

Original Article

MicroRNA-34a inhibits proliferation and stimulates apoptosis in human keratinocyte through activation of Smac-mediated mitochondrial apoptotic pathway: a potential therapeutic target for psoriasis

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Abstract: This study aimed to investigate the effects of miR-34a and the possible underlying molecular mechanisms on human keratinocyte cell (HaCaT) proliferation and apoptosis. We used keratinocyte growth factor (KGF) to induce HaCaT cell proliferation. Then, HaCaT cells were transfected with miR-34a mimic, si-miR-34a, and control (siNC). The cell proliferative and invasive capacities were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay and a Matrigel invasion chamber assay, respectively. Cells apoptosis was detected by Annexin V-FITC/PI Kit. Besides, the expression changes in Smac-mediated mitochondrial apoptotic pathway key proteins were measured by Western blot analysis. These key proteins included anti-apoptotic protein (Bcl-2) and pro-apoptotic proteins (Cyt C, cleaved/pro-caspase-3, and cleaved/pro-caspase-9). HaCaT cell proliferation increased with increase in the concentration of KGF, with maximum proliferation at 20 ng/mL concentration ($P < 0.01$). miR-34a overexpression significantly suppressed HaCaT cell proliferation ($P < 0.05$) and increased apoptosis ($P < 0.05$). Moreover, miR-34a overexpression significantly down-regulated the expression of Bcl-2 ($P < 0.05$), and up-regulated the expression of Cyt C ($P < 0.01$), cleaved-caspase-3 ($P < 0.01$), and cleaved-caspase-9 ($P < 0.05$). However, the effects of miR-34a suppression on HaCaT cells proliferation, apoptosis and on these four protein expressions were completely opposite to those found out in miR-34a overexpression. miR-34a overexpression inhibits human keratinocyte (HaCaT cells) proliferation and induces apoptosis through activation of Smac-mediated mitochondrial apoptotic pathway. Therefore, miR-34a might be used as a therapeutic target for treating psoriasis.

Keywords: microRNA-34a, psoriasis, cell proliferation, apoptosis, mitochondrial apoptotic pathway

Introduction

Psoriasis is a common chronic inflammatory skin disease affecting about 2% to 3% of the European population. The common symptoms of psoriasis include inflammation and raised and scaly lesions [1]. It is characterized by the hyperproliferation and aberrant differentiation of keratinocytes, increased vascularity in the dermis, and infiltration of inflammatory cells. These processes are immune-mediated; therefore, immune dysfunction is an important factor in the pathogenesis and progression of psoriasis [2-4].

Keratinocytes and immune cells in psoriatic skin are dependent on each other. Keratinocytes

secrete cytokines and chemokines, such as interleukin-1b (IL-1b), tumor necrosis factor- α (TNF- α), chemokine (C-X-C motif) ligand 1 (CXCL1)/growth-related oncogene-a, CXCL5/epithelial-derived neutrophil-activating peptide 78, and CXCL8/IL-8, which activate and attract immune cells to migrate into epidermis and dermis. These immune cell-derived cytokines in turn, act on keratinocytes to increase the expression of inflammatory genes, promote keratinocyte proliferation, and impair keratinocyte differentiation [2].

Recent studies have indicated that microRNAs (miRNAs) play important roles in psoriasis as a novel regulator of gene expression [5]. miRNAs are small short endogenous RNAs that

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regulate the expression of complementary messenger RNAs (mRNAs) at the post-transcriptional level [6]. It is well known that miRNAs play an important role in regulating various cell functions such as cell proliferation, differentiation, migration, invasion, and apoptosis [7]. Dysregulation of miRNAs and their regulated targets has been implicated in the pathogenesis of psoriasis [5, 8]. Several miRNAs with specific functions in skin morphogenesis and homeostasis have been studied; these include miR-203, miR-34a/c, miR-125b, and miR-200/miR-205 [5]. Of these miRNAs, we chose miR-34a to evaluate its potential role in psoriasis as miR-34a is repressed by p63 in epidermal cells to maintain cell cycle progression and expression of cyclin D1 and Cdk 4 [9]. Basically, miR-34a is a tumor suppressor [10, 11] which is expressed in all tissues, with the highest level of expression in the brain [12-14].

In the current study, we used keratinocyte growth factor (KGF) to induce the excessive proliferation of spontaneously immortalized human keratinocyte line (HaCaT cells), simulating the abnormal proliferation of psoriatic epidermis. Thereafter, we investigated the functional role of miR-34a in regulation of excessive proliferation of human keratinocytes.

Materials and methods

Cell culture and KGF treatment

HaCaT cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1.4 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (Seromed-Biochrom KG, Germany) at 37°C in 5% CO₂. HaCaT cells were serum-starved overnight and incubated with bacterial recombinant human KGF (K1757; Sigma-Adrich, St. Luis, MO, USA) for indicated periods.

siRNAs transfection

HaCaT cells were transfected with miR-34a mimic, si-miR-34a, control (siNC), and si-Smac (GenePharma, Shanghai, China) using Lipofectamine 3000 (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. miR-34a mimic cells show expression of miR-34a, si-miR-34a are interfer-

ing RNA which induces short-term silencing of miR-34a expression, siNC served as a negative control, and si-Smac cells were used to silent the expression of Smac.

MTT analysis

The cell proliferative and invasive capacities were determined using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2Htetrazolium bromide (MTT) colorimetric assay and Matrigel invasion chamber assay, respectively, according to standard methods described earlier. Each experiment was performed three times.

Apoptosis assay

Cells apoptosis was detected by Annexin V-FITC/PI Kit (Beijing Biosea Biotechnology, Beijing, China), according to the manuals. Briefly, the cells (100,000 cells/well) were seeded in 6 well-plate. Treated cells were washed twice with cold phosphate buffer saline (PBS) and resuspended in buffer. The adherent and floating cells were combined and treated according to the manufacturer's instruction and measured with flow cytometer (Beckman Coulter, USA) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from transfected cells using Trizol™ (Invitrogen) and treated with DNase I (Promega, Madison, WI, USA). Reverse transcription was performed using the Multiscribe RT kit (Applied Biosystems) and random hexamers or oligo (dT). The reverse transcription conditions were 10 min at 25°C, 30 min at 48°C, and a final step of 5 min at 95°C.

Western blot analysis

Smac-mediated mitochondrial apoptotic pathway key proteins, Bcl-2, Cyt C, cleaved/pro-caspase-3, and cleaved/pro-caspase-9, used for Western blotting were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China), and supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The Western blot system was established using a Bio-Rad Bis-

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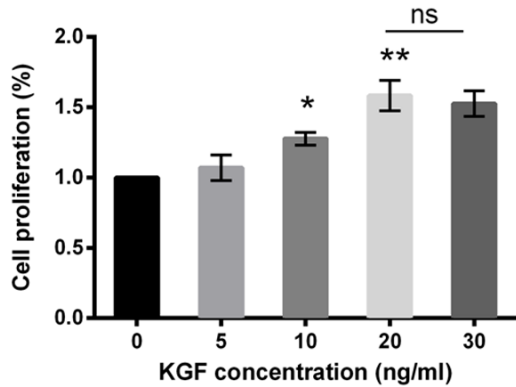


Figure 1. HaCaT cells proliferation induced by KGF at different concentrations. The concentrations of KGF used were 0, 5, 10, 20, and 30 ng/ml. Cell proliferation ability is presented as percentage (%) of the control. KGF: keratinocyte growth factor; ns: not significant. * $P < 0.05$, ** $P < 0.01$.

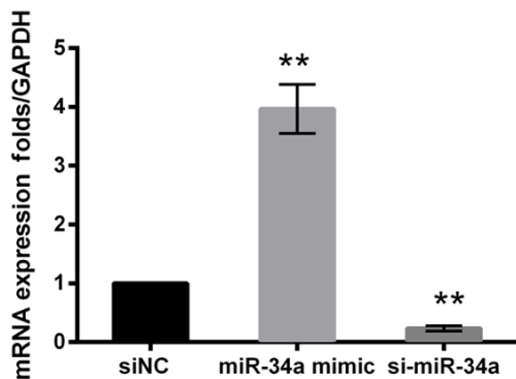


Figure 2. Transfection efficiency of miR-34a in HaCaT cells. The transfection efficiency of miR34a mimics, inhibitors, and control was confirmed by qRT-PCR using GAPDH. The transfection efficiency is presented as mRNA expression folds. GAPDH: glyceraldehyde-3-phosphate dehydrogenase. ** $P < 0.01$.

Tris Gel system, according to the manufacturer's instructions. Rabbit anti-human Smac antibody for chromatin immunoprecipitation was purchased from Abcam (Shanghai, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Sigma. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1000. Primary antibodies were incubated with the membrane at 4°C overnight, followed by wash and incubation with secondary antibodies marked by horseradish peroxidase for 1 hour at room temperature. After rinsing, the polyvinylidene difluoride (PVDF) membrane-carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™

XRS system, and then 200 μ L Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

Statistical analysis

All experiments were repeated three times. All data are expressed as mean \pm standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) in Graphpad statistical software. A P -value of < 0.05 was considered to be statistically significant.

Results

KGF-induced HaCaT cells proliferation

We used different concentrations of KGF (0, 5, 10, 20, and 30 ng/mL) to induce HaCaT cell proliferation. The results showed that HaCaT cell proliferation increases in a dose-dependent manner with the increasing concentration of KGF. The results were statistically significant at 10 ng/mL ($P < 0.05$) and 20 ng/mL ($P < 0.01$) concentrations. As evident from **Figure 1**, the cell proliferation was highest at the KGF concentration of 20 ng/mL. Therefore, 20 ng/mL was chosen in the following experiments.

Transfection efficiency of miR-34a in HaCaT cells

HaCaT cells were transfected with miR-34a mimic, si-miR-34a, and control (siNC). The transfection efficiency of miR-34a was confirmed by qRT-PCR using GAPDH antibody. As evident from **Figure 2**, mRNA expression was highest in the miR-34a mimic cells (overexpression of miR-34a) and lowest in cells transfected with si-miR-34a (knockdown of miR-34a expression); both the results were statistically significant ($P < 0.01$).

Effects of miR-34a aberrant expression on HaCaT cell proliferation

The effects of the altered miR-34a expression on HaCaT cells proliferation were measured by MTT assay. As per **Figure 3**, miR-34a overexpression significantly inhibited the proliferation of HaCaT cells compared with the control group

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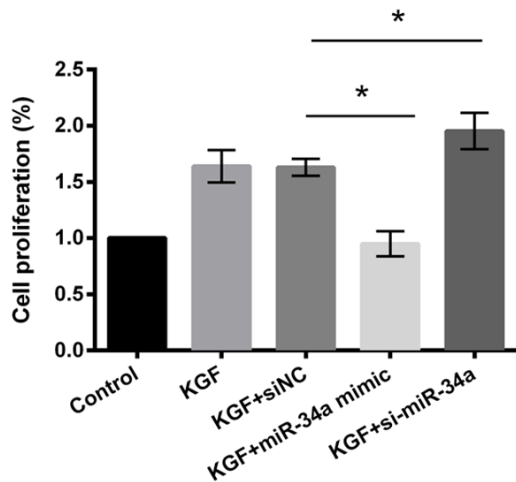


Figure 3. Effects of miR-34a aberrant expression on HaCaT cell proliferation. Cell proliferation ability of the control, KGF (20 ng/ml), KGF+siNC, KGF+miR-34a mimic, and KGF+si-miR-34a is presented as percentage (%) of the control. KGF: keratinocyte growth factor; ns: not significant. * $P < 0.05$.

containing KGF+siNC ($P < 0.05$). However, miR-34a suppression (miR-34a knockdown) significantly increased the proliferation of HaCaT cells ($P < 0.05$) in comparison to control group of cells.

Effects of miR-34a aberrant expression on HaCaT cell apoptosis

After HaCaT cells were transfected with miR-34a mimics, inhibitors or control, flow cytometry was used to detect the apoptotic cells, aimed to reveal the effects of miR-34a on HaCaT cells apoptosis. **Figure 4** shows that miR-34a overexpression significantly increased cell apoptosis compared with the control group containing KGF+siNC ($P < 0.05$), while miR-34a suppression decreased apoptosis, although it was not statistically significant.

Effects of miR-34a aberrant expression on Smac-mediated mitochondrial apoptotic pathway

To investigate the possible underlying molecular mechanisms of miR-34a-induced apoptosis in HaCaT cells, Smac-mediated mitochondrial apoptotic pathway key proteins - Bcl-2, Cyt C, cleaved/pro-caspase-3, and cleaved/pro-caspase-9 - were selected and the expression levels of these proteins in the transfected cells were detected by Western blot. Bcl-2 is anti-

apoptotic protein while Cyt C, cleaved/pro-caspase-3, and cleaved/pro-caspase-9 are pro-apoptotic proteins. **Figure 5** shows that miR-34a overexpression significantly down-regulated the expression of Bcl-2 ($P < 0.05$), and up-regulated the expression of Cyt C ($P < 0.01$), cleaved-caspase-3 ($P < 0.01$), and cleaved-caspase-9 ($P < 0.05$) compared with the control group. miR-34a knockdown HaCaT cells showed just the opposite outcomes to that observed with miR-34a overexpression. To further verify the effects of miR-34a overexpression on Smac-mediated mitochondrial apoptotic pathway, we evaluated the effect of miR-34a overexpression by silencing the expression of Smac using si-Smac. The results were same as observed with miR-34a knockdown cells, although the changes in the expression of levels of these factors were not significant when compared with control group of cells.

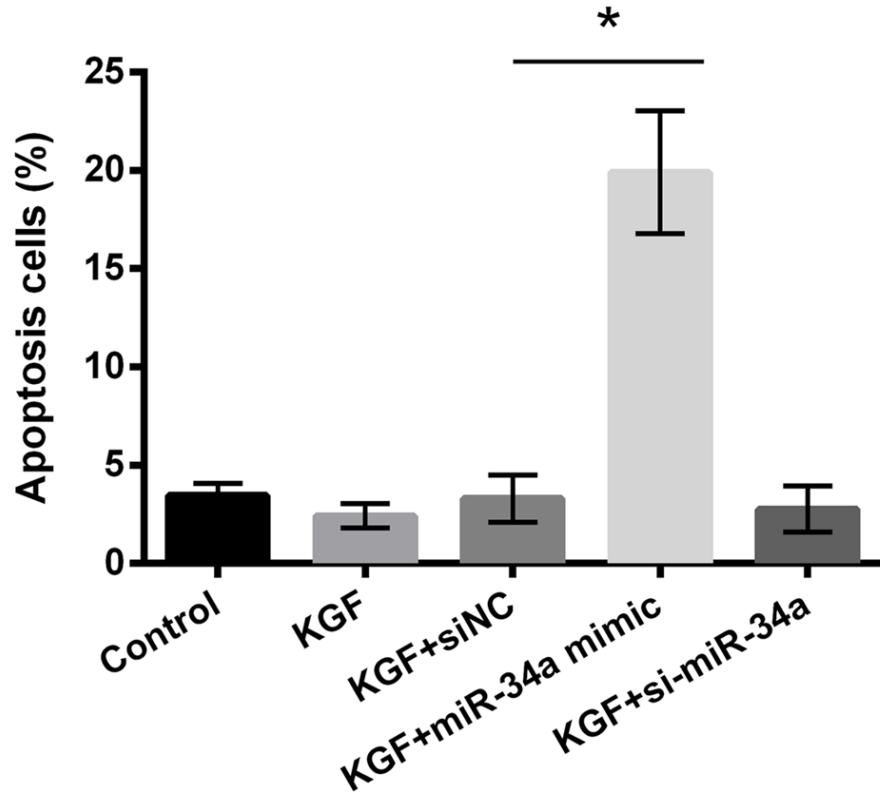
Discussion

Roles of miR-34a in the biology of many kinds of cancers are well known [10, 11]; however, its role in psoriasis is not much explored. In the present study, we explored the functional role of miR-34a in regulation of excessive proliferation of human keratinocyte cell line (HaCaT cells) as occurs in psoriasis. We used KGF to induce excessive proliferation of HaCaT cells. The role of KGF in inducing cell proliferation is well established [15-17]. In our study, cell proliferation increased with increasing concentrations of KGF. Similar results were observed in the study conducted by Andreadis et al [17]. We showed that overexpression of miR-34a can significantly inhibit the excessive proliferation of HaCaT cells and promote cell apoptosis. However, suppression of miR-34a reversed these results.

Furthermore, we observed that miR-34a overexpression can decrease anti-apoptotic protein (Bcl2) expression and increase pro-apoptotic proteins (Cyt C, pro-caspase-3, and pro-caspase-9) expression and activate mitochondrial apoptotic pathway, leading to cell apoptosis and inhibition of keratinocyte excessive proliferation.

HaCaT cells are extensively used to study the epidermal homeostasis and its pathophysiology [18]. HaCaT cells contain a mutant p53 [19, 20]. The p53 protein is a transcription factor

A



B

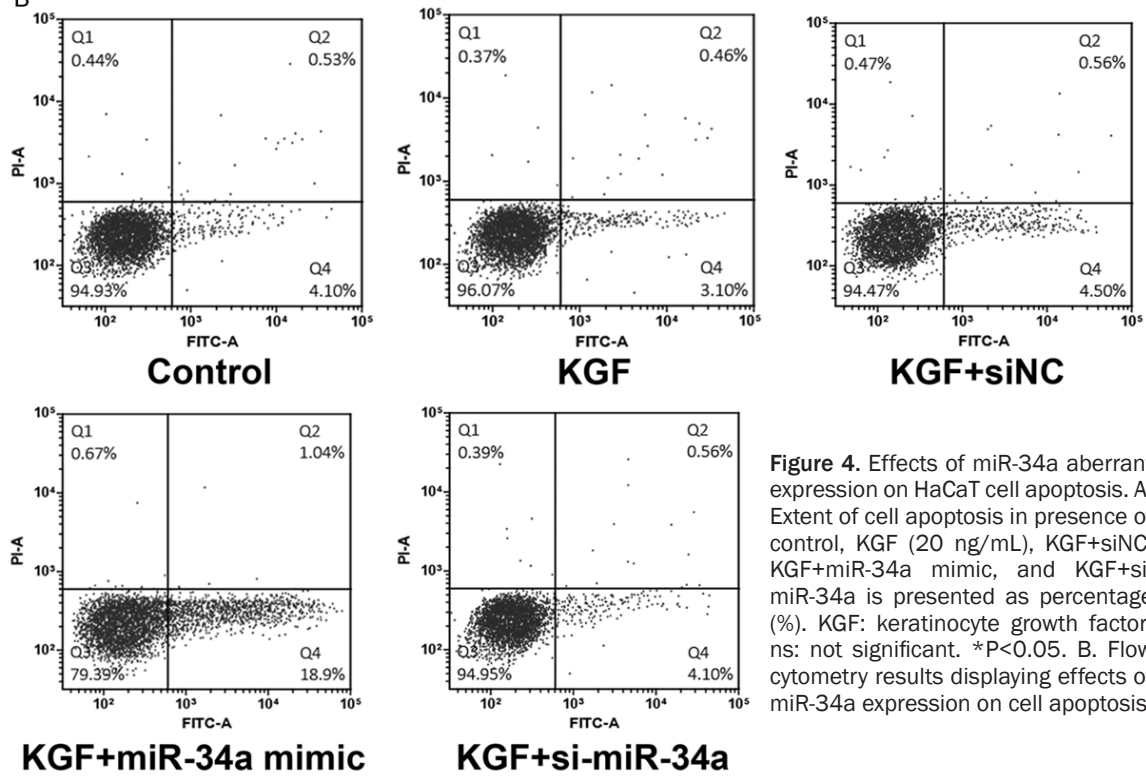


Figure 4. Effects of miR-34a aberrant expression on HaCaT cell apoptosis. A. Extent of cell apoptosis in presence of control, KGF (20 ng/mL), KGF+siNC, KGF+miR-34a mimic, and KGF+si-miR-34a is presented as percentage (%). KGF: keratinocyte growth factor; ns: not significant. *P<0.05. B. Flow cytometry results displaying effects of miR-34a expression on cell apoptosis.

which inhibits cell proliferation and stimulates cell death. miRNA is one of the pathways th-

rough which p53 regulates cell growth. Cellular stress stabilizes p53 which in turn regulates

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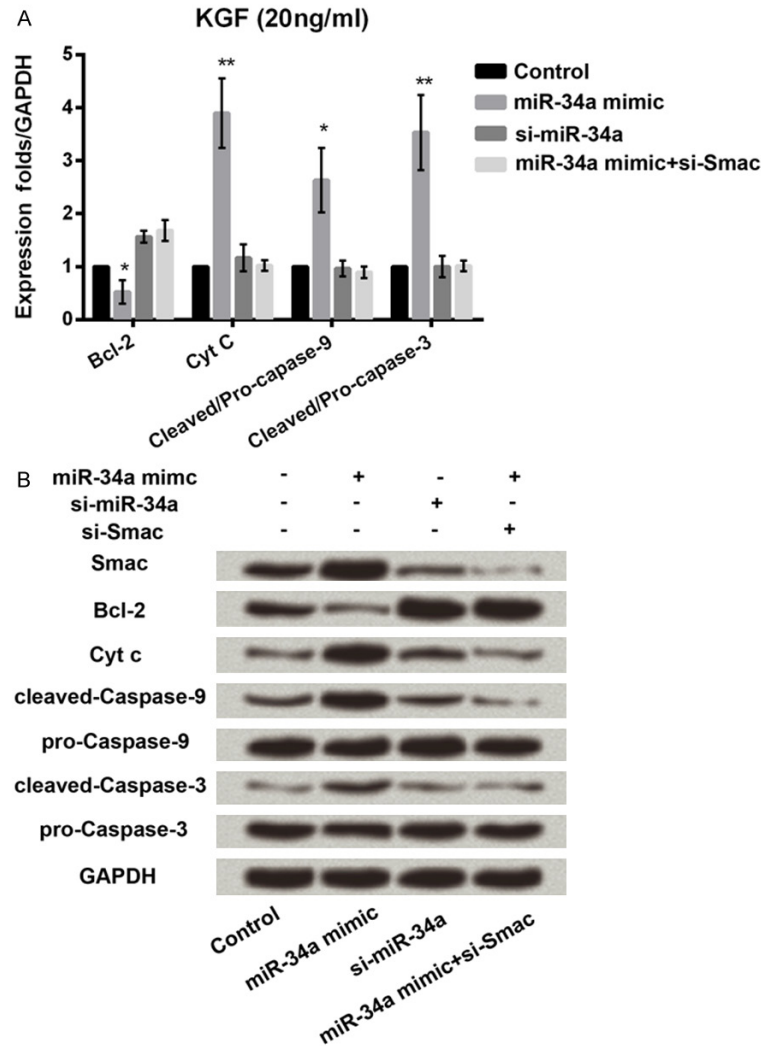


Figure 5. Effects of miR-34a aberrant expression on Smac-mediated mitochondrial apoptotic pathway key proteins. A. The expression levels of Smac-mediated mitochondrial apoptotic pathway key proteins, Bcl-2, Cyt C, cleaved/pro-caspase-3, and cleaved/pro-caspase-9, in the transfected cells were detected by Western blot analysis using GAPDH. * $P < 0.05$, ** $P < 0.01$. B. Western blot analysis results displaying effects of miR-34a expression on Smac-mediated mitochondrial pathway proteins. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

the expression of a set of miRNA. These miRNAs control apoptosis and senescence. miR-34a is one of the miRNAs that is activated by p53 [11, 21-24].

A previous study has demonstrated that miR-34a promotes tumor cell growth arrest and apoptosis by targeting and inhibiting expression of silent mating type information regulation 2 homolog-1 (SIRT1; 10). SIRT1 is an NAD-dependent deacetylase which regulates apoptosis in response to oxidative and genotoxic stress [25, 26]. In the present study, overex-

pression of miR-34a led to suppressed HaCaT cells proliferation, and suppression of miR-34a promoted cell proliferation. Further, we studied the effect of miR-34a aberrant expression on HaCaT cell apoptosis and found that miR-34a overexpression could induce HaCaT cells apoptosis, while miR-34a suppression could inhibit apoptosis.

Similar findings have been reported in previous studies, albeit for different diseases. Sun et al found that miR-34a suppresses cell proliferation and induces apoptosis in U87 glioma stem cells [27]; Duan et al found that miR-34a inhibits cell proliferation and induces apoptosis of glioma cells via targeting Bcl-2 [28]. Possible role of miR-34a in neuropathy is also assessed. In a study done by Lin et al, miR-34a induced apoptosis in PC12 cells by reducing B-cell lymphoma 2 and sirtuin-1 expression [29].

We also studied the effect of miR-34a aberrant expression on Smac-mediated mitochondrial apoptotic pathway key proteins. Mitochondrial protein, Smac (second mitochondria-derived activator of caspases), is located at the intermembrane space of mitochondria. Upon apoptotic stimuli, Smac is released to-

gether with Cyt c from mitochondria into the cytosol; this process is regulated by Bcl-2. Cyt c directly activates Apaf-1 and caspase-9. On the other hand, Smac interacts with multiple inhibitors of apoptosis (IAPs) and inhibits caspase-9 and caspase-3 [30]. It has been shown that miR-34 family members (miR-34a, miR-34b, and miR-34c) target Bcl-2 in many cancers [31]; and ectopic expression of miR-34a can induce apoptosis [32]. In our study, miR-34a inhibited Bcl-2, and promoted Cyt C, pro-caspase-3, and pro-caspase-9 in HaCaT cells. Similar findings were reported by Lei et al [33], wherein com-

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bined expression of miR-34a and Smac mediated by oncolytic vaccinia virus synergistically promoted anti-tumor effects in multiple myeloma.

Furthermore, in our study, silencing the expression of Smac using si-Smac could not activate mitochondrial apoptotic pathway even during overexpression of miR-34a. This indicates that miR-34a activates mitochondrial apoptotic pathway by upregulating the expression of Smac. Combined together, these results imply potential functional mechanism of miR-34a in psoriasis.

In conclusion, this study revealed that overexpressed miR-34a could inhibit keratinocyte proliferation and induce cell apoptosis through activation of Smac-mediated mitochondrial apoptotic pathway. Our results provide a new insight into clinical treatment of psoriasis, and therefore miR-34a might be considered as a potential therapeutic target for the treatment of psoriasis. However, further research is needed to provide more insight regarding other possible the molecular mechanisms of overexpression of miR-34a in psoriasis.

Disclosure of conflict of interest

None.

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