

Calcipotriol inhibits proliferation of human keratinocytes by downregulating STAT1 and STAT3 signaling

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ABSTRACT

Psoriasis is an autoimmune disease, which is characterized by aberrantly high levels of inflammation, but the underlying pathogenic mechanisms are still not fully understood. Signal transducer and activator of transcription 1 (STAT1) and STAT3, and the downstream proteins suppressor of cytokine signaling 1 (SOCS1) and SOCS3, have been implicated in psoriasis disease progression. Calcipotriol, a synthetic derivative of vitamin D, has been used clinically to treat psoriasis, but the mechanism of action that underlies the beneficial effects of calcipotriol is still being explored. The objective of this study was to determine whether STAT1 and STAT3 signaling is involved in calcipotriol treatment. Using an in vitro immortal human keratinocyte cell line, HaCaT cells, as a psoriasis model, we examined the molecular signaling induced by calcipotriol treatment. We found that calcipotriol treatment or silencing of either STAT1 or STAT3 inhibited proliferation of HaCaT cells. Calcipotriol downregulated the expression of STAT1 and STAT3 at the messenger RNA (mRNA) and protein levels. The levels of phosphorylated STAT1 and STAT3 were also decreased, suggesting calcipotriol treatment inhibited STAT1 and STAT3 activation. Calcipotriol-mediated STAT inhibition was further substantiated by the downregulation of SOCS1 and SOCS3 at the mRNA and protein expression levels. Taken together, our results suggest a novel molecular mechanism for calcipotriol-mediated treatment effects in psoriasis.

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease characterized by epidermal hyperplasia and altered keratinocyte differentiation.¹ Psoriasis affects ~0.1–3% of the population worldwide.² Common treatments for psoriasis include topical, systemic, phototherapy, combination, and herbal therapy treatments that inhibit excessive keratinocyte proliferation.³ Despite the fact multiple psoriasis therapies are available, no cure has been developed to date, and the relapsing–remitting nature of psoriasis adversely affects patient quality of life.^{4–5}

Although considerable evidence indicates psoriasis is immunologically mediated and has a complex genetic basis, the basic pathogenic mechanisms have not yet been fully elucidated.^{6–8}

Significance of this study

What is already known about this subject?

- ▶ Psoriasis is an inflammatory disease with aberrant epidermal hyperplasia and keratinocyte differentiation.
- ▶ Activation of signal transducer and activator of transcription 1 (STAT1) and STAT3 has been implicated in psoriasis.
- ▶ Calcipotriol is a synthetic derivative of vitamin D and used in the treatment of psoriasis.

What are the new findings?

- ▶ Calcipotriol inhibits activation of STAT1 and STAT3.
- ▶ Calcipotriol downregulates messenger RNA (mRNA) and protein expression of STAT1 and STAT3.
- ▶ Calcipotriol downregulates mRNA and protein expression of suppressor of cytokine signaling 1 (SOCS1) and SOCS3.

How might these results change the focus of research or clinical practice?

- ▶ Our results suggested a new mechanism of calcipotriol in treating psoriasis.

Owing to the lack of a widely accepted animal model, our understanding of psoriasis pathogenesis is primarily derived from clinical studies and translational science conducted in patients.⁹ Histological examinations indicate that abnormal angiogenesis and inflammatory infiltrates are closely related to the pathogenesis of psoriasis.^{6–10} There is a complex interplay between dendritic cells, T cells, and keratinocytes that results in chronic skin inflammation in psoriasis. These cells produce cytokines such as, tumor necrosis factor, interferons (IFNs), interleukin-6 (IL-6), IL-23, and IL-17, in the psoriatic lesions that play important roles in disease progression.^{11–12} Signal transducer and activator of transcription (STAT) signaling downstream of IFNs and IL-6 has been implicated in psoriasis.^{13–14} In psoriatic skin lesions, expression and activation of STAT1 and STAT3 has been documented, and in vitro in normal human keratinocytes IFNs and IL-6



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induce STAT1 and STAT3 activation, respectively.^{15 16} In addition, keratin 17, which is regarded as a hallmark of psoriasis because it is strongly expressed in psoriatic lesions but not in normal skin, is upregulated in keratinocytes by IL-17A through STAT1-dependent and STAT3-dependent mechanisms.¹⁷ Transgenic mice that express constitutively active STAT3 in their keratinocytes develop a skin phenotype spontaneously, and in response to wounding, that closely resembles psoriasis.¹⁸ Given the role of STATs in the development of psoriasis, the feasibility of inhibiting the upstream Janus kinases (JAK) to prevent STAT activation has been extensively tested as a novel therapeutic strategy.^{19 20}

Calcipotriol is a vitamin D3 analog that is used as a topical treatment for psoriasis alone or in combination with betamethasone.^{21 22} Calcipotriol inhibits proliferation and normalizes keratinocyte differentiation by targeting the vitamin D receptor expressed on the keratinocytes in the lower epidermis.²³ Information regarding the molecular mediators underlying the mechanism of action of calcipotriol in psoriasis is still limited. In the HaCaT keratinocyte cell line and in primary human keratinocytes, the antiproliferative effect of calcipotriol was accompanied by an increase in sphingomyelin hydrolysis and the antiproliferative lipid, ceramide.^{24 25} In the presence of extracellular calcium, calcipotriol mediated the dephosphorylation of the epidermal growth factor receptor, which might be the mechanism underlying the inhibition of cell proliferation in HaCaT cells.²⁶

To date, a possible role for signaling mediated by STAT1 and STAT3 in the pharmacological action of calcipotriol has not yet been investigated. The aim of this study was to identify the signaling molecules that mediate the calcipotriol-induced inhibition of cell proliferation in HaCaT cells.

MATERIALS AND METHODS

Cells and reagents

The HaCaT cell line was obtained from the Shanghai Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Calcipotriol was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega (Madison, Wisconsin, USA). STAT1, STAT3, phosphorylated STAT1 (p-STAT1), p-STAT3, suppressor of cytokine signaling 1 (SOCS1), SOCS3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from ebioe (Guangzhou, China). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Boster Biological Technology (Wuhan, China).

Calcipotriol treatment

HaCaT cells were cultured in Dulbecco's Modified Eagle's Minimal essential medium with 10% fetal bovine serum (Hyclone, USA) at 37°C in a 5% CO₂ humidified environment. HaCaT cells were collected during the logarithmic growth phase and aliquoted in a 96-well plate (1×10⁴ cells/well). The cells were treated with 10 nM calcipotriol or left untreated (control group). The cells were allowed to grow overnight and then the culture medium was replaced with fresh medium containing 10 nM calcipotriol, an

equivalent volume of fresh media was replaced in the control group.

MTS assay

After treatment, 10 µL of MTS solution (Promega, USA) was added into each well and the plate was incubated at 37 °C for 4 hours. The optical density was determined at 490 nm using a quantitative automatic microplate reader (Multiskan MK3, Thermo Fisher Scientific).

Downregulation of STAT1 and STAT3

HaCaT cells were transfected with 50 nM small interfering RNA (siRNA) oligonucleotides (Sigma-Aldrich) for human STAT1 and STAT3 or negative control (NC)-siRNA by using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). The siRNA sequences were: STAT1-siRNA (SASI_Hs02_00343388), forward-5'-CUGUGAAGUUGAGACUGUUDtT-3' and reverse-5'-AACAGUCUACAACUUCACAGdTT-3'; STAT3-siRNA (SASI_Hs01_00121206), forward-5'-GGUAACGUCUAUAGCAGAdTT-3' and reverse-5'-UCUGCUAAUGACGUAUACCCdTT-3'; NC-siRNA, forward-5'-UUCUCCGACGUGUCACGUTT-3' and reverse-5'-ACGUGACACGUUCGGAGAATT-3'.

RNA isolation, reverse-transcription PCR (RT-PCR), and real-time quantitative PCR (RT-qPCR)

RNA was isolated from each cell sample using TRIzol (Invitrogen, California, USA) according to the manufacturer's protocol. A 1 µg aliquot of total RNA from every sample was used for complementary DNA (cDNA) synthesis using the First-Strand cDNA Synthesis Kit (Beijing ComWin Biotech, China). RT-qPCR was performed using SYBR Green qPCR SuperMix (Invitrogen, California, USA) and analyzed on an Applied Biosystems ViiA 7 Real-Time PCR System (Life Technologies, USA). β-actin (*ACTB*) was used as an internal control. The difference in the real-time PCR cycle number (Ct value) between the target gene and *ACTB* was quantified using the ΔΔCt method. All commercially available kits were used according to the manufacturer's protocol.

Western blot analyses

Western blotting was performed according to standard protocols. Briefly, cells were harvested and lysed in a lysis buffer containing a cocktail of protease inhibitors. After centrifugation at 14000 rpm for 15 min at 4°C, supernatants were collected, mixed with sample buffer containing dithiothreitol, and boiled for 8 min. Equal amounts of proteins were separated by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 10% skim milk for 2 hours or overnight, incubated with the primary antibody for 1.5 hours, and incubated with the secondary antibody for 1 hour. The blots were washed and the proteins were visualized by chemiluminescence. Primary antibodies (all purchased from Abcam) against STAT1 (rabbit polyclonal, Cat. number ab31369, 1:500–1:1000 dilution), STAT3 (Rabbit polyclonal, Cat. number ab31370, 1:500–1:1000 dilution), p-STAT1 (Tyr-701) (rabbit polyclonal, Cat. number ab30645, 1:500–1:1000 dilution), and p-STAT3 (Ser-727) (rabbit polyclonal, Cat. number ab30647, 1:500–

1:1000 dilution) were used. The films were developed and scanned. The Western blot images were then analyzed using an UVIpro Gel documentation and analysis system (UVItc, UK). GAPDH was used as an internal control.

Statistical analyses

At least three independent experiments were performed, and all experiments were performed in at least triplicate. Data are presented as the mean \pm SD and analyzed with SPSS statistical software (V.13.0; SPSS, Chicago, Illinois, USA). One-way analysis of variance was used for mean comparisons. The $p < 0.05$ was considered statistically significant.

RESULTS

Effect of calcipotriol and STAT1/3 silencing on proliferation of HaCaT cells

To determine the effect of calcipotriol treatment or the role of STAT1 and STAT3 on HaCaT cells, we used the MTS assay to quantify cellular proliferation. As shown in

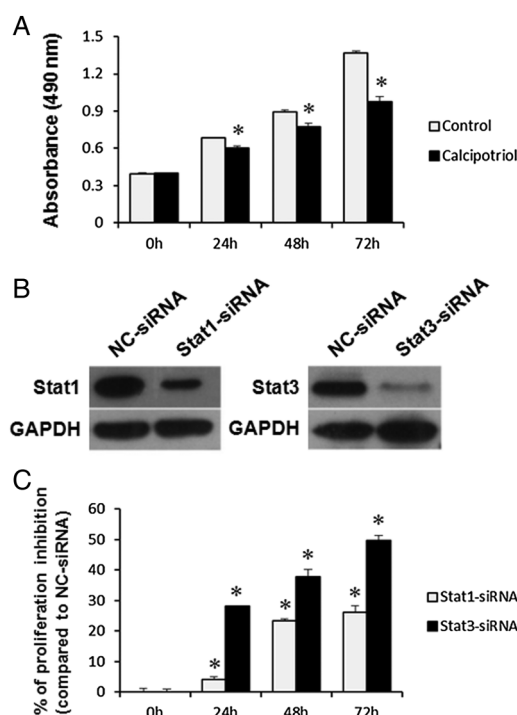


Figure 1 Calcipotriol or STAT1 and STAT3 silencing reduced proliferation in HaCaT cells. (A) HaCaT cells were treated with 10 nM calcipotriol for the indicated time. Cell proliferation was determined using the MTS assay; the absorbance at 490 nm was recorded. * $p < 0.05$ compared with control. (B) HaCaT cells were transfected with 50 nM of STAT1-siRNA, STAT3-siRNA, or NC-siRNA for 24 hours. Total protein was harvested for Western blot analysis to determine STAT1, STAT3, and GAPDH protein expression levels. GAPDH was the internal control. (C) HaCaT cells were transfected with 50 nM of STAT1-siRNA, STAT3-siRNA, or NC-siRNA for the indicated time. Cell proliferation was determined using the MTS assay, the per cent of proliferation inhibition was calculated as $(1 - \text{mean proliferation of treated group} / \text{mean proliferation of NC-siRNA group}) \times 100$. * $p < 0.05$ compared with 0 hours. NC, negative control; siRNA, small interfering RNA; STAT1, signal transducer and activator of transcription 1.

figure 1A, HaCaT cells in the control group (without calcipotriol treatment) proliferated according to the culture time. However, calcipotriol significantly ($p < 0.05$) reduced the amount of cell proliferation compared with the control group (vehicle control) in a time-dependent manner, suggesting an antiproliferative effect in HaCaT cells. Furthermore, we silenced the expression of STAT1 or STAT3 using siRNA in HaCaT cells (figure 1B), and the silenced cells were subjected to MTS assay to determine the time-dependent proliferation (figure 1C). We found significant proliferation inhibition of STAT1-siRNA or STAT3-siRNA transfected HaCaT cells. The results suggested the indispensable role of STAT1 and STAT3 in the proliferation of HaCaT cells.

Effect of calcipotriol on STAT1 and STAT3 messenger RNA (mRNA) and protein expression in HaCaT cells

Given that STAT1 and STAT3 signaling has been implicated as a potentially new therapeutic target for psoriasis,¹³ we sought to determine whether STAT1 and STAT3 were involved in the antiproliferative effect of calcipotriol in HaCaT cells. To determine whether calcipotriol treatment affects STAT1 and STAT3 expression, we first examined the STAT1 and STAT3 mRNA expression levels by RT-qPCR analysis. As shown in figure 2A, B, the expression levels of STAT1 and STAT3 were significantly reduced in calcipotriol-treated HaCaT cells ($p < 0.05$).

Moreover, Western blot analysis showed that the expression of p-STAT1, STAT1, p-STAT3, and STAT3 were all downregulated by calcipotriol treatment (figures 3A and 4A). To see whether the downregulation of p-STAT1 and p-STAT3 was direct or secondary effects of calcipotriol treatment, the mean density ratios of p-STAT1 and p-STAT3 relative to total protein were calculated (figures 3B and 4B). Results showed a significant ($p < 0.05$) downregulated p-STAT3/STAT3 ratio but not p-STAT1/STAT1 in calcipotriol-treated HaCaT cells. As shown in figure 3C–D and figure 4C–D, calcipotriol treatment significantly decreased the mean density ratio of p-STAT1, STAT1, p-STAT3, and STAT3 relative to GAPDH compared with the ratio in the control group ($p < 0.05$). Together, these results suggest that calcipotriol might regulate STAT1 and STAT3 transcriptionally, post-transcriptionally, and post-translationally.

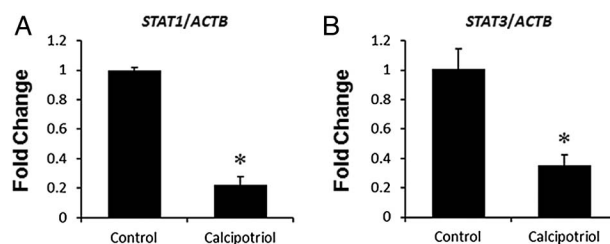


Figure 2 Calcipotriol reduced mRNA expression of STAT1 and STAT3. HaCaT cells were treated with 10 nM calcipotriol for 48 hours. Total mRNA was extracted and used for RT-PCR and RT-qPCR to determine STAT1 (A) and STAT3 (B) mRNA expression levels. Results were calculated with $\Delta\Delta C_t$ methods, normalized to an internal control (actin), and are shown as the fold change. * $p < 0.05$ compared with control. ACTB, β -actin; Ct, cycle threshold; mRNA, messenger RNA; RT-PCR, reverse-transcription PCR; RT-qPCR, real-time quantitative PCR; STAT1, signal transducer and activator of transcription 1.

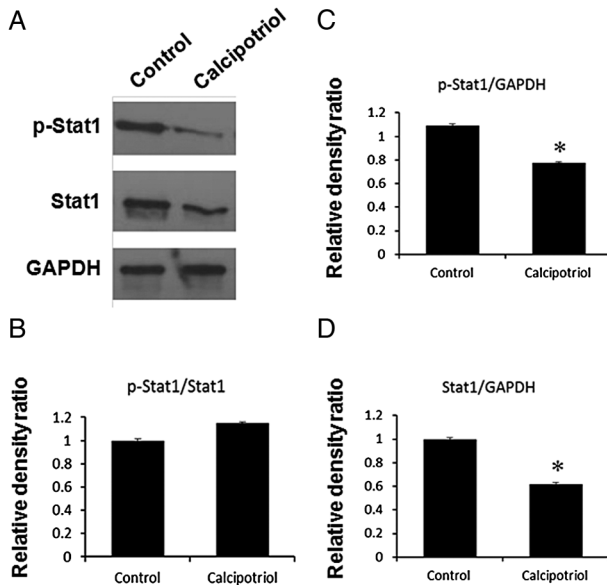


Figure 3 Calcipotriol reduced protein expression levels of signal transducer and activator of transcription 1 (STAT1) and phosphorylated STAT1 (p-STAT1). HaCaT cells were treated with 10 nM calcipotriol for 48 hours, and total protein was harvested for Western blot analysis to determine STAT1, p-STAT1, and GAPDH protein expression levels (A). GAPDH was used as internal control. Protein bands were quantified with densitometry and the relative density ratio of p-STAT1/STAT1 (B), p-STAT1/GAPDH (C), and STAT1/GAPDH (D) were shown. * $p < 0.05$ compared with control.

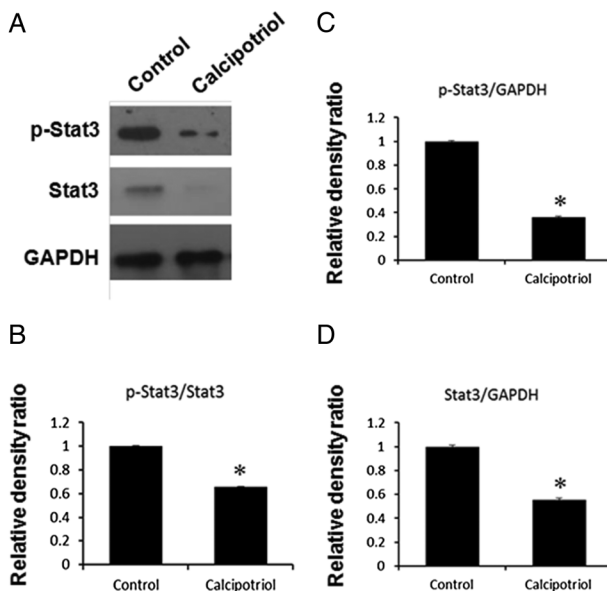


Figure 4 Calcipotriol reduced protein expression levels of signal transducer and activator of transcription 3 (STAT3) and phosphorylated STAT3 (p-STAT3). HaCaT cells were treated with 10 nM calcipotriol for 48 hours and total protein was harvested for Western blot analysis to determine STAT1, p-STAT1, and GAPDH protein expression levels (A). GAPDH was as internal control. Protein bands were quantified with densitometry and the relative density ratio of p-STAT3/STAT3 (B), p-STAT3/GAPDH (C), and STAT3/GAPDH (D) were shown. * $p < 0.05$ compared with control.

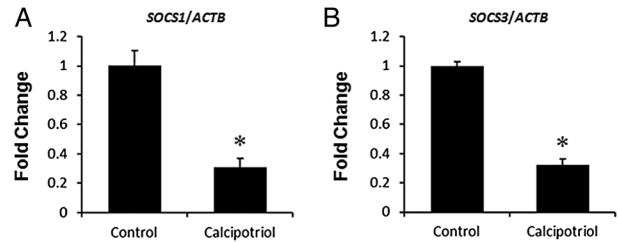


Figure 5 Calcipotriol reduced mRNA expression of SOCS1 and SOCS3. HaCaT cells were treated with 10 nM calcipotriol for 48 hours. Total mRNA was extracted and used for RT-PCR, and RT-qPCR to determine SOCS1 (A) and SOCS3 (B) mRNA expression levels. Results were calculated with $\Delta\Delta Ct$ methods, normalized to internal control (actin), and shown as fold change. * $p < 0.05$ compared with control. ACTB, β -actin; Ct, cycle threshold; mRNA, messenger RNA; RT-PCR, reverse-transcription PCR; RT-qPCR, real-time quantitative PCR; SOCS1, suppressor of cytokine signaling 1.

Effect of calcipotriol on SOCS1 and SOCS3 mRNA and protein expression in HaCaT cells

To further elucidate the effects of calcipotriol on the STAT1 and STAT3 signaling pathway the expression of their downstream molecules SOCS1 and SOCS3 was assessed. As shown in figure 5A, B, RT-qPCR analysis indicated that the mRNA expression levels of SOCS1 and SOCS3 were significantly reduced in calcipotriol-treated HaCaT cells compared with the control group ($p < 0.05$). Western blot analysis showed that calcipotriol treatment significantly reduced the protein expression of SOCS1 and SOCS3 (figure 6A). The density ratio of SOCS1 and SOCS3 relative to GAPDH was significantly reduced compared with the control group in the calcipotriol-treated HaCaT cells ($p < 0.05$) (figure 6B, C). Taken together, these results suggest that calcipotriol inhibited HaCaT cell proliferation, possibly via a mechanism associated with downregulation of STAT1 and STAT3 and the SOCS1 and SOCS3 signaling pathway.

DISCUSSION

Accumulating studies implicated that STAT1 and STAT3 play crucial roles in the pathogenesis of psoriasis and might serve as new therapeutic targets for the development of psoriasis therapies.¹³ In this study, we sought to assess the effects of calcipotriol treatment on cellular proliferation and molecular signaling pathways in the immortalized human psoriatic keratinocyte HaCaT cell line. While calcipotriol is widely used in clinical practice for psoriasis treatment, its mechanism of action is still unclear.^{27 28} We show that after calcipotriol treatment, HaCaT cell proliferation was inhibited, and p-STAT1 and p-STAT3, and their downstream proteins SOCS1 and SOCS3 were downregulated. Therefore, we propose that calcipotriol-mediated inhibition of HaCaT cell proliferation is mediated by inactivating STAT1 and STAT3. To the best of our knowledge, this study is the first to suggest this link between calcipotriol treatment and STATs/SOCSs signaling in psoriasis.

Both our work and other studies have suggested that STAT1 and STAT3 are aberrantly activated in the epidermis of psoriatic lesions.^{16 17 29} STAT1 and STAT3 apart from

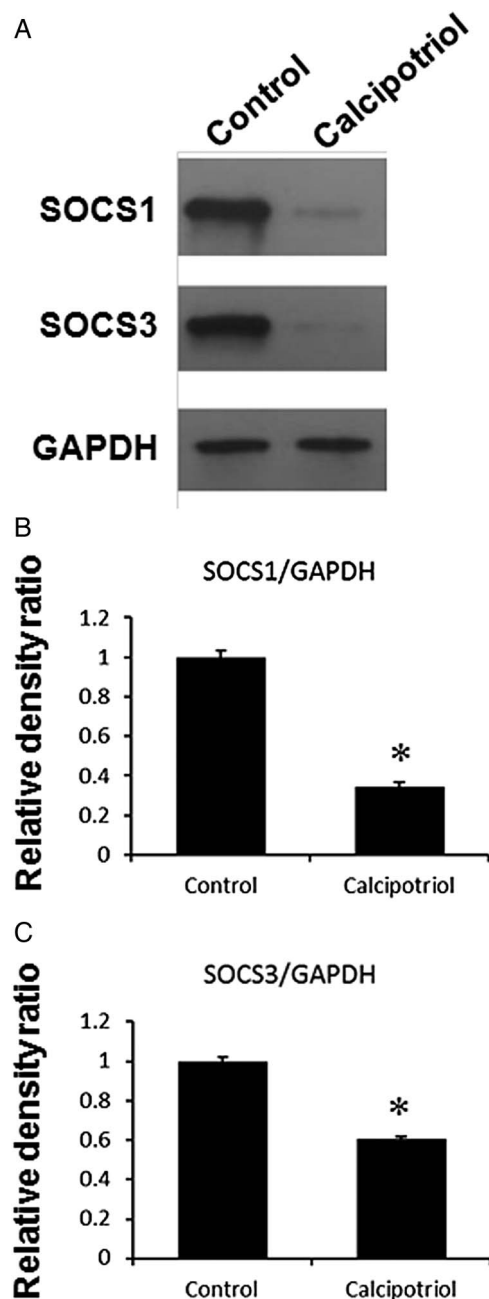


Figure 6 Calcipotriol reduced protein expression of suppressor of cytokine signaling 1 (SOCS1) and SOCS3. HaCaT cells were treated with 10 nM calcipotriol for 48 hours, and total protein was harvested for Western blot analysis to determine SOCS1, SOCS3, and GAPDH protein expression levels (A). GAPDH was as internal control. Protein bands were quantified with densitometry and the relative density ratios of SOCS1/GAPDH (B) and SOCS3/GAPDH (C) were shown. * $p < 0.05$ compared with control.

regulating cell cycle progression genes such as cyclin D1, PCNA, and p-RB, also control the production of the chemokines (CCL2, CXCL10, and CXCL8) and human β -defensins (HBD-2 and HBD-3) in keratinocytes.^{30–31} Thus, by downregulating STAT1 and STAT3, calcipotriol could potentially provide treatment for the inflammatory process and keratinocyte proliferation that characterize psoriasis. The JAK pathway is also involved in the signal

transduction pathway downstream of many of the cytokines implicated in the pathogenesis of psoriasis.^{13–32} A preclinical study showed a small molecule inhibitor of JAK1 and JAK2 exhibits clinical activity for the topical treatment of psoriasis.³³ Other inhibitors such as tofacitinib and lestaurtinib are in phase II trials to treat psoriasis.^{34–36} Given that JAKs are the primary activators of STAT proteins,³⁷ the effect of calcipotriol treatment on JAK activation during psoriasis warrants further investigation.

The SOCS family of proteins negatively regulates many of the cytokine signal-transduction pathways that are involved in the immunopathogenesis of inflammatory disease.³⁸ SOCS expression is rapidly induced by the JAK/STAT pathway, and then triggers a negative feedback process to inhibit JAK/STAT signaling. The SOCS proteins can inhibit JAK/STAT signaling via several inhibitory mechanisms, including directly binding to the catalytic domain of JAKs and inhibiting the recruitment and phosphorylation of STAT proteins.^{39–40} Among the SOCS family members, SOCS1 and SOCS3 are most well characterized with regard to inflammation and cancer.^{41–42} SOCS1 inhibits STAT1 activation in response to IFN- γ signaling, while SOCS3 is a major negative regulator of IL-6 induced STAT3 signaling.³⁹ Increasingly, the roles of SOCS1 and SOCS3 in psoriasis are being recognized, but they are still largely unknown.³⁸ SOCS1 and SOCS3 proteins are highly expressed in the epidermis of patients with psoriasis, which might associate with impaired IFN- γ signaling.⁴³ T cells from patients with psoriasis are deficient in SOCS3 expression, which leads to an increased sensitivity to IFN- γ .⁴⁴ In transgenic mice, specific deletion of SOCS3 in keratinocytes caused severe psoriasis-like skin inflammation, although SOCS1 deletion caused no inflammation.⁴⁵ Another study showed that SOCS1 and SOCS3 suppressed cytokine-induced apoptosis by sustaining the activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway in keratinocytes, which results in the thickening of psoriatic skin.⁴⁶ Further investigation is necessary to determine whether the calcipotriol-induced downregulation of SOCS1 and SOCS3 in our study has similar biological functions.

In conclusion, our data support that calcipotriol-mediated inhibition of cell proliferation may be associated with downregulation of the STAT1 and STAT3 signaling pathway. Consistent with previous findings, inhibiting STAT1 and STAT3 exerts an anti-inflammatory and anti-proliferative effect in psoriasis. We provide evidence suggesting that calcipotriol might have a dual mechanism of action in the treatment of psoriasis.

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Contributors LS designed the study and wrote the protocol. LS, WL and ZL performed research/study. ZL, LZ, XQ and YZ managed the literature searches and analyses. WL and LS wrote the first draft of the manuscript. LS took overall responsibility. All authors have read and approved the final draft of this article.

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Competing interests None declared.

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