benign tumor extending beyond the confines of the

original wound and invading into surrounding healthy skin.³ These scars are not only esthetically displeasing, but also painful and functionally disabling, casting severely physical and psychological distress to the patients.^{4,5} Although keloids are benign dermal tumors, their management also presents a great challenge in clinic. The scars do not regress over time, and sole surgical excision is associated with a high rate of recurrence. Although numerous therapies are available nowadays, definite and effective treatment modalities have not yet been established.^{6,7} To help with the development of new therapeutic strategies to improve the prevention or treatment of the disorder, a better understanding of pathogenesis of keloids is critical. Whereas the pathophysiological mechanism finally leading to keloid formation remains partially revealed,⁸ it is commonly recognized that keloid is a result of the unbalanced cellular dynamics caused by overabundant fibroblast

proliferation and insufficient fibroblast apoptosis.⁹ In this regard, several studies infer that factors related to tumor formation and cytokine function may play crucial roles in the formation of keloids and influence their development.^{10,11}

MicroRNAs (miRs) belong to an endogenous class of small non-coding RNAs which function through inhibition of translation levels or fracture of targeted mRNAs.^{12,13} The role of miRs in keloids has been confirmed in previous studies: Liu *et al.* conducted an investigation on the miR expression profiles in keloid tissue and corresponding normal skin tissue and identified several dysexpressed miRs in keloids tissues.¹⁴ Additionally, study of Wu *et al.* showed that keloid fibroblasts transfected with miR-199a-5p mimics exhibited impaired cell proliferation and an altered cell cycle.¹⁵ Thus, targeting the dynamics of miRs in keloid holds great promise for developing novel therapeutic strategies.

The studies focusing on the wound healing cascade have revealed a critical role of growth factors, such as vascular endothelial growth factor (VEGF), transforming growth factor β , insulin growth factor, etc. in the regulation of scar development.^{16,17} Based on this theory, Wu *et al.* applied dexamethasone to suppress the expression of VEGF and achieved considerable treatment effect against keloid.¹⁸ As an angiogenic peptide composed a variety of isoforms, VEGF can promote neovascularization and cell growth^{19,20} by activating multiple early signaling cascades, including ERK1/2, PI3K-dependent Akt/PKB pathway, and phospholipase C- γ .^{21–24} Regarding keloid, VEGF is proved to be elevated in keloid fibroblasts²⁵ and accounts for the increased level of plasminogen activator inhibitor-1 via activation of ERK1/2 pathways. Thus, considering the central function of miRs and VEGF in the onset and development of keloids, exploration on the possible interaction between the two factors in this disease is of great value for a better understanding of the pathogenesis of keloids.

In the current study, miR-205-5p, which was proved to be suppressor of VEGFA in human glioblastoma cells,²⁶ was selected as a regulator of VEGF in keloids. We hypothesized that miR-205-5p was capable of suppressing keloids formation by inhibiting the VEGF-mediated wound healing cascade. To test such hypothesis, the expression levels of miR-205-5p and VEGF in clinical keloid samples and human keloid cell line human keloid fibroblast (HKF) were investigated. Then the regulation of miR-205-5p on VEGF was assessed using dual-luciferase assay. Afterward, orchestrated treatments on HKF with miR-205-5p mimic, specific VEGF siRNA, Akt inhibitor, and Akt agonist were conducted to elucidate the mechanism through which miR-205-5p acted on VEGF-mediated signaling transductions.

Methods

Chemicals and cell cultures

Antibodies against VEGF, phosphorylated Akt (p-Akt), fibrous protein, α -SMA, and GAPDH were purchased from Promega (Madison, WI, USA). HKF was purchased from Bioleaf Corporation (Shanghai, China), and human

embryonic skin fibroblasts obtained from Beijing Union Medical College cell bank. Cells were cultured in DMEM medium complemented with 15% FBS and 1% (v/v) antibiotics mixture in an atmosphere of 95% air and 5% CO₂ at 37°C. Mimic of miR-205-5p and non-targeting mimic were purchased from GenePharma (Shanghai, China). 740 Y-P (ApexBio, Houston, TX) and LY294002 (Selleck Chemicals, Houston, TX) were used in this research as the agonist and inhibitor, respectively, in PI3K/Akt pathway.

Patients and tissue specimen collection

Investigation of the expression status of miR-205-5p was conducted. Keloid tissues were collected from keloid patient, and matched normal skin samples were collected from patients. Corresponding information of patients were listed in Table 1. All samples were preserved in liquid nitrogen for further detection. All the diagnosis of keloids was confirmed by histology tests. Keloid tissue and normal tissue were carefully excised. All the cases involved in the analysis should possess detailed information of clinical pathological and prognostic characteristics. The study was approved by ethics committee. The ethics committee approved the relating screening, inspection, and data

Table 1 The profile of each sample for primary culture

No.	Sex	Age (years)	Biopsy	Duration (Months)	Etiology
1	F	24	KS: Shoulder NS: Shoulder	20	Trauma
2	F	16	KS: Neck NS: Neck	6	scald
3	M	5	KS: Foot NS: Leg	16	Flame burn
4	F	21	KS: Chest NS: Chest	22	Trauma
5	F	47	KS: Earlobe NS: Neck	17	Trauma
6	M	37	KS: Chest NS: Chest	12	Oil burn
7	M	62	KS: Chest NS: Chest	15	Operation
8	F	40	KS: the back of the hand NS: forearm	28	Flame burn
9	F	20	KS: Back NS: Back	10	Trauma
10	M	27	KS: elbow hand NS: the upper arm	11	scald
11	F	29	KS: Abdomen NS: Abdomen	12	operation
12	M	35	KS: Shoulder NS: Shoulder	17	Trauma
13	M	15	KS: Forearm NS: Forearm	12	Trauma
14	M	26	KS: Foot NS: Leg	20	Flame burn

F: female; KS: keloid skin; M: male; NS: normal skin.

collection of the patients, and all subjects signed a written informed consent form. All works were undertaken following the provisions of the Declaration of Helsinki. The expression of miR-205-5p and VEGF in different samples was detected using reverse transcription quantitative PCR (RT-qPCR) and western blotting assay, respectively. Then the expression statuses of miR-205-5p, miR200b, and miR200c in HKF were also assessed using RT-qPCR.

Transfection of miR-205-5p mimics into HKF

To explore the effect of miR205 overexpression on keloid cells, HKFs were transfected with different versions of mimics using Lipofectamine 2000 reagent according to the manufacturer's instruction (Lipofectamine 2000) and grouped into two groups: (a) NC group, cells were transfected with non-targeting mimics. (b) Mimics group, cells were transfected with miR-205-5p mimics. Then the action of miR-205-5p on cell viability, cell apoptosis, cell cycle distribution, and cell migration and invasion ability was detected as described following.

Knockdown of VEGF gene in HKF

VEGF specific (5'-GAAGUUCAUGGAUGUCUAUCA-3') and non-targeting (5'-ACGUGACACGUUCGGAGAATT-3') siRNAs were synthesized by Genepharm (Shanghai, China). Transfection was conducted using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instruction (Lipofectamine 2000). To detect the effect of VEGF knockdown on biological processes in HKF, two experimental groups were set up: (a) NC group, HKF transfected with NC siRNA. (b) siVEGF group, HKF transfected with VEGF siRNA. Each treatment was represented by at least three replicates. The knockdown efficiency of VEGF by siRNA was assessed using RT-qPCR. Stable transfected cells for further experiments were screened in medium with the presence of G418 (0.5 µg/µL). Effect of VEGF knockdown on cell viability, cell apoptosis and cell cycle distribution, and cell migration and invasion ability was assessed as described following.

Activation and inhibition of PI3K/Akt pathways

Cells transfected with miR-205-5p mimics were grouped into three groups to further reveal the pathway through which miR-205-5p exerted its function in keloid: (a) Control group, HKF transfected with miR-205-5p mimics. (b) Agonist group, miR-205-5p overexpressed HKF incubated with 20 µM 740 Y-P for 24 h. (c) Inhibitor group, miR-205-5p overexpressed HKF incubated with 25 µM LY294002 for 24 h. Then effect of different treatments on the expression of VEGF, p-Akt, fibrous protein, and α-SMA was quantified using western blotting assay. The cell viability, cell apoptosis, cell cycle distribution, and cell migration and invasion ability in different groups were detected as described below.

Dual luciferase assay

Moreover, fragments of the 3'UTR of VEGF and mutant 3'UTR of VEGF cDNAs were inserted into psiCHECK-2

vector (vector containing firefly luciferase under the control of SV40 promoter, Promega) to form WT VEGF and MUT VEGF plasmids. Concentration of HKF was adjusted to 1×10^4 /mL and incubated on slides in one well of 24-well plates for 24 h before co-transfection with different combinations of vectors using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol, and subsequent selection was conducted using 400 µg/mL ampicillin. Grouping of cells was as follows: (a) WT + NC group, cells were co-transfected with WT VEGF plasmid + NC mimics; (b) WT + Mimics, cells were co-transfected with WT VEGF plasmid and miR-205-5p mimics group; (c) MUT + NC group, cells were co-transfected with MUT VEGF plasmid + NC mimics; (d) MUT + Mimics group, cells were co-transfected with MUT VEGF plasmid + miR-205-5p mimics. Dual luciferase assay was conducted to measure the firefly luciferase activity using Luciferase Report Gene Assay Kit (E1910, Promega).

RT-qPCR

Whole RNA in samples was extracted using RNA simple Total RNA Kit according to the manufacturers' instruction (No. DP419, TIANGEN, Beijing, China). β-actin was selected as the reference gene. Then the RNA was reversely transcribed to cDNA templates using Super M-MLV reverse transcriptase (No. RP6502, BioTeke, Beijing, China). The final RT-qPCR reaction mixture of volume 20 µL consisted of 10 µL of SYBR GREEN mastermix, 0.5 µL of each primers (miR-205-5p, forward: ACACTCCAGCTGGGTCCTTCAT TCC, RT: CTCAACTGGTGTCTGGAGTCGGCAATTCA GTTGAGCAGACT; miR200b, forward: ACACTCCAGCT GGGTAATACTGCCT; RT: CTCAACTGGTGTCTGGAG TCGGCAATTCAGTTGAGTCCAAT; miR200c, forward: ACACTCCAGCTGGGTAATACTGCCG, RT: CTCAACTG GTGTCGTGGAGTCGGCAATTCAGTTGAGCCAAAC; U6, forward: CTCGCTTCGGCAGCACA, reverse: AACGCT TCACGAATTTGCGT, VEGF, forward: GCCTTGCTTG CTGCTCTAC, reverse: CACCAGGGTCTCGATTGGAT; GAPDH forward: TGTTCGTCATGGGTGTGAAC, reverse: ATGGCATGGACTGTGGTCAT), 1 µL of the cDNA template, and 8 µL of RNase-free H₂O. Thermal cycling parameters for the amplification were set up as following: a denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s. Relative expression level of targeted gene was calculated with Exicycler™ 96 (BIONEER, South Korea) according to the expression of $2^{-\Delta\Delta Ct}$.

CCK-8 assay

Cell viabilities of HKF under different treatments at different time points were measured using a CCK-8 method. Hundred microliters of CCK-8 solution was added to the cultures and incubated at 37°C for 1 h. The OD values at 450 nm of different treatments were recorded using a microplate reader.

Flow cytometry assay

The cell cycle distributions and apoptotic rates in different groups were determined with flow cytometry. Cells in different groups were collected with centrifugation at 2000 rpm for 5 min. Cell cycle distribution was detected according to standard procedure: briefly, cells were fixed with 70% alcohol at 4°C for 2 h. Then 500 μ L propidium iodide (PI) was added to different samples to stain DNA in the dark at 4°C for 30 min. After 20 min incubation at room temperature, the DNA contents of the cells were analyzed using a flow cytometer (Accuri C6, BD, USA). Then the cell apoptotic rates were also measured using an Annexin V-FITC Apoptosis Detection Kit (WLA001c, Wanleibio, Shenyang, China) according to the instructions for manufacturers: briefly, 5 μ L Annexin V was added to different wells. After incubation with Annexin V for 10 min at room temperature, the cells were resuspended with 1 $\times$ binding buffer and added with 5 μ L PI. Then the apoptotic rates were analyzed using a FACSscan flow cytometry (Accuri C6, BD, USA). The apoptotic cell rate (UR+LR-all apoptosis cell percentage) was equal to the sum of the late apoptotic rate (UR, upper right quadrant-advanced stage apoptosis cell percentage) and the early apoptotic rate (LR, lower right quadrant-prophase apoptosis cell percentage).

Transwell experiment

The transwell experiment which evaluated the migration ability of HKF cells in different groups was performed: 200 μ L incubation (with 1 mM MgCl₂) medium containing 1 $\times$ 10⁴ cells were seeded into the upper chamber transwell chambers (Corning star, Cambridge, MA). Then cells were incubated at 37°C for 24 h to allow the migration through the porous membrane. Upon completion of the culture, cells remaining at the upper surface of the chamber were completely removed. The lower surfaces of the membranes were fixed with 4% paraformaldehyde for 20 min and stained in a solution containing 0.5% (w/v) crystal violet for 5 min. After being washed using ddH₂O, numbers of cells in different groups were determined using Image-Pro Plus 6.0 software (Nikon). Then the invasion ability of HKF was measured as described above just with polycarbonate membranes being previously coated with 40 μ L matrigel (1.5 mg/mL; BD Biosciences, San Jose, CA, USA) at 37°C for 2 h to form a reconstituted basement membrane.

Western blotting assay

Protein product of different samples was extracted using Whole Protein Extraction Kit according to the manufacturers' instruction (WLA019, Wanleibio, China) and GAPDH was used as reference protein. The concentration of the extracted protein samples was determined according to the BCA method. Then 40 μ g protein in 20 μ L solution was subject to a 13% sodium dodecylsulfate polyacrylamide gel electrophoresis and subsequently transferred onto polyvinylidene difluoride sheets. Then the membranes were washed in TTBS for 5 min and incubated with 5% skim milk powder solution for 1 h. Primary antibody against VEGF, p-Akt, fibrous protein, α -SMA, or GAPDH

(primary antibodies from Abcam, Cambridge, MA) was added into the solution and incubated with the membranes at 4°C overnight. After 24 h incubation, the membranes were washed with TTBS for four times and incubated with secondary IgG-HRP antibodies (Santa Cruz Biotech, USA) for 45 min at 37°C. After the final six washes with TTBS, the blots were developed using Beyo ECL Plus reagent and the results were observed in the Gel Imaging System. The relative expression levels of protein in different groups were calculated with Gel-Pro-Analyzer (Media Cybernetics, USA).

Statistical analysis

All the data were expressed in the form of mean $\pm$ SD. Student's *t*-test and multiple comparisons using the least significant difference (LSD) method was performed with a significant level of 0.05 with GraphPad Prism 6 (GraphPad Software, San Diego, CA).

Results

Expression miR-205-5p was decreased and expression of VEGF was increased in clinical keloid samples

Based on the investigation of the miR-205-5p expression status in clinical samples, levels of miR-205-5p in keloid samples were all lower than those in corresponding normal skin samples, and the average difference between keloid tissue and normal skin samples was statistically significant ($P < 0.001$) (Figure 1(a)). Contrary to the changing pattern of miR-205-5p, the expression of VEGF in keloid patients was significantly upregulated both at mRNA ($P < 0.001$) and protein levels (Figure 1(b) and 1(c)).

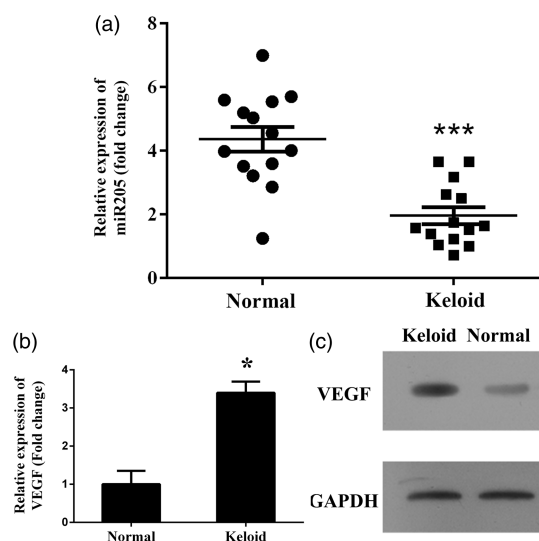


Figure 1 Investigation of expression status of miR-205-5p and VEGF in clinical keloids and HKF. (a) Quantitative analysis results of miR-205-5p levels as detected by RT-qPCR, levels of miR-205-5p were reduced in keloid tissues compared to normal tissues. (b) Representative analysis results of VEGF levels as detected by RT-qPCR, mRNA level of VEGF was increased in keloid tissues compared to normal tissues. "****", $P < 0.05$ compared with normal tissues or cells. "*****", $P < 0.001$ compared with normal tissues or cells. (c) Representative image of protein level of VEGF as detected by western blotting assay, protein level of VEGF was increased in keloid tissues compared to normal tissues

Expression of miR-205-5p, miR200b, and miR200c and overexpression of miR-205-5p inhibited cell proliferation

Transcription levels miR-205-5p, miR200b, and miR200c were also detected with HKF. All the three miRs were proved to be inhibited in keloids. As shown in Figure 2(a), expression of the three miRs was also downregulated, the difference between normal skin cells and HKF was statistically significant ($P < 0.05$). The *in vitro* growth ability of HKF was determined by CCK-8 assay at three time points (24, 48, 72 h) (Figure 2(b)). For cell viability measured at 48 h, the OD₄₅₀ values for NC and Mimics groups were 0.82 ± 0.04 and 0.53 ± 0.04 , respectively. And for the detection at 72 h, the OD₄₅₀ values for NC and Mimics groups were 0.91 ± 0.07 and 0.56 ± 0.05 , respectively. Significant differences in cell viability between NC and Mimics groups were detected for the last two time points ($P < 0.05$).

Overexpression of miR-205-5p affects apoptosis percentage and cell cycle distribution in HKF

The effect of miR-205-5p overexpression on the apoptosis and cell cycle distribution in HKF was also analyzed by conducting Annexin V and PI double staining. The early phase (26.8%) and late-phase apoptotic cells (9.4%) were both dramatically increased in cells transfected with miR-205-5p mimics when compared with scramble control cells (11.5% for early phase apoptosis and 2.1% for late-phase apoptosis) (Figure 2(c) and 2(d)), representing an apoptosis-induced action of miR-205-5p in keloid cells. However, no obvious effect of miR-205-5p overexpression

on cell cycle distribution was detected in the current assay (Figure 3(b)).

Overexpression miR-205-5p suppressed the invasion and migration ability of HKF

Invasive growth is an important biological characteristic of malignant tumor cells. To evaluate the impact of miR-205-5p on the invasion and migration abilities of HKF, transwell assays (with or without matrigel) were performed. As shown in Figure 3(a), the numbers of cells moving through the porous membrane were decreased in Mimics groups for both assays. The results demonstrated that miR-205-5p was capable of reducing the invasion and migration abilities of keloid cells.

VEGF is potential target of miR-205-5p in HKF

To further explore the interaction between VEGF gene and miR-205-5p, dual luciferase assay was also performed. Reporter assay revealed that overexpression of miRNA-205 significantly suppressed the activity of WT VEGF plasmid in HKF ($P < 0.001$), without change in luciferase activity of MUT VEGF plasmid (Figure 3(c)), which indicated that miR-205-5p directly modulate VEGF expression by binding to 3'UTR of VEGF in keloid cells.

VEGF is crucial to cell viability and cell invasion and migration growth of keloid cells

VEGF gene in HKF was knockdown by specific siRNA (Figure 4). To explore the pathway involving VEGF in keloid cells, p-AKT, fibrous protein, and α -SMA

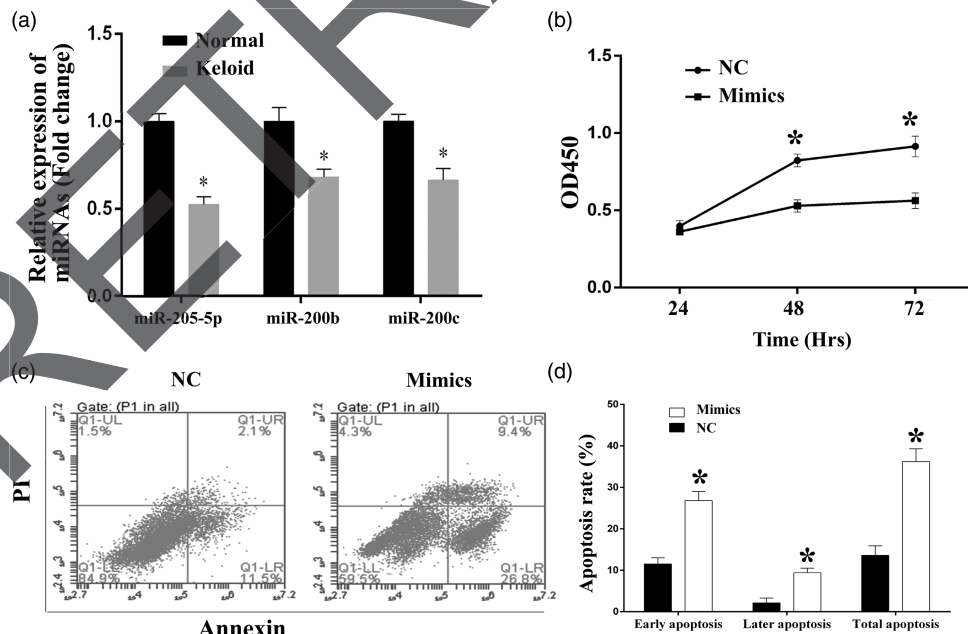


Figure 2 Overexpression of miR-205-5p impaired the cell viability, induced cell apoptosis, and reduced the cell invasion and migration ability in HKF. (a) Quantitative analysis results of levels of miR-205-5p, miR200b, and miR200c as detected by RT-qPCR, mRNA levels were reduced in HKF compared to normal skin cells. (b) Quantitative analysis results of CCK-8 assay, transfection of miR-205-5p mimics inhibited the cell proliferation in HKF. (c) Representative images of cell apoptosis as detected by flow cytometry, transfection of miR-205-5p mimics induced cell apoptosis in HKF. (d) The percentage of early, late phase, and total apoptosis cells of each group. ****, $P < 0.05$ compared with NC group. LR (lower right): Early apoptosis cells, UR (upper right): Later phase apoptosis cells, LL (lower left): Viable cells, UL (upper left): Necrosis cells. (A color version of this figure is available in the online journal.)

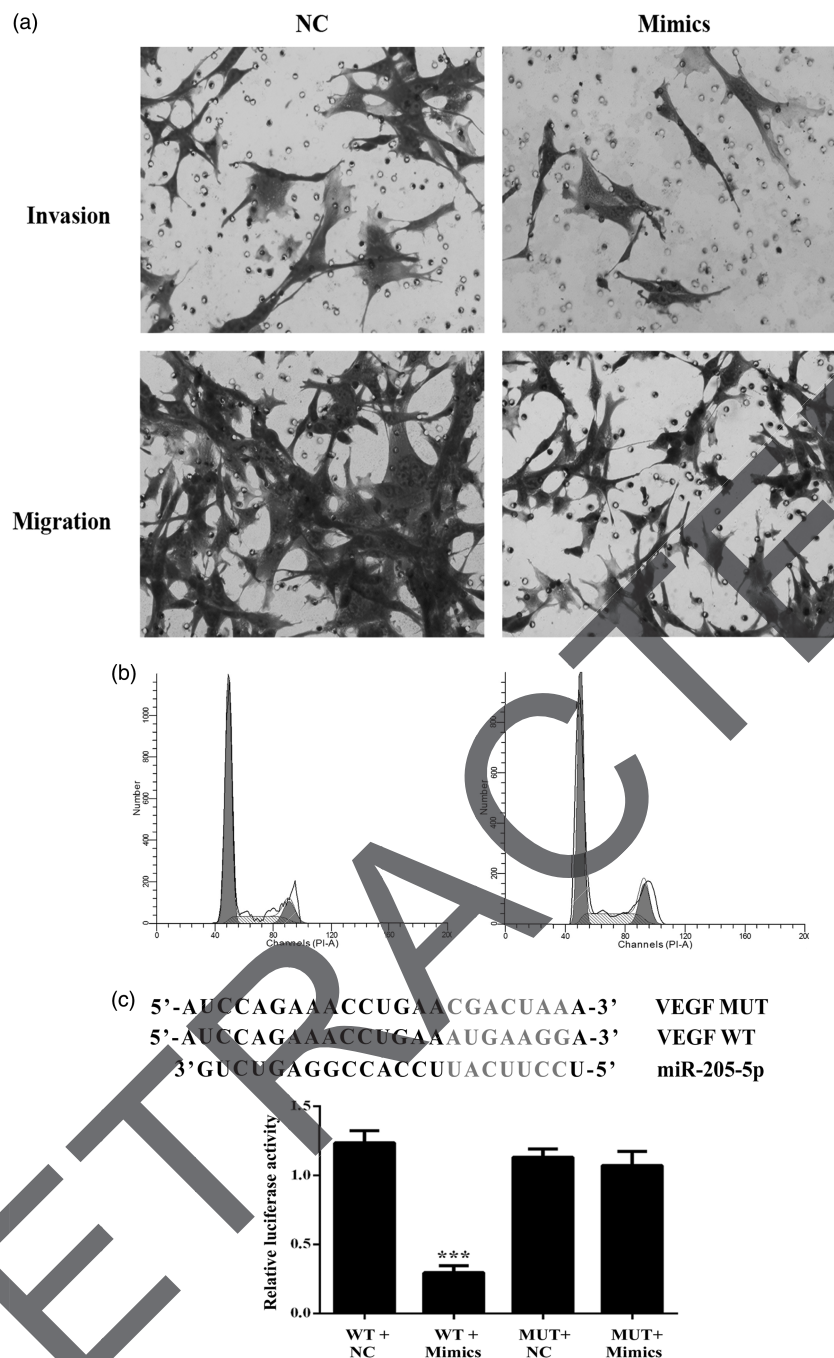


Figure 3 Overexpression of miR-205-5p reduced the cell invasion and migration ability through targeting VEGF in HKF. (a) Representative images of transwell assays, transfection of miR-205-5p mimics reduced the invasion and migration ability of HKF. (b) Representative images of cell cycle distribution as detected by flow cytometry, transfection of miR-205-5p mimics had no impact on the cell cycle distribution in HKF. (c) VEGF is a target of miR-205-5p. Sequence alignment of miR-205-5p and 3'-UTR of VEGF using TargetScan. (d) Luciferase activity in the VEGF-3'UTR-WT group was significantly decreased after miR-205-5p mimics transfection. *** $P < 0.01$. (A color version of this figure is available in the online journal.)

expression level were measured. Compared to NC group, cells treated with siRNA had a lower expression of protein level mentioned above (Figure 4(a)). Then the cell proliferation ability, the cell apoptosis and cell cycle distribution, and the cell invasion and migration abilities were all measured. As seen from Figure 4(b), the cell invasion and migration ability were impaired in siVEGF group. Corresponding to the effect of miR-205-5p overexpression, VEGF knockdown significantly inhibited

the cell proliferation ability in ($P < 0.05$) (Figure 4(e)) induced cell apoptosis (17.0% for NC group and 40.1% for siVEGF group) (Figure 4(c)). Cell cycle assays were presented in Figure 4(d), more cells were blocked in S-phase in siVEGF group compared to NC. Considering the direct regulation effect of miR-205-5p on transcription of VEGF, miR-205-5p might take action in keloid cells by inhibiting the activity of VEGF-related signaling transduction.

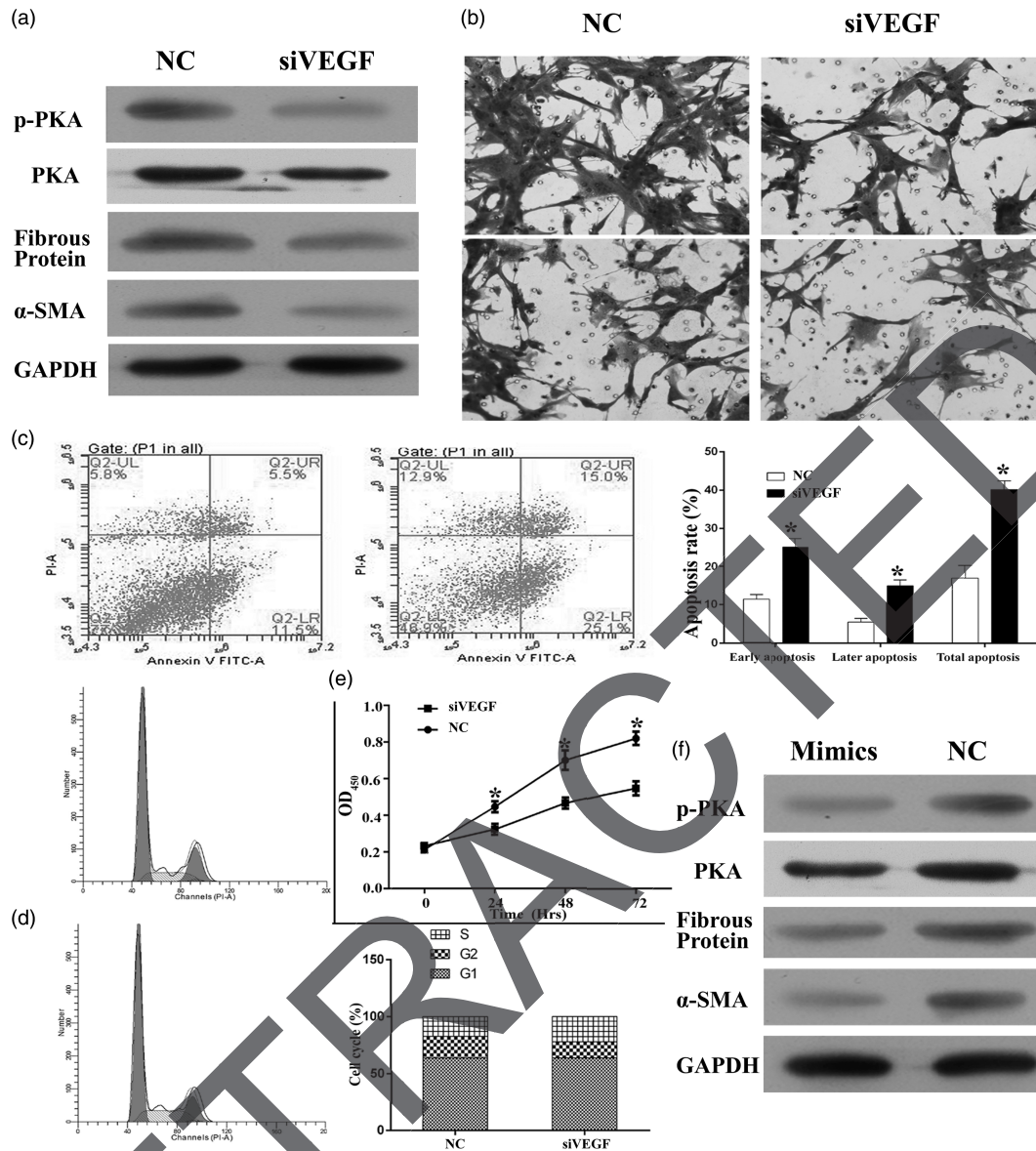


Figure 4 Knockdown of VEGF reduced the cell viability, induced cell apoptosis, and reduced the cell invasion and migration ability in HKF. (a) Expression level of p-Akt, Akt, fibrous protein, and α-SMA treated with siVEGF. (b) Representative images of transwell assays, knockdown of VEGF reduced the invasion and migration ability of HKF. (c) Representative images of cell apoptosis as detected by flow cytometry, knockdown of VEGF induced cell apoptosis in HKF. Quantitative analysis results of VEGF knockdown efficiency as detected by RT-qPCR, level of VEGF mRNA was significantly reduced after transfection of siVEGF. (d) Representative images of cell cycle distribution as detected by flow cytometry, knockdown of VEGF had no impact on the cell cycle distribution in HKF. (e) Quantitative analysis results of CCK-8 assay, knockdown of VEGF inhibited the cell proliferation in HKF. (f) Overexpression of miR-205-5p-induced relative protein downregulation. ****, $P < 0.05$ compared with NC group. *****, $P < 0.001$ compared with NC group. (A color version of this figure is available in the online journal.)

MiR-205-5p antagonized keloids through a VEGF/Akt inhibition manner

To further explore the mechanism through which miR-205-5p exerted its function in keloid cells, the expression of downstream effector of VEGF, including p-Akt, fibrous protein, and α-SMA was quantified with western blotting assay. The results showed that overexpression of miR-205-5p significantly downregulated the expression of the three indicators (Figure 4(f)). Moreover, the possible VEGF/Akt-dependent manner of miR-205-5p regulation in HKF was subsequently tested by incubating miR-205-5p overexpressed cells with PI3K/Akt agonist and inhibitor. It was

found that 740 Y-P, the activator of PI3K, upregulated expression status of p-AKT. Activation of PI3K/Akt pathway counteracted the impairments of miR-205-5p on the cell invasion and migration ability (Figure 5(a)); cell viability (Figure 5(b)); and the expression level of p-AKT, fibrous protein, and α-SMA (Figure 5(c)). Cell apoptosis rate was enhanced following inhibitor treatment (Figure 6(a)), while it decreased in the agonist treated group (Figure 6(b)). These results indicated that PI3K/Akt can mediate the profile of HKF. Cell cycle assay presented a higher percentage of S-phase in cell with LY294002, and contrary trend was found when cell was treated with 740 Y-P (Figure 6(c) and (d)). The

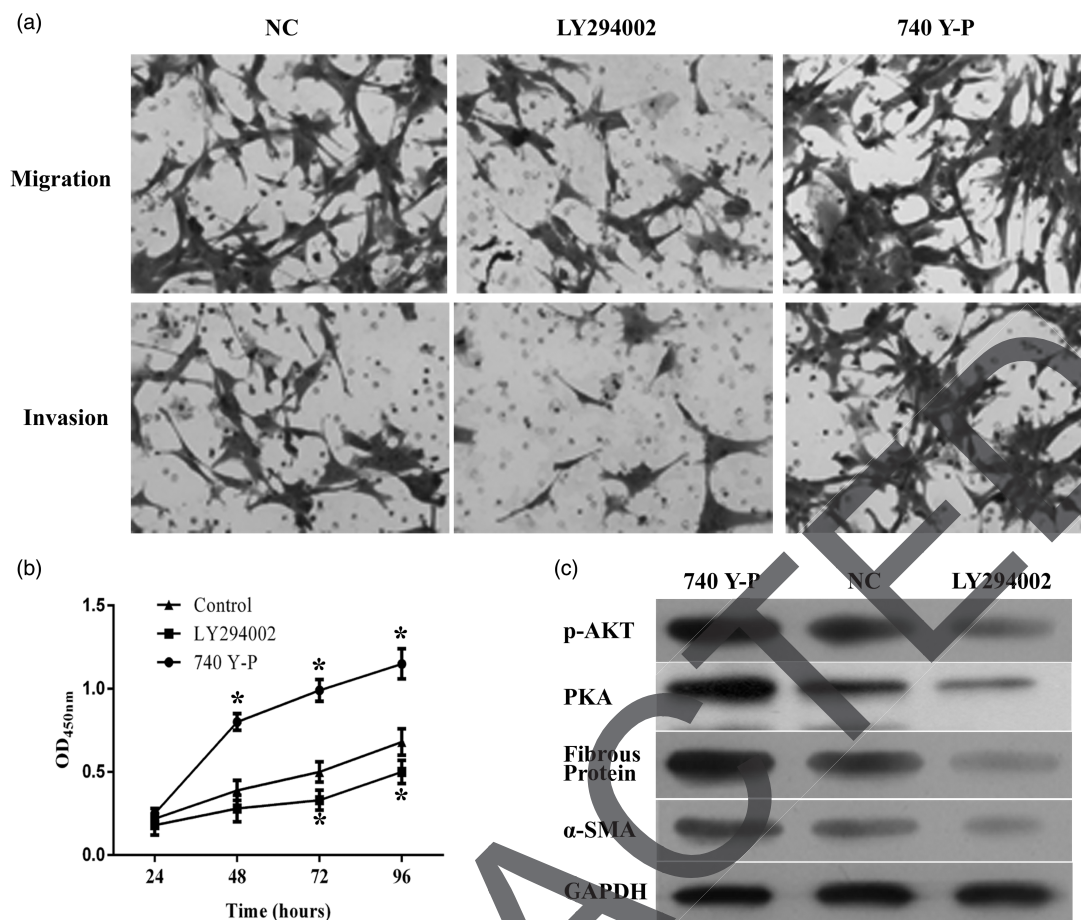


Figure 5 Administration of PI3K/Akt agonist 740 Y-P and inhibitor LY294002 affected the cell invasion and migration ability, viability, apoptosis in miR-205-5p HKF. (a) Representative images of transwell assays, administration of 740 Y-P and LY294002 affected migration and invasion ability in HKF. (b) Quantitative analysis results of CCK-8 assay, administration of 740 Y-P and LY294002 affected viability in HKF. (c) Expression level of p-AKT, fibrous protein, α-SMA in HKF treated with 740 Y-P and LY294002 compared to NC. Representative images of cell apoptosis as detected by flow cytometry, administration of 740 Y-P inhibited cell apoptosis in HKF. (d) Representative images of cell cycle distribution as detected by flow cytometry, administration of 740 Y-P had no impact on the cell cycle distribution in HKF. ****, $P < 0.05$ compared with control group. (A color version of this figure is available in the online journal.)

results clearly demonstrated a VEGF/Akt signaling transduction sequence during the action of miR-205-5p.

Discussion

Keloids are abnormal wound-healing process after skin injury, representing a connective tissue response to trauma, inflammation, surgery, or burns.²⁷ In keloids, the synthesis of collagen is approximately 20 times as high as that in normal skin tissues,^{16,27} which leads simple total excision of a keloid to simulation of additional collagen synthesis, sometimes even prompting quick recurrence of a keloid larger than the initial one.^{28,29} Thus, surgical therapies for keloids should be combined some adjuvant treatments. In the current study, for the first time, miR-205-5p was characterized as a signature to differentiate between keloids and normal fibroblasts. The expression levels of miR-205-5p in clinical keloid samples and human keloid cell HKF were both downregulated and overexpression of miR-205-5p significantly impaired the activity of HKF. Our data also inferred that the action of miR-205-5p

overexpression in keloids might be taken through a VEGF/Akt inhibition manner.

Previous study of Li *et al.* has demonstrated that expression profile of miRs is altered in keloids compared with that in fetal and adult dermal fibroblasts.³⁰ Given the diverse of miRs, Lu and his colleague show that a small subset of miRs may define keloid entities better than microarray expression data from bunches of mRNAs,³¹ especially those playing critical roles in tissue fibrosis and extracellular cell matrix metabolism have been reported.^{32,33} In the current study, it was found that overexpression of miR-205-5p significantly inhibited the production of fibrous protein and α-SMA. Fibrous protein contributes to strength and flexibility of tissues and α-SMA forms the materials basis for shrinkage of keloids.^{34,35} The negative regulation of miR-205-5p overexpression on the two indicators was corresponding to the antagonizing effect of miR-205-5p mimics on cell viability and cell invasion and migration ability in HKF, solidly indicating the central role of miR-205-5p in the formation of keloids. Previous researches regarding the function of miR-205-5p indicated that miR-205-5p was

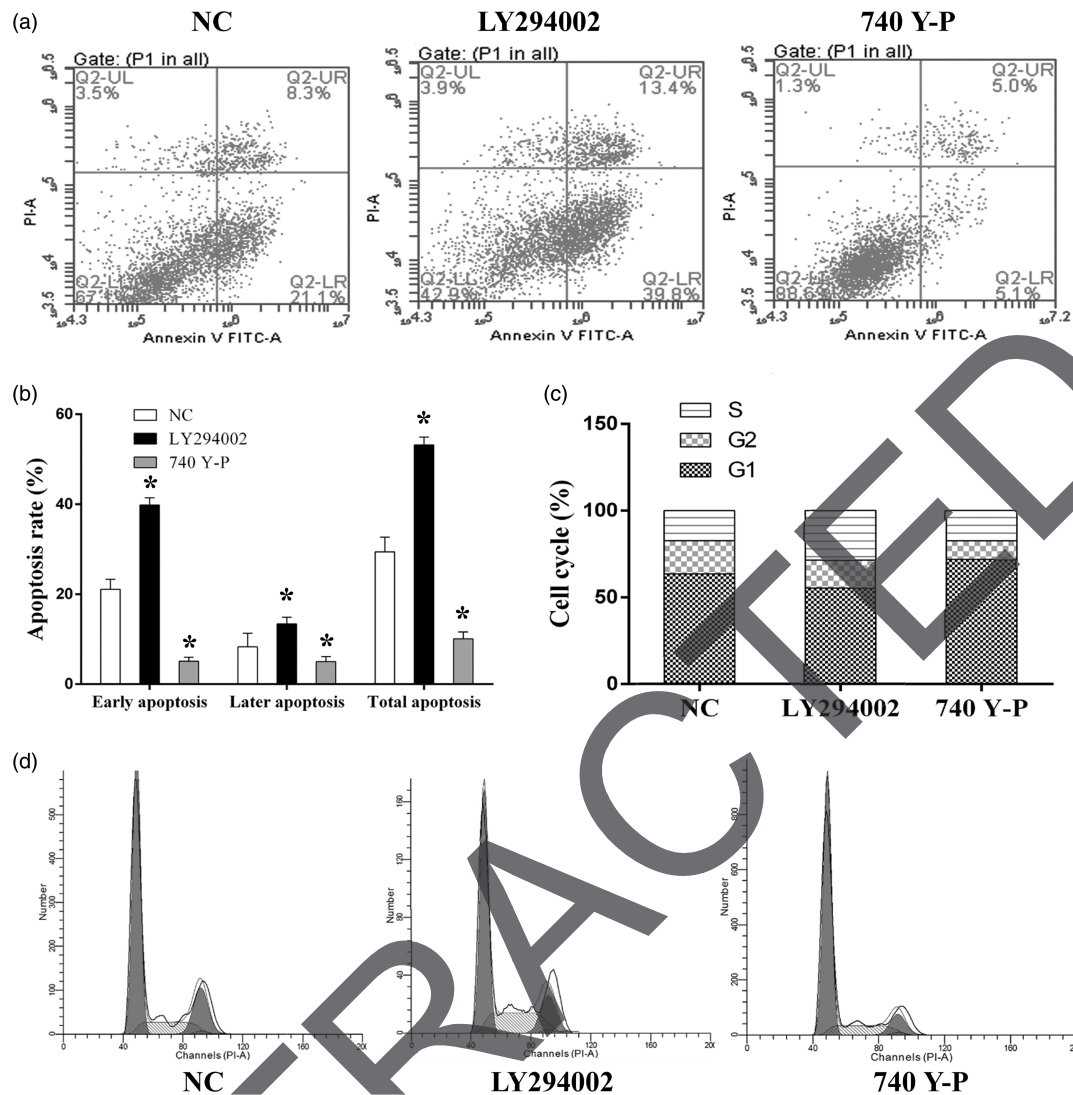


Figure 6 Administration of PI3K/Akt agonist 740 Y-P affected the cell apoptosis and cell cycle in miR-205-5p HKF. (a) Representative images of cell apoptosis as detected by flow cytometry, administration of 740 Y-P and LY294002 affected cell apoptosis in HKF. (b) The percentage of early, late phase, and total apoptosis cells of each group. (c) and (d) Representative images of cell cycle distribution as detected by flow cytometry, administration of 740 Y-P and LY294002 affected cell cycle distribution in HKF. (A color version of this figure is available in the online journal.)

negatively correlated with the increased malignancy of glioma but upregulated in various other cancer types, including lung cancer, bladder cancer, ovarian cancer, etc.^{36–41} Thus, the effect of miR-205-5p may be tissue sensitive.

In the current study, the mechanism which drives the function of miR-205-5p in keloids was elucidated by investigating its interaction with VEGF-related signaling. As a matter of fact, multiple miRs members, including miR-196, miR-21, and miR-199a-5p, have shown their potential in antagonizing keloids,^{15,42,43} but few ones are capable of directly interacting with VEGF in these skin disorders. The theory commonly accepted is that onset of keloids is associated with an abundance of growth factors and cytokines,⁴⁴ especially VEGF which favors blood vessels hyperpermeable and continued induction of tissue granulation of the healing wounds.⁴⁴ Our data showed that clinical keloid tissues consistently expressed higher level of VEGF mRNA

and protein than normal skins. Then VEGF gene in HKF was knockdown with specific siRNA, resulting in significant decrease in cell viability and cell invasion and migration ability in keloid cells, which represented a key role of VEGF in the attack of keloids as being previously validated.^{18,25} Previous study of Yue *et al.* has revealed that the VEGF transcription in glioma was directly regulated by miR-205.²⁶ To verify the conclusion, dual luciferase assay was performed in our study and the result demonstrated that in human keloid cells, miR-205-5p directly modulated VEGF expression by binding to 3'UTR of VEGF promoter. Although Yue and his colleague first reported the direct regulating function of miR-205 on VEGF, the authors failed to provide a further explanation on the downstream pathways following the modulation of miR-205 on VEGF. Moreover, the study was conducted with glioblastoma cells instead of keloid. Thus, to uncovering the downstream signaling following miR-205 and VEGF

interaction in keloid, a more comprehensive exploration was conducted in the current work.

Generally, VEGF exerts its function by binding to two receptor tyrosine kinases, VEGF receptor (VEGFR)-1/Flt-1 and VEGFR-2/KDR,⁴⁴ and activates multiple downstream pathways, including Akt signaling transduction.⁴⁴ Akt becomes activated via the phosphoinositide-3-OH kinase (PI3K) pathway⁴⁵ and inhibits cell death pathways by directly phosphorylating and inactivating proteins involved in apoptosis, including Bad, procaspase 9, and members of the Forkhead transcription factor family.⁴⁵ Administration of HKF with PI3K/Akt agonist significantly increased the cell viability and cell migration and invasion ability as well as upregulating the expression of fibrous protein and α -SMA. However, the treatment of PI3K/Akt agonist and inhibitor had no impact on the suppression of VEGF due to miR-205-5p mimics. Such results evidently revealed a VEGF→Akt signaling transduction sequence during the action of miR-205-5p in keloids, which is supplement to the mechanism by which miR-205-5p functions in keloid. And considering the important function of VEGF in the progression of keloid, the direct regulating effect of miR-205 on VEGF offers a promising strategy for treatment and prevention of keloid in clinic.

In summary, findings outlined in the current study showed that there is a significant low expression of miR-205-5p in keloid tissue specimens and cell line. Moreover, miR-205-5p plays a determinant role in the formation and invasion growth of keloids. The molecule suppresses the cell viability and cell migration and invasion by directly targeting VEGF expression and subsequently inhibiting PI3K/Akt pathway. In addition, this is the first study demonstrating that miR-205-5p inhibits the pathogenesis of keloids. Still, we believe that miR-205-5p should exert its function in keloid in a more complicated pattern. Therefore, along with the key role of VEGF/Akt pathway in the onset of keloid, the potential of miR-205-5p as an anti-keloid agent should be further investigated in the future.

Authors' contributions: GA finished the experimental design; GA, SL, CS, and YL participated in the entire research. GA and SL collected the essential materials and methods. Manuscript was drafted by GA. All authors read and approved the final manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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