

miR-203 contributes to IL-17-induced VEGF secretion by targeting SOCS3 in keratinocytes

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Abstract. Interleukin (IL)-17 signaling serves an important role in the development and pathogenesis of psoriasis; a chronic skin disease characterized by increased dermal vascularity and the hyperproliferation of keratinocytes. microRNA (miR)-203 is preferentially expressed in the skin and is an important regulator of keratinocyte differentiation. miR-203 has been implicated in a number of skin diseases, including psoriasis. However, the role of miR-203 in IL-17-induced vascular endothelial growth factor (VEGF) secretion has yet to be elucidated. The present study demonstrated that miR-203 expression was upregulated in the ears of IL-17-stimulated mice and IL-17-treated HaCaT cells. In addition, the IL-17-induced increase in miR-203 expression activated the Janus kinase/signal transducer and activator of transcription signaling pathway and promoted VEGF secretion in HaCaT cells. Furthermore, miR-203 was observed to bind to the 3'-untranslated region of suppressor of cytokine signaling 3 (SOCS3) and inhibited SOCS3 expression. The results suggest that miR-203 expression may be upregulated by IL-17 stimulation, and miR-203 is a positive regulator of IL-17-induced VEGF secretion. The present study may support potential therapeutic strategies for the treatment of psoriasis.

Introduction

Psoriasis is a complex skin disease involving reciprocity between immune cells and keratinocytes, and is characterized by the infiltration of multiple inflammatory cells, upregulation dermal vascularity and keratinocyte proliferation (1-4). Angiogenesis commences with early psoriatic alterations and disappears when the disease is cured. A number of pro-angiogenic mediators, including vascular

endothelial growth factor (VEGF), tumor necrosis factor, hypoxia-inducible factor, interleukin (IL)-8, angiopoietins and IL-17, are upregulated during the development of psoriasis (5-8).

Initially, psoriasis was considered to be a T-helper (Th)1-mediated skin disease (9). However, a number of subsequent studies later demonstrated that Th17 cells, which are activated upon exposure to IL-23, IL-6 and transforming growth factor- β and produce IL-17, IL-21 and IL-22 cytokines, serves a key role in the pathogenesis of psoriasis (9-12). IL-17 is one of the most potent proinflammatory cytokines, and is secreted by Th17 cells (9). A previous study indicated that IL-17 may induce keratinocytes to produce VEGF, which is an important angiogenic mediator (13). Therefore, elucidation of the mechanisms by which IL-17 signaling in keratinocytes is regulated may facilitate the development of novel treatments for Th17-mediated angiogenesis.

The Janus kinase/signal transducer and activator of transcription signaling (JAK/STAT) signaling pathway controls a number of important biological responses, including cellular differentiation, immune functions, hematopoiesis and cellular growth, and it is initiated when a receptor is bound by its corresponding cytokine and subsequently transmits signals from the cell surface membrane to the target genes (14). A previous study confirmed that IL-17 induced upregulation of VEGF expression via activation of the JAK2/STAT3 signaling pathway (15). However, the precise mechanisms involved required further investigation. An increasing number of studies have indicated that microRNAs (miRNAs/miRs) maybe involved in the pathogenesis of psoriasis, and miRNAs associated with psoriasis have been identified by comparing their expression in normal and psoriatic skin samples (16,17). However, whether specific miRNAs influence the process of IL-17-induced VEGF expression remains unclear.

miR-203 is preferentially expressed in the skin and is an important regulator of keratinocyte differentiation. miR-203 has been implicated in a number of skin diseases, particularly psoriasis (18). However, the role of miR-203 in IL-17-induced VEGF secretion has yet to be elucidated. The aim of the present study was to investigate the role of miR-203 in IL-17-induced VEGF secretion to further elucidate the mechanism of miR-203 in psoriasis.

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Materials and methods

Animals and stimulation with IL-17. A total of 16 BALB/c mice (age, 6–8 weeks; weight, 18–22 g) were obtained from the Center of Experimental Animals of China Medical University (Shenyang, China). The mice were maintained in cages (4 mice/cage) under controlled conditions (temperature, 20–25°C; humidity 40–70%) with daily 12-h light/dark cycles. Mice were provided with food and water *ad libitum*. All animal experiments were approved by the Institutional Animal Ethics Committee of China Medical University, and were performed in accordance with the Animal Care Guidelines for Experimental Animals (19). Mice were divided into the IL-17 stimulated group and control group ($n=8$ mice/group), then the mice were injected intradermally into each ear with 30 μ l PBS, either alone or with 3 μ g recombinant mouse IL-17A (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) using a 30-gauge needle once a day for two consecutive days.

Immunohistochemical staining. Following 1 week, all mice were sacrificed and mouse ear tissues were fixed in 4% paraformaldehyde for 48 h at 4°C, and embedded in paraffin wax. Sections were cut at 4 μ m and mounted onto slides. The tissue sections were dewaxed in xylene, re-hydrated using a descending ethanol series, and subjected to antigen retrieval in 0.01 M citrate buffer. Following inhibition of endogenous peroxidase by incubating samples with 3% H₂O₂ at 37°C for 30 min, the sections were subsequently incubated with anti-VEGF (cat no. sc-80442; 1:200 dilution) and cluster of differentiation (CD)34 monoclonal antibodies (cat no. sc-74499; 1:200 dilution) (both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C. Following washing with PBS, tissue sections were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG1 secondary antibody (cat no. ab97240; 1:1,000 dilution; Abcam, Cambridge, MA, USA) at 37°C for 30 min. Reaction products were visualized by incubation with diaminobenzidine for 60 sec at room temperature and then counterstained with hematoxylin for 5 min at room temperature. As a negative control, tissue samples were subject to the same staining procedures without incubation with primary antibodies. Regions of positive staining were quantified by calculating the pixel density using analysis LS Research image analysis software v5.0 (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The mice were injected intradermally into each ear with 30 μ l PBS, either alone or with 3 μ g recombinant mouse IL-17A once a day for two consecutive days. Following 1 week, the ear tissues were collected and the expression of miR-203a-3p.1 in ear tissues was determined by RT-qPCR. Total RNA from ear tissues was extracted using TRIzol (Takara Biotechnology Co., Ltd., Dalian, China) following the manufacturer's instructions. Single strand cDNA was synthesized using the PrimeScript miRNA cDNA Synthesis kit (Takara Biotechnology Co., Ltd.) using 2 μ g of total RNA as a template according to the manufacturer's instructions. qPCR was performed using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. The thermocycling conditions were as follows:

95°C for 30 sec, followed by 45 cycles of 95°C for 5 sec and 53°C for 20 sec. The expression of U6 small nuclear B non-coding RNA was used as an internal normalization control. The primers used were as follows: miR-203a-3p.1 primer, 5'-TAC GAGTGAAATGTTTAGGACCACTAG-3'; U6, 5'-ATTGGA ACGATACAGAGAAGATT-3'.

HaCaT cells were treated with medium only or with IL-17 for 48 h, in the presence or absence of miR-203 inhibitor for 24 h. The expression of suppressor of cytokine signaling 3 (SOCS3) in HaCaT cells was then determined by RT-qPCR. cDNA was synthesized using the PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd.) using 2 μ g of total RNA as a template according to the manufacturer's instructions. qPCR was performed using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. The following thermocycling conditions were used: 95°C for 30 sec, followed by 45 cycles of 95°C for 5 sec and 60°C for 20 sec. β -actin was used to normalize the expression levels of SOCS3. The primers used were as follows: SOCS3 forward, 5'-TGGATGGAGCGGGAGGCT-3', and reverse, 5'-ACGGACATCTTTCACCTCAGGCTCCT-3'; β -actin forward, 5'-GACAGGATGCAGAAGGAGATTACT-3' and reverse, 5'-TGATCCACATCTGCTGGAAGGT-3'. All RT-qPCR experiments were performed in triplicate using the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All primers were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Samples were analyzed in triplicate, and the mean quantification cycle (C_q) was calculated. Gene expression levels were analyzed by comparing the ΔC_q values of samples [where $\Delta C_q = C_q(\text{target gene}) - C_q(\text{housekeeping gene})$] transformed to a linear scale ($2^{-\Delta\Delta C_q}$) (20).

Cell culture. The human umbilical vein endothelial cells (HUVECs) cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin, and maintained in a humidified atmosphere of 5% CO₂ at 37°C. The human keratinocyte cell lines, HaCaT and HEK293T (both from ATCC), were incubated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin, and maintained in a humidified atmosphere of 5% CO₂ at 37°C.

Cell transfection. HaCaT cells were seeded in 6-well plates (2×10^5 /well) for 24 h prior to transfection. Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect HaCaT cells with 50 nmol miR-203 mimic, 150 nmol miR-203 inhibitor or the same concentration of their respective negative controls (Guangzhou RiboBio Co., Ltd.), according to the manufacturer's instructions. The sequence of miR-203 mimic, miR-203 inhibitor and their respective negative controls were as follows: miR-203a-3p.1 mimic, 5'-GUGAAAUGUUUAGGACCACUAG-3' and 5'-AGUGGUCCUAAACAUUUCACUU-3'; miR-203a-3p.1 mimic control, 5'-UUCUCCCAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3'; miR-203a-3p.1

inhibitor, 5'-CUAGUGGUCCUAACA AUUCAC-3'; miR-203a-3p.1 inhibitor control, 5'-CAGUACUUUGU GUAGUACAA-3'. HaCaT cells were co-transfected with 150 nmol SOCS3 small interfering (si)RNA or scrambled siRNA (Shanghai GenePharma Co., Ltd., Shanghai, China) and 150 nmol miR-203 inhibitor using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). At 24 h following transfection, HaCaT cells were treated with 80 ng/ml IL-17 (PeproTech, Inc., Rocky Hill, NJ, USA) for 48 h at 37°C, then cells (~1.5x10⁶/well) were harvested for analysis. HUVECs were treated with conditioned HaCaT cell medium only at 37°C for 7 h.

Luciferase reporter assays. For luciferase activity analysis, the 3'-UTR sequence of SOCS3 or the mutant SOCS3 3'-UTR sequence, which included a mutation in the miR-203 binding site, was cloned into a pGL3-promoter vector (Promega Corporation, Madison, WI, USA). Then HEK293T cells were co-transfected with 500 ng wild-type pGL3-SOCS3-3'UTR or mutant pGL3-SOCS3-3'UTR and 10 pmol miR-203 mimic or 10 pmol miRNA control using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). At 24 h following transfection, luciferase activity was detected using a dual-luciferase reporter assay system according to the manufacturer's instructions (Promega Corporation). Luciferase activity was normalized to *Renilla* luciferase activity.

Computational prediction. The target gene of miR-203 was predicted using TargetScan Release 7.1 software (www.targetscan.org/vert_71/).

Western blot analysis. HaCaT cells treated with medium alone or with IL-17 for 48 h, in the presence or absence of miR-203 inhibitor for 24 h. Total protein was extracted using a protein extraction kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. Protein concentration was determined using a bicinchoninic protein assay kit (Beyotime Institute of Biotechnology). For western blotting analysis, an equal quantity of total protein (30 µg/lane) was loaded, and separated by 8% SDS-PAGE. Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane and blocked for 1 h at room temperature with 5% milk in TBS with 0.1% Tween-20. The membrane was incubated with primary antibodies against the IL-17 receptor (cat no. ab180904; 1:1,000 dilution; Abcam), phosphorylated (p)-JAK2 (cat no. 4406; 1:1,000 dilution), p-STAT3 (cat no. 9145; 1:1,000 dilution), β-actin (cat no. 4970; 1:1,000 dilution), SOCS1 (cat no. 3950; 1:1,000 dilution) and SOCS3 (cat no. 2932; 1:1,000 dilution) (all from Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight. The blots were subsequently incubated with a horseradish peroxidase-conjugated secondary antibody (cat no. E030120-02; 1:2,000 dilution; EarthOx Life Sciences, Millbrae, CA, USA) for 1 h at room temperature. Proteins were visualized using Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. Densitometry analysis of the western blots was achieved using ImageJ v1.48 software (National Institutes of Health, Bethesda, MD, USA).

Tube formation assay. The tube formation assay was performed as described previously (21). Briefly, a 96-well plate was coated with 60 µl Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), which was allowed to polymerize and solidify at 37°C for 30 min. HUVECs (2x10⁴ cells) were seeded onto the Matrigel layer in the presence or absence of conditioned medium from HaCaT cells treated with IL-17 (80 ng/ml for 48 h), miR-203 inhibitor (150 nmol for 24 h) or inhibitor control (150 nmol for 24 h), and SOCS3-siRNA (150 nmol for 24 h) or siRNA-control (150 nmol for 24 h), and cells were incubated at 37°C for 7 h. The number of blood-vessel-like tubules from six fields of view selected at random were counted, and images were captured using an inverted light microscope (Nikon Corporation, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA). HaCaT cells were treated with IL-17 (80 ng/ml for 48 h), miR-203 inhibitor (150 nmol for 24 h) or inhibitor control (150 nmol for 24 h), and SOCS3-siRNA (150 nmol for 24 h) or siRNA-control (150 nmol for 24 h). The cell culture media were centrifuged at 1,500 x g for 10 min at 4°C, the supernatants were collected and stored at -80°C prior to ELISA analysis. The level of VEGF secretion in HaCaT cell cultures were measured by an ELISA kit (cat no. DVE00; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using SPSS v13.0 software (SPSS, Inc., Chicago, IL, USA). The results were analyzed by one-way analysis of variance followed by a Student-Newman-Keuls post hoc test, and presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated three times.

Results

miR-203 is upregulated in the ears of IL-17-stimulated mice and IL-17-treated HaCaT cells. In order to investigate the effect of miR-203 on IL-17-induced VEGF expression, miR-203, VEGF and CD34 expression in the ears of untreated and IL-17-stimulated mice were first examined. The results demonstrated that the expression of miR-203, VEGF and CD34 were significantly upregulated in the ears of IL-17-stimulated mice compared with the normal untreated group (Fig. 1A-C). The expression of miR-203 and VEGF secretion in IL-17-stimulated HaCaT cells was then investigated. Consistent with the *in vivo* results, miR-203 expression and VEGF secretion levels were significantly increased in HaCaT cells stimulated with IL-17 (Fig. 1D and E).

Suppression of miR-203 inhibits IL-17-induced VEGF secretion in HaCaT cells. To explore the role of miR-203 in IL-17-induced VEGF secretion, an miR-203 inhibitor was transfected into the HaCaT cells prior to stimulation with IL-17. As demonstrated in Fig. 2A, transfection of HaCaT cells with the miR-203 inhibitor was associated with a significant reduction in relative miR-203 expression levels when compared with cells transfected with the inhibitor control. IL-17-stimulation was associated with a significant increase in VEGF mRNA and the levels of VEGF in the supernatant of HaCaT cells,

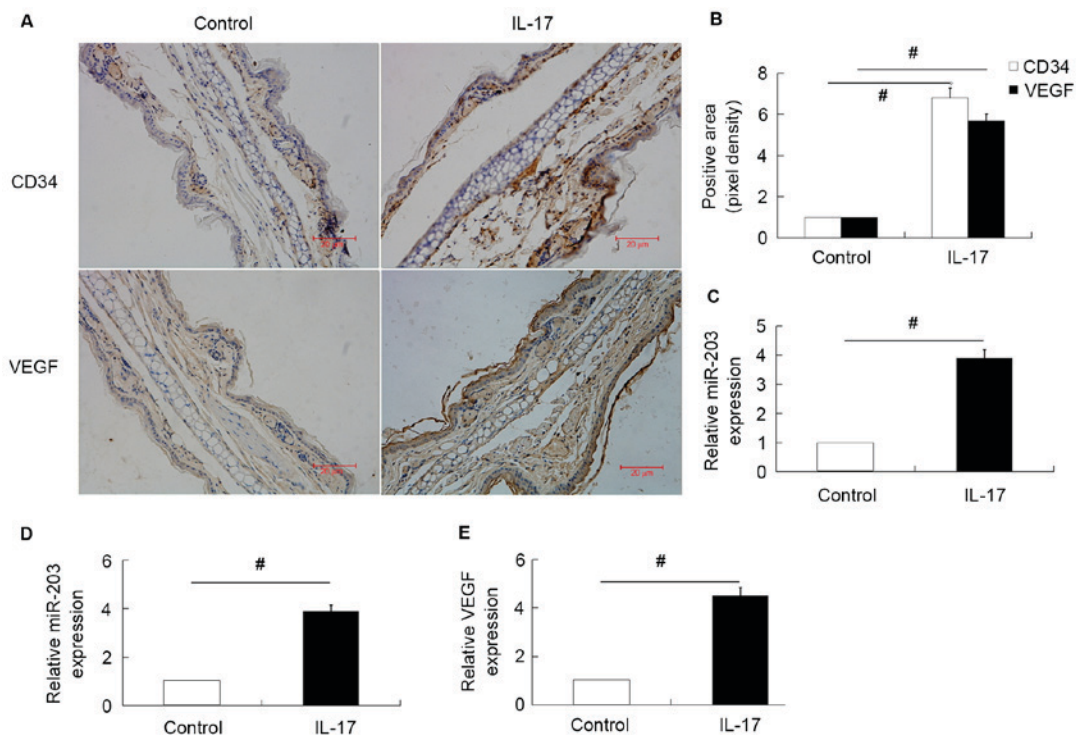


Figure 1. miR-203 is upregulated in the ears of IL-17-stimulated mice and IL-17-treated HaCaT cells. A total of 3 μ g recombinant mouse IL-17A was intradermally injected into the ears of mice for two consecutive days. (A) Ear tissues were collected at 24 h following the final administration of IL-17 and the expression of CD34 and VEGF in normal untreated mice and IL-17-stimulated mice was determined by immunohistochemistry (scale bar, 20 μ m) and (B) quantified. (C) The relative expression of miR-203 in the ear tissues of normal untreated and IL-17-stimulated mice was determined by RT-qPCR. The results are presented as the mean \pm standard deviation (n=8). (D) HaCaT cells were treated with 80 ng/ml IL-17 for 48 h, and the relative expression level of miR-203 in normal and IL-17-treated HaCaT cells was determined by RT-qPCR. The expression of miR-203 was normalized to that of U6. (E) VEGF secretion level in normal and IL-17-treated HaCaT cells was determined by enzyme-linked immunosorbent assay analysis. The results are presented as the mean \pm standard deviation (n=3). $^{\#}P<0.05$ as indicated. miR, microRNA; IL, interleukin; CD, cluster of differentiation; VEGF, vascular endothelial growth factor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

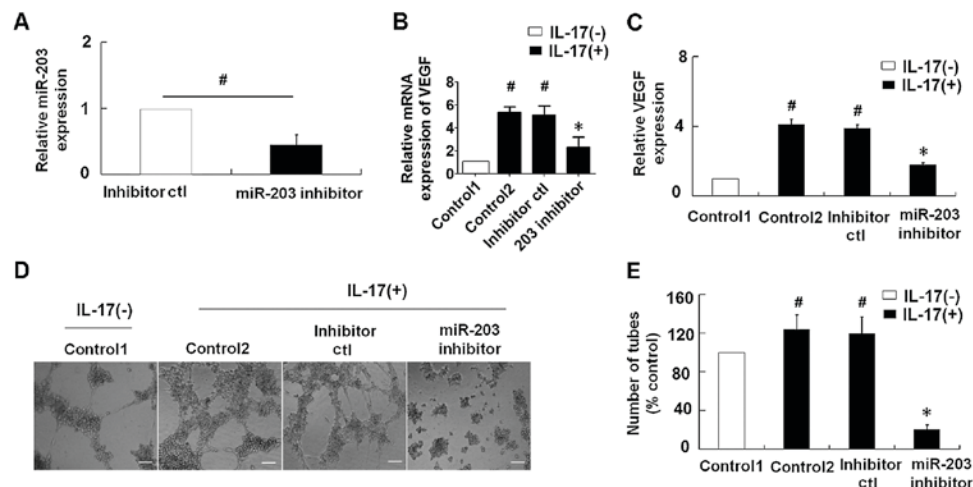


Figure 2. Suppression of miR-203 inhibits IL-17-induced VEGF secretion in HaCaT cells. Relative expression of (A) miR-203 and (B) VEGF mRNA in HaCaT cells transfected with an miR-203 inhibitor or an inhibitor ctl at 48 h following IL-17 stimulation, as determined by reverse transcription-quantitative polymerase chain reaction analysis. U6 and β -actin were used as endogenous controls for miR-203 and VEGF expression, respectively. (C) VEGF levels in the supernatant of HaCaT cells were determined using an enzyme-linked immunosorbent assay. HUVECs were seeded onto a Matrigel layer in the presence of conditioned medium from different groups of HaCaT cells for 7 h. (D) Blood-vessel-like tubules from six fields of view selected at random were visualized under an inverted light microscope (scale bar, 100 μ m) and (E) counted. The control1 group consisted of untransfected HaCaT cells that were not treated with IL-17, whereas the control2 group consisted of untransfected HaCaT cells that were treated with IL-17. The results are presented as the mean \pm standard deviation. All experiments were repeated three times. $^{\#}P<0.05$ vs. control1; $^*P<0.05$ vs. control2. miR, microRNA; IL, interleukin; VEGF, vascular endothelial growth factor; ctl, control.

while repression of miR-203 significantly inhibited the IL-17-induced upregulation of VEGF levels (Fig. 2B and C). In

addition, the effect of miR-203 on IL-17-induced VEGF secretion was assessed using a tube formation assay in HUVECs.

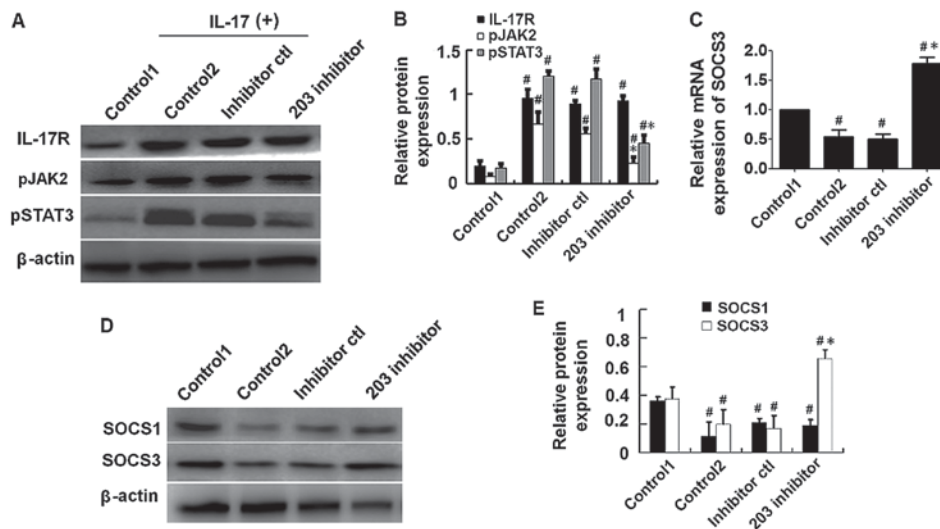


Figure 3. Repression of miR-203 inhibited IL-17-induced activation of the JAK2/STAT3 signaling pathway. HaCaT cells were transfected with miR-203 inhibitor for 24 h and subsequently treated with 80 ng/ml IL-17 for a further 48 h. (A) Western blot analysis of the protein expression levels of IL-17R, pJAK2 and pSTAT3, and (B) quantification of the results by densitometry analysis using Image J software. (C) The mRNA levels of SOCS3 were measured by reverse transcription-quantitative polymerase chain reaction analysis, and β -actin was used as an endogenous control. (D) Western blot analysis of the protein expression levels of SOCS1 and SOCS3 inhibitors of the JAK2/STAT3 signaling pathway, and (E) quantification of the results by densitometry analysis using ImageJ software. Target protein levels were normalized to β -actin. The control1 group consisted of untransfected HaCaT cells that were not treated with IL-17, whereas the control2 group consisted of untransfected HaCaT cells that were treated with IL-17. The results are presented as the mean \pm standard deviation ($n=3$). * $P<0.05$ vs. control1; ** $P<0.05$ vs. control2. miR, microRNA; IL, interleukin; JAK, Janus kinase; STAT, signal transducer and activator of transcription; SOCS, suppressor of cytokine signaling; p-, phosphorylated; R, receptor.

Consistent with the results observed in HaCaT cells, suppression of miR-203 significantly attenuated the IL-17-induced upregulation of VEGF levels and significantly inhibited tube formation of HUVECs (Fig. 2B-E).

Repression of miR-203 inhibits IL-17-induced activation of the JAK2/STAT3 signaling pathway. In order to explore the effect of miR-203 on IL-17-induced activation of the JAK2/STAT3 signaling pathway, HaCaT cells were transfected with miR-203 inhibitor or controls for 24 h, then treated with 80 ng/ml IL-17 for a further 48 h. Protein expression levels were subsequently detected by western blot analysis. The results demonstrated that the expression levels of p-JAK2 and p-STAT3 were significantly reduced in miR-203 inhibitor-transfected cells when compared with IL-17-stimulated and inhibitor control-transfected cells (Fig. 3A and B). The next aim was to identify targets of miR-203. As demonstrated in (Fig. 3C-E), the mRNA and protein expression levels of SOCS3, an inhibitor of the JAK2/STAT3 signaling pathway, was significantly upregulated in miR-203 inhibitor-transfected cells when compared with IL-17-stimulated and inhibitor control-transfected cells. By contrast, western blot analysis demonstrated that the protein expression levels of SOCS1, an additional inhibitor of the JAK2/STAT3 signaling pathway, were not significantly affected by miR-203 inhibition (Fig. 3D and E). These results suggest that miR-203 may inhibit JAK2/STAT3 signaling via targeting of SOCS3 expression.

miR-203 directly targets SOCS3. To assess the role of miR-203 in regulating the JAK2/STAT3 signaling pathway, computational analysis (TargetScan v7.1) was performed to predict the potential target genes of miR-203. The results indicated

that miR-203 may target sequences in 3'-untranslated region (UTR) of SOCS3 (Fig. 4A). The 3'-UTR sequence of SOCS3 was then cloned into a luciferase reporter plasmid. A mutant SOCS3 3'-UTR sequence, which included a mutation in the miR-203 binding site, was additionally cloned into the luciferase reporter plasmid (Fig. 4A). The dual-luciferase reporter assay was performed using HEK293T cells. The results demonstrated that the luciferase activity in HEK293T cells co-transfected with an miR-203 mimic and a luciferase reporter plasmid containing the wild type SOCS3 3'-UTR sequence was significantly reduced when compared with the mimic control (Fig. 4B). By contrast, no significant alterations in luciferase activity were observed HEK293T cells transfected with miR-203 mimic and a luciferase reporter plasmid containing the mutant SOCS3 3'-UTR sequence (Fig. 4B). In addition, transfection of HaCaT cells with miR-203 mimics was associated with a significant reduction in the mRNA and protein expression levels of SOCS3 (Fig. 4C-E). These results provide evidence to suggest that SOCS3 may be a direct target of miR-203.

Repression of miR-203 attenuates IL-17-induced VEGF secretion in HaCaT cells via targeting SOCS3. To further confirm the role of SOCS3 in miR-203-mediated VEGF secretion in response to IL-17 stimulation, HaCaT cells were co-transfected with an miR-203 inhibitor together with an siRNA targeting SOCS3, and then stimulated with IL-17. The results demonstrated that treatment of cells with the SOCS3 siRNA significantly attenuated the effects of the miR-203 inhibitor on the IL-17-induced upregulation of VEGF levels and increase in tube formation (Fig. 5). These results suggest that IL-17 may induce miR-203 expression, which may subsequently increase VEGF secretion via targeting of SOCS3.

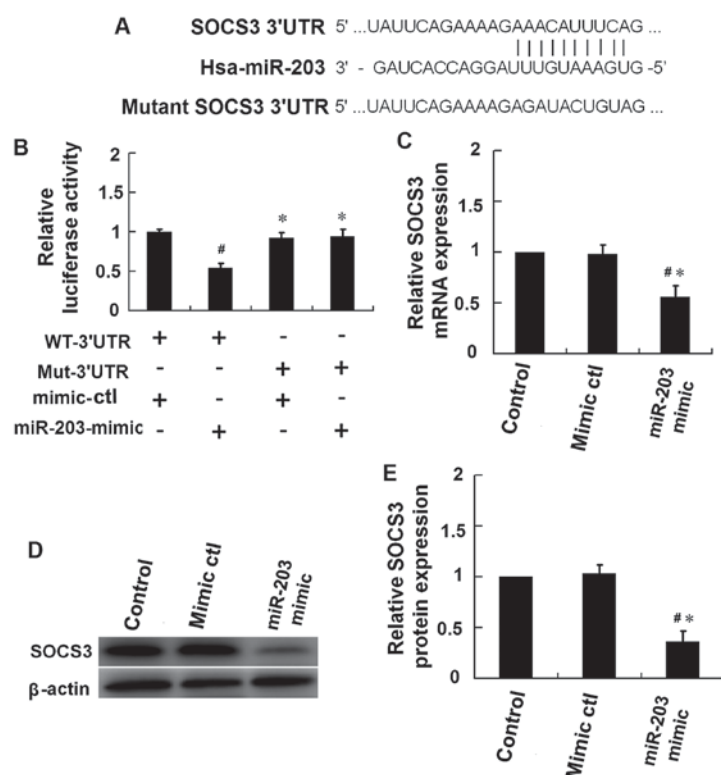


Figure 4. SOCS3 is the molecular target of miR-203. (A) Schematic of the predicted miR-203 binding site in the 3'-UTR region of SOCS3, as detected using TargetScan v7.1 software. The mutant SOCS3 3'-UTR sequence included several mutations in the miR-203 binding site. (B) A dual-luciferase reporter assay was performed using HEK293T cells. Cells were co-transfected with a reporter vector containing the WT-3'UTR or the Mut-3'UTR, in addition to a miR-203-mimic or a mimic-ctl. The firefly/*Renilla* activity ratio was calculated to determine the luciferase activity ($^{\#}P < 0.05$ vs. HaCaT cells transfected with WT-3'UTR plus mimic-ctl; $^{*}P < 0.05$ vs. HaCaT cells transfected with WT-3'UTR plus miR-203 mimic). Following 48 h of IL-17 stimulation, HaCaT cells were then transfected with an miR-203 mimic or mimic-ctl, and cells were harvested at 24 h following transfection. The (C) mRNA and (D) protein levels of SOCS3 were measured by western blotting and reverse transcription-quantitative polymerase chain reaction analysis, respectively, and (E) densitometry analysis of the western blots was performed using ImageJ software. SOCS3 mRNA and protein expression levels were normalized to that of β -actin ($^{\#}P < 0.05$ vs. control; $^{*}P < 0.05$ vs. mimic-ctl). The results are presented as the mean \pm standard deviation ($n=3$). SOCS, suppressor of cytokine signaling; miR, microRNA; UTR, untranslated region; WT-3'UTR, pGL3 vector containing the wild-type SOCS3 3'-UTR; Mut-3'UTR, pGL3 vector containing the mutant SOCS3 3'-UTR; ctl, control.

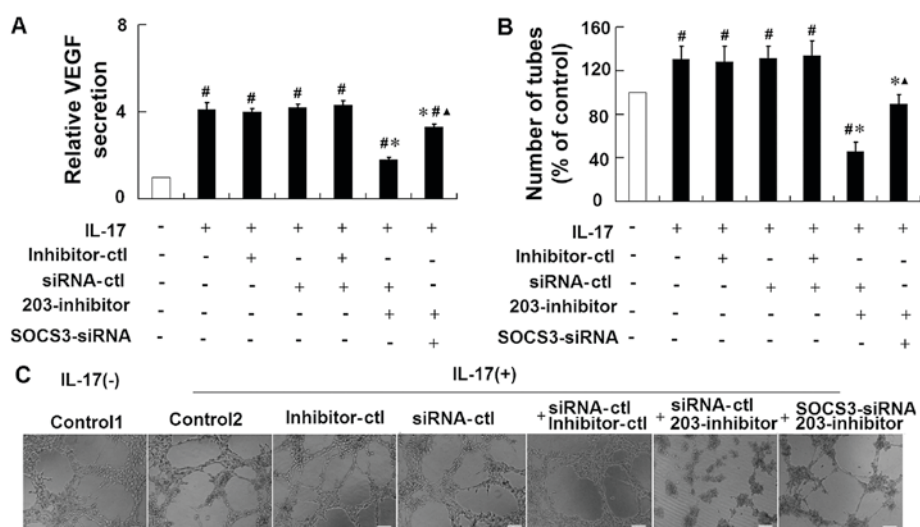


Figure 5. Repression of miR-203 attenuates IL-17-induced VEGF secretion in HaCaT cells via targeting of SOCS3. (A) VEGF secretion by HaCaT cells transfected with miR-203 inhibitor alone or in combination with SOCS3 siRNA at 48 h following IL-17 stimulation, as determined by enzyme-linked immunosorbent assay analysis. The level of VEGF secretion in treated cells was normalized to that of untransfected cells that were not treated with IL-17. HUVECs were then seeded onto a Matrigel layer in the presence of conditioned medium from HaCaT cells from different groups for 7 h. (B) Blood-vessel-like tubules from six fields of view selected at random were counted and (C) photographed under an inverted light microscope (scale bar, 100 μ m). The results are presented as the mean \pm standard deviation. All experiments were repeated three times. $^{\#}P < 0.05$ vs. untreated cells; $^{*}P < 0.05$ vs. IL-17-only treated cells; $^{\Delta}P < 0.05$ vs. IL-17-treated cells transfected with siRNA ctl and a miR-203 inhibitor. miR, microRNA; IL, interleukin; VEGF, vascular endothelial growth factor; SOCS, suppressor of cytokine signaling; siRNA, small interfering RNA; HUVEC, human umbilical vein endothelial cells; ctl, control.

Discussion

The results of the present study demonstrated that miR-203 expression is significantly upregulated in mice and HaCaT cells stimulated with IL-17. In addition, VEGF levels were observed to increase in the ears of IL-17-stimulated mice and in the supernatant of IL-17-treated HaCaT cells. These results suggest that IL-17 may induce VEGF expression, and that miR-203 may be involved in mediating this effect. In addition, the results indicated that inhibition of miR-203 reversed the IL-17-induced increase in VEGF secretion and inhibited the IL-17-induced activation of JAK2/STAT3 signaling. Furthermore, the present study provided evidence to suggest that SOCS3, a repressor of the JAK2/STAT3 signaling pathway, may be a direct target of miR-203.

miRNAs are a class of abundant non-coding RNA molecules that modulate mRNA degradation or inhibition of translation by specifically binding to the 3'-UTR of target mRNA sequences (22-24). miRNAs serve key roles in the regulation of cell differentiation, growth and death in normal and malignant tissues (25,26). miR-203 is a miRNA preferentially expressed in the skin, and is an important regulator of keratinocyte differentiation (18). miR-203 has been implicated in a number of skin diseases, particularly psoriasis, via regulation of pro-inflammatory cytokines (27,28). However, a complete understanding of the mechanisms underlying the involvement of miR-203 in psoriasis is lacking. The results of the present study demonstrated that miR-203 expression is significantly upregulated in mice and HaCaT cells stimulated with IL-17. In addition, VEGF levels were increased in the ears of IL-17-stimulated mice and in the supernatant of IL-17-treated HaCaT cells. These results implied that miR-203 may be involved in IL-17-induced VEGF expression. In addition, the results of the present study demonstrated that miR-203 may function as a positive effector of IL-17-induced VEGF secretion, as inhibition of miR-203 effectively reversed IL-17-induced VEGF secretion.

Further investigation of the mechanisms by which miR-203 functions to mediate the IL-17-induced increase in VEGF secretion demonstrated that SOCS3 may be a direct target of miR-203 via binding to its 3'-UTR, thus inhibiting SOCS3 expression. An inverse association between miR-203 and SOCS3 expression in HaCaT cells stimulated by IL-17 was observed. These results suggest that SOCS3 may be a direct target of miR-203, and potentially mediate the process of IL-17-induced VEGF expression in HaCaT cells.

In order to prevent the adverse effects of over-activation, the duration and intensity of JAK/STAT signaling pathway activation is strictly controlled by negative regulators (29). SOCS proteins are induced by growth factors or cytokines, and regulate the duration and magnitude of inflammatory responses activated by these cytokines via the inhibition of JAK proteins in a negative feedback loop (30). Previous studies have demonstrated that SOCS1 and SOCS3 proteins are the major regulators of the JAK2/STAT3 signaling pathway (31-33). The results of the present study demonstrated that SOCS1 and SOCS3 expression was significantly down-regulated in HaCaT cells following stimulation with IL-17. However, only SOCS3 protein expression was upregulated

following inhibition of miR-203 in IL-17-treated HaCaT cells. This provides additional evidence to suggest that SOCS3 may be a target of miR-203.

Angiogenesis is an important pathological feature of psoriasis, and the expression of a number of angiogenic mediators, including VEGF, increase during the development of psoriasis (34,35). The present study demonstrated that IL-17 induces the expression of VEGF *in vitro* and *in vivo*, which is consistent with the results of previous studies (13,15). Notably, suppression of miR-203 reversed IL-17-induced VEGF expression in HaCaT cells. These results are consistent with the observed upregulation of SOCS3 expression and the reduction in JAK/STAT signalling pathway activation in the IL-17 signaling process following inhibition of miR-203, which indicated that miR-203 may be involved in mediating IL-17-induced VEGF expression.

In conclusion, the results of the current study suggest that miR-203 may inhibit the expression of SOCS3 by directly binding to the 3'-UTR of SOCS3 and promoting the degradation of the SOCS3 mRNA, thus activating the JAK2/STAT3 signaling pathway and mediating IL-17-induced VEGF secretion. Future studies that aim to investigate the role of miR-203 further, may provide novel insights into its mechanisms of action, as well as identify potential therapeutic strategies for the treatment of psoriasis.

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