

## Research paper

# MicroRNA-124 alleviates chronic skin inflammation in atopic eczema via suppressing innate immune responses in keratinocytes

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## ARTICLE INFO

## Keywords:

MicroRNA 124  
Atopic eczema  
Inflammation  
NF- $\kappa$ B (p65)  
Keratinocytes

## ABSTRACT

Chronic skin inflammation in atopic eczema is associated with elevated expression of proinflammatory genes and activation of innate immune responses in keratinocytes. MicroRNAs (miRNAs) are short, single-stranded RNA molecules that silence genes via the degradation of target mRNAs or inhibition of translation. Recent studies have demonstrated that miR-124 is associated with regulation of inflammation factors in several diseases. The aim of this study was to investigate the role of miR-124 in skin inflammation of atopic eczema. We showed that miR-124 expression is decreased in chronic lesional skin of patients with atopic eczema, and could be strongly inhibited by IFN- $\gamma$  and TNF- $\alpha$ . Through Western blot, real-time PCR and luciferase assays, we revealed that miR-124 inhibited the expression of p65, a member of NF- $\kappa$ B family which can regulate many factors involved in the immune response and inflammatory reactions, through direct targeting. Further, upon IFN- $\gamma$  or TNF- $\alpha$  stimulation, IL8, CCL5 and CCL8 showed to be significantly upregulated by IFN- $\gamma$  or TNF- $\alpha$ , down-regulated by miR-124; the promotive effect of IFN- $\gamma$  and TNF- $\alpha$  could be partially reversed by miR-124. The levels of IL8, CCL5 and CCL8 could be significantly downregulated by p65 knockdown, upregulated by miR-124 inhibition; the suppressive effect of p65 knockdown could be partially reversed by miR-124. Moreover, contrary to miR-124, p65, IL8, CCL5 and CCL8 mRNA expression was upregulated in chronic lesional skin of patients with atopic eczema, and all inversely correlated with miR-124. Taken together, our data demonstrate that miR-124 controls NF- $\kappa$ B-dependent inflammatory responses in keratinocytes and chronic skin inflammation in atopic eczema; rescuing miR-124 expression presents a promising strategy for atopic eczema treatment.

## 1. Introduction

Eczema, a skin inflammatory reaction mediated by antigen-specific T cells [1], occurs in 15%–20% of infants and young children and persists throughout adulthood in 1%–3% of the cases [2]. The most common type of eczema is atopic eczema, in which a dry, itchy rash develops [3]. Recurrent exacerbations, activation of the immune system, and hyperplasia and keratinocyte apoptosis in the chronic phase represent cardinal features of atopic eczema [2,4,5].

Skin inflammation during an exacerbation can persist for several weeks and is nearly exclusively complicated with secondary infections that contribute to the activation of innate immune responses and the nuclear factor kappa B (NF- $\kappa$ B) pathway in keratinocytes [6,7]. Several studies have reported that NF- $\kappa$ B plays a critical role in the production of chemokines in TNF- $\alpha$  and/or IFN- $\gamma$ -stimulated keratinocytes [8–10]. IFN- $\gamma$  can induce several characteristic chemokines in keratinocytes, including CCL5 (also known as RANTES) and CCL8 (also known as MCP-2) [11,12]. CCL5 promoter polymorphisms and enhanced levels

have been shown to be associated with atopic dermatitis [13–15]. CCL8 mediates the recruitment of IL-5-enriched TH2 cells in the inflamed skin in a mouse model of chronic atopic dermatitis [16]. In addition, the presence of TNF- $\alpha$  could increase the secretion of GM-CSF, CCL5, and IL-10 [17].

Regulation of inflammatory responses in diseases is mediated by coordinated control of gene expression, which is also modulated by microRNAs (miRNAs). MiRNAs silence genes by triggering the degradation of target mRNAs and inhibition of translation [18,19]. Several miRNAs, including miR-124, function in the regulation of various inflammatory processes [20–22]. In glioma cells, the restoration of miR-124 can reduce the activation of NF- $\kappa$ B [23]. Additionally, an activated NF- $\kappa$ B-centered inflammatory loop in the highly aggressive non-small cell lung cancer cells leads to down-regulation of miR-124 [24]. The transfection of PC12 neurons with miR-124 counteracted the inhibition of neurites mediated by both recombinant TNF- $\alpha$  and macrophages, suggesting that miR-124 might be a valuable tool for desensitizing neurons to a repulsive inflammatory environment [25]. However, the

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<http://dx.doi.org/10.1016/j.cellimm.2017.08.003>

Received 28 April 2017; Received in revised form 3 August 2017; Accepted 20 August 2017  
0008-8749/ © 2017 Published by Elsevier Inc.

function of miR-124 in the normal skin and keratinocytes and in atopic eczema has not been previously explored.

Here, we demonstrate that the level of miR-124 is decreased in lesions tissue from patients with atopic eczema and also decreased in keratinocytes in response to inflammation factors, in turn, controls chronic inflammatory processes that are triggered by TNF- $\alpha$  and/or IFN- $\gamma$  though direct targeting NF- $\kappa$ B and regulation of CCL5, CCL8 and IL-8 expression in keratinocytes.

## 2. Materials and methods

### 2.1. Tissues, cell lines and cell transfection

We collected 37 paired atopic eczema skin lesional tissues and normal non-lesional tissues with approval of the Ethical Review Committees on Hunan University of Chinese Medicine. All patients signed a written informed consent form and were diagnosed by dermatologists according to the diagnostic standard for atopic eczema. The criteria for diagnosis and disease phase of these patients were Eczema Area and Severity Index (EASI) [26].

For functional studies, pooled, normal human epidermal keratinocytes (Promocell, Heidelberg, Germany) were obtained and cultured in EpiLife® basal medium (Gibco, USA) supplemented with  $1 \times$  Human Keratinocyte Growth Supplement (Gibco, USA), 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin (Beyotime, China) in a 37 °C incubator with 5% CO<sub>2</sub>. Keratinocytes were stimulated with different cytokines (R & D Systems, USA) for 48 h, including IL-13 (50 ng/ml), IL-1 $\beta$  (10 ng/ml), IL-17 A (10 ng/ml), IL-4 (40 ng/ml), IFN- $\gamma$  (10 ng/ml) or TNF- $\alpha$  (25 ng/ml). p65 knockdown is achieved by si-p65 (Genepharma, China) transfection. MiR-124 mimics or miR-124 inhibitor (Genepharma, China) was transfected into indicated target cells to achieve miR-124 overexpression or miR-124 inhibition by using Lipofectamine 2000 (Invitrogen).

### 2.2. Quantitative real time PCR (qPCR) for miRNA and mRNA detection

Trizol reagent (Invitrogen) was used for total RNA extraction following the manufacturer's instructions. As previously described [17], total RNA (20 ng) was converted to cDNA using the miScriptRT kit (Qiagen, German). qRT-PCR was performed using a miScript SYBR Green PCR kit (Qiagen, German) on the ABI 7900HT Real-time PCR System (Applied Biosystem, USA) following the  $2^{-\Delta\Delta Ct}$  method. The

expression of miRNAs or mRNA was normalized using the RNU6 and GAPDH as endogenous control, respectively. Primers were showed in Table 1. The samples were amplified in triplicate and each experiment was repeated three times.

### 2.3. Western blotting

Keratinocytes were incubated with TNF- $\alpha$ /INF- $\gamma$  (25 ng/ml or 10 ng/ml, respectively) in the presence or absence of miR-124 for 24 h. After 24 h incubation, cell samples were lysed for 30 min on ice with Pro-Prep™ protein extraction solution according to the protocol provided. After 30 min of incubation, whole cell lysates were centrifuged at 13,000 rpm at 4 °C for 5–10 min and clear supernatants were collected in new Eppendorf tubes and stored at -70 °C. For immunoblotting, proteins in cell lysates were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 8–10% gels and then transferred to nitrocellulose membranes (Millipore) for 1.5 h. After blocking in 5% skim milk, membranes were blocked with the following antibodies: anti-p65 (Cat# E379, Abcam, USA), anti-GAPDH (Cat# 6C5, Abcam) 4 °C overnight, and incubated with HRP-conjugated secondary antibody (1:5000). Signals were visualized using ECL Substrates (Millipore, USA). The protein expression was normalized to endogenous GAPDH.

### 2.4. Enzyme-linked immunosorbent assay (ELISA)

Keratinocytes ( $1 \times 10^6$ /well) were transfected with miR-124 mimics or mimics NC and then stimulated with TNF- $\alpha$  or IFN- $\gamma$  for 24 h. CCL5, CCL8 and IL-8 production was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Abcam Inc.) according to the manufacturer's instructions.

### 2.5. Luciferase activity

A wt-RELA 3'UTR luciferase reporter gene vector and a mut-RELA 3'UTR luciferase reporter gene vector containing a 6 bp mutation on the predicted binding site of miR-124 in the 3'UTR of RELA was obtained from Yrbio Co, ltd (Changsha, China). Keratinocytes were cultured overnight after being seeded into a 24-well plate, co-transfected with the indicated vectors and miR-124 mimics or miR-124 inhibitor. 48 h after transfection, Dual Luciferase Reporter Assay System (Promega, USA) was used to determine the luciferase activity.

Table 1

| Gene name | Forward                   | Reverse                   |
|-----------|---------------------------|---------------------------|
| MiR-124   | GTCGCCTAAGGCACGCGGTG      | GTGCAGGGTCCGAGGT          |
| U6        | CTCGCTTCGGCAGCACA         | AACGCTTCACGAATTGCGT       |
| ICAM1     | GTATGAACTGAGCAATGTGCAAG   | GTCCACCCGTCTGGAGTC        |
| CXCL2     | CTGCGCTGCCAGTGCTTG        | CGATGCGGGTTGAGACA         |
| IRAK1     | AGGTTTCGTACCCAAACATT      | CGGGCTGTACCCAGAAGGA       |
| RELB      | TTCCGAGCCGCTCTATGACAA     | CGTCTTGAACACAATGGCAATC    |
| PLAU      | GCTTGTCGAAGAGTGATGGT      | CAGGGCTGGTTCTCGATGG       |
| IL8       | GCAGCTCTGTGTAAGGTGCAGTT   | TTCTGTGTGGCGCAGTGTGGTC    |
| NFKB1     | GAAGCACGAATGACAGAGGC      | GCTTGGCGGATTAGCTCTTTT     |
| NFKB1A    | CTCCGAGACTTTCGAGGAAATAC   | GCCATTGTAGTTGGTAGCCTCA    |
| NFKB2     | ATGGAGAGTTGCTACAACCCA     | CTGTTCCACGATCACCAGGTA     |
| TNFAIP3   | TCCTCAGGCTTTGTATTTGAGC    | TGTGTATCGGTGCATGGTTTAA    |
| BCAR1     | ATGGGCAGTACGAGAACAGC      | GGCCAGGTCGTGGTCTATG       |
| CCL8      | AGGGACTTGCTCAGCCAGATTCACT | TCTTGTGTAGCTCTCCAGCCTCTGG |
| CCL5      | AGTCGCTTTGTACCCGAAA       | TCTCCATCCTAGCTCATCTCCAA   |
| STAT2     | GAGCCAGCAACATGAGATTGA     | GCCTGGATCTTATATCGGAAGCA   |
| CXCL5     | AGCTGCGTTGCGTTTGTATAC     | TGGCGAACACTTGCAGATTAC     |
| RAC2      | TCTGCTTCTCCCTCGTCAG       | TCACCGAGTCAATCTCCTTGG     |
| CXCL1     | CACGTGCTGCTCCTGCTCCT      | GGCTATGACTTCGGTTTGG       |
| CCL20     | ACCGTATCTTTCATCCTAA       | TTACCCCAAGTCTGTTTT        |
| PXN       | CCCTCAATGGCACAATCC        | GGCGGTGAGTTTCAGAAAG       |
| PPBP      | CTCCACCAAAGGACAAAC        | ATTCATCACCTGCCAATT        |
| GAPDH     | GGAGCGAGATCCCTCCAAAAT     | GGCTGTTGTCATCTTCATGG      |

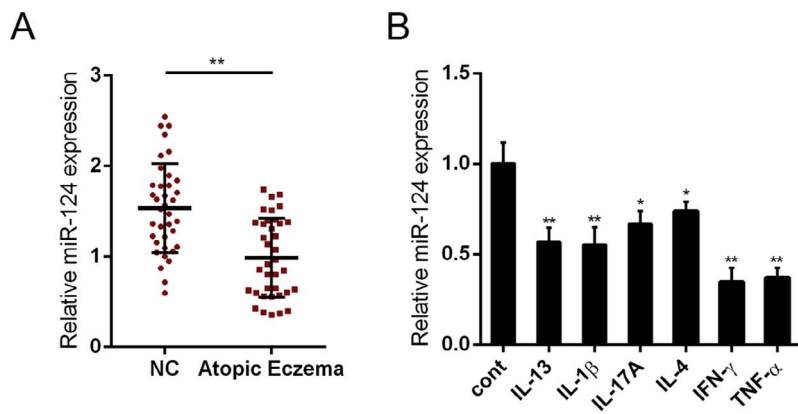


**Fig. 1.** MiR-124 expression was downregulated in atopic eczema tissues and in response to inflammatory factor stimulation. (A) miR-124 expression in atopic eczema lesional and non-lesional tissues was determined using real-time PCR assays. (B) Human primary keratinocytes were treated with IL-13, IL-1 $\beta$ , IL-17A, IL-4, IFN- $\gamma$  and TNF- $\alpha$ ; miR-124 expression in response to the indicated inflammatory factors was determined using real-time PCR assays. The data are presented as mean  $\pm$  SD of three independent experiments. \* $P$  < 0.05, \*\* $P$  < 0.01.

## 2.6. Statistical analysis

All data are presented as means  $\pm$  SD of at least three separate experiments. Student's  $t$  test and two-way analysis of variance (ANOVA) were used to determine the statistical significance of differences among values for the treated and untreated (control) groups. Differences with  $P$  values < 0.05 were considered significant.

## 3. Results

### 3.1. MiR-124 expression was downregulated in atopic eczema tissues and in response to inflammatory factor stimulation

Previous studies have reported the association between miR-124 and inflammatory factors [25,27]. Here we firstly evaluated miR-124 expression in atopic eczema lesional tissues and non-lesional normal tissues ( $n = 37$ ). Results from real-time PCR assays showed that miR-124 expression was downregulated in atopic eczema tissues, compared to the non-lesional normal tissues (Fig. 1A). Next, we conducted a series of inflammatory factor treatment, including IL-13, IL-1 $\beta$ , IL-17A, IL-4, IFN- $\gamma$  and TNF- $\alpha$ , to evaluate miR-124 expression in response to these inflammatory factors. Results showed that miR-124 expression could be downregulated by all the indicated inflammatory factors, and more strongly downregulated by IFN- $\gamma$  and TNF- $\alpha$  (Fig. 1B). These data suggested that miR-124 might play a role in skin inflammation in atopic eczema.

### 3.2. MiR-124 inhibited NF- $\kappa$ B (p65) expression through direct binding to the 3'UTR of RELA

NF- $\kappa$ B plays a critical role in the production of chemokines in TNF- $\alpha$  and/or IFN- $\gamma$ -stimulated keratinocytes [8–10]. Given the potential role of miR-124 in inflammatory skin of atopic eczema, we further investigated whether miR-124 is associated with NF- $\kappa$ B. Si-p65 was transfected into keratinocytes to achieve p65 knockdown, as verified using Western blot assays (Fig. 2A). Next, miR-124 mimics or miR-124 inhibitor was transfected into keratinocytes to achieve ectopic miR-124 expression or miR-124 inhibition, as verified using real-time PCR assays (Fig. 2B). In response to ectopic miR-124 expression, p65 protein level was significantly reduced, whereas increased by miR-124 inhibition (Fig. 2C). These data suggested that miR-124 could inversely regulate p65 protein level.

Further, we employed luciferase assays to investigate the mechanism by which miR-124 regulates p65 expression. A wt-RELA (gene name of p65) 3'UTR luciferase reporter gene vector and a mut-RELA 3'UTR luciferase reporter gene vector containing a 6 bp mutation on the predicted binding site of miR-124 in the 3'UTR of RELA was constructed (Fig. 2D). The indicated vectors were co-transfected into keratinocytes with miR-124 mimics or miR-124 inhibitor, and then the luciferase

activity changes were monitored. Results showed that the luciferase activity of wild-type vectors was significantly suppressed by miR-124 mimics, amplified by miR-124 inhibitor; after mutation in the predicted binding site, the changes of luciferase were abolished (Fig. 2E). These data indicated that miR-124 regulates p65 expression through direct binding to the 3'UTR of RELA.

### 3.3. Overexpression of miR-124 inhibits NF- $\kappa$ B-dependent genes in human primary keratinocytes

To study miR-124 function in keratinocytes, we overexpressed miR-124 in un-stimulated (us) and IFN- $\gamma$ - or TNF- $\alpha$ -stimulated keratinocytes and performed qRT-PCR analysis. As showed in the heatmaps, miR-124 overexpression resulted in modulation of a large number of NF- $\kappa$ B-dependent and chemokine signaling associated genes [6,28,29] in IFN- $\gamma$ - or TNF- $\alpha$ -stimulated keratinocytes (Fig. 3A); among these genes, IL-8, CCL5 and CCL8 were significantly downregulated by miR-124 in IFN- $\gamma$ - or TNF- $\alpha$ -stimulated keratinocytes (Fig. 3B).

To further confirm miR-124 regulation of IL-8, CCL5 and CCL8 in IFN- $\gamma$ - or TNF- $\alpha$ -stimulated keratinocytes, ELISA assays were employed to evaluate protein levels of IL-8, CCL5 and CCL8. Results showed that IL-8 secretion could be induced by TNF- $\alpha$  stimulation, reduced by miR-124 overexpression (Fig. 3C), compared to us + control (mimics NC) group; CCL5 and CCL8 secretion could be induced by TNF- $\alpha$  stimulation, reduced by miR-124 overexpression (Fig. 3C), compared to us + control (mimics NC) group. Moreover, the promotive effect of IFN- $\gamma$  or TNF- $\alpha$  on IL-8 or CCL5 and CCL8 could be partially reversed by miR-124. These data suggested that miR-124 regulates p65 expression, thus to affect the production of chemokines in TNF- $\alpha$  and/or IFN- $\gamma$ -stimulated keratinocytes.

### 3.4. MiR-124 could reverse the effect of p65 on downstream inflammatory factors

Given that miR-124 overexpression inhibits NF- $\kappa$ B-dependent genes in human primary keratinocytes; we further investigated whether miR-124 could reverse the effect of p65 on the indicated chemokines. Si-p65 and miR-124 inhibitor was co-transfected into human primary keratinocytes; the protein levels of IL-8, CCL5 and CCL8 were then evaluated using ELISA assays. Results showed that the protein levels of IL-8, CCL5 and CCL8 were significantly reduced after p65 knockdown, whereas increased by miR-124 inhibition; the inhibitory effect of si-p65 on the indicated chemokines could be partially reversed by miR-124 inhibition (Fig. 4A).

Finally, we monitored the mRNA expression levels of p65, IL-8, CCL5 and CCL8 in atopic eczema lesional tissues and non-lesional tissues. Results showed that the mRNA expression of p65, IL-8, CCL5 and CCL8 was significantly upregulated in atopic eczema lesional tissues, compared to non-lesional tissues (Fig. 4B,  $P$  < 0.01). By performing

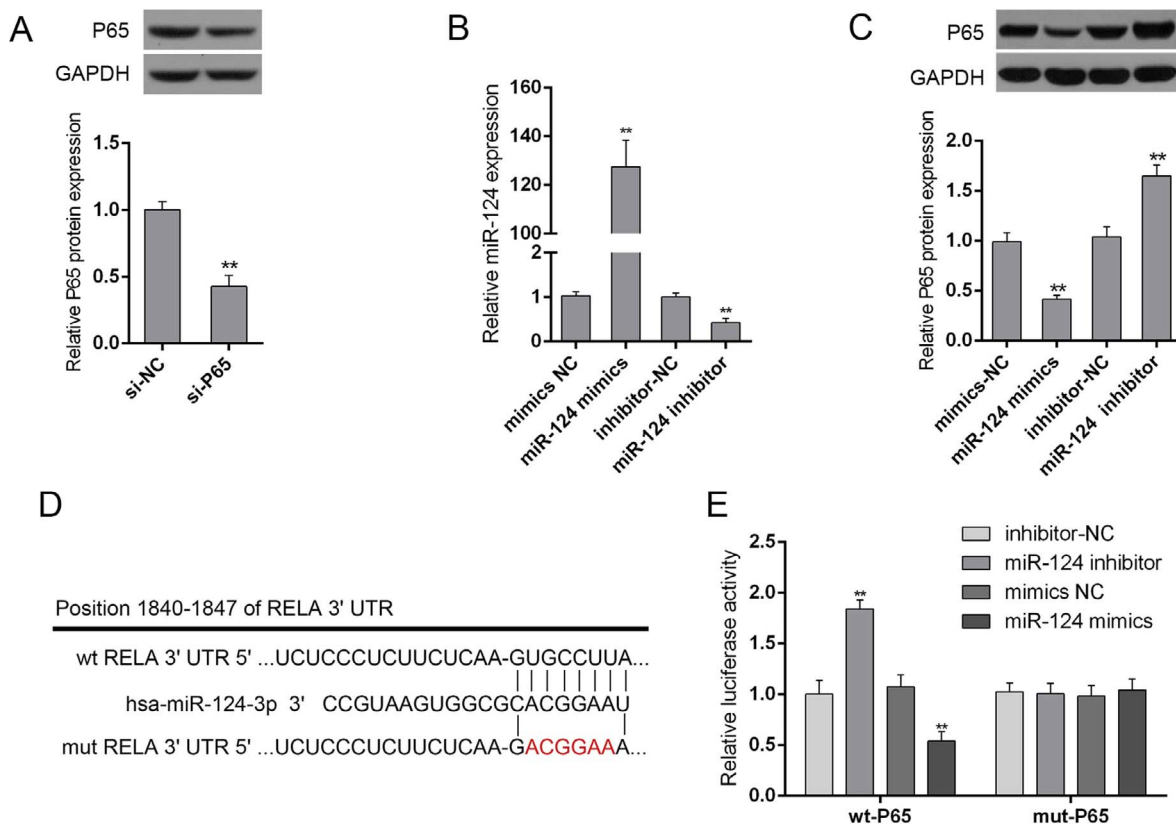


**Fig. 2.** MiR-124 inhibited NF- $\kappa$ B (p65) expression through direct binding to the 3'UTR of RELA. (A) si-p65 was transfected into keratinocytes to achieve p65 knockdown, as verified using Western blot assays. (B) miR-124 mimics or miR-124 inhibitor was transfected into keratinocytes to achieve ectopic miR-124 expression or miR-124 inhibition, as verified using real-time PCR assays. (C) p65 protein in miR-124 mimics- or miR-124 inhibitor-transfected keratinocytes was determined using Western blot assays. (D) A wt-RELA 3'UTR luciferase reporter gene vector and a mut-RELA 3'UTR luciferase reporter gene vector containing a 6 bp mutation on the predicted binding site of miR-124 in the 3'UTR of RELA was constructed. (E) Keratinocytes were co-transfected with the indicated vectors and miR-124 mimics or miR-124 inhibitor. 48 h after transfection, Dual Luciferase Reporter Assay System was used to determine the luciferase activity. The data are presented as mean  $\pm$  SD of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

Spearman's rank correlation analysis, we observed an inverse correlation between miR-124 expression and p65, IL-8, CCL5 and CCL8 expression in atopic eczema lesional tissues, respectively (Fig. 4C), which further suggested that rescuing miR-124 expression, thus to suppress p65 expression and the production of downstream chemokines present a promising strategy for atopic eczema treatment.

#### 4. Discussion

Information regarding the role of miRNAs in allergic diseases is limited [30,31]. The present study demonstrates an essential immune regulatory function of miR-124 in the inflammatory skin of atopic eczema. miR-124 is downregulated in the lesional skin of patients with atopic eczema and inhibits the expression of p65 through direct binding to the 3'UTR of *RELA*, thus to suppress numerous proinflammatory factors, including IFN- $\gamma$ - or TNF- $\alpha$ -inducible genes IL-8, CCL5 and CCL8 in human primary keratinocytes. Through achieving p65 knockdown and miR-124 inhibition, we also revealed that miR-124-mediated suppression of the indicated chemokines occurs partially through regulation of p65 in keratinocytes. Our data together show that the expression of p65, IL-8, CCL5 and CCL8 is increased during skin inflammation in atopic eczema; thus rescuing miR-124 to reduce p65 expression and the production of the indicated chemokines helps to control chronic inflammatory processes triggered by IFN- $\gamma$  or TNF- $\alpha$  in keratinocytes.

MicroRNAs (miRNAs) are an abundant class of short (22 nucleotides), non-protein-coding RNAs that regulate the expression of protein-coding genes at the posttranscriptional level. Alteration of miRNA expression plays a role in various human disorders such as cancer [32–34], developmental abnormalities [35], cardiovascular disorders

[36] and schizophrenia [37]. MicroRNAs are also involved in the regulation of the normal development of the immune system [38,39] and in the pathogenesis of chronic inflammatory disorders [40]. Recently, a set of miRNAs were found to be dysregulated in atopic eczema, implying a general role for them in skin inflammation [41]. Lv et al. showed that serum miR-203 and miR-483-5p were significantly upregulated in children with atopic dermatitis compared with the control group, whereas miRNA-203 was significantly downregulated in urine from patients with atopic dermatitis [42]. In addition, miR-124 has been reported to reduce the activation of NF- $\kappa$ B in glioma [23]. MiR-124 directly controls Rac1 expression, thus to regulate p65 NF- $\kappa$ B phosphorylation and the TNF- $\alpha$  release from retinal microglial cells [27]. In the present study, we firstly demonstrated that miR-124 expression was significantly downregulated in atopic eczema lesional, compared to non-lesional tissues. Further, we observed that in response to a series of inflammatory factors, miR-124 expression was also downregulated, implying a potential association of miR-124 with skin inflammation in atopic eczema.

Keratinocytes express numerous pattern-recognition receptors, the stimulation of which leads to the activation of the NF- $\kappa$ B pathway. In B-cell lymphomas, miR-124 directly targets NF- $\kappa$ B (p65), and this interaction accounts for the miR-124-mediated suppression of MYC and BCL2 [43]. Moreover, an activated NF- $\kappa$ B-centered inflammatory loop in the highly aggressive non-small lung cancer cells has been reported to lead to down-regulation of miR-124 [44]. In the present study, we also observed an inverse regulation of p65 by miR-124. In keratinocytes, ectopic miR-124 significantly reduced p65 protein, whereas miR-124 inhibition increased p65 protein. In addition, p65 knockdown promoted miR-124 expression. By employing luciferase assays, we

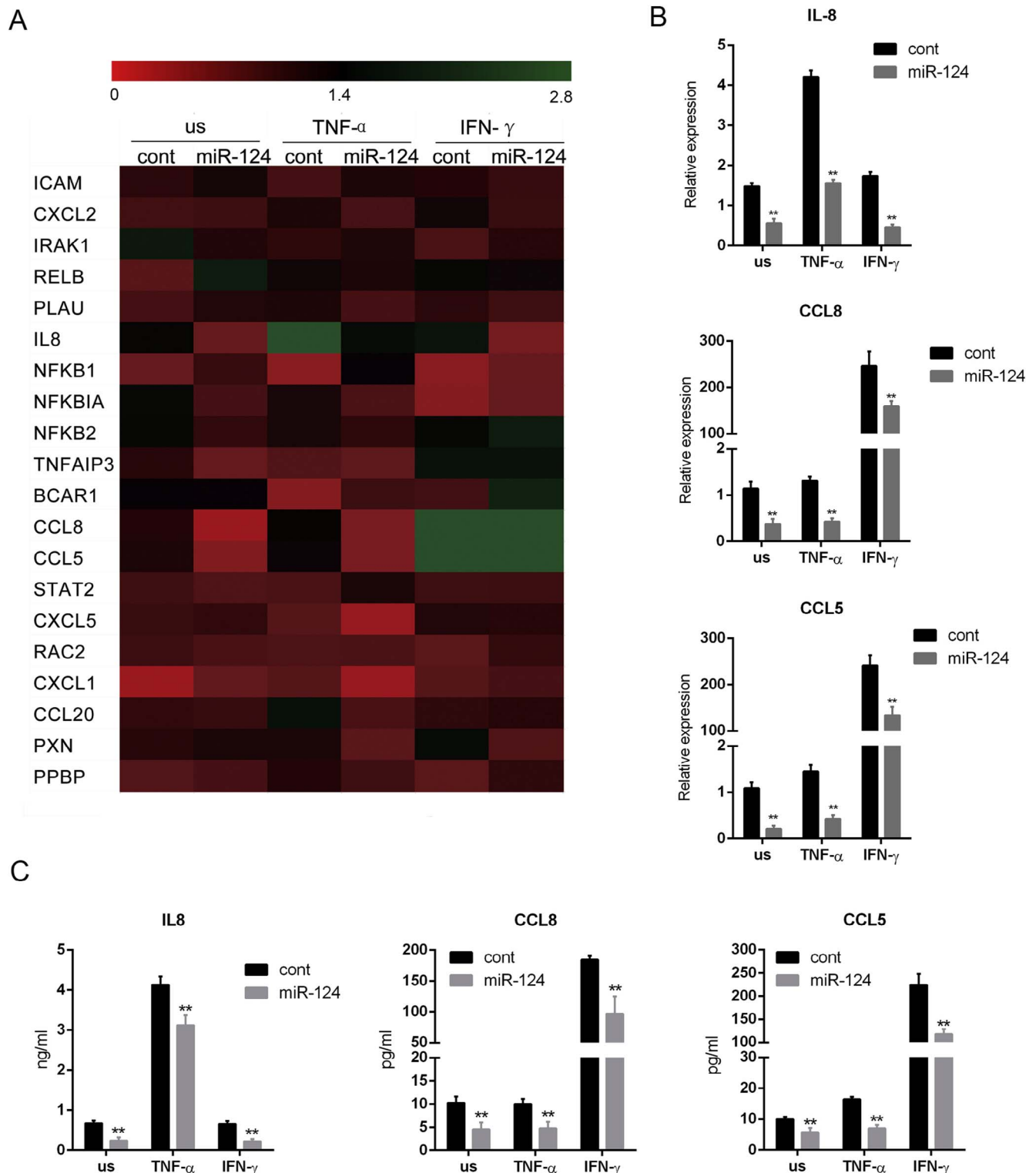


**Fig. 3.** Overexpression of miR-124 inhibits NF- $\kappa$ B-dependent genes in human primary keratinocytes. Keratinocytes were transfected either with control (cont) or miR-124 mimics (miR-124) for 24 h and then stimulated with IFN- $\gamma$  or TNF- $\alpha$  for 48 h or left un-stimulated (us). (A) The miR-124-affected genes from the selected signaling pathways were shown. Log2 values of mRNA expression signals are mean-centered for each gene separately. Color scale from red (lower) to green (higher) represents deviation from the mean (black). (B) The mRNA expression of IL-8, CCL5 and CCL8 was determined using real-time PCR assays. The data are presented as mean  $\pm$  SD of three independent experiments. \*\* $P$  < 0.01. (C) The protein levels of IL8, CCL5 and CCL8 were determined using ELISA assays. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

confirmed that miR-124 could directly bind to the 3'UTR of *RELA* to inhibit p65 expression in keratinocytes, which is consistent with previous studies. These further suggested the possible role of miR-124 in regulation of skin inflammation of atopic eczema.

Chronic skin inflammation in atopic eczema is affected by numerous factors, including secondary infections and activation of keratinocytes by TNF- $\alpha$  or IFN- $\gamma$  [45,46]. TNF- $\alpha$  or IFN- $\gamma$  also induces expression changes of thousands of genes in keratinocytes, including cytokines

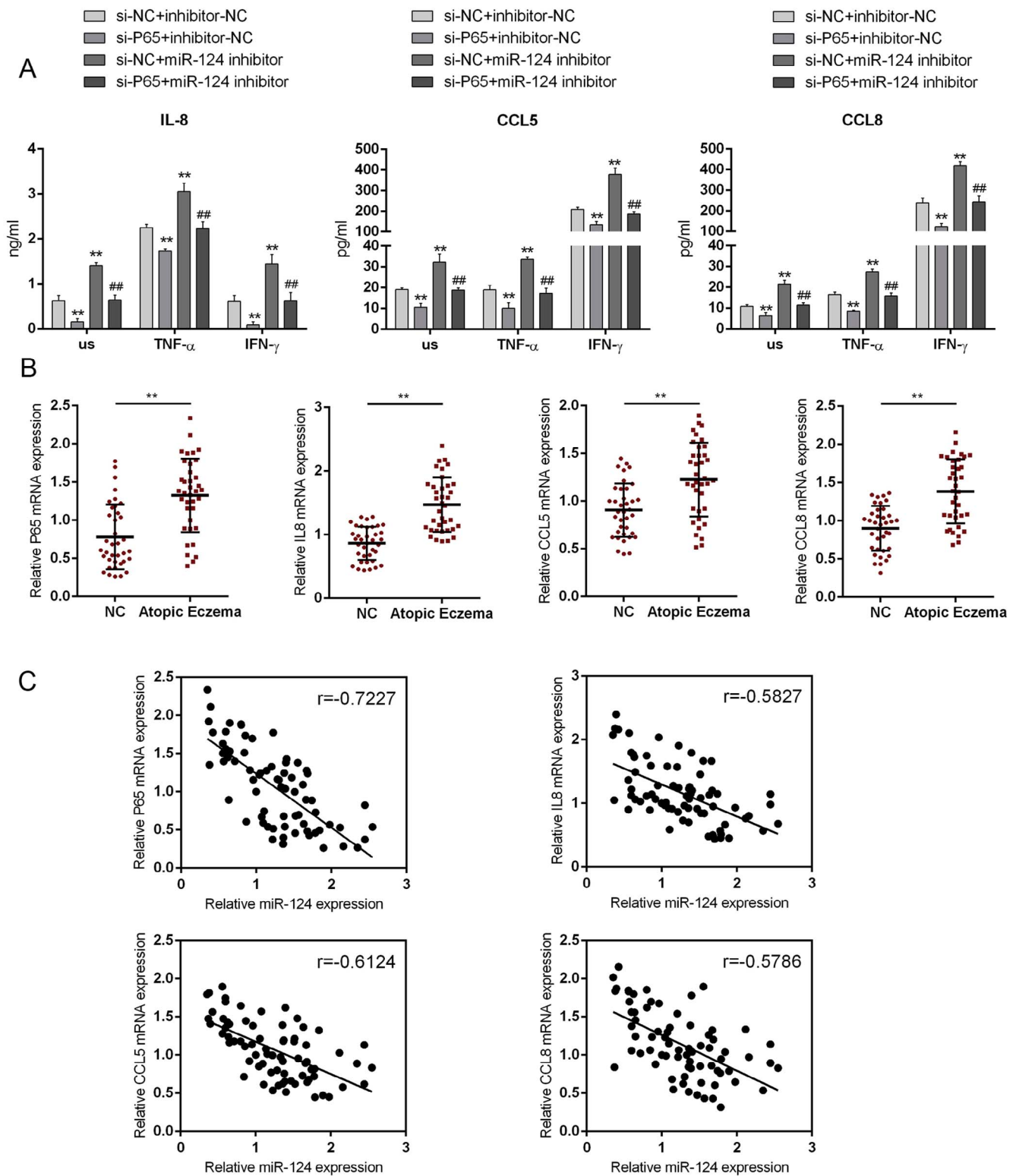


**Fig. 4.** MiR-124 could reverse the effect of p65 on downstream inflammatory factors. (A) Keratinocytes were co-transfected with si-p65 and miR-124 inhibitor; the protein levels of IL-8, CCL5 and CCL8 were determined using ELISA assays. (B) The mRNA expression of p65, IL-8, CCL5 and CCL8 in atopic eczema lesions and non-lesional tissues was determined using real-time PCR assays. The data are presented as mean  $\pm$  SD of three independent experiments. \*\* $P < 0.01$ . (C) The correlation between miR-124 and p65, IL-8, CCL5 and CCL8 in atopic eczema lesions was analyzed using Spearman's rank correlation analysis.

such as IL-2, IL-4, IL-15, CCL20, CCL5, CCL8, and so on [6,46]. To study the function of miR-124 in keratinocytes, we used stimulations with TNF- $\alpha$  and IFN- $\gamma$ . MiR-124 downregulated a large number of genes in TNF- $\alpha$ - or IFN- $\gamma$ -stimulated keratinocytes, pointing to a strong anti-inflammatory function of miR-124 in atopic eczema, among which IL-8,

CCL5 and CCL8 are associated with NF- $\kappa$ B signaling [47–49]. Consistent with previous studies, we further observed that in TNF- $\alpha$ - or IFN- $\gamma$ -stimulated keratinocytes, miR-124 overexpression significantly reduced both mRNA and protein levels of IL-8, CCL5 and CCL8. Since we demonstrated that miR-124 regulates p65 expression through direct

binding to the 3'UTR of RELA, here we further evaluated the combined effect of miR-124 and p65 on IL-8, CCL5 and CCL8 expression. The protein levels of IL-8, CCL5 and CCL8 were significantly reduced after p65 knockdown, whereas increased by miR-124 inhibition; the inhibitory effect of p65 on the indicated chemokines could be partially reversed by miR-124 inhibition. These data further indicated that miR-124 directly inhibits p65 expression, thus to reduce the production of IL-8, CCL5 and CCL8 in TNF- $\alpha$ - or IFN- $\gamma$ -stimulated keratinocytes.

Finally, we monitored the mRNA expression of p65, IL-8, CCL5 and CCL8 in lesional and non-lesional tissues in patients with atopic eczema. In lesional tissues, p65, IL-8, CCL5 and CCL8 mRNA showed significantly upregulated. Moreover, miR-124 expression was inversely correlated with p65, IL-8, CCL5 and CCL8 expression in atopic eczema lesional tissues, respectively. Taken together, we demonstrated that miR-124 controls NF- $\kappa$ B-dependent inflammatory responses in keratinocytes and chronic skin inflammation in atopic eczema; rescuing miR-124 expression presents a promising strategy for atopic eczema treatment.

## Acknowledgments

This work was supported by the National Natural Sciences Foundation of China (No: 81573985) and Chinese medicine research project of Hunan Province (No: 2016137 and 2016127).

## Author disclosure statement

There is no conflict of interest.

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