'Psoriasis 1' reduces psoriasis-like skin inflammation by inhibiting the VDR-mediated nuclear NF-κB and STAT signaling pathways

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Abstract. 'Psoriasis 1', a Chinese herbal medicine (CHM) formulation, is extensively used to treat psoriasis in China. Although this CHM formulation yields good therapeutic effect, the underlying mechanism of how this works remains unknown. The present study aimed to test the hypothesis that the CHM formulation 'psoriasis 1' inhibits vitamin D receptor (VDR)-mediated inflammation in psoriasis. To test this, a model of psoriasis was established by stimulating keratinocytes (HaCaT cells) with tumor necrosis factor (TNF)-α; these cells were subsequently transfected with a lentiviral VDR RNA interference expression vector. The expression levels of 25-hydroxyvitamin D3 (25HVD3), TNF-α, interleukin (IL)-4, IL-1, IL-17C, IL-23 and IL-6 were measured using ELISA, and the expression levels of VDR, inhibitor of nuclear factor (NF)-κB (IKK), NF-κB, signal transducer and activator of transcription (STAT) 3 and STAT4 were measured using reverse transcription-quantitative polymerase chain reaction analysis and western blotting. It was observed that 'psoriasis 1' downregulated the concentrations of TNF-α, IFN-γ, IL-22, IL-17C, IL-1β and IL-4, and upregulated the concentration of 25HVD3; furthermore, 'psoriasis 1' downregulated the expression levels of NF-κB, phosphorylated (p)-NF-κB, IKK, p-IKK, STAT3, p-STAT3, STAT4 and p-STAT4, and upregulated the expression level of VDR in TNF-α-induced HaCaT cells. These results suggested that 'psoriasis 1' suppressed the inflammatory response and the activation of the NF-κB and STAT signaling pathways. In addition, it was identified that silencing VDR expression decreased the levels of TNF-α, IFN-γ, IL-22, IL-17C, IL-1β and IL-4, and increased the level of 25HVD3; silencing VDR expression additionally downregulated the expression levels of NF-κB, p-NF-κB, IKK, p-IKK, STAT3, p-STAT3, STAT4 and p-STAT4, and upregulated the level of VDR in TNF-α-induced HaCaT cells. It was concluded that 'psoriasis 1' exerts inflammation-suppressive effects in psoriasis by suppressing the NF-κB and STAT signaling pathways.

Introduction

Psoriasis is a common autoimmune disease affecting ~3% of the global population (1). Psoriasis is characterized by the abnormal proliferation of epidermal cells and the infiltration of inflammatory cells (2,3). Patients with psoriasis frequently suffer from disfigurement and complications, including painful arthritis, cardiovascular disease and metabolic syndrome (4,5). Although the pathophysiology of psoriasis remains unclear, increasing evidence suggests that the imbalance between pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-22 and IL-17C, and anti-inflammatory mediators, including IL-10, contributes to the underlying disease etiology (6). Nuclear factor-κB (NF-κB) (7) and signal transducers and activators of transcription (STAT) (8) are thought to be the principal effectors that produce a large number of pro-inflammatory cytokines, including TNF-α, IL-22, interferon-γ (IFN-γ) and IL-1. In addition, a previous study reported that the NF-κB and STAT signaling pathways are involved in the development and progression of psoriasis (9). Currently, few effective therapeutic strategies exist, in part due to the high recurrence

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rate of the disease. Furthermore, the majority of the current anti-psoriatic drugs are associated with serious side effects.

Traditional Chinese medicine (TCM), practiced in China for centuries, is considered to be an alternative medical system in Western countries (10,11). According to TCM, psoriasis is characterized by three predominant syndromes: Blood heat, blood stasis and blood dryness (12,13). Different prescriptions of Chinese herbal medicine (CHM) with few side effects are routinely prescribed in China (13,14). For example, for the past few decades, the CHM formulation 'psoriasis 1', which comprises 13 Chinese herbs, has successfully treated a large number of patients with psoriasis in hospitals in China (15). The majority of patients exhibit an improvement in skin lesions following application of 'psoriasis 1', although the underlying molecular mechanism of action this formulation in psoriasis is unknown (16,17).

In the present study, HaCaT cells were stimulated with TNF-α and transfected with a lentiviral vitamin D receptor (VDR) RNA interference (RNAi) expression vector. The effect of 'psoriasis 1' on psoriasis with or without *Tripterygium wilfordii* polyglycoside (TWP) was investigated. The impact of VDR inhibition on the expression levels of cytokines, and NF-κB and STAT signaling pathway components was additionally observed. It was demonstrated that 'psoriasis 1' and combined with the inhibition of VDR decreased the concentrations of TNF-α, IFN-γ, IL-22, IL-17C, IL-1β and IL-4, and increased the concentration of 25-hydroxyvitamin D3 (25HVD3). Furthermore, this treatment downregulated the expression levels of NF-κB, phosphorylated (p)-NF-κB, inhibitor of NF-κB (IKK), p-IKK, STAT3, p-STAT3, STAT4 and p-STAT4, and upregulated the expression of VDR in TNF-α-induced HaCaT cells. It was observed that 'psoriasis 1' and silencing of VDR suppressed the inflammatory response, and the activation of the NF-κB and STAT signaling pathways. Therefore, it was hypothesized that 'psoriasis 1' may alleviate psoriasis-like skin inflammation by inhibiting the VDR-mediated nuclear NF-κB and STAT signaling pathways.

Materials and methods

Components of the 'psoriasis 1' formulation. 'Psoriasis 1' was provided by The First Affiliated Hospital of Guangzhou University (Guangzhou, China), and was comprised of *rhizoma Smilacis glabrae* (30 g), *Folium isatidis* (30 g), *radix isatidis* (15 g), *Angelica sinensis* (15 g), *Hedyotis diffusa* (15 g), Sichuan lovage rhizome (12 g), plantain herb (12 g), *fructus kochiae* (12 g), Chinese lobelia (15 g), *nidus vespae*, *rhizoma alismatis* (12 g), *cortex dictamni* (12 g) and *radix glycyrrhizae* (6 g). In addition, TWP (Fujian Huitian Biological Pharmaceutical Co., Ltd., Sanming, China; 10 mg/tablet) was used as a positive control.

Preparation of the serum containing 'psoriasis 1'. Specific pathogen free level Sprague-Dawley male rats were purchased and raised at Guangzhou University of Chinese Medicine, Guangzhou, China (license no. SCXK 20130020; animal qualified no. 44005900002507). The rats were maintained in environmentally controlled rooms at 20-25˚C with a relative humidity of 55% and 12-15 air changes/h, under a 12-h light-dark cycle (artificial lighting between 8:00 am and 8:00 pm). The rats were fed with standard laboratory food and water *ad libitum*. All animal experiments were approved by the International Committee on Laboratory Animals of Jingmen First People's Hospital (Jingmen, China).

For the treatments, 18 SD male rats with SPF level weighing between 220 and 260 g were randomly divided into a negative control group (NC group, treated with an equal volume of normal saline, n=3), low dosage Chinese medicine group (LD group; 3.09 g/kg; n=3), medium dosage Chinese medicine group (MD group; 4.64 g/kg; n=3), high dosage Chinese medicine group (HD group; 6.17 g/kg; n=3), Western medicine control group (TWP group; 0.4 mg/kg; n=3), and a combined curative group with medium dosage and TWP (MD+TWP group; 0.4 mg/kg, n=3). Animals were treated daily at 9:00 am by gavage, once a day for 7 consecutive days. Prior to administration, animals were marked, fasted overnight and weighed. Following the final drug treatment, all animals were sacrificed and serum was collected from the rat hearts.

Cell culture. The immortalized human keratinocyte (HaCaT) and 293T cell lines were obtained from the Chinese Academy of Sciences (Shanghai, China), and were maintained in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere of 5% CO_2. For construction of the cellular model of psoriasis, HaCaT cells were treated with TNF-a (10 ng/ml) for 48 h.

RNA interference (RNAi). Negative control (NC) and VDR siRNAs were synthesized by Shanghai GenePharma, Co., Ltd. (Shanghai, China) and the sequences are as follows: NC, 5'-GUACCGCACGUCAUUCGUAUC-3' (forward), 5'-UACGAAUGACGUGCGGUACGU-3' (reverse); VDR siRNA: 5'-CCCUUCAAUGGAGAUUGCCGCAUCA-3' (forward), 5'-UGAUGCGGCAAUCUCCAUUGAAGGG-3' (reverse). HaCaT cells ($2x10^4$) were seeded into each well of a 6-well plate in 2 ml of Opti-MEM I reduced serum medium (Thermo Fisher Scientific, Inc.) overnight at 37˚C in a humidified atmosphere of 5% CO_2. Next day, cells were transfected with 50 μM NC siRNA and 50 μM VDR siRNAs for 48 h using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Lentiviral construction and infection. LV3-NC (Lot number: E11AZ), VDR-Homo-544 (Lot number: 160502DZ), VDR-Homo-433 (Lot number: 160510IZ) and VDR-Homo-775 (Lot number: 160502EZ) were synthesized by Suzhou GenePharma Co., Ltd. (Suzhou, China). Briefly, VDR full-length cDNA was amplified by quantitative polymerase chain reaction (qPCR) from 293T cells and were inserted in to a lentiviral vector and identified by sequencing as described in previous studies (18,19). These lentiviral vectors were packaged in 293T cells ($1x10^7$ in each 10-cm culture dish) by co-transfection with packaging vectors (pCMV-VSVG, pMDLg/pRRE and pRSV-REV) to produce the viral particles for lentiviral transduction. Subsequently, the purification was performed using ultracentrifugation (25,000 x g for 2 h at 4˚C). HaCaT cells ($1x10^5$ cells/well) were seeded in 6-well

plates and transduced with lentiviruses supplemented with 8 mg/ml polybrene (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). G418 (Life Technologies; Thermo Fisher Scientific, Inc.; 0.8 mg/ml) was used to select the stable expression cell lines.

Infection [multiplicity of infection (MOI)] detection. HaCaT cells ($1x10^4$ cells/well) were seeded in 96-well plates at 37˚C in a humidified atmosphere of 5% CO_2. The cells were transduced the following day with lentiviruses at 37˚C, and were divided into three groups: 3.75 μl viral suspension $4x10^8$ transducing units (TU)/ml+300 μl completely cultured with polybrene (total MOI=50); 7.5 μl viral suspension ($4x10^8$ TU/ml)+300 μl completely cultured with polybrene (total MOI=100); and 15 μl viral suspension ($4x10^8$ TU/ml)+300 μl completely cultured with polybrene (total MOI=200). Following 24 h, the medium was replaced with 100 μl complete culture medium at 37˚C. After 48 h, the cell transfection efficiency was observed under an inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan; x40 magnification), and was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR; see below) to determine the virus transfection MOI.

ELISA analysis. The concentrations of TNF-α, IFN-γ, IL-22, IL-17C, IL-1β, IL-4 and 25HVD3 in the HaCaT culture medium were determined using ELISA kits according to the manufacturer's protocol. The ELISA kits were IL-1β ELISA kit (EK0392), TNF-α ELISA kit (k EK0525), IL-4 ELISA kit (EK0404), IL-17C ELISA kit (EK0789), IL-22 ELISA kit (EK0933), INF-γ ELISA kit (EK0373; all from Wuhan Boster Biological Technology, Ltd., Wuhan, China), and 25HVD3 ELISA kit (CSB-E08097h; Cusabio Technology LLC, Houston, TX, USA). A 96-well plate was coated with monoclonal anti-TNF-α, anti-IFN-γ, anti-IL-22, anti-IL-17C, anti-IL-1β, anti-IL-4 and anti-25HVD3. The captured cytokines were detected using a secondary antibody conjugated to horseradish peroxidase. The absorbance was determined at 450 nm using a microtiter reader (Multiskan Go microplate reader; Thermo Fisher Scientific, Inc.). The concentrations of cytokines were determined by comparing the absorbance values with those of the standards.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. First-strand cDNAs were reverse transcribed from total RNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). For cDNA synthesis with random primers, the thermocycling conditions were: Incubation for 5 min at 25˚C followed by 60 min at 42˚C. The expression levels of cytokines were detected by qPCR using SYBR Premix Ex Taq (Takara Bio, Inc., Otsu, Japan). The reaction system was performed in a volume of 20 μl and the thermocycling conditions were as follows: Initial denaturation at 95˚C for 30 sec; 40 cycles of 95˚C for 5 sec, 60˚C for 34 sec. The primers for GAPDH, TNF-α, IFN-γ, IL-22, IL-17C, IL-1β, IL-4 and 25HVD3 were obtained from Sangon, China. The primers of the genes of interest and GAPDH (internal loading control) are presented in Table I. The data were analyzed using $2^{-\Delta\Delta Cq}$ method (20).

Table I. Specific primer sequences for reverse transcription-quantitative polymerase chain reaction analysis.

Name	Sequence, 5'→3'	Product length, bp
GAPDH F	TGTTCGTCATGGGTGTGAAC	154
GAPDH R	ATGGCATGGACTGTGGTCAT	
VDR F	GTGGACATCGGCATGATGAAG	181
VDR R	GGTCGTAGGTCTTATGGTGGG	
IKK F	ATGAAGAAGTTGAACCATGCCA	110
IKK R	CCTCCAGAACAGTATTCCATTGC	
NF-κB F	ATGTGGAGATCATTGAGCAGC	151
NF-κB R	CCTGGTCCTGTGTAGCCATT	
STAT3 F	ATCACGCCTTCTACAGACTGC	176
STAT3 R	CATCCTGGAGATTCTCTACCACT	
STAT4 F	TGTTGGCCCAATGGATTGAAA	119
STAT4 R	GGAAACACGACCTAACTGTTCAT	

F, forward; R, reverse; VDR, vitamin D receptor; NF-κB, nuclear factor-κB; IKK, inhibitor of NF-κB; IKB, NF-κB essential modulator; STAT, signal transducer and activator of transcription.

Western blotting. Total proteins were extracted using 250 μl radioimmunoprecipitation assay buffer (cat no. P0013; Beyotime Institute of Biotechnology, Shanghai, China) at 4˚C for 30 min. Nuclear protein was extracted with NE-PER Nuclear Extraction Reagents (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The protein concentrations were determined by using a bicinchoninic acid (BCA) Protein Assay kit (cat. no. 23225; Thermo Fisher Scientific, Inc.). Total proteins (50 μg) were separated by 8% SDS-PAGE and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Subsequent to the nitrocellulose membranes being blocked with 5% skimmed milk in TBS-Tween 20 solution (100 mmol/l Tris-Cl, pH 7.5; 150 mmol/l NaCl; and 0.1% Tween 20), the primary antibodies were incubated overnight at 4˚C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse; dilution, 1:2,000; cat no. SC-2005, and goat anti-rabbit; dilution, 1:2,000; cat no. SC-2004) at room temperature for 2 h (both Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The results were detected by using an enhanced chemiluminescence (ECL) detection kit (cat. no. 345818; EMD Millipore). The primary antibodies used in the present study included anti-GAPDH antibody (1:2,000; cat. no. sc-47724, Santa Cruz Biotechnology, Inc.), anti-NF-κB essential modulator antibody (1:2,000; cat. no. 8242, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-p-STAT3 antibody (1:100; cat. no. sc-81523, Santa Cruz Biotechnology, Inc.), anti-STAT3 antibody (1:1,000; Abcam, Cambridge, UK; cat. no. ab5073), anti-p-STAT4 antibody (1:100; cat. no. sc-28296, Santa Cruz Biotechnology, Inc.), anti-STAT4 antibody (1:1,000; Abcam; cat. no. ab68156), anti-VDR antibody (1:2,500; Abcam;

cat. no. ab134826), anti-NF-κB antibody (1:2,500; Abcam; cat. no. ab131493), anti-p-NF-κB antibody (1:1,000; Abcam; cat. no. ab28849), anti-p-IKK antibody (1:1,000; Abcam; cat. no. ab38515) and anti-IKK antibody (1:1,000; cat. no. 2682; Cell Signaling Technology, Inc.).

Statistical analysis. All quantitative data are presented as the mean ± standard deviation from at least three independent experiments. One-way analysis of variance was used to analyze the differences between the groups followed by the Dunnett test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference. SPSS 18.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) software was used for data analysis.

Results

'Psoriasis 1' downregulates the expression of TNF-α, IFN-γ, IL-22, IL-17C, IL-1β and IL-4, and upregulates the expression of 25HVD3 in TNF-α-induced psoriatic models. TNF-α is one of the primary cytokines involved in psoriasis-like inflammation. To investigate the effects of 'psoriasis 1' on psoriasis, a HaCaT cell psoriasis model was established, wherein cells were treated with TNF-α for 1 h. As presented in Fig. 1, the concentrations of TNF-α, IFN-γ, IL-22, IL-17C, IL-1β and IL-4 were increased, and the concentration of 25HVD3 was decreased in TNF-α-induced psoriasis-like cells compared with the normal control (NC) group. In addition, the ELISA results demonstrated that the concentrations of TNF-α (Fig. 1A), IFN-γ (Fig. 1C), IL-22 (Fig. 1D), IL-17C (Fig. 1E), IL-1β (Fig. 1F) and IL-4 (Fig. 1G) were downregulated, and the concentration of 25HVD3 (Fig. 1B) was upregulated in the LD, MD, HD, TWP and MD+TWP groups compared with the TNF-α group. These results suggested that 'psoriasis 1' may inhibit the inflammatory effects observed in TNF-α-induced psoriatic models.

'Psoriasis 1' downregulates NF-κB, p-NF-κB, IKK, p-IKK, STAT3, p-STAT3, STAT4 and p-STAT4 expression, and upregulates VDR expression in TNF-α-induced psoriatic models. The NF-κB and STAT signaling pathways regulate gene expression by responding to certain cellular stimulants. In addition, these two pathways are reported to be involved in the development of psoriasis (21,22). A model of psoriasis was established by treating HaCaT cells with TNF-α. The effects of 'psoriasis 1' on the NF-κB and STAT signaling pathways were subsequently investigated. The mRNA and protein expression levels of IKK, VDR, STAT3, STAT4 and nuclear NF-κB were analyzed by RT-qPCR and western blotting, respectively. As presented in Fig. 2, the mRNA expression levels of IKK, nuclear NF-κB, STAT3 and STAT4 were significantly upregulated, and the mRNA expression of VDR was significantly downregulated in TNF-α induced psoriasis-like cells compared with the NC group. Furthermore, the results indicated that the mRNA expression levels of IKK (Fig. 2A), nuclear NF-κB (Fig. 2C), STAT3 (Fig. 2D) and STAT4 (Fig. 2E) were significantly decreased, and the mRNA expression level of VDR (Fig. 2B) was significantly increased in the LD, MD, HD, TWP and the combined curative (MD+TWP) groups, compared with the TNF-α group. These results suggested that the NF-κB and STAT signaling pathways were activated by 'psoriasis 1' in the psoriasis-like cells. Consistent with these data, western blot analysis demonstrated that 'psoriasis 1' downregulated the expression of NF-κB, p-NF-κB, IKK, p-IKK, STAT3, p-STAT3, STAT4 and p-STAT4, and upregulated the expression of VDR in TNF-α-induced psoriatic models (Fig. 2F).

Silencing the VDR via lentiviral RNAi expression vector in HaCaT cells. 25HVD3 modulates the gene expression levels of NF-κB, STAT3 and STAT4 by binding to the VDR (23-25). To investigate whether the inhibitory effects of 'psoriasis 1' on NF-κB, STAT3 and STAT4 are dependent on VDR expression, VDR expression was knocked down using a lentiviral RNAi expression vector, and the expression levels of VDR were detected using RT-qPCR and western blot analyses. The results indicated that VDR was inhibited in HaCaT cells transfected with the lentiviral RNAi expression vector compared with the NC group, and VDR775 exhibited the greatest knockdown effect (Fig. 3A and B). The infectivity (MOI) of VDR775 was detected. As observed in the control group, the cell transfection efficiency was ~10%, 20-30 and 50-60% when the MOI was 100, 200 and 300, respectively, and the cells were in good condition. Regarding the transfection efficiency of the VDR755 lentivirus: At an MOI of 50, the cell transfection efficiency was ~30%; at an MOI of 100, the cell transfection efficiency was 50-60%, and the cells grew well; and at an MOI of 200, the cell transfection efficiency was ~80% (the transfection efficiency was detected by RT-qPCR assay; data not shown), although the state of the cells was poor and there was evidence of pycnosis, which is not conducive to the selection of stable cells. Therefore, HaCaT cells with stably silenced VDR at an MOI of 100 were used for the subsequent experiments (Fig. 3C).

Silencing VDR decreases the expression levels of TNF-α, IFN-γ, IL-22, IL-17C, IL-1β and IL-4, and increases the expression of 25HVD3 in TNF-α-induced HaCaT cells. To investigate the impact of VDR expression on cytokine levels, cytokine levels were measured in HaCaT cells with stably silenced VDR. The concentrations of TNF-α, 25HVD3, IFN-γ, IL-22, IL-17C, IL-1β and IL-4 were detected by ELISA (Fig. 4). The results revealed that the concentrations of TNF-α (Fig. 4A), IFN-γ (Fig. 4C), IL-22 (Fig. 4D), IL-17C (Fig. 4E), IL-1β (Fig. 4F) and IL-4 (Fig. 4G) were increased, and the concentration of 25HVD3 (Fig. 4B) was decreased in HaCaT cells transfected with VDR RNAi compared with NC. Therefore, it may be hypothesized that inhibiting VDR expression decreased TNF-α, IFN-γ, IL-22, IL-17C, IL-1β and IL-4 expression levels, and increased the 25HVD3 level in TNF-α-induced HaCaT cells.

Silencing VDR downregulates the expression of NF-κB, p-NF-κB, IKK, p-IKK, STAT3, p-STAT3, STAT4 and p-STAT4, and upregulates the expression of VDR in TNF-α-induced HaCaT cells. To demonstrate the impact of VDR on the nuclear NF-κB and STAT signaling pathways, the expression levels of IKK, VDR, STAT3, STAT4 and NF-κB (nuclear) were measured in HaCaT cells with stably silenced VDR using RT-qPCR and western blot analysis (Fig. 5). The

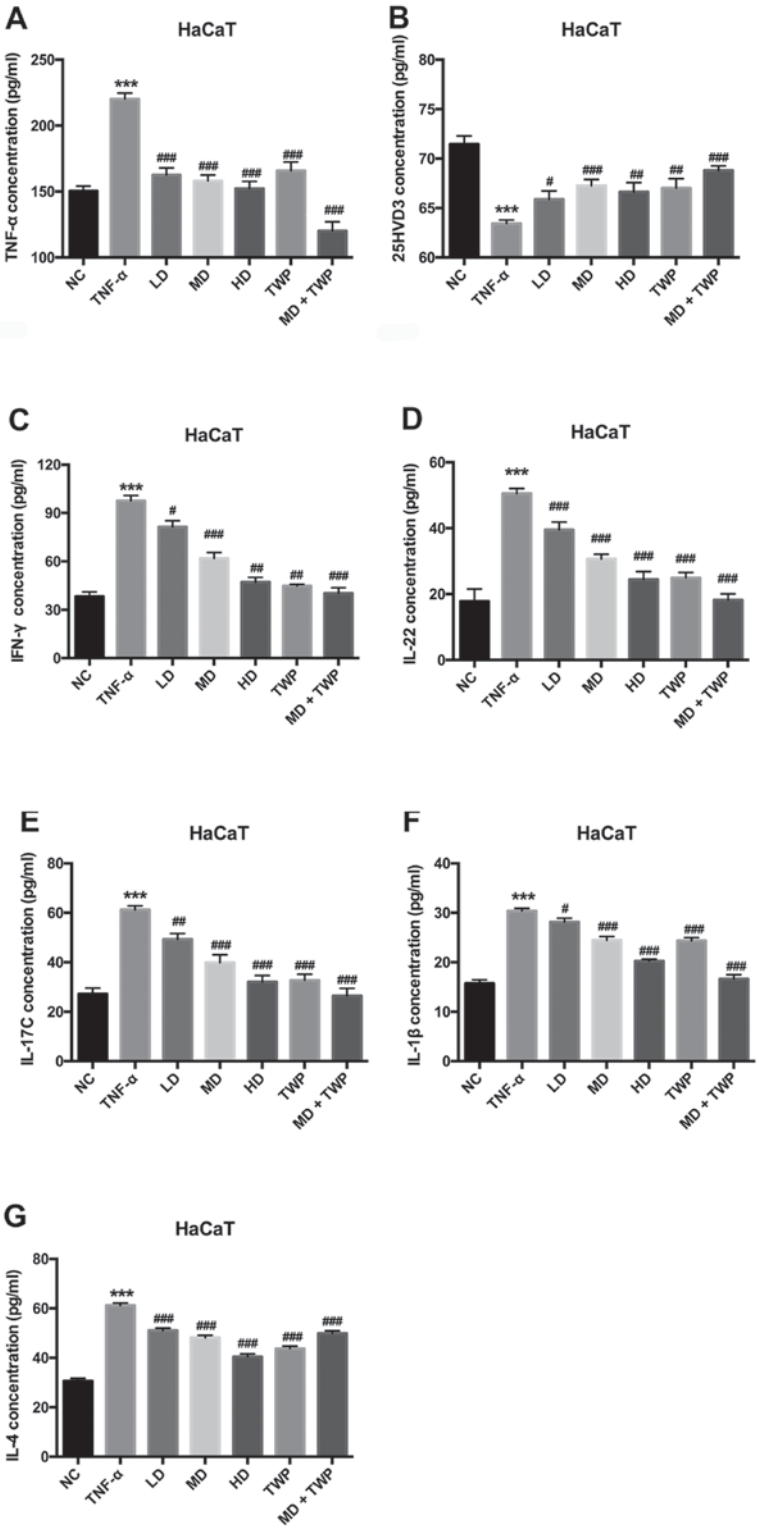

Figure 1. 'Psoriasis 1' downregulates the expression of TNF-α, IFN-γ, IL-22, IL-17C, IL-1β and IL-4, and upregulates the expression of 25HVD3 in TNF-α-induced psoriatic models. The concentrations of (A) TNF-α, (B) 25HVD3, (C) IFN-γ, (D) IL-22, (E) IL-17C, (F) IL-1β and (G) IL-4 were detected by ELISA. ***P<0.001 vs. NC group; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. TNF-α group. TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL, interleukin; 25HVD3, 25-hydroxyvitamin D3; LD, low dose; MD, medium dose; HD, high dose; TWP, *Tripterygium wilfordii* polyglycoside; NC, normal control.

results indicated that the mRNA expression levels of NF-κB in the nuclei (Fig. 5A), IKK (Fig. 5B), STAT3 (Fig. 5D) and STAT4 (Fig. 5E) were significantly increased, and VDR (Fig. 5C) was significantly decreased in HaCaT cells transfected with stably silenced VDR compared with NC. In addition, the results of the present study indicated that the inhibition of VDR upregulated the expression of NF-κB, p-NF-κB, IKK, p-IKK, STAT3, p-STAT3, STAT4

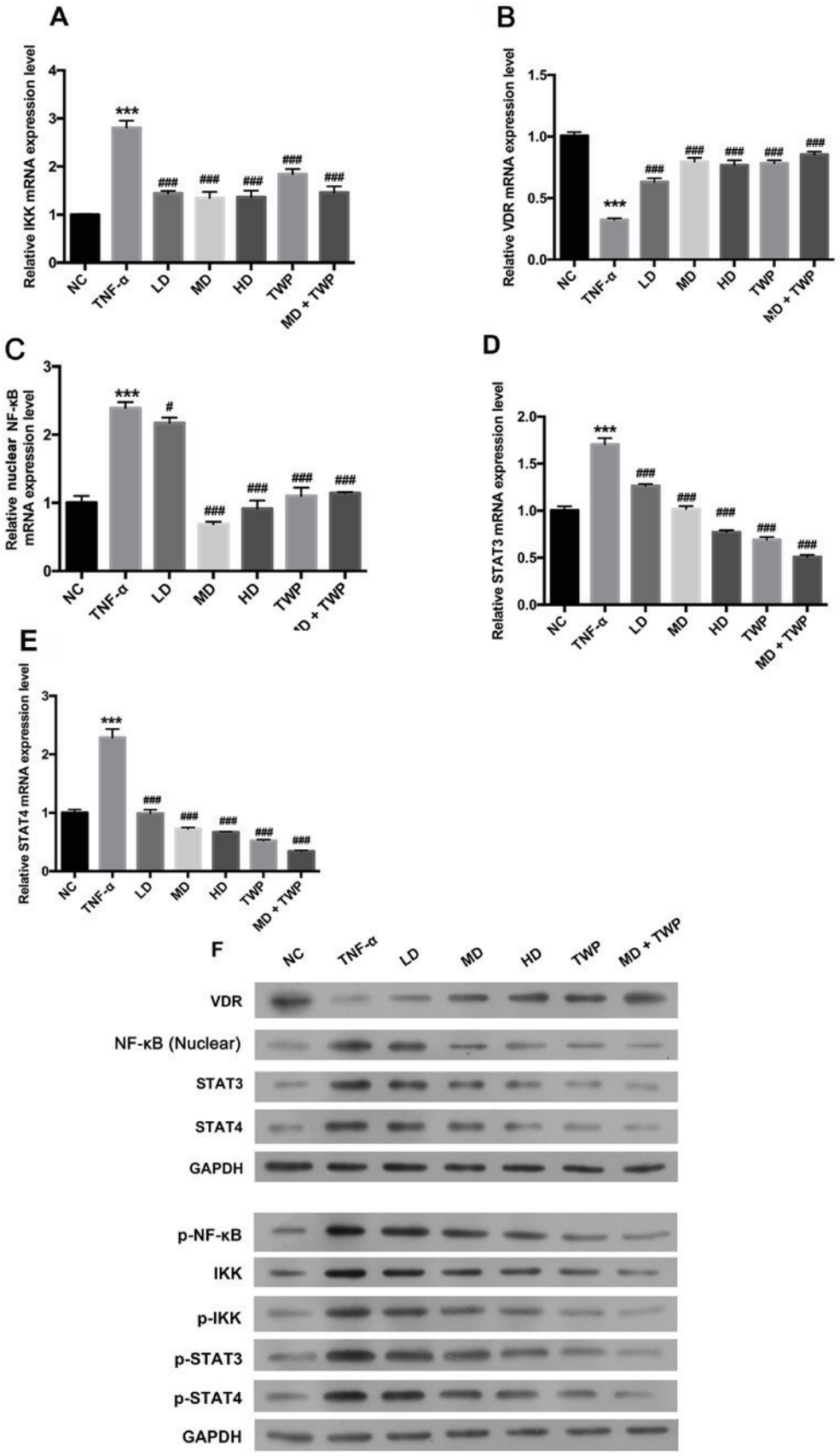

Figure 2. 'Psoriasis 1' downregulates NF-κB, p-NF-κB, IKK, p-IKK, STAT3, p-STAT3, STAT4 and p-STAT4 expression, and upregulates VDR expression in TNF-α-induced psoriatic models. The mRNA expression levels of (A) IKK, (B) VDR, (C) nuclear NF-κB, (D) STAT3 and (E) STAT4 were analyzed by reverse transcription-quantitative polymerase chain reaction assay. (F) Total proteins were extracted using radioimmunoprecipitation assay buffer; nuclear protein was extracted using NE-PER Nuclear Extraction Reagents. Western blotting was used to detect the expression levels of NF-κB and p-NF-κB in the nuclei, IKK, p-IKK, VDR, STAT3, p-STAT3, STAT4 and p-STAT4. GAPDH was used as a loading control. ***P<0.001 vs. NC group; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. TNF-α group. TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; p, phosphorylated; IKK, inhibitor of NF-κB; VDR, vitamin D receptor; STAT, signal transducer and activator of transcription; NC, normal control; LD, low dose; MD, medium dose; HD, high dose; TWP, *Tripterygium wilfordii* polyglycoside.

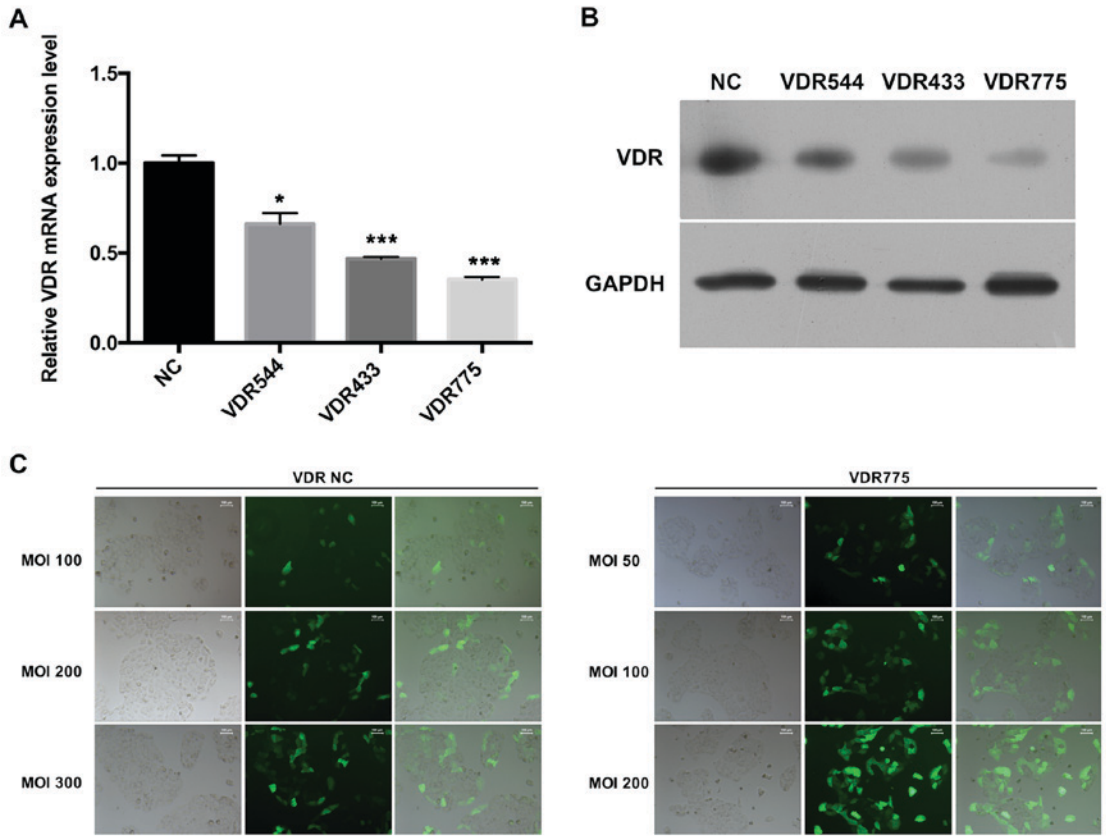

Figure 3. Silencing VDR using a lentiviral RNAi expression vector in HaCaT cells. HaCaT cells were transfected with the VDR RNAi lentiviruses (VDR544, VDR433, VDR775). (A) The mRNA expression level of VDR was detected by reverse transcription-quantitative polymerase chain reaction assay. (B) The protein expression level of VDR was detected by western blot analysis. GAPDH was used as a loading control. (C) The cell transfection efficiency was determined by infectivity (MOI) detection. Magnification, x200. Scale bars, 100 μm. *P<0.05, ***P<0.001 vs. NC group. VDR, vitamin D receptor; RNAi, RNA interference; MOI, multiplicity of infection; NC, normal control.

and p-STAT4, and downregulated VDR expression in TNF-α-induced HaCaT cells (Fig. 5F and G).

Discussion

Psoriasis is an inflammatory skin disease characterized by a significant elevation in the concentration of cytokines, including TNF-α (26,27). The annual cost of psoriasis in China alone is estimated to be over <1 billions of yuan, and the socioeconomic burden of psoriasis has an important influence on the Chinese healthcare system (28). However, because the underlying mechanism remains undetermined, current treatments have not yet fully met the needs of patients. TNF-α is a critical pro-inflammatory cytokine which is produced by various cell types, including macrophages, monocytes and activated T cells (29). It is now widely accepted that TNF-α is a principal inducer of psoriasis and a number of drugs targeting TNF-α are currently being assessed for the treatment of psoriasis (30,31). TNF-α mediates the gene expression of numerous cytokines, including IL-1, IL-6 and IL-22 (32), by activating the NF-κB and STAT signaling pathways.

TCM has been developed and applied by the Chinese over a long period of time, and includes acupuncture, Chinese traumatology and CHM; CHM is considered to be the most popular type of TCM worldwide (33,34). It has been reported that various CHM formulations have been used for the treatment of a number of diseases, including gastrointestinal disease, diabetes mellitus and psoriasis (28,35,36). In China, numerous patients with psoriasis accept treatment with CHM, and ~2% of patients receive similar therapy in the USA (37). Although the effects of various CHM formulations are positive in patients with psoriasis, the underlying mechanism of these therapies remains unknown.

For decades, 'psoriasis 1', a formulation originating in CHM, has been used to effectively treat patients with psoriasis in China (38,39). In the present study, it was identified that the concentrations of TNF-α, IFN-γ, IL-22, IL-17C, IL-1β and IL-4 were increased, and the concentration of 25HVD3 was decreased in TNF-α-induced psoriasis-like cells compared with the NC group; the concentrations of TNF-α, IFN-γ, IL-22, IL-17C, IL-1β and IL-4 were decreased, and the concentration of 25HVD3 was increased in the LD, MD, HD, TWP and combined curative (MD+TWP) groups, compared with the TNF-α group. The data indicated that TNF-α promoted inflammation, and 'psoriasis 1' was able to block these inflammatory effects in TNF-α-induced psoriatic models.

Previous work demonstrated that the NF-κB and STAT signaling pathways are involved in the gene expression of certain cytokines during an inflammatory response. TNF-α was used to stimulate the formation of the psoriasis cell model. TNF-α activates the NF-κB signaling pathway, and increases p-IKK and p-NF-κB expression levels; the activated

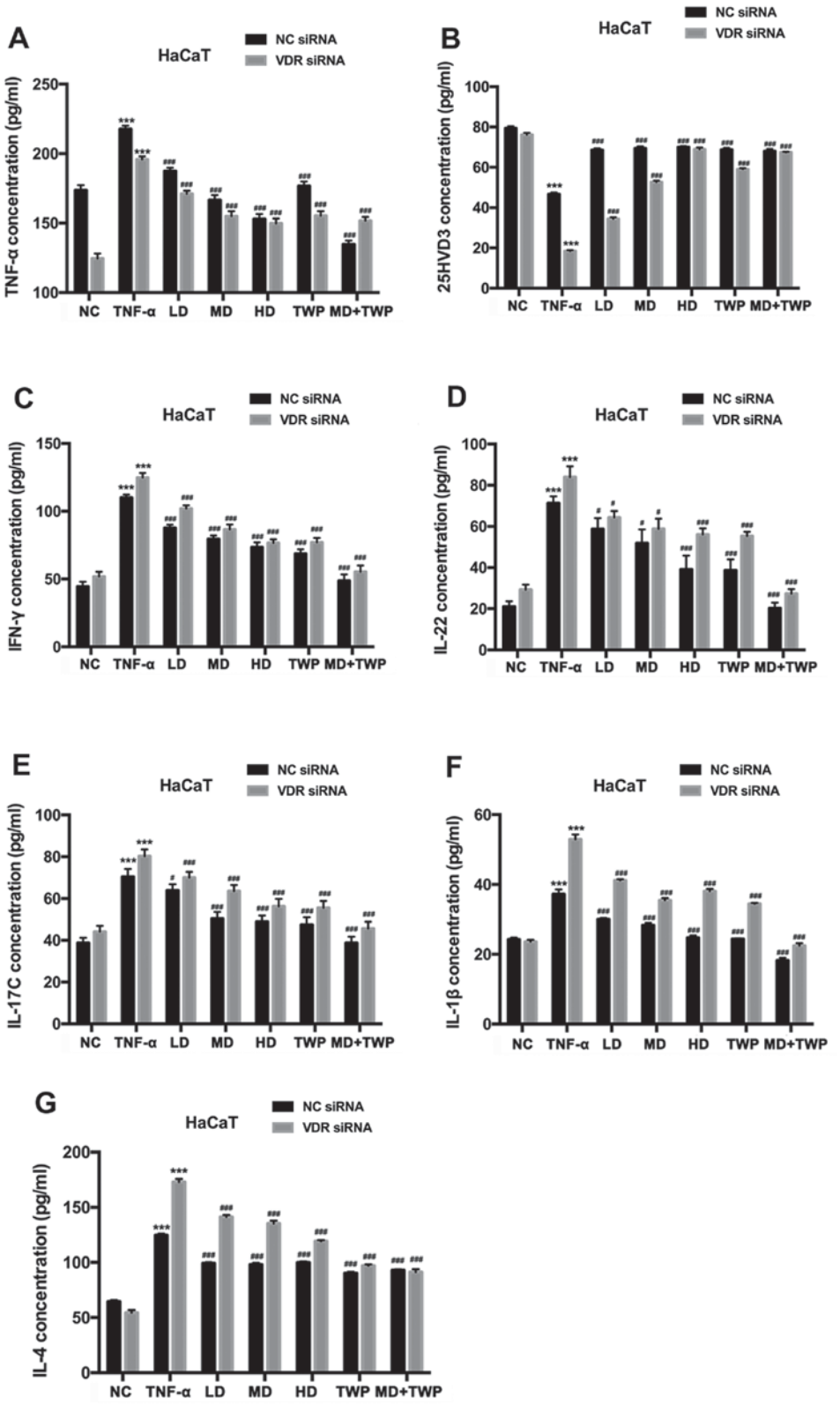

Figure 4. Silencing VDR decreases TNF-α, IFN-γ, IL-22, IL-17C, IL-1β and IL-4 expression levels, and increases the 25HVD3 expression level in TNF-α-induced HaCaT cells with stably silenced VDR. The concentrations of (A) TNF-α, (B) 25HVD3, (C) IFN-γ, (D) IL-22, (E) IL-17C, (F) IL-1β and (G) IL-4 were detected by ELISA. ***P<0.001 vs. NC group; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. TNF-α group. VDR, vitamin D receptor; TNF-α, tumor necrosis factor; IFN-γ, interferon-γ; IL, interleukin; 25HVD3, 25-hydroxyvitamin D3; NC, normal control; LD, low dose; MD, medium dose; HD, high dose; TWP, *Tripterygium wilfordii* polyglycoside; siRNA, small interfering RNA.

NF-κB is transferred to the nucleus, promotes the expression levels of STAT4 and STAT3, and activates p-STAT3 and p-STAT4 (40-43). Studies have demonstrated that VDR may form complexes with NF-κB subunits which exist stably in the cytoplasm, and inhibits the activation of the NF-κB signaling pathway (44,45). Therefore, the expression level of VDR was

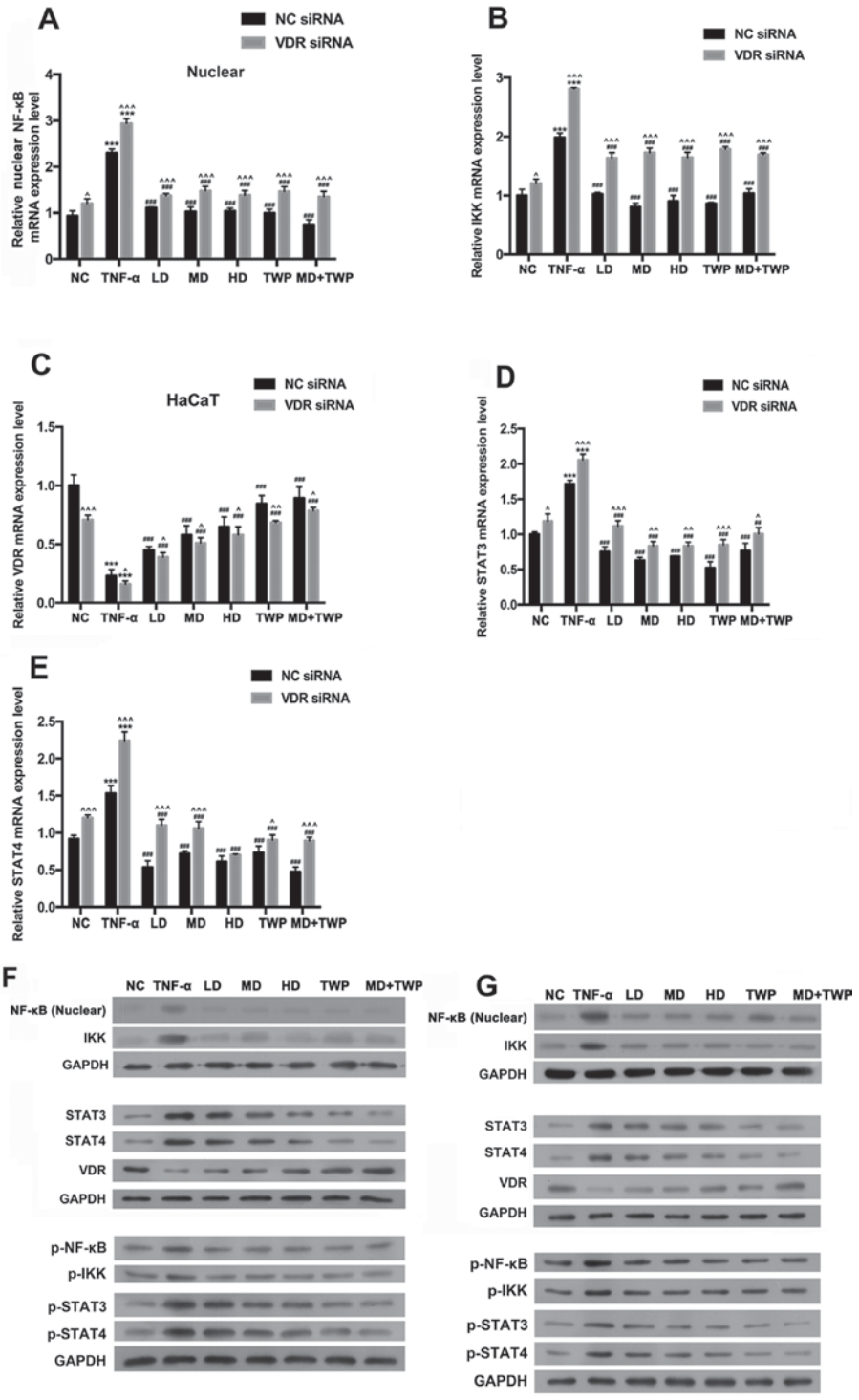

Figure 5. Silencing of VDR upregulates NF-κB, p-NF-κB, IKK, p-IKK, STAT3, p-STAT3, STAT4 and p-STAT4 expression levels, and downregulates VDR expression in TNF-α-induced HaCaT cells. The reverse transcription-quantitative polymerase chain reaction was used to analyze the mRNA expression levels of (A) NF-κB, (B) IKK, (C) VDR (D) STAT3 and (E) STAT4. Total proteins were extracted using radioimmunoprecipitation assay buffer; Nuclear protein was extracted using NE-PER Nuclear Extraction Reagents. Western blotting was used to detect the expression levels of NF-κB and p-NF-κB in the nuclei, and IKK, p-IKK, VDR, STAT3, p-STAT3, STAT4 and p-STAT4, in the (F) empty vector NC group and (G) HaCaT cells with stably silenced VDR. GAPDH was used as a loading control. ***P<0.001 vs. NC group; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. TNF-α group; $^{\wedge}$P<0.05, $^{\wedge\wedge}$P<0.01, $^{\wedge\wedge\wedge}$P<0.001 vs. respective NC siRNA group. TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; p, phosphorylated; IKK, inhibitor of NF-κB; VDR, vitamin D receptor; STAT, signal transducer and activator of transcription; NC, normal control; LD, low dose; MD, medium dose; HD, high dose; TWP, *Tripterygium wilfordii* polyglycoside; siRNA, small interfering RNA.

reduced in the psoriasis cell model, and silencing of VDR with small interfering RNA was able to further activate the NF-κB signaling pathway, promoting STAT4 and STAT3 expression. Furthermore, 'psoriasis 1' was able to partially inhibit the activation of the VDR-mediated NF-κB signaling pathway. Alternatively, STAT3, STAT4 and NF-κB may be considered

to be parallel signaling pathways, and the psoriasis cell model and VDR intervention was able to increase inflammatory factors, including IFN-γ, IL-22, IL-17C, IL-1β and IL-4, in addition to activating the NF-κB/STAT signaling pathway.

The present study examined whether 'psoriasis 1' induced the downregulation of cytokines associated with the NF-κB and STAT signalling pathways. It was identified that the expression levels of NF-κB, p-NF-κB, IKK, p-IKK, STAT3, p-STAT3, STAT4 and p-STAT4 were significantly upregulated, and VDR was significantly downregulated in TNF-α-induced psoriasis-like cells compared with the NC group, suggesting that the nuclear NF-κB and STAT signaling pathways were activated in the psoriasis-like cell model. Furthermore, it was demonstrated that 'psoriasis 1' downregulated NF-κB, p-NF-κB, IKK, p-IKK, STAT3, p-STAT3, STAT4 and p-STAT4 expression, and upregulated VDR expression in TNF-α-induced psoriatic models. Lentiviral VDR RNAi expression vectors were transduced into model HaCaT cells, to eventually construct cells with stably silenced VDR expression *in vitro*. The effect on these cells of 'psoriasis 1' with or without TWP was investigated, in addition to the effect of silencing VDR expression. Silencing of VDR increased the concentrations of, IFN-γ, IL-22, IL-17C, IL-1β, and IL-4, and decreased the concentration of TNF-α and 25HVD3. In addition, silencing of VDR upregulated the expression levels of NF-κB, p-NF-κB, IKK, p-IKK, STAT3, p-STAT3, STAT4 and p-STAT4, and downregulated the expression level of VDR in TNF-α-induced HaCaT cells. It was demonstrated that 'psoriasis 1' and silencing of VDR suppressed the inflammatory reaction, and the activation of the NF-κB and STAT signaling pathways. Therefore, it was concluded that 'psoriasis 1' alleviated psoriasis-like inflammation by inhibiting the VDR-mediated nuclear NF-κB and STAT signaling pathways.

In conclusion, the results of the present study demonstrated that 'psoriasis 1' suppressed the inflammatory reaction, and the activation of the NF-κB and STAT signaling pathways through VDR, suggesting that 'psoriasis 1' inhibited psoriasis-like skin inflammation by suppressing the VDR-mediated nuclear NF-κB and STAT signaling pathways.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WS, YG, XY and XC designed the experiments. WS, YG, YY, JY, ZZ, YC, YL and XP performed the experiments and conducted data analysis.

Ethics approval and consent to participate

All animal experiments were approved by the International Committee on Laboratory Animals of Jingmen First People's Hospital (Jingmen, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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