

## Original Article

# MicroRNA-139-5p alleviates UVB-induced injuries by regulating TLR4 in si-IL-6-treated keratinocyte cells

Bo Yu<sup>1\*</sup>, Hui Dong<sup>2\*</sup>, Yunshu Zang<sup>1</sup>, Shui Jiang<sup>1</sup>, Hong Luan<sup>2</sup>, Yue Zhang<sup>2</sup>

Departments of <sup>1</sup>Dermatology, <sup>2</sup>Nephrology, The Affiliated Hospital of Qingdao University, Qingdao, China. \*Equal contributors.

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**Abstract:** *Background:* Lupus erythematosus (LE) is an autoimmune disease that is often exacerbated by ultraviolet B (UVB) irradiation. MicroRNAs (miRNAs) play vital roles in response to UVB damage to keratinocyte cells. Herein, our study aimed to explore the functions of miR-139-5p in UVB-induced injuries in keratinocyte cells. *Methods:* Human keratinocyte cell line HaCaT was pre-treated with 20 ng/ml si-IL-6 and transfected with miR-139-5p mimic, pc-TLR4 and corresponding controls. These cells were underwent 30 mJ/cm<sup>2</sup> UVB irradiation and incubated for 24 h. Thereafter, cell viability and apoptosis were detected by trypan blue staining and flow cytometry assays. Furthermore, the expression levels of miR-139-5p, TLR4, apoptosis-associated factors, Notch and PI3K/AKT pathways factors were examined by qRT-PCR and western blot. *Results:* Our results showed that UVB irradiation pronouncedly enhanced si-IL-6-induced cell injuries, as decreased cell viability and promoted apoptosis in HaCaT cells. In addition, miR-139-5p was up-regulated in UVB-exposed HaCaT cells and overexpression of miR-139-5p attenuated UVB-induced injuries in si-IL-6-treated HaCaT cells. Further study we found that overexpression of TLR4 significantly abolished the protective effects of miR-139-5p overexpression against UVB-induced injuries in si-IL-6-treated HaCaT cells. Besides, western blot results demonstrated that overexpression of miR-139-5p inactivated Notch and PI3K/AKT pathways by down-regulation of TLR4. *Conclusions:* These results indicated that miR-139-5p alleviated UVB-induced injuries by regulation of TLR4 in si-IL-6-treated HaCaT cells. The study might provide new therapeutic strategies for treatment of LE.

**Keywords:** MicroRNA-139-5p, lupus erythematosus, ultraviolet B (UVB) irradiation, TLR4, Notch/PI3K/AKT

## Introduction

Lupus erythematosus (LE) is an autoimmune disease, which is characterized by immune system abnormally attacks itself normal tissues [1]. The symptoms of LE maybe implicate the whole body, including joint, skin and heart [2, 3]. To date, more than 80% patients of LE with skin damage, such as erythema and rash [4]. Ultraviolet B (UVB) irradiation is a pivotal reason for cause skin damage. Furthermore, excessive UVB irradiation could lead to skin aging, suppress skin immunity, even initiates skin carcinoma [5]. In terms of LE, recent studies demonstrated that UVB irradiation could damage keratinocytes cells, trigger cutaneous inflammatory response and stimulate or aggravate LE [6-8]. However, there is currently no effective treatment to against the skin damage of UVB irradiation in LE. Therefore, clarification of the molecular mechanisms of UVB-induced injuries

in LE will be contributed to finding novel therapeutic strategies.

MicroRNAs (miRNAs), are conserved, non-coding RNAs, that combined with the 3' untranslated region (3'UTR) of target mRNAs to induce mRNA degradation or suppress translation [9]. Several studies have indicated that miRNAs play important roles in various cellular reactions induced by UVB irradiation, and are associated with UVB-induced skin damage [10, 11]. And emerging miRNA-based studies have manifested that miR-34c-5p and miR-22 were UVB-response miRNAs and up-regulation of these miRNAs expressions were able to modulate UVB-induced dermal fibroblasts cells senescence and apoptosis [12, 13]. MiR-139-5p as a tumor suppressor has been found in various cancers, such as acute myeloid leukemia (AML), lung cancer and colorectal cancer [14-16]. Moreover, miR-139-5p was involved in al-

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most all cell biological processes, such as cell viability, apoptosis, metastasis and inflammatory immune responses [17]. However, the role and mechanism of miR-139-5p on UVB-induced injuries in LE are still ambiguous.

Therefore, our study investigated the effects of miR-139-5p on UVB-induced injuries in keratinocyte cells. Firstly, HaCaT cells were pre-treated with 20 ng/ml si-IL-6 and transfected with miR-139-5p mimic, pc-Toll-like receptor 4 (TLR4) and corresponding controls. Then these cells were underwent 30 mJ/cm<sup>2</sup> UVB irradiation and incubated for 24 h. After this, cell viability and apoptosis were detected by trypan blue staining and flow cytometry assays. The expression levels of miR-139-5p, TLR4, apoptosis-associated factors, Notch and phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) pathways factors were examined by qRT-PCR and western blot. The study will be helpful to finding potential therapeutic strategies for UVB injury during LE.

### Materials and methods

#### *Cell culture and treatment*

Human keratinocyte cell line of HaCaT was purchased from the Chinese Academy of Sciences (Kunming, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, Life Technologies), and then these cells were treated by different concentrations (0, 10, 20 and 50 ng/ml) of IL-6 small interfering RNA (si-IL-6) (R&D Systems, Inc., Minneapolis, MN) at 37°C under an atmosphere of 5% CO<sub>2</sub> and 95% air. In addition, 1 × 10<sup>5</sup> HaCaT cells were plated on culture dishes and were irradiated in phosphate-buffered saline (PBS) with 30 mJ/cm<sup>2</sup> of UVB (280-320 nm) from a bank of lamps (Spectronics Corp., Westbury, NY, USA) placed 25 cm. The irradiance of the lamps was calculated by a calibrated photometer (Spectronics Corp., Westbury, NY, USA), the cells supplied with fresh culture medium after exposure to UVB were incubated for the indicated times.

#### *Cell transfection*

MiR-139-5p mimic and scramble were synthesized by GenePharma Co. (Shanghai, China) and transfected into HaCaT cells. Plasmids overexpression TRL4 was ligated into the pc-

DNA3.1 and was referred as to pc-TRL4. The empty pcDNA3.1 plasmid was used as its control. All cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol.

#### *Cell viability assay*

Trypan blue staining was used to analyze cell viability. In brief, 1 × 10<sup>5</sup> HaCaT cells per well were plated in 24-wells plates at 37°C, 5% CO<sub>2</sub>. After incubation for 24 h, cells were trypsinized and stained by 0.4% trypan blue (Invitrogen, Carlsbad, CA, USA) for 4 min. The viable cells were counted using cell counting chamber (Hausser Scientific, Horsham, PA). The dead cells were stained blue, and the viable cells were not stained.

#### *Cell apoptosis assay*

Flow cytometry analysis was performed to identify and quantify the apoptotic cells with Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). Briefly, the cells (1 × 10<sup>5</sup> cells/well) were seeded in 6-well plate, washed twice with cold PBS and re-suspended in buffer. Subsequently, 10 μl Annexin-V-FITC (20 μg/ml) was added to solution and incubated in a dark at room temperature for 30 min. Then 5 μl PI (50 μg/ml) and 300 μl PBS were added, and the apoptotic cells were measured by flow cytometer (Beckman Coulter, USA) according to the manufacturer's instruction.

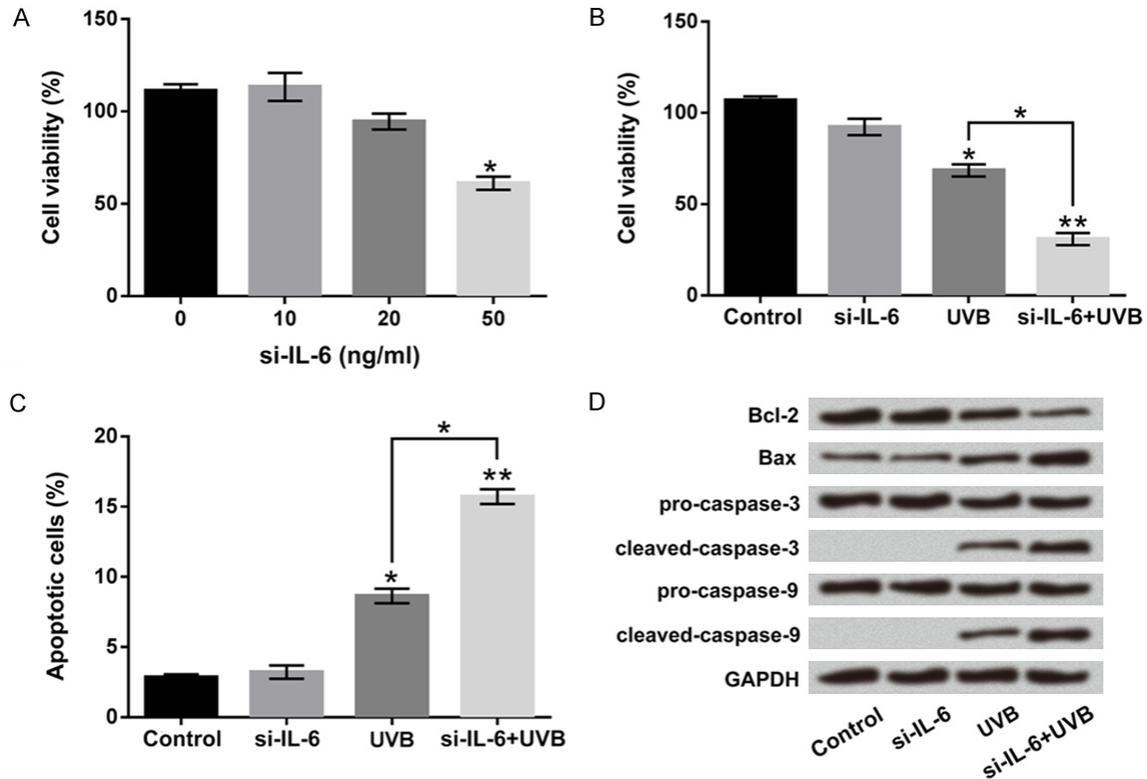
#### *Quantitative real-time PCR (qRT-PCR)*

Total RNAs were isolated from transfected cells by using TRIzol reagent (Invitrogen) and treated with DNaseI (Promega, Madison, WI, USA) to remove the DNA contamination. The complementary DNA (cDNA) was synthesized using MultiScribe Reverse Transcriptase (RT) kit and random hexamers or oligo (dT) (Applied Biosystems, Toronto, Canada). qRT-PCR was conducted using the ABI Prism 7700 Sequence Detection system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. All data were analyzed using 2<sup>-ΔΔCt</sup> method.

#### *Western blot*

The protein samples which were used for western blot was extracted using RIPA lysis buffer

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**Figure 1.** UVB irradiation enhanced si-IL-6-induced HaCaT cells injuries. The different concentrations of si-IL-6 (0, 10, 20, and 50 ng/ml) were used to treat HaCaT cells, then (A) the percentage of HaCaT cells viability in different concentrations of si-IL-6 was detected by trypan blue staining; Next, 20 ng/ml si-IL-6 were selected for treated HaCaT cells, and underwent 30 mJ/cm<sup>2</sup> UVB irradiation for 24 h. (B) cell viability (C) apoptosis and (D) the protein levels of apoptosis-associated factors were respectively analyzed by trypan blue staining, flow cytometry and western blot. UVB: ultraviolet radiation B; si-IL-6: interleukin-6 small interfering RNA; \**P* < 0.05, \*\**P* < 0.01.

(Beyotime Biotechnology, Shanghai, China) and quantified by using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) according to the manufacturer's instructions. These samples were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) membrane (Roche, Basel, Switzerland) and transfected onto polyvinylidene difluoride (PVDF) membrane. The primary antibodies of B-cell lymphoma 2 (Bcl-2; ab32124), BCL-2-Associated X (Bax, ab32503), pro-caspase-3 (ab32150), cleaved caspase-3 (ab13585), pro-caspase-9 (ab32068), TLR4 (ab13556), Notch1 (ab65297), Notch 2 (ab8926), Notch 3 (ab23426), GAPDH (ab8245, Abcam, Cambridge, UK), cleaved caspase-9 (#9501), PI3K (#4292) and phosphorylated (p)-PI3K (#13857), AKT (#9272) and phosphorylated (p)-AKT (#4060, Cell Signaling Technology) were prepared in 5% blocking buffer at a dilution of 1:1,000 and incubated overnight at 4°C. Then the blots were incubated with secondary antibody (dilution of 1:5000) marked by horserad-

ish peroxidase for 1 h at room temperature. Finally, the signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

### Statistical analysis

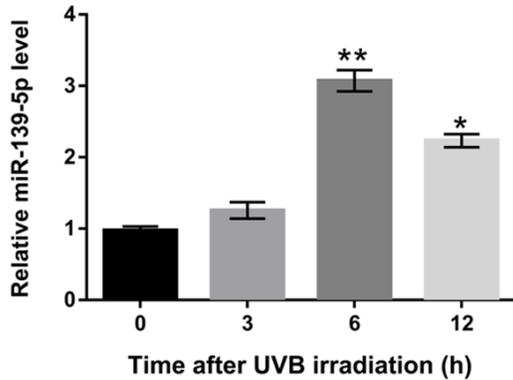
Data in the present study were presented as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA). The *P*-values were calculated using a one-way analysis of variance (ANOVA). *P* < 0.05 was considered to indicate a statistically significant result.

### Results

#### UVB irradiation enhanced si-IL-6-induced cells injuries in HaCaT cells

To distinct the effect of UVB irradiation on si-IL-6-induced cells injuries in HaCaT cells, the dif-

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**Figure 2.** MiR-139-5p was up-regulated in UVB irradiation HaCaT cells. Relative expression of miR-139-5p changes in HaCaT cells 0, 3, 6 and 12 h after UVB irradiation was examined by qRT-PCR. MiR: microRNA; UVB: ultraviolet radiation B; qRT-PCR: quantitative real-time PCR; \* $P < 0.05$ , \*\* $P < 0.01$ .

ferent concentrations of si-IL-6 (0, 10, 20 and 50 ng/ml) were used to treat HaCaT cells. Then these cells were underwent 30 mJ/cm<sup>2</sup> UVB irradiation and incubated for 24 h. As results showed in **Figure 1A**, the cell viability was reduced at 20 ng/ml and 50 ng/ml of si-IL-6 as compared to control group ( $P < 0.05$ ). Therefore, 20 ng/ml of si-IL-6 was selected for subsequent study. Moreover, UVB irradiation significantly strengthened si-IL-6-induced HaCaT cells injuries, as further inhibited cell viability and promoted apoptosis ( $P < 0.05$  or  $P < 0.01$ , **Figure 1B** and **1C**). Western blot results showed that UVB irradiation inhibited Bcl-2 expression, but promoted Bax, cleaved caspase-3 and cleaved caspase-9 expressions in si-IL-6-treated cells. There were no obviously changes of pro-caspase-3 and pro-caspase-9 in different groups (**Figure 1D**). These data indicated that UVB irradiation enhanced si-IL-6-induced cells injuries in HaCaT cells.

### *MiR-139-5p was up-regulated in UVB-exposed HaCaT cells*

Next, we used qRT-PCR assay to analyze the expression of miR-139-5p changes in HaCaT cells 0, 3, 6 and 12 h after UVB irradiation. The results revealed that miR-139-5p was up-regulated in HaCaT cells both 6 and 12 h after UVB irradiation ( $P < 0.05$  or  $P < 0.01$ , **Figure 2**). These data demonstrated that UVB irradiation might alter the expression of miR-139-5p in HaCaT cells.

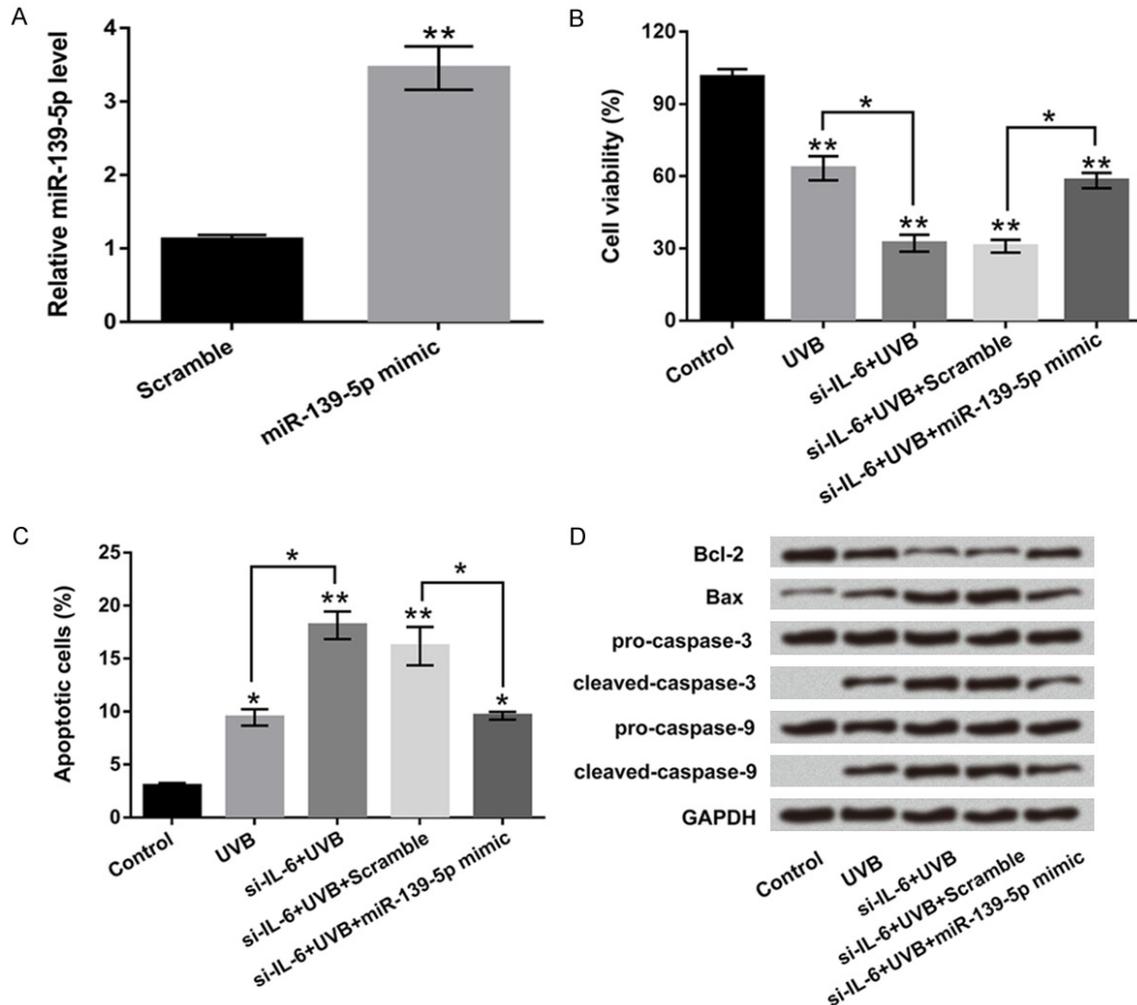
### *MiR-139-5p alleviated UVB-induced injuries in si-IL-6-treated HaCaT cells*

To further investigate whether miR-139-5p was involved in regulating UVB-induced cell injuries, HaCaT cells were transfected with miR-139-5p mimic and control, then these transfected cells were treated with 20 ng/ml si-IL-6 and underwent 30 mJ/cm<sup>2</sup> UVB irradiation for 24 h. The results displayed that miR-139-5p was obviously up-regulated in transfected miR-139-5p mimic cells compared with control ( $P < 0.01$ , **Figure 3A**), suggested that the transfection efficiency was well and could use for further studies. In **Figure 3B** and **3C**, the results displayed that overexpression of miR-139-5p remarkably increased cell viability and declined apoptosis after UVB irradiation in si-IL-6-treated cells compared with scramble control group ( $P < 0.05$ ). Furthermore, we found that miR-139-5p overexpression obviously up-regulated the protein expressions of Bcl-2, down-regulated Bax, cleaved-caspase-3 and cleaved-caspase-9 as showed in **Figure 3D**. Taken together, these data indicated that miR-139-5p as a protector that alleviated UVB-induced injuries in si-IL-6-treated HaCaT cells.

### *TLR4 overexpression abolished the protective effects of miR-139-5p on UVB-induced injuries in si-IL-6-treated HaCaT cells*

To confirm whether TLR4 was associated with miR-139-5p modulated UVB-induced injuries, pc-TLR4, miR-139-5p mimic, co-transfected pc-TLR4 and miR-139-5p mimic and corresponding controls were transfected into HaCaT cells. All these transfected cells were treated with 20 ng/ml si-IL-6 and underwent 30 mJ/cm<sup>2</sup> UVB irradiation for 24 h. Results showed in **Figure 4A**, the mRNA and protein expression levels of TLR4 were remarkably down-regulated by miR-139-5p overexpression compared with control group ( $P < 0.05$ ). Then we examined the transfection efficiency of pc-TLR4, as results displayed in **Figure 4B**, the mRNA and protein levels of TLR4 were obviously increased after treatment with pc-TLR4 ( $P < 0.01$ ). Interestingly, we found that TLR4 overexpression significantly abolished the protective effects of miR-139-5p overexpression on UVB-induced injuries in si-IL-6-treated HaCaT cells via reducing cell viability and increasing apoptosis ( $P < 0.05$  or  $P < 0.01$ , **Figure 4C** and **4D**). And western blot results showed that co-transfected pc-TLR4 and miR-139-5p mimic remarkably suppressed

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**Figure 3.** Overexpression of miR-139-5p protected UVB-induced injuries in si-IL-6-treated cells. HaCaT cells were transfected with miR-139-5p mimic and control, then these transfection cells were treated with 20 ng/ml si-IL-6 and underwent 30 mJ/cm<sup>2</sup> UVB irradiation for 24 h. (A) The expression of miR-139-5p in miR-139-5p mimic-transfected cells were detected by qRT-PCR; (B) cell viability (C) apoptosis and (D) the protein levels of apoptosis-associated factors were respectively analyzed by trypan blue staining, flow cytometry and western blot. MiR: microRNA; UVB: ultraviolet radiation B; si-IL-6: interleukin-6 small interfering RNA; qRT-PCR: quantitative real-time PCR; \**P* < 0.05, \*\**P* < 0.01.

Bcl-2 expression, but promoted Bax, cleaved-caspase-3 and cleaved-caspase-9 expressions (Figure 4E). These data indicated that TLR4 was involved in UVB-induced cell injuries, and overexpression of TLR4 abolished the protective effects of miR-139-5p on UVB-induced injuries in si-IL-6-treated HaCaT cells.

### *MiR-139-5p inactivated Notch and PI3K/AKT pathways by down-regulation of TLR4*

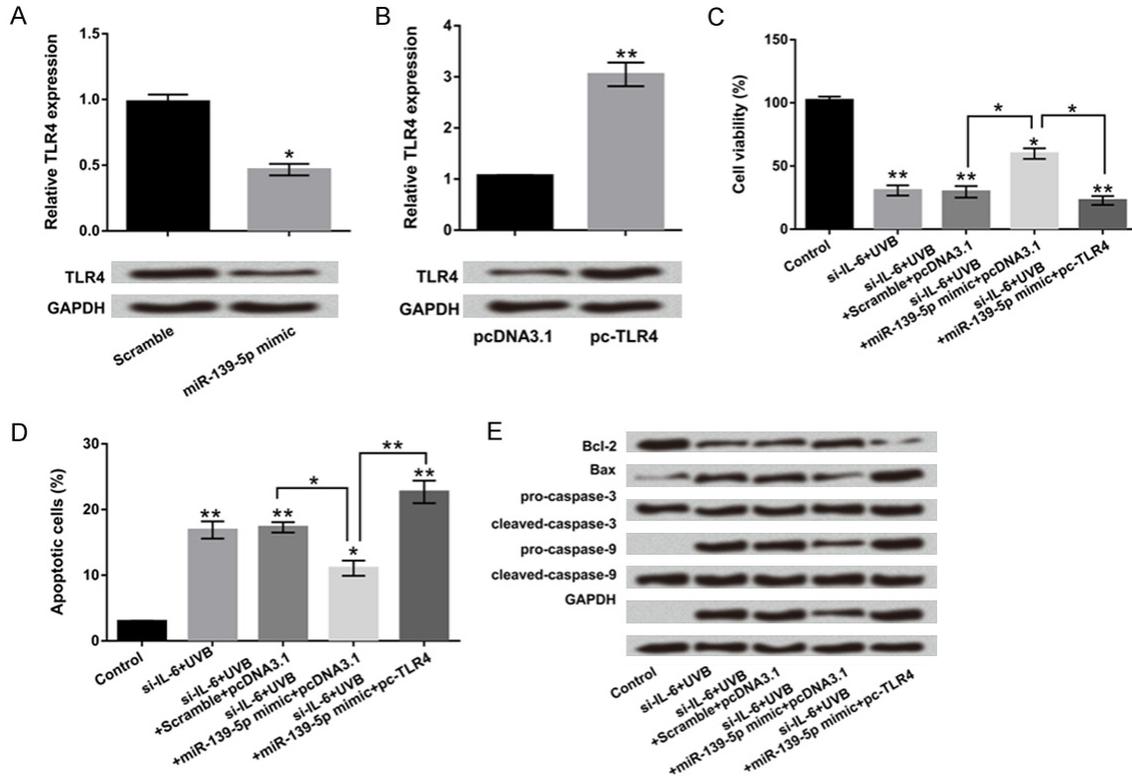
Finally, we detected the expression levels of Notch and PI3K/AKT to explore the relationship of miR-139-5p, TLR4 and Notch and PI3K/AKT pathways in UVB-induced cell injuries. Western blot results showed in Figure 5A and 5B, over-

expression of miR-139-5p blocked Notch and PI3K/AKT pathways through inhibiting Notch1, Notch2, Notch3, p-PI3K, p-AKT expressions. However, co-transfected with overexpression of miR-139-5p and TLR4 significantly promoted these five factors expressions. Therefore, we conferred that miR-139-5p inactivated Notch and PI3K/AKT pathways by regulation of TLR4, thereby regulating UVB-induced injuries in si-IL-6-treated HaCaT cells.

### Discussion

In present study, these results indicated that UVB irradiation enhanced si-IL-6-induced cell injuries in HaCaT cells. Moreover, miR-139-5p

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**Figure 4.** Overexpression of TLR4 abolished the protective effect of miR-139-5p overexpression on UVB-induced injuries in si-IL-6-treated cells. HaCaT cells were transfected with miR-139-5p mimic, pc-TLR4, and corresponding controls. Subsequently, these transfected cells were treated with 20 ng/ml si-IL-6 and underwent 30 mJ/cm<sup>2</sup> UVB irradiation for 24 h. The mRNA and protein levels of TLR4 in (A) miR-139-5p mimic-transfected cells and (B) pc-TLR4-transfected cells were detected by qRT-PCR; (C) cell viability (D) apoptosis and (E) the protein levels of apoptosis-associated factors were respectively determined by trypan blue staining, flow cytometry and western blot. TLR4: Toll-like receptor 4; MiR: microRNA; UVB: ultraviolet radiation B; si-IL-6: interleukin-6 small interfering RNA; qRT-PCR: quantitative real-time PCR; \**P* < 0.05, \*\**P* < 0.01.

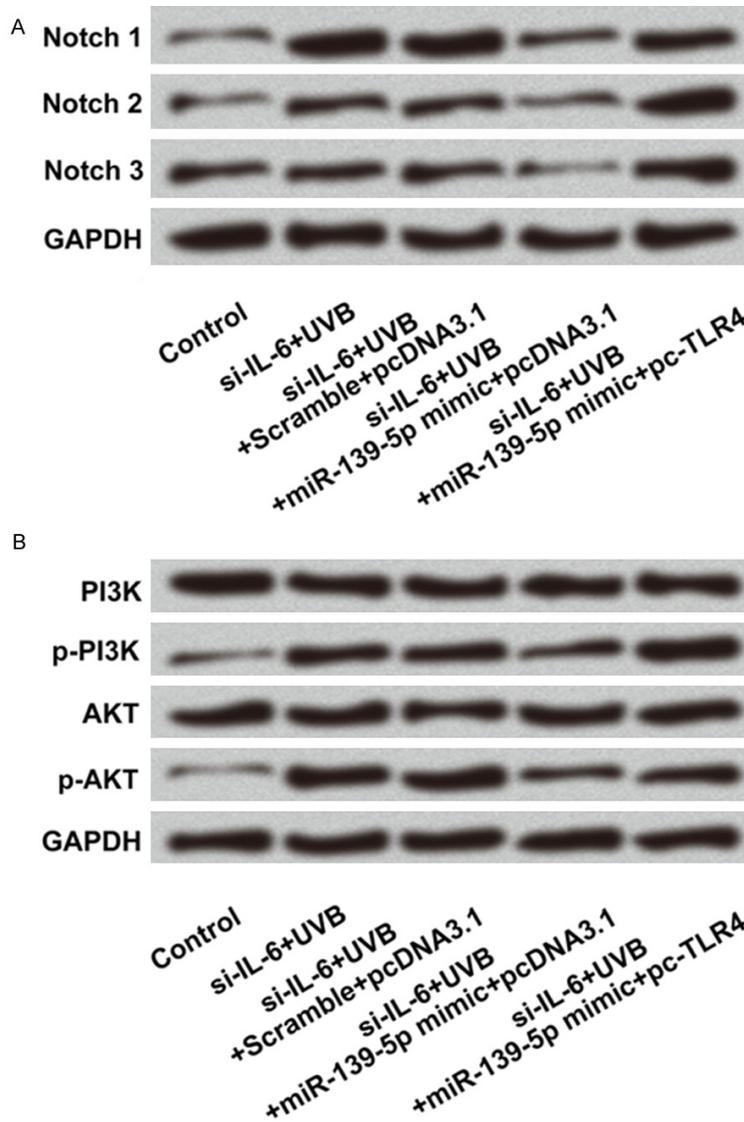
was up-regulated in UVB-exposed HaCaT cells and overexpression of miR-139-5p attenuated UVB-induced injuries in si-IL-6-treated HaCaT cells. Further study we found that overexpression of TLR4 significantly abolished the protective effects of miR-139-5p overexpression against UVB-induced injuries in si-IL-6-treated HaCaT cells. Besides, overexpression of miR-139-5p inactivated Notch and PI3K/AKT pathways by regulation of TLR4.

LE is an autoimmune system disease that is often accompanied by skin damage. Inflammation is an important feature in skin damage process [18]. IL-6 is a multifunctional cytokine, which is involved in the regulation of cell viability, apoptosis, immune response and inflammation [19]. Several studies demonstrated that UVB irradiation could modulate pro-inflammatory cytokines expressions in irradiated skin of

LE [20, 21]. Moreover, UVB irradiation often aggravated skin damage of LE patients [22]. Based on these previous studies, we used si-IL-6 to treat HaCaT cells to explore the effect of UVB-induced cell injuries on LE. Our study found that UVB-irradiation pronouncedly enhanced si-IL-6-induced injuries, as decreased cell viability and promoted apoptosis in HaCaT cells.

Emerging evidences have hinted that UVB irradiation could alter miRNA expression [23]. For instance, Dziunycz *et al.* reported that UVB irradiation increased miR-203 expression, but decreased miR-205 expression in skin keratinocytes [24]. Additionally, Guo *et al.* exposed that miR-23 expression was up-regulated after the UVB irradiation of HaCaT cells [25]. In our study, we also discovered that miR-139-5p expression was up-regulated in HaCaT cells after UVB

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**Figure 5.** MiR-139-5p inactivated Notch and PI3K/AKT pathways by regulation of TLR4. HaCaT cells were pre-treated with 20 ng/ml si-IL-6, and transfected with miR-139-5p mimic, pc-TLR4, and corresponding controls, then these cells were underwent 30 mJ/cm<sup>2</sup> UVB irradiation for 24 h. The protein expressions of (A) Notch and (B) PI3K/AKT signal pathways were examined by western blot. MiR: microRNA; PI3K: phosphatidylinositol 3 kinase; AKT: protein kinase B; TLR4: Toll-like receptor 4; si-IL-6: interleukin-6 small interfering RNA; UVB: ultraviolet radiation B.

irradiation. The result was similar with Kramer *et al.* reported that miR-139-5p expression was up-regulated in primary keratinocytes after UVB irradiation [26].

Recently, dysregulated miRNAs have been confirmed to be associated with cell proliferation, apoptosis, immune inflammation response, and UVB irradiation response [27]. As Ninio-Many *et al.* and He *et al.* reported that miR-

125a-3p suppressed cell proliferation and migration by targeting Fyn kinase, which was activated by UVB irradiation in HaCaT cells [28, 29]. Furthermore, Guo *et al.* demonstrated that protection of HaCaT cells against UVB irradiation was regulated by miR-23a [25]. Overall, these studies indicated that miRNAs may be a critical regulator of multiple cellular processes and further suggest that miRNAs may have protective effect in response to UVB. However, the effects of miR-139-5p on UVB-induced injuries in LE have not been investigated. Similar with these previous studies, our study found that miR-139-5p as a protector against UVB-induced injuries in si-IL-6-treated cells.

Toll-like receptor 4 (TLR4) is a transmembrane protein, lead to inflammatory cytokine production, and which is an essential modulator of innate immune system [30]. Increasing evidence indicated that UVB induced cutaneous immunosuppression by TLR4 [31]. Moreover, TLR4 enhanced UVB-induced DNA damage and inflammation [32]. In terms of LE, one study found that TLR4 could influence susceptibility and severity and as a protector against autoimmune disorders [33]. Another report demonstrated that up-regulation of TLR4 play a critical role in the pathogenesis of skin damage [34]. However, whether TL-

R4 participated in UVB-induced injuries in si-IL-6 treated HaCaT cells remain unclear. In our study, we found that TLR4 was down-regulated by miR-139-5p overexpression, and TLR4 overexpression abolished the protective effect of miR-139-5p on UVB-induced injuries in si-IL-6 treated HaCaT cells.

Notch and PI3K/AKT pathways regulate various cell biological processes and are important

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mediators of UVB-induced cellular responses [35]. Recent evidence have proved that activation of Notch, and PI3K/AKT pathways could prevent UVB-induced skin damage [35, 36]. In addition, AKT serve as survival signals to fight against UV-induced cell death and apoptosis in HaCaT cells [37]. Importantly, miRNA-based studies demonstrated that miR-139-5p suppresses cells differentiation via regulating Notch and PI3K/AKT pathways in pre-adipocyte [38]. Whereas, whether miR-139-5p and TLR4 affected UVB-induced cell injuries through modulating Notch and PI3K/AKT pathways have not been investigated. In our study, we found that overexpression of miR-139-5p inactivated the Notch and PI3K/AKT pathways via down-regulation of TLR4, indicating that Notch and PI3K/AKT pathways were involved in regulating UVB-induced injuries in si-IL-6-treated HaCaT cells.

Collectively, the study demonstrated that miR-139-5p as a protector that attenuated UVB-induced cell injuries in IL-6-treated HaCaT cells, and activated Notch and PI3K/AKT pathways by regulating TLR4. The study provided new therapeutic strategies for the treatment of LE. However, further studies are needed to detect the exact mechanisms of miR-139-5p regulated UVB-induced cell injuries in LE.

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### Disclosure of conflict of interest

None.

**Address correspondence to:** Hui Dong, Department of Nephrology, The Affiliated Hospital of Qingdao University, 16 Jiangsu Road, Qingdao 266003, China. E-mail: leonjackson7085@gmail.com

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