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MiR-223 regulates proliferation and apoptosis of IL-22-stimulated HaCat human keratinocyte cell lines via the PTEN/Akt pathway

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ABSTRACT

Psoriasis, a chronic inflammatory skin disorder disease, is closely associated with hyperproliferation of keratinocytes. Upregulated miR-223 has been found in peripheral blood mononuclear cells from patients with psoriasis and from psoriatic skin. However, its role in keratinocytes remains unknown. We thus aimed to investigate the function of miR-223 in psoriasis. Interleukin-22 (IL-22) is a crucial keratinocyte trigger in the T-cell-mediated immune response to psoriasis. We found miR-223 to be overexpressed in psoriatic lesions and in IL-22-stimulated HaCaT cells. HaCaT cells then were transfected with a miR-223 mimic or inhibitor to overexpress or inhibit expression of miR-223, respectively. A Cell Counting Kit-8 assay revealed that miR-223 overexpression promoted and miR-223 downregulation inhibited proliferation in IL-22-stimulated HaCaT cells. Flow cytometry analysis certified that miR-223 overexpression decreased HaCaT cell apoptosis, whereas miR-223 downregulation increased it. A dual-luciferase reporter assay demonstrated that miR-223 directly targeted the phosphatase and tensin homolog (PTEN) gene. MiR-223 also negatively regulated mRNA and protein expression of PTEN and modulated the PTEN/Akt pathway in IL-22-stimulated HaCaT cells. PTEN silencing attenuated the activity of the miR-223 inhibitor in these cells via the PTEN/Akt pathway. Overall, the results showed that miR-223 increased proliferation and inhibited apoptosis of IL-22-stimulated keratinocytes via the PTEN/Akt pathway.

1. Introduction

Psoriasis is a chronic inflammatory skin disorder disease with adverse patient health outcomes [1]. It is a complex pathological dysfunction characterized by aberrant proliferation and differentiation of keratinocytes and infiltration of inflammatory cells into the dermis and epidermis [2–5]. T cells and keratinocytes participate in the immune response of this disease [6]. However, the pathogenetic mechanism of psoriasis remains unclear.

Multiple inflammatory cytokines, such as interleukin (IL)-1, IL-17, IL-22, and tumor necrosis factor (TNF)-a are regarded as vital pathological factors of psoriasis [7]. IL-22 is produced by several immune cells, such as T helper 17 (Th17) and Th22 [8]. It is closely related to psoriatic pathology [9,10]. Studies have shown that psoriatic severity correlates with high serum levels of IL-22 [11,12]. IL-22 also may cause the characteristic epidermal alterations in psoriasis [13]. In a mouse model of imiquimod-induced psoriasis-like disease, IL-22 was linked to psoriasis-like lesions [14]. IL-22 induces proliferation [15], migration, and proinflammatory gene expression of human keratinocytes [16]. This evidence illustrates the vital role of IL-22 in psoriatic pathology.

MicroRNAs (miRNAs), a class of small noncoding RNAs, regulate gene expression by binding to the 3′-UTR of target mRNA. MiRNAs help regulate cellular processes, such as proliferation, apoptosis, differentiation, invasion, and metabolism [17]. Evidence has demonstrated the crucial role of microRNAs in psoriasis, including modulating hyperproliferation of keratinocytes, production of cytokines and chemokines in keratinocytes, and mediation of immune dysfunction [1,18]. Recently, several miRNAs, such as miR-155 [19], miR-194 [20], and miR-181b [21], have been found to be abnormally expressed and to regulate keratinocyte proliferation in psoriasis. MiR-155 silencing inhibits inflammation in psoriasis via NLRP3 regulation [22]. MiR-138 regulates the balance of Th1 and Th2 in psoriasis [23] and modulates proliferation and apoptosis of HaCaT cells in psoriasis vulgaris [24]. MiR-330 inhibits IL-22-stimulated HaCaT cell proliferation via β-catenin gene [15]. Jiang et al. [25] report that miR-486-3p downregulation in psoriatic epidermis leads to keratin 17 overexpression and keratinocyte hyperproliferation. MiR-217 promotes keratinocyte differentiation in psoriasis by targeting GRHL2 [26]. Increasing evidence confirms the association of miR-223 with immune response regulation and different inflammation disorders [27,28]. Also, miR-223 is

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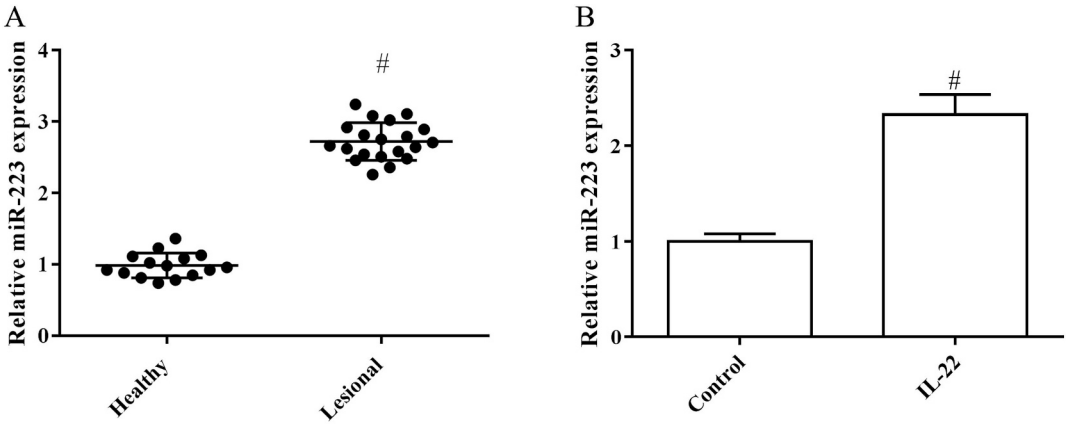

Fig. 1. MiR-223 is upregulated in psoriatic skin lesions and IL-22 stimulated keratinocytes. (A) RT-qPCR analysis of miR-223 level in healthy skin from healthy individuals (N = 15) and lesions from patients with psoriasis (N = 20). Results represented mean ± standard deviation (SD), $^{\#}P < 0.05$ vs. healthy. (B) Expression of miR-223 in IL-22 stimulated HaCaT cells. HaCaT cells were treated with or without IL-22 (100 ng/ml) for 24 h. N = 3, $^{\#}P < 0.05$ vs. control.

significantly upregulated in psoriatic skin [29] and in peripheral blood mononuclear cells from patients with psoriasis [30]. However, its role in psoriasis remains largely unknown.

In this study, we explored the function of miR-223 in the proliferation and apoptosis of IL-22-stimulated keratinocytes and found it to be overexpressed in these cells and in psoriatic skin samples. We found that miR-223 increased proliferation and inhibited apoptosis of IL-22-stimulated keratinocytes by regulating the PTEN/Akt pathway. These results indicate that miR-223 may be a molecular target for treating psoriasis.

2. Materials and methods

2.1. Patients and tissues specimens

We recruited 20 patients with psoriasis who had no previous treatment for psoriasis and 15 healthy volunteers. We then collected 4-mm punch biopsies from psoriatic lesions and from the skin of the healthy individuals. The ethics committee of our institute approved this study, which was performed according to the Declaration of Helsinki. All participants provided informed consent.

2.2. Cell culture

Human keratinocyte HaCaT cells (China Center for Type Culture Collection, Wuhan, China) and human embryo kidney 293 (HEK293) cells (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Life Technologies, Grand Island, NY, USA) at 37 °C with 5% CO_2.

2.3. IL-22 stimulation

HaCaT cells were seeded onto a 6-well plate (4×10^5 cells/well) until reaching 80% to 90% confluence. Cells were starved in serum-free DMEM for 24 h, followed by stimulation with a 100 ng/ml concentration of IL-22 (Sangon Biological Engineering Technology Company, Shanghai, China) [15] in serum-free DMEM for another 24 h.

2.4. Transfection

HaCaT cells were seeded into a 12-well plate (2×10^5/well) before miRNA transfection. At 80% confluence, cells were transfected with a miR-223 mimic (100 nM) [31] (sequence: 5′-UGUCAGUUUGUCAAAUACCCCA-3′), miR-223 inhibitor (50 nM) [32] (sequence: 5′-UGGGUAUUUGACAAACUGACA-3′) or their respective scramble controls (Genepharma, Shanghai, China) using Lipofectamine 3000 (Invitrogen). After 6 h of incubation in serum-free medium, cells were cultured in DMEM with serum for 48 h. To assess transfection efficiency, cells were harvested, and the miR-223 level was confirmed by real-time quantitative (qRT-PCR). To silence PTEN, HaCaT cells were transfected with a 100 nmol/L concentration of PTEN siRNA (siPTEN; Dharmacon, Lafayette, CO, USA) using Lipofectamine 3000. Scramble siRNA transfection was used as the control.

2.5. CCK-8 assay

Cells were seeded into 96-well plates (2×10^4 cells/well) after the previously described transfection. Cell Counting Kit-8 reagents (CCK-8; Beyotime Technology, Jiangsu, China) at 10 μL/well were added for 1 h to each well at different time intervals (24, 48, 72 h). Optical density (OD) at 450 nm was detected using a microplate reader (Bio-Rad, Hercules, CA, USA).

$$\text{Cell proliferation (\%)} = [(OD_{test} - OD_{blank})/(OD_{control} - OD_{blank})] \times 100\%$$

2.6. Flow cytometry

After washing with PBS, HaCaT cells were stained with Annexin V/PI (BD Bioscience, Franklin Lakes, NJ, USA) for 15 min in darkness. Apoptotic cells were measured using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The percentage of early apoptotic cells (Annexin V+/PI−) and late apoptotic cells (Annexin V+/PI+) were calculated.

2.7. qRT-PCR

After extraction of total RNA with TRIzol (Invitrogen), 1 μg of RNA was reverse-transcribed into cDNA using SuperScript II reverse transcriptase (Takara, Dalian, China) for mRNA detection or a miScript reverse transcription kit (Qiagen, Dusseldorf, Germany) for miRNA detection. Then, qRT-PCR was conducted using a 7500 Real-Time PCR system (Applied Biosystems, Mannheim, Germany). Relative gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method. U6 was used as the internal control. MiR-223 primers were as follows: forward 5′-TGGATCCGTGTCACTCGGGCTTTACCTG-3′ and reverse 5′-CGAATTCGTAGACACAGCCCAGGGCTGT-3′. PTEN primers were as follows: forward 5′-GTTTACCGGCAGCATCAAAT-3′ and reverse 5′-CCCCCACTTTAGTGCACAGT-3′.

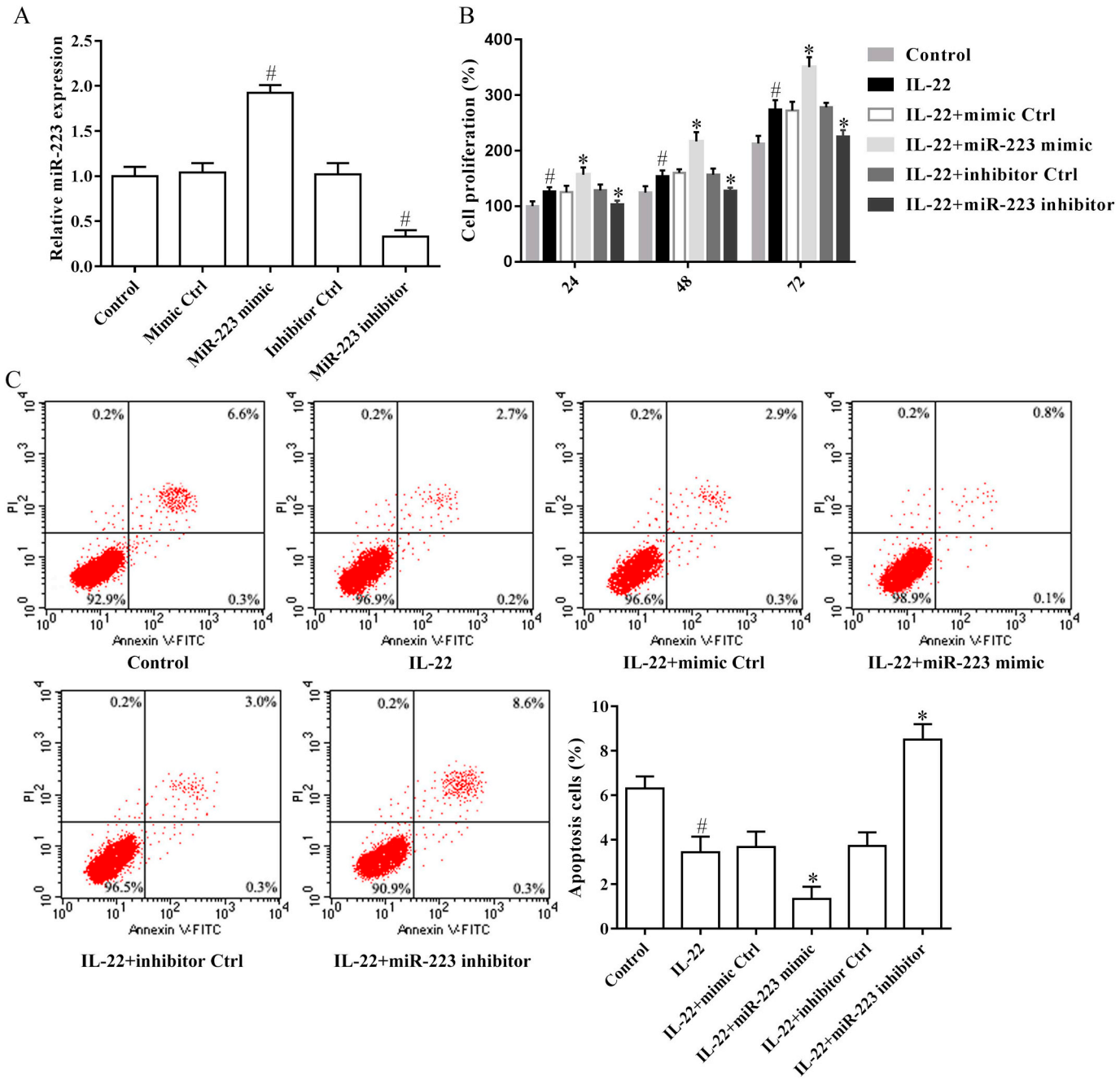

Fig. 2. Effect of miR-223 on the proliferation and apoptosis of IL-22 stimulated HaCaT cells. HaCaT cells were transfected with a miR-223 mimic, inhibitor, or respective negative control. (A) QRT-PCR examination of miR-223 level. After transfecting with miR-223 mimic or miR-223 inhibitor, HaCaT cells were stimulated with IL-22 (100 ng/mL) for 24 h. (B) Cell proliferation of HaCaT cells, as detected by CCK-8 assay. (C) Cell apoptosis of HaCaT cells, as detected by flow cytometry. Results represented mean ± SD, N = 3, $^{\#}P < 0.05$ vs. control; $^{*}P < 0.05$ vs. IL-22 group.

2.8. Western blot

Cells were lysed in RIPA buffer (Pierce, Shanghai, China), and protein concentrations were determined using a BCA kit (Beyotime). Total protein was separated via 10% SDS-PAGE and then transferred onto polyvinylidene fluoride membranes. After probing with the antibodies PTEN (1:10,000; Abcam, Cambridge, MA, USA; ab32199), Protein kinase B (Akt; 1:500; Abcam; ab8805), p-Akt phospho T308 (1:1000; Abcam; ab38449), and β-actin (1:2000; Abcam; ab8227) overnight at 4 °C, the blots were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000; Abcam; ab7090) at room temperature for 1 h. After three washes, protein bands were visualized using an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Protein levels were quantified using Image J software, and endogenous control β-actin was used for normalization.

2.9. Dual-luciferase reporter assay

HEK293 cells were seeded into 24-well plates (1×10^5 cells/well). The PTEN cDNA fragment was cloned into the pGL3 luciferase promoter vector (Promega, Madison, WI, USA). A wild-type (wt-PTEN) and mutant-type PTEN (mut-PTEN) 3′ UTR and a miR-223 mimic were co-transfected into HEK293 cells for 48 h using Lipofectamine 3000. Luciferase activity was implemented via a dual-luciferase reporter system (Promega).

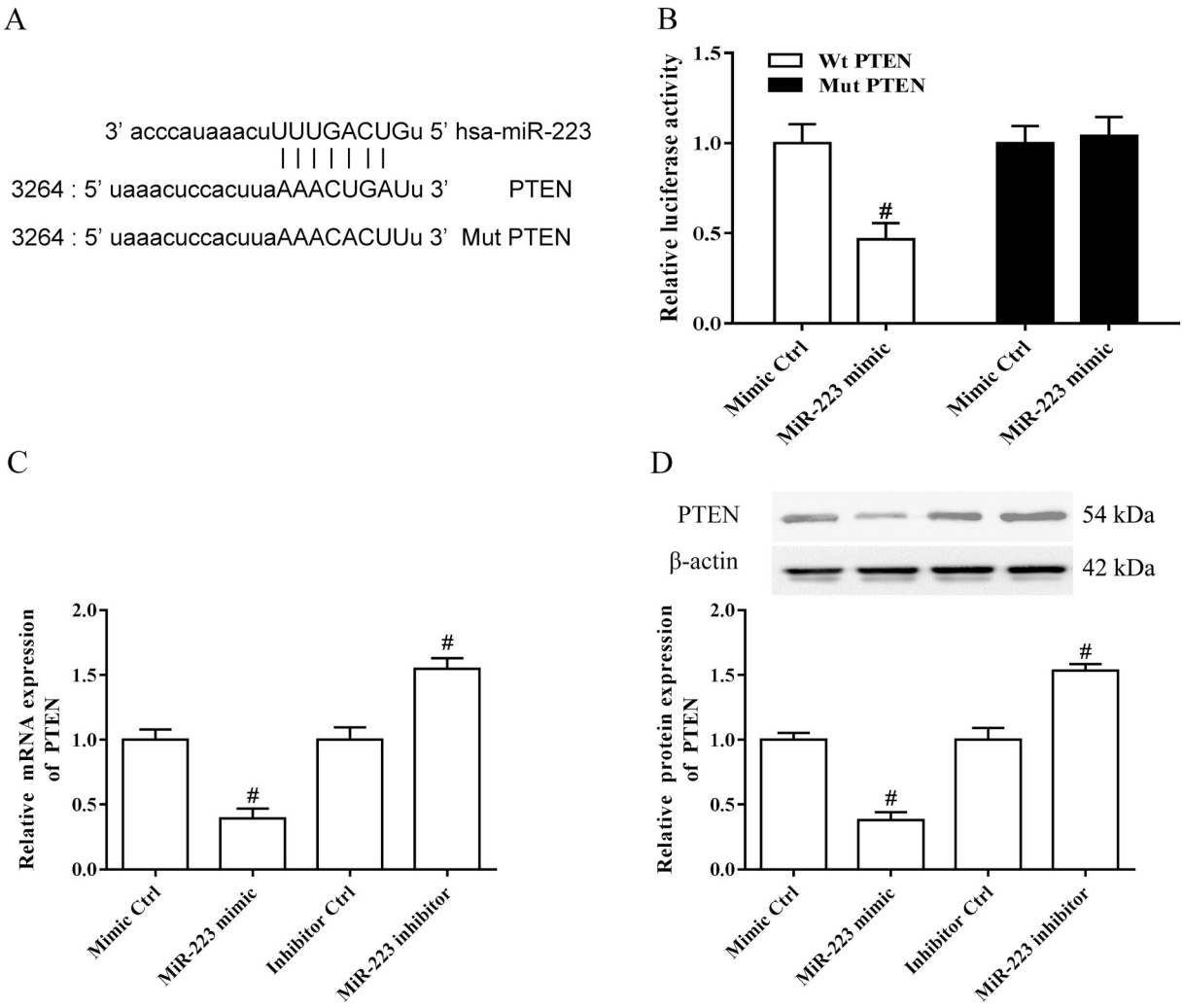

Fig. 3. MiR-223 targeted the PTEN gene. (A) Schematic representation of the binding sites of PTEN with miR-223 predicted by microRNA.org. (B) Dual-luciferase reporter assay results of the interaction between miR-223 and the 3′-UTR of PTEN. The (C) mRNA and (D) protein expression of PTEN in HaCaT cells, as detected by qPCR and western blot after transfection with miR-223 mimic or miR-223 inhibitor without IL-22 stimulation. The bars represent the mean ± SD, N = 3, $^{\#}P < 0.05$ vs. control.

2.10. Statistical analysis

Data are shown as the mean ± standard deviation (SD) and processed using SPSS 22.0 statistical software (Chicago, IL, USA). Statistical significance was evaluated by ANOVA with Tukey correction to compare differences among multiple groups or *t*-test to assess statistical significance of differences between two groups. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. MiR-223 was upregulated in psoriatic skin and IL-22-stimulated HaCaT cells

We measured miR-223 expression in lesions from patients with psoriasis using qRT-PCR and found it be higher than that in samples from healthy volunteers ($P < 0.05$; Fig. 1A). To explore IL-22's pivotal role in psoriatic pathogenesis, we exposed HaCaT cells to IL-22 for 24 h. The qRT-RCT results showed miR-223 to be more upregulated in these cells than in the control group ($P < 0.05$; Fig. 1B). These findings indicate the vital role of miR-223 in psoriasis.

3.2. Effect of miR-223 on the proliferation and apoptosis of HaCaT cells under IL-22 stimulation

To explore the effects of miR-223 in psoriasis, we artificially transfected HaCaT cells with a miR-223 mimic or miR-223 inhibitor to upregulate or downregulate miR-223, respectively ($P < 0.05$; Fig. 2A). We observed that the miR-223 mimic notably accelerated cell proliferation and that the inhibitor inhibited proliferation of IL-22-stimulated HaCaT cells ($P < 0.05$; Fig. 2B). The miR-223 mimic decreased and the miR-223 inhibitor promoted apoptosis in IL-22 treated HaCaT cells ($P < 0.05$; Fig. 2C). These data suggest that miR-223 regulated IL-22-induced keratinocyte proliferation and HaCaT cell apoptosis.

3.3. MiR-223 inhibited PTEN expression by directly targeting the 3′-UTR

To elucidate the mechanism of miR-223 in regulating IL-22-induced keratinocyte proliferation and apoptosis, we screened the putative targets of miR-223 using bioinformatics technique-microRNA.org and identified PTEN as a target gene (Fig. 3A). Luciferase activity dramatically decreased after miR-223 overexpression but remained substantially unchanged by miR-223 with the pGL3-PTEN-Mut 3′-UTR (Fig. 3B). Moreover, miR-223 overexpression markedly repressed the mRNA and protein levels of PTEN, whereas miR-223 downregulation

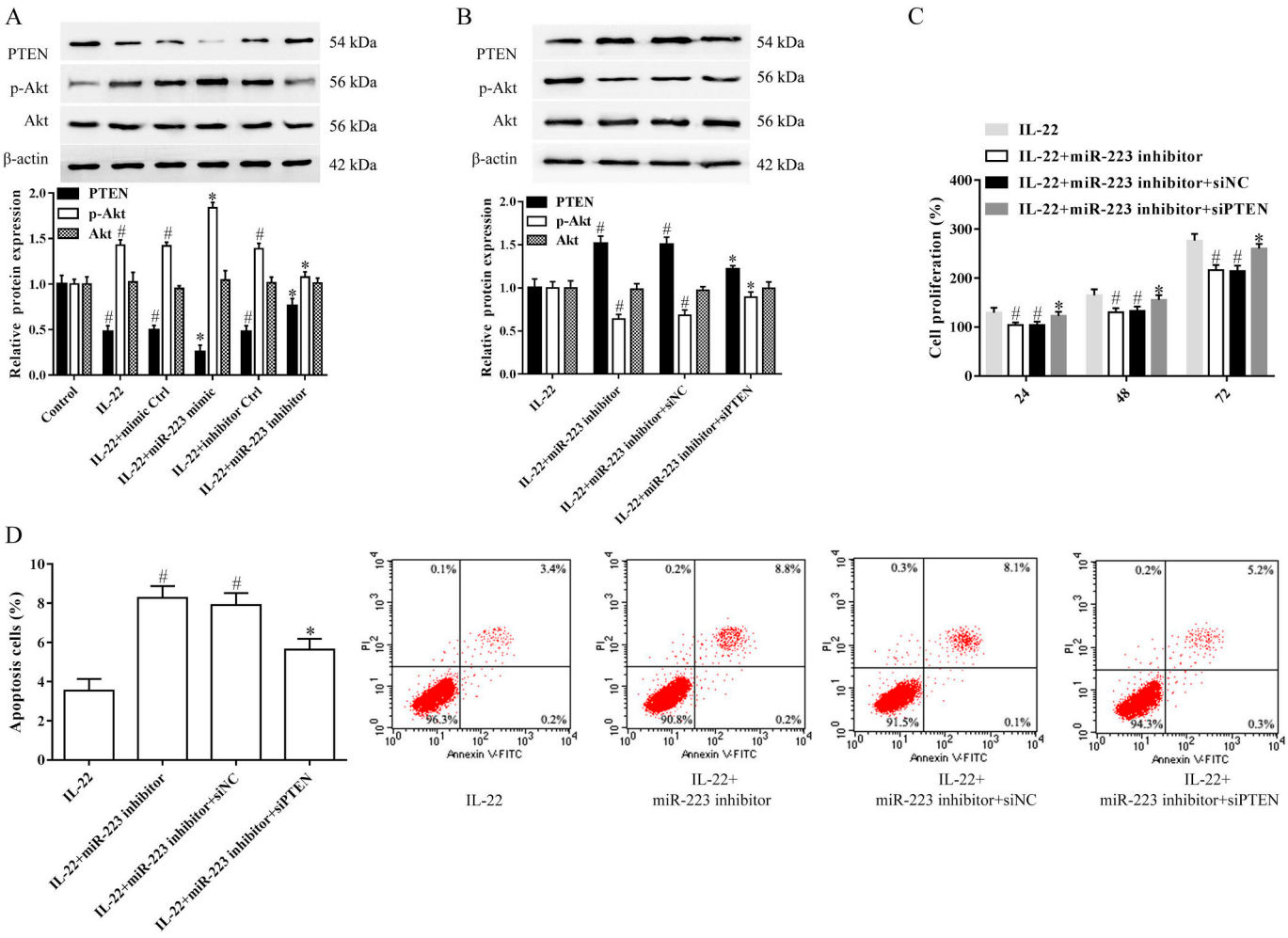

Fig. 4. MiR-223 regulated cell proliferation and apoptosis via the PTEN/p-Akt pathway in IL-22 treated HaCaT cells. (A) The protein expression of PTEN and p-Akt after transfection with miR-223 mimic or miR-223 inhibitor in IL-22 treated HaCaT cells. The bars represent the mean ± SD, N = 3, $^{\#}P < 0.05$ vs. control; $^{*}P < 0.05$ vs. IL-22. (B) Western blot analysis of PTEN and p-Ak after transfection with miR-223 inhibitor and (or) siPTEN for 48 h in IL-22 treated HaCaT cells. (C) CCK-8 analysis of cell proliferation after transfection with miR-223 inhibitor and (or) siPTEN for 48 h in IL-22-treated HaCaT cells. (D) Flow cytometry results for cell apoptosis after transfection with miR-223 inhibitor and (or) siPTEN for 48 h in IL-22-treated HaCaT cells. The bars represent the mean ± SD, N = 3, $^{\#}P < 0.05$ vs. IL-22; $^{*}P < 0.05$ vs. IL-22 + miR-223 inhibitor or IL-22 + miR-223 inhibitor + siNC.

significantly increased them ($P < 0.05$; Fig. 3C and D). These data demonstrate that miR-223 directly targeted PTEN.

3.4. MiR-223 modulated the PTEN/Akt pathway in IL-22-stimulated HaCaT cells

PTEN and its downstream Akt signaling play an important role in hyperproliferation of psoriatic keratinocytes [33]. To determine if this signaling participates in miR-223's regulatory effect on keratinocytes, we examined expression of the PTEN/Akt pathway protein in IL-22-stimulated HaCaT cells and found PTEN to be decreased and p-Akt to be increased ($P < 0.05$; Fig. 4A). MiR-223 overexpression resulted in a significant decrease of PTEN protein expression and increased p-Akt protein expression in IL-22 treated HaCaT cells ($P < 0.05$; Fig. 4A). Inhibition of miR-223 significantly upregulated PTEN expression and downregulated p-Akt in IL-22-stimulated HaCaT cells ($P < 0.05$; Fig. 4A). These results suggest that miR-223 directly modulated the activity of the PTEN/Akt pathway.

3.5. MiR-223 regulated keratinocyte proliferation and apoptosis via the PTEN/Akt pathway

To further clarify the mechanisms of miR-223 in IL-22-induced keratinocyte proliferation, we co-transfected HaCaT cells with a miR-223 inhibitor and PTEN siRNA, followed by IL-22 treatment. The results revealed that PTEN siRNA suppressed the upregulation of PTEN that was induced by the miR-223 inhibitor in IL-22–treated HaCaT cells ($P < 0.05$; Fig. 4B). Co-transfection with the miR-223 inhibitor and PTEN siRNA upregulated the protein level of p-Akt more in IL-22-stimulated HaCaT cells than in cells transfected with the miR-223 inhibitor only ($P < 0.05$; Fig. 4B). PTEN siRNA also counteracted the inhibitory effects of the miR-223 inhibitor on cell proliferation (Fig. 4C) and apoptosis (Fig. 4D). The data indicate that miR-223 regulated IL-22-induced proliferation and apoptosis of keratinocytes via the PTEN/Akt pathway.

4. Discussion

We investigated the function of miR-223 in the pathogenesis of psoriasis and observed upregulated miR-223 in psoriatic epidermis samples and in IL-22-stimulated HaCaT cells. We also demonstrated that miR-223 improved proliferation and suppressed apoptosis of IL-22-stimulated keratinocytes via the PTEN/Akt pathway. Overall, our results demonstrate the critical role of miR-223 in these keratinocytes.

Previous studies have indicated a link between dysfunctional miRNAs and psoriasis [1,18]. Targeting miR-21 has been reported as a

potential treatment for psoriasis [34]. It has been shown that miR-146a has a crucial role in psoriasiform skin inflammation [35]. By targeting grainyhead-like 2, miR-194 regulates keratinocyte proliferation and differentiation in psoriasis [20]. Recent studies have shown upregulated miR-223 expression in PBMCs from patients with psoriasis [30] and in human psoriatic skin [29]. Thus, we speculated that miR-223 may be implicated in psoriasis. In our study, we detected the expression and effect of miR-223 in IL-22-stimulated HaCaT cells. Consistent with [29], we found miR-223 to be markedly upregulated in psoriatic skin. Similarly, miR-223 was overexpressed in HaCaT cells stimulated by IL-22, indicating that miR-223 may play a crucial role in the pathogenesis of psoriasis in these conditions.

Next, we investigated the biological function of miR-223 in keratinocyte proliferation and apoptosis caused by IL-22. Keratinocytes activate, maintain, and amplify inflammation by expressing molecules involved in recruiting, retaining, and activating T cells. Thus, they play an important role in psoriasis. Abnormal proliferation of keratinocytes is a predominant feature and important mechanism of psoriasis that leads to epidermal hyperplasia and morphologic characteristics of psoriasis [36]. Apoptosis counteracts keratinocyte proliferation and regulates stratum corneum formation. Psoriatic keratinocytes have strong anti-apoptotic ability and may be a crucial factor in the disease. [37]. Apoptotic resistance of keratinocytes leads to uncontrolled proliferation of keratinocytes in patients with psoriasis [38]. Restoring apoptosis to a normal state thus could minimize severity of the disease [39].

To further understand the function of miR-223 in IL-22-stimulated keratinocyte proliferation and apoptosis in psoriasis, we upregulated or downregulated miR-223 expression in HaCaT cells via transfection with its mimic or inhibitor, respectively. Whereas the miR-223 mimic amplified proliferation and decreased apoptosis, the miR-223 inhibitor produced the opposite results, suggesting that miR-223 contributes to IL-22-induced proliferation and apoptosis of keratinocytes.

PTEN, a major tumor suppressor, exerts lipid and protein phosphatase activities. It dephosphorylates the D3 position of phosphatidylinositol 3,4,5-trisphosphate (PIP3) by blocking the PI3K/Akt pathway and regulating cell proliferation, survival, migration, and differentiation [40]. PTEN expression is markedly downregulated in psoriatic lesions [33]. In our study, miR-223 directly targeted PTEN, and PTEN was downregulated in HaCaT cells stimulated by IL-22. MiR-223 also negatively regulated expression of PTEN in these cells. Subsequently, miR-223 downregulation suppressed the downstream gene p-Akt, and miR-223 overexpression increased it. Consistently, increased PI3K/AKT signal activation by the PTEN reduction mediated by miR-223 was also detected in other cells, such as epithelial ovarian cancer cells [41]. Furthermore, downregulation of PTEN via PTEN siRNA transfection reversed the miR-223 inhibitor's effect on the proliferation and apoptosis of IL-22-stimulated HaCaT cells. MiR-223's role thus appears to be linked to the PTEN/Akt pathway in these cells. Taken together, our results suggest that miR-223 regulated the proliferation and apoptosis of IL-22-stimulated HaCaT cells by targeting PTEN and subsequently affecting the downstream factor Akt. These findings suggest a novel target for psoriasis treatment. However, some limitations remain in this study, such as the detailed mechanism of miR-223 on keratinocytes and whether other signaling pathways are also participated in this effect. Further studies about this will be done in the future.

5. Conclusion

Our study showed that miR-223 promoted proliferation and inhibited apoptosis in IL-22-stimulated keratinocytes via the PTEN/Akt pathway. MiR-223 may influence the pathogenesis of psoriasis and thus is a potential target for psoriasis treatment.

Declaration of Competing Interest

The authors declare no conflict of interest.

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