Effect of RNA Interference Targeting *STAT3* Gene Combined with Ultrasonic Irradiation and SonoVue Microbubbles on Proliferation and Apoptosis in Keratinocytes of Psoriatic Lesions

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Abstract

Background: Signal transducer and activator of transcription 3 (STAT3) was strongly expressed and activated in psoriatic keratinocytes (KCs) and correlated with the severity of psoriasis. The study aimed to investigate the effects of *STAT3* small interfering RNA (siRNA) combined with ultrasonic irradiation and SonoVue microbubbles on the proliferation and apoptosis in KCs of psoriatic lesions and the relative mechanisms.

Methods: Psoriatic KCs were transfected under four experimental conditions: (1) *STAT3* siRNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles (LUS group); (2) *STAT3* siRNA only carried by Lipofectamine 3000 (L group); (3) the negative control of siRNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles (siRNA-NC); (4) not treated as Blank. Cell Counting Kit-8 assay was used to evaluate the cell proliferation. Cell cycle analysis was detected with cycle test Plus DNA reagent kit associated with flow cytometer. FITC Annexin V apoptosis detection kit associated with flow cytometer was applied for apoptosis analysis. Fluo calcium indicator associated with flow cytometer was used to analyze intracellular free calcium concentration ($[Ca^{2+}]_i$). The expressions of cyclin D1 and Bcl-xL were detected both at the mRNA level by real-time reverse transcription-polymerase chain reaction (RT-PCR) and at the protein level by Western blotting. The obtained data were statistically evaluated by two-way analysis of variance.

Results: *STAT3* siRNA inhibited the growth of KCs in a time-dependent manner showing the highest proliferation inhibition in LUS group with proliferation ratio of 45.38% ± 5.85% at 72h ($P < 0.05$ vs. L group, siRNA-NC, or Blank). *STAT3* siRNA induced an altered cell cycle distribution of KCs showing the highest increases in G2/M-phase population up to 18.06% ± 0.36% in LUS group ($P < 0.05$ vs. L group, siRNA-NC, or Blank). *STAT3* siRNA induced late apoptosis of KCs with the highest late apoptosis percentage of 22.87% ± 1.28% in LUS group ($P < 0.05$ vs. L group, siRNA-NC, or Blank). *STAT3* siRNA induced the elevation of $[Ca^{2+}]_i$ of KCs with the highest calcium fluorescence intensity mean of 1213.67 ± 60.51 in LUS group ($P < 0.05$ vs. L group, siRNA-NC, or Blank). *STAT3* siRNA induced the downregulation of cyclin D1 and Bcl-xL expressions of KCs at mRNA and protein levels with the lowest expressions in LUS group with cyclin D1 expression of 51.81% ± 9.58% and 70.17% ± 4.22% at mRNA level and at protein level, respectively, and with Bcl-xL expression of 37.58% ± 4.92% and 64.06% ± 7.78% at mRNA level and at protein level, respectively ($P < 0.05$ vs. L group, siRNA-NC, or Blank).

Conclusions: *STAT3* siRNA inhibited the growth and induced the apoptosis in psoriatic KCs likely partly through altering cell cycle distribution, elevating $[Ca^{2+}]_i$, and downregulating cyclin D1 and Bcl-xL expressions. Silencing the target gene *STAT3* in psoriatic KCs with siRNA combined with ultrasonic irradiation and microbubbles would contribute to a significant innovation as a new clinical therapy for psoriasis.

Key words: Apoptosis; Cell Proliferation; Keratinocytes; Microbubbles; Psoriasis; RNA Interference; Small Interfering RNA; STAT3 Transcription Factor

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Introduction

Psoriasis is an immune-mediated, chronic inflammatory skin disease with a complex etiology involving both genetic and environmental risk factors which affects approximately 1–3% of the population worldwide.[1] It had been reported that signal transducer and activator of transcription 3 (STAT3) was strongly expressed and activated in psoriatic keratinocytes (KCs) and participated in the hyperproliferation of KCs which was the main characteristic of psoriasis, and *STAT3* could be recognized as a therapeutic target for psoriasis treatment.[2-4] Some studies discovered that small interfering RNA (siRNA) combined with ultrasonic irradiation and SonoVue microbubbles could trigger RNA interference (RNAi), enhance gene transfer efficiency, and be applied for the tissue-specific delivery.[5,6] Our previous studies had screened out the most effective sequence of siRNA targeted to the *STAT3* gene of psoriatic KCs and also showed the optimal dose- and time-response of the siRNA combined with ultrasonic irradiation and SonoVue microbubbles on knocking down the *STAT3* expression of psoriatic KCs.[7] In this study, the effects of *STAT3* siRNA combined with ultrasonic irradiation and SonoVue microbubbles on the proliferation and apoptosis of psoriatic KCs were investigated, and the relative mechanisms were discussed. This study aimed to provide a new way of exploring gene therapy in the treatment of psoriasis.

Methods

Patients and ethical approval

The study was approved by the Ethics Committee of Beijing Chaoyang Hospital, and the informed consent was obtained from all participants. Patients with active chronic plaque psoriasis were recruited for the study. They had not received any topical treatment for at least 4 weeks or any systemic treatment for at least 3 months before sampling. The biopsies were taken under local anesthesia from chronic psoriatic plaques. The biopsy specimens were used for isolating and culturing KCs.

Skin biopsy processing and keratinocytes culturing

Biopsies were rinsed with PBS containing antibiotics and incubated in 0.25% Dispase solution (Gibco, Thermo Fisher Scientific, USA) overnight at 4°C. On the following day, the epidermis was peeled from the dermis and incubated in the solution with 0.25% Trypsin-EDTA (Gibco) at 37°C for 10–15 min, and then the digestion was terminated with DMEM solution (Gibco) containing 10% fetal bovine serum (Gibco). The epidermis was blown and filtered, and then the KCs suspension was collected. The psoriatic KCs were seeded in culture flask filled with defined KC serum-free medium supplemented with growth supplement and bovine pituitary extract (Gibco). Cells were grown in the incubator at 37°C with 50 ml/L CO_2 and 95% relative humidity. At flaky confluence, KCs passage was made. The third generational KCs were applied for later experiments. Each experiment described below was performed three times using the same lot of KCs.

Selected small interfering RNA and transfection condition

The preliminary studies of the project showed that the siRNA targeting to *STAT3* (target sequence: CCGTGGAACCATACACAAA, sense: 5'- CCGUGGAACCAUACACAAAdTdT-3' and antisense: 3'- dTdTGGCACCUUGGUAUGUGUUU-5') achieved the highest knockdown effect among three designed siRNAs and was selected for the following experiments. The negative control of siRNA (siRNA-NC), with random sequences, did not target any known mammalian gene. The siRNAs were chemically synthesized by Guangzhou Ribobio (Ribobio, China). The preliminary studies of the project also showed that 100 nmol/L siRNA carried by Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, USA) combined with ultrasonic irradiation at the parameters of 0.5 W/cm^2 and 30 s and 2.5 μg/μl SonoVue microbubbles (Bracco Suisse SA, Switzerland) could significantly downregulate the protein expression of the *STAT3* gene in KCs at 72 h after transfection, and thus the same transfection conditions were used in this research. The ultrasonic transfer apparatus was from Institute of Ultrasound Imaging of Chongqing Medical University.

Evaluation of cell proliferation (Cell Counting Kit-8 assay)

Growth curves were plotted with Cell Counting Kit-8 (Dojindo, Japan) according to the manufacturer's instructions. KCs were seeded into sterilized 96-well plastic plates made of polystyrene and transfected with 100 nmol/L *STAT3* siRNA (LUS group) or siRNA-NC carried by Lipofectamine 3000 associated with SonoVue microbubbles and then irradiated by the probe of ultrasonic transfer apparatus through ultrasound couplant (Yalian, China) from the bottom of the plates. At the same time, one group of cells was transfected with 100 nmol/L *STAT3* siRNA only carried by Lipofectamine 3000 (L group), and one group of cells was not treated (Blank). KCs were incubated for 24, 48, 72, or 96 h. Ten microliters Cell Counting Kit-8 (CCK-8) reagent was added to the cells, and KCs were incubated for 2 h. Absorbance at 450 nm was measured using the Rayto RT-6000 enzyme microplate reader (Rayto, China). Results were derived from the mean of triplicate wells. Cell proliferation was expressed as a percentage ratio of exposed cells to blank control cells.

Cell cycle analysis

KCs were seeded into sterilized plastic dish made of polystyrene and transfected under four experimental conditions: (1) LUS group, (2) L group, (3) siRNA-NC, and (4) Blank. KCs were incubated for 72 h. Attached cells were trypsinized and pooled with the detached population, and subsequently, the cell cycle was detected with cycle test Plus DNA reagent kit (BD Biosciences, USA) according to the manufacturer's instructions. Cell cycle analysis was performed using a FACSCalibur flow cytometer (BD, USA), and the percentage of cells in G0/G1, S, and G2/M phases was determined using ModFit LT analysis program (Verity Software House, USA).

Annexin V-FITC apoptosis analysis

KCs were seeded into sterilized plastic dish and transfected under four experimental conditions: (1) LUS group, (2) L group, (3) siRNA-NC, and (4) Blank. KCs were incubated for 72 h. Attached cells were trypsinized, pooled with the detached population, and subsequently treated according to the manufacturer's instructions of FITC Annexin V apoptosis detection kit (BD Biosciences). The cells were analyzed by flow cytometer using the CellQuest analysis program (BD Biosciences). There were four populations of cells that were, respectively, distributed in four quadrants. Upper-left, upper-right, lower-left, and lower-right quadrants indicated, respectively, the percentages of cells damaged, in late apoptosis, viable, and in early apoptosis.

Assays for intracellular free calcium concentration ($[Ca^{2+}]_i$)

KCs were seeded into sterilized plastic dish and transfected under four experimental conditions: (1) LUS group, (2) L group, (3) siRNA-NC, and (4) Blank. KCs were incubated for 72 h. The incubated cells were harvested and $[Ca^{2+}]_i$ was detected according to the manufacturer's instructions of Fluo Calcium Indicators kit (Fluo-4/AM) (Invitrogen, UK). The cells were analyzed by flow cytometer using CellQuest analysis program.

Real-time reverse transcription-polymerase chain reaction and Western blotting assays for cyclin D1 and Bcl-xL expressions

KCs were seeded into sterilized plastic dish and transfected under four experimental conditions: (1) LUS group, (2) L group, (3) siRNA-NC, and (4) Blank. KCs were incubated for 72 h. The incubated cells were harvested and the expressions of cyclin D1 and Bcl-xL were detected at mRNA level by real-time reverse transcription-polymerase chain reaction (RT-PCR) and at protein level by Western blotting.

RNA extraction and real-time reverse transcription-polymerase chain reaction

The total RNA was extracted from KCs using TRIzol reagent (Ambion, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized using the TIANScript RT-PCR reagent kit (TIANGEN, China) according to the manufacturer's instructions. The specific primers for cyclin D1 (forward: 5'-TAGCAGCGAGCAGCAGAGTC-3', reverse: 5'-TTTCCACTTCGCAGCACAGG-3', 174bp) and for Bcl-xL (forward: 5'-AGCTT TGAACAGGATACTTTTGTGG-3', reverse: 5'-GGTGGGAGGGTAGAGTGGAT-3', 183 bp) were designed according to the BatchPrimer3 software (NCBI Reference Sequence: NM_053056.2 and NM_001191.2, respectively) and synthesized by Sangon Biotech (Sangon, China). The specific primer for β-Actin was provided by Sangon Biotech. Real-time PCR was prepared using the SYBR Premix Ex Taq kit (TAKARA, China) according to the manufacturer's protocol, and amplification was performed on an ABI Prism 7000 detection system (Applied Biosystems, USA) according to the conditions recommended by the manufacturer, with an initial denaturation step at 95°C (2 min), followed by 40 cycles of denaturation at 95°C (15 s) and annealing at 60°C (40 s). At the end of the amplification, a melting curve (disassociation curve) was run to ensure that only a single specific product was amplified. The cycle threshold values were determined. The modification of the $2^{-\Delta\Delta Ct}$ method was used to calculate changes of the relative expressions of cyclin D1 and Bcl-xL normalized against β-Actin.

Protein extraction and Western blotting

The total protein was extracted from the KCs using RIPA lysis buffer and 1:100 dilution of a protease inhibitor cocktail (Sigma, USA). A total of 30 μg of the protein lysate was resolved electrophoretically on SDS-PAGE and blotted onto PVDF membrane (Immobilon, USA). After being blocked for 1 h in blocking buffer (5% nonfat dried milk and 0.1% Tween-20 in Tris-buffered saline [TBS]) and separately incubated with an antihuman cyclin D1 rabbit monoclonal antibody (1:1000) (Cell Signaling, USA), an antihuman Bcl-xL rabbit monoclonal antibody (1:1000) (Cell Signaling, USA), and an antihuman β-Actin rabbit monoclonal antibody (1:1000) (Cell Signaling, USA) for 2 h at room temperature, the blots were washed three times with TBST (0.1% Tween in TBS) and incubated for 1.5 h with goat antirabbit IgG/HRP (1:3000) (ZSJB-BIO, China) at room temperature, followed by washing three times with TBST. The signals were visualized with the enhanced chemiluminescence method and detected by Tanon 4200 chemiluminescence imaging analysis system (Tanon, China). The band density was measured by ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized against the density of the respective housekeeping β-Actin.

Statistical analysis

The obtained data were statistically evaluated by two-way analysis of variance (ANOVA) to compare the difference among groups and presented as mean ± standard deviation (SD) from three independent experiments. A value of $P < 0.05$ was considered statistically significant. All analyses were carried out using SPSS 22.0 version statistical software (SPSS Inc., Chicago, IL, USA).

Results

STAT3 small interfering RNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles inhibited the growth of psoriatic keratinocytes

The results were obtained in CCK-8 assays, revealing that *STAT3* siRNA inhibited the growth of KCs in a time-dependent manner when compared with siRNA-NC and Blank, showing the highest proliferation inhibition in LUS group with proliferation ratio of 45.38% ± 5.85% at 72 h ($P < 0.05$ vs. L group, siRNA-NC, or Blank), the next in L group with proliferation ratio of 64.82% ± 7.17% at 72 h ($P < 0.05$ vs. siRNA-NC and Blank), and no significant difference between siRNA-NC and blank at 72 h ($P = 0.54$) [Figure 1].

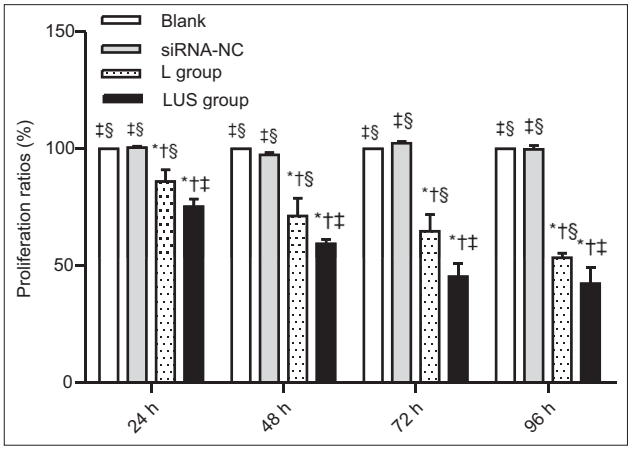

Figure 1: Proliferation ratios of psoriatic KCs obtained by CCK-8 assays. *P < 0.05 versus Blank. †P < 0.05 versus siRNA-NC. ‡P < 0.05 versus L group. §P < 0.05 versus LUS group. KCs: Keratinocytes; CCK: Cell Counting Kit-8; siRNA-NC: Negative control of small interfering RNA.

STAT3 siRNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles altered cell cycle distribution of psoriatic keratinocytes

STAT3 siRNA induced an altered cell cycle distribution of KCs when compared with siRNA-NC and Blank, showing increases in G2/M-phase population up to 18.06% ± 0.36% (P < 0.05 vs. L group, siRNA-NC, or Blank) in LUS group and up to 13.28% ± 2.26% (P < 0.05 vs. siRNA-NC and Blank) in L group, respectively, no significant difference in G2/M-phase population between siRNA-NC and blank (P = 0.89), and no significant differences in G0/G1-phase and S-phase populations among the four groups (P = 0.94, P = 0.42, respectively) [Figure 2].

STAT3 small interfering RNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles mediated apoptosis of psoriatic keratinocytes

STAT3 siRNA induced late apoptosis of KCs significantly when compared with siRNA-NC and blank, with late apoptosis percentage located in the upper-right quadrant of 22.87% ± 1.28% (P < 0.05 vs. L group, siRNA-NC, or Blank) in LUS group and with late apoptosis percentage of 13.96% ± 4.98% (P < 0.05 vs. siRNA-NC and blank) in L group, respectively, without significant difference in late apoptosis percentage between siRNA-NC and Blank (P = 0.99), and there were no significant differences in the percentages of cells in the damaged and in early apoptosis among the four groups (P = 0.43 and P = 0.06, respectively) [Figure 3]. There were 2×10^4 cells analyzed in every group.

STAT3 small interfering RNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles induced the elevation of $[Ca^{2+}]_i$ of psoriatic keratinocytes

STAT3 siRNA induced the elevation of $[Ca^{2+}]_i$ of KCs significantly when compared with siRNA-NC and blank, with calcium fluorescence intensity mean of 1213.67 ± 60.51 (P < 0.05 vs. L group, siRNA-NC, or Blank) which indicated $[Ca^{2+}]_i$ in LUS group and the calcium fluorescence intensity mean of 1028.20 ± 111.40 (P < 0.05 vs. siRNA-NC and Blank) in L group, respectively, and without significant difference between siRNA-NC and blank (P = 0.92) [Figure 4].

STAT3 small interfering RNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles induced the downregulation of cyclin D1 expression of psoriatic keratinocytes

STAT3 siRNA induced the downregulation of cyclin D1 expression of KCs significantly when compared with siRNA-NC and Blank, with 51.81% ± 9.58% cyclin D1 expression at mRNA level (P < 0.05 vs. L group, siRNA-NC, or blank) and 70.17% ± 4.22% cyclin D1 expression at protein level (P < 0.05 vs. L group, siRNA-NC, or Blank) in LUS group, with 67.01% ± 10.06% cyclin D1 expression at mRNA level (P < 0.05 vs. siRNA-NC and Blank) and 80.87% ± 3.45% cyclin D1 expression at protein level (P < 0.05 vs. siRNA-NC and Blank) in L group, respectively, and without significant difference in cyclin D1 expression at mRNA or protein level between siRNA-NC and blank (P = 0.52, P = 0.61, respectively) [Figure 5].

STAT3 small interfering RNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles induced the downregulation of Bcl-xL expression of psoriatic keratinocytes

STAT3 siRNA induced the downregulation of Bcl-xL expression of KCs significantly when compared with siRNA-NC and Blank, with 37.58% ± 4.92% Bcl-xL expression at mRNA level (P < 0.05 vs. L group, siRNA-NC, or blank) and 64.06% ± 7.78% Bcl-xL expression at protein level (P < 0.05 vs. L group, siRNA-NC, or Blank) in LUS group, with 51.49% ± 13.90% Bcl-xL expression at mRNA level (P < 0.05 vs. siRNA-NC and Blank) and 77.02% ± 6.26% Bcl-xL expression at protein level (P < 0.05 vs. siRNA-NC and blank) in L group, respectively, and without significant difference in Bcl-xL expression at mRNA or protein level between siRNA-NC and blank (P = 0.83 and P = 0.20, respectively) [Figure 5].

Discussion

Psoriatic skin lesions are typically characterized by the redness, thickness, and scaling induced by inflammation and epidermal hyperproliferation with disordered KCs differentiation, and these symptoms can severely impair patient quality of life.[1,8] It is generally accepted that various cytokines including TNF-α, IFN-γ, IL-6, IL-12, IL-17, IL-20, IL-22, and IL-23 result in the excessive proliferation and aberrant differentiation of KCs which produce various kinds of immune-related cytokines such as IL-1α, IL-1β, and IL-8, leading to an amplified immune response.[9-11] The cytokines play roles through signaling pathways. It has been demonstrated that Janus kinase (JAK)/STAT signaling pathway is altered in psoriatic skin, specifically

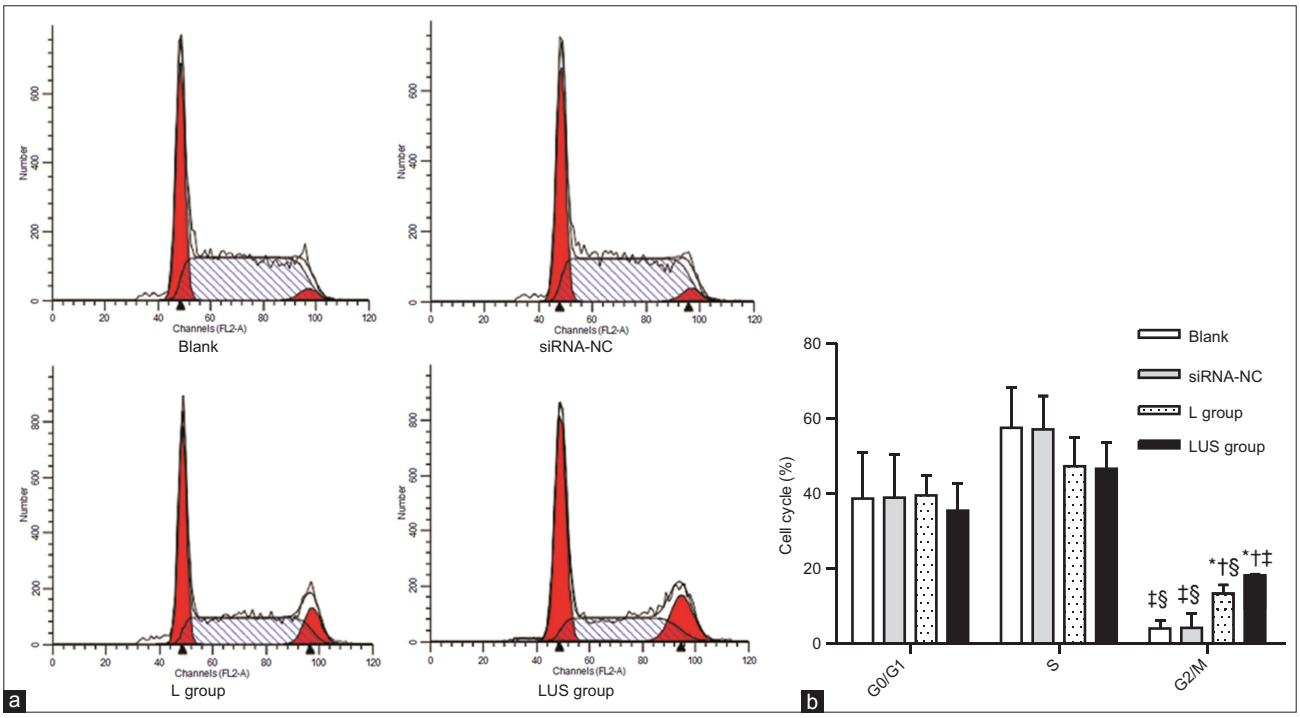

Figure 2: Cell cycle of psoriatic KCs. (a) cell cycle distributions. (b) Cell cycle percentage. *$P < 0.05$ versus Blank. †$P < 0.05$ versus siRNA-NC. ‡$P < 0.05$ versus L group. §$P < 0.05$ versus LUS group. KCs: Keratinocytes; siRNA-NC: Negative control of small interfering RNA.

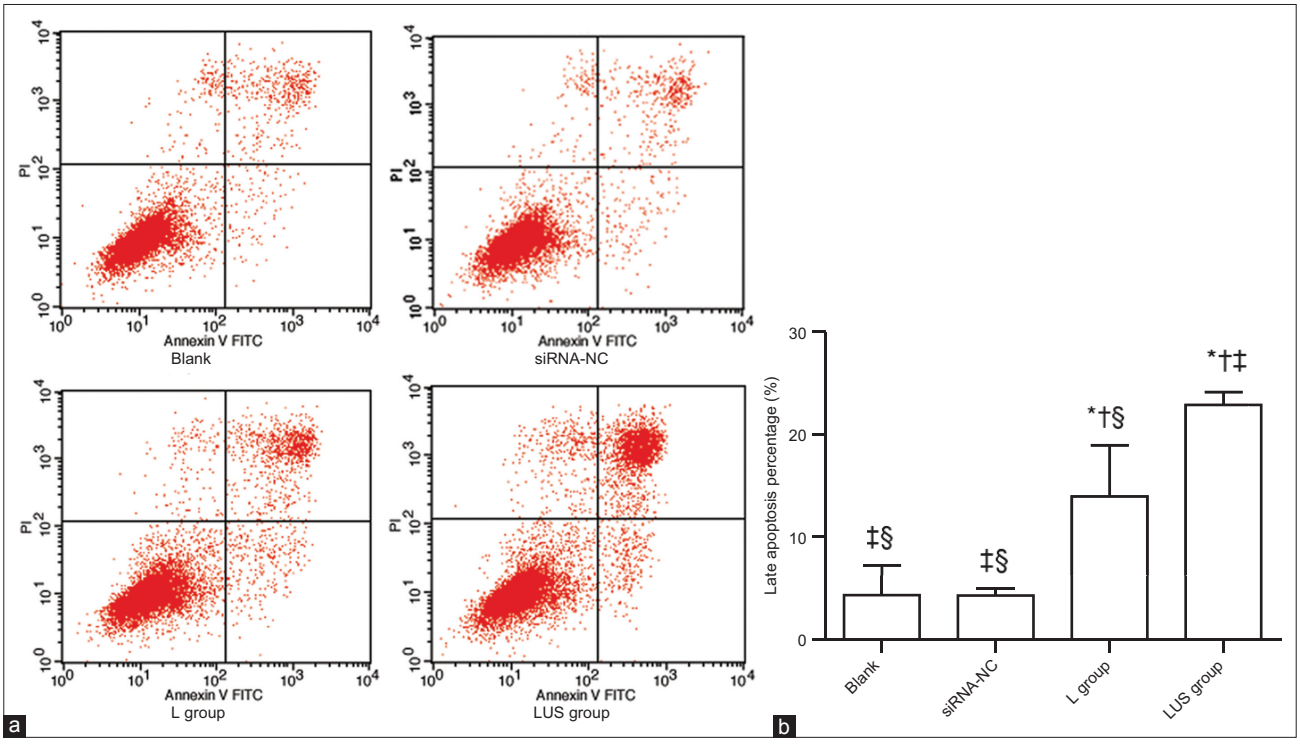

Figure 3: Annexin V-FITC apoptosis analysis of psoriatic KCs. (a) Annexin V-FITC apoptosis distributions. (b) Late apoptosis percentage. *$P < 0.05$ versus Blank. †$P < 0.05$ versus siRNA-NC. ‡$P < 0.05$ versus L group. §$P < 0.05$ versus LUS group. KC: Keratinocytes; siRNA-NC; Negative control of small interfering RNA.

the expression of *STAT3* is correlated with the severity of psoriasis, *STAT3* participates in the hyperproliferation of KCs and inflammatory infiltration, and *STAT3* is a possible important link between KCs and immunocytes which is crucial to the development of psoriasis.[2,4,10,12-14] Thus, blockading the JAK-STAT signaling pathway, especially *STAT3*, could be a potential strategy for the treatment of psoriasis so that the immunocytes/cytokines/KCs network cannot be formed.[2,10,12,15] *STAT3* is a potential therapeutic target for psoriasis.

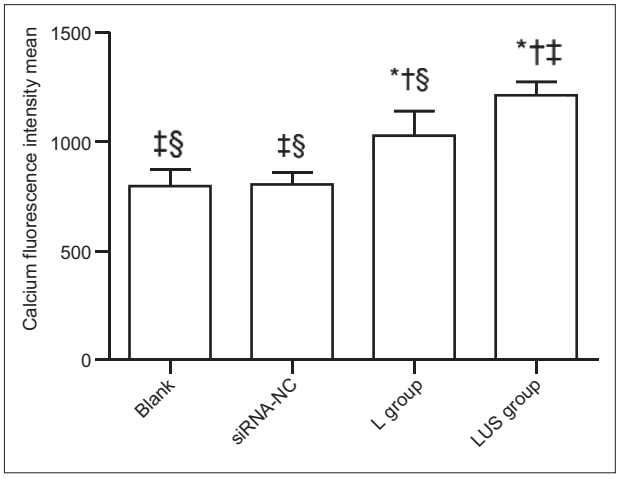

Figure 4: Calcium fluorescence intensity mean which indicated $[Ca^{2+}]_i$ of psoriatic KCs. *$P < 0.05$ versus Blank. †$P < 0.05$ versus siRNA-NC. ‡$P < 0.05$ versus L group. §$P < 0.05$ versus LUS group. KCs: Keratinocytes; siRNA-NC: Negative control of small interfering RNA.

Over the last decade, targeted gene suppression by antisense DNA and siRNA has shown promising preclinical results.[16] Gene silencing through RNAi could inhibit all classes of gene targets with high selectivity and potency, unlike traditional drugs.[17] However, the use of viral vectors is a major concern because this vector system is associated with numerous safety concerns including toxicity, immunogenicity, and tumorigenicity.[5] The present studies show that ultrasound-mediated microbubble destruction may have the potential to become a new approach for targeted gene transfection.[5] Our previous studies had shown *STAT3* siRNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles could effectively knock down and downregulate the protein expression of *STAT3* gene in psoriatic KCs.[7] Subsequently, we applied this kind of experimental method to investigate the effects about *STAT3* gene silencing on the proliferation and apoptosis of psoriatic KCs and the relative mechanisms. The same study was not found in previous reports.

Previous studies have shown that *STAT3* is a key transcriptional factor involved in the regulation of cell proliferation, and the inhibitions of *STAT3* phosphorylation or expression can result in decreased proliferation of normal human KCs or HaCaT cells.[12,18] In our study, *STAT3* siRNA inhibited the growth of psoriatic KCs in a time-dependent manner. The siRNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles showed higher proliferation inhibition compared with that only carried by Lipofectamine 3000.

Several studies have suggested that *STAT3* promotes the cell cycle, and the inhibitions of *STAT3* phosphorylation or phosphorylated *STAT3* translocation may be related with accumulation of cells in the G1 or G2/M phase in normal human KCs or HaCaT cells.[15,19] In the present study, *STAT3* siRNA induced an altered cell cycle distribution of psoriatic KCs showing increases in G2/M-phase population

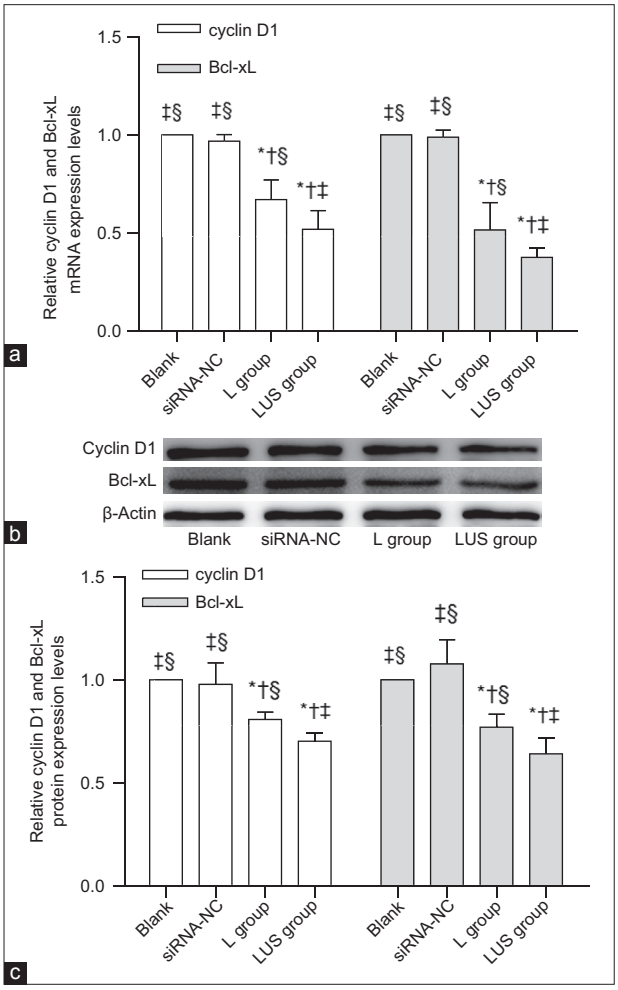

Figure 5: Expressions of Cyclin D1 and Bcl-xL in psoriatic KCs. (a) Relative cyclin D1 and Bcl-xL mRNA expression ratios by real-time RT-PCR and normalized against β-Actin. (b) Cyclin D1 and Bcl-xL protein expressions by Western blotting. (c) Relative cyclin D1 and Bcl-xL protein expression ratios by Western blotting and normalized against β-Actin. *$P < 0.05$ versus Blank. †$P < 0.05$ versus siRNA-NC. ‡$P < 0.05$ versus L group. §$P < 0.05$ versus LUS group. KCs: Keratinocytes; RT-PCR: Reverse transcription-polymerase chain reaction.

and no significant alterations in G0/G1-phase and S-phase populations. The siRNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles showed higher G2/M-phase cell percentage compared with that only carried by Lipofectamine 3000. It can be said that *STAT3* siRNA influences the cycle progression of psoriatic KCs and causes the accumulation of cells in G2/M-phase and the inhibition of DNA synthesis: one of the possible mechanisms about proliferation arrest of psoriatic KCs elicited by *STAT3* siRNA.

Previous studies suggest that the ability of psoriatic KCs to resist apoptosis is a key mechanism in psoriatic pathogenesis, *STAT3* activation is involved in modulating cell apoptosis, and the inhibition of *STAT3* signaling or dephosphorylation of *STAT3* induces apoptosis of HaCaT KCs or normal KCs.[4,14,20] In our study, *STAT3* siRNA

induced the late apoptosis of psoriatic KCs significantly. The siRNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles showed higher late apoptosis percentage compared with that only carried by Lipofectamine 3000. Further elucidation about the effects of *STAT3* RNAi on apoptosis pathway needs subsequent research.

Some reports suggest that the psoriatic epidermis shows an altered calcium metabolism, there exist specific defect of calcium influx and reduced $[Ca^{2+}]_i$ in psoriatic KCs, calcium can significantly inhibit the proliferation of KCs, and high calcium can result in a reduced proliferation of psoriatic KCs.[21,22] Our results indicated that *STAT3* siRNA induced the elevation of $[Ca^{2+}]_i$ of psoriatic KCs significantly. The siRNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles showed higher calcium fluorescence intensity mean which indicated $[Ca^{2+}]_i$ compared with that only carried by Lipofectamine 3000. It can be suggested that the inhibition of *STAT3* expression of psoriatic KCs induces the release of the calcium in intracellular calcium store and the inflowing of the calcium from extracellular to intracellular compartments. This elevation of $[Ca^{2+}]_i$ may be one of the mechanisms causing proliferation arrest of psoriatic KCs due to knocking down of *STAT3*. Further elucidation of the mechanism about the elevation of $[Ca^{2+}]_i$ regulating proliferation arrest of psoriatic KCs needs subsequent research.

Potential target genes of *STAT3* are cell survival genes including Bcl-xL and Bcl-2 and cell cycle regulators such as cyclin D1, and the downregulation of *STAT3* has been shown to downregulate cyclin D1 and Bcl-xL of HaCaT KCs.[14,23] The present study demonstrated that *STAT3* siRNA induced the significant downregulations of cyclin D1 and Bcl-xL expressions in psoriatic KCs at both mRNA and protein levels. The siRNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles showed lower cyclin D1 and Bcl-xL expressions compared with that only carried by Lipofectamine 3000. The data suggest that the apoptosis mediated by silencing *STAT3* of psoriatic KCs may be induced partly through downregulating the Bcl-xL expression as an antiapoptotic protein and the suppressed G2/M-phase switching mediated by silencing *STAT3* of psoriatic KCs may be induced partly through downregulating the cyclin D1 expression as a gene regulating cell cycle progression, although the mechanisms about apoptosis and cell cycle are complicated and further investigations are necessary to elucidate them.

In conclusion, the results suggest that *STAT3* siRNA inhibited the growth and induced the apoptosis of psoriatic KCs likely partly through altering the cell cycle distribution, elevating $[Ca^{2+}]_i$, and downregulating cyclin D1 and Bcl-xL expressions. The siRNA carried by Lipofectamine 3000 combined with ultrasonic irradiation and SonoVue microbubbles showed more significant effects than that carried by Lipofectamine 3000 alone. We conclude that silencing the target gene *STAT3* in psoriatic KCs with siRNA combined with ultrasonic irradiation and microbubbles would contribute to a significant innovation as a new clinical therapy for psoriasis. The experiment needs to be performed *in vivo* to further support the conclusion, and imiquimod-induced psoriasis mice model may be applied for the relative study.

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Conflicts of interest

There are no conflicts of interest.

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联合超声辐照和声诺维微泡靶向STAT3基因的RNA干扰对银屑病皮损角质形成细胞增殖和凋亡的影响

摘要

背景：信号传导子和转录活化子3（Signal transducer and activator of transcription 3, STAT3）在银屑病角质形成细胞（keratinocytes, KCs）中表达增加并活化，与银屑病严重程度相关。本研究目的是调查联合超声辐照和声诺维微泡的STAT3小分子干扰RNA（small interfering RNA, siRNA）对银屑病皮损KCs增殖和凋亡的影响及其机制。

方法：在以下4种实验条件下对银屑病KCs进行转染：①联合超声辐照和声诺维微泡的以Lipo3000为载体的STAT3 siRNA作为LUS组；②以Lipo3000为载体的STAT3 siRNA作为L组；③联合超声辐照和声诺维微泡的以Lipo3000为载体的阴性对照siRNA作为siRNA-NC组; ④不作处理的作为Blank组。CCK-8实验用于检测细胞增殖。细胞周期检测试剂盒联合流式细胞术分析细胞周期。FITC Annexin V凋亡检测试剂盒联合流式细胞术分析细胞凋亡。钙荧光探针联合流式细胞术分析胞内游离钙离子浓度($[Ca^{2+}]_i$)。应用实时RT-PCR 和Western blotting 分别在mRNA 和蛋白水平检测cyclin D1和Bcl-xL的表达。应用两因素方差分析对数据进行统计学分析。

结果：STAT3 siRNA以时间依赖方式抑制KCs增殖且尤以LUS组为著，72h时其增殖比率为45.38% ± 5.85% (与L 组、siRNA-NC组及Blank组相比，均P<0.05)。STAT3 siRNA 改变KCs细胞周期进程，LUS组G2/M期细胞百分比最高，为18.06% ± 0.36% (与L 组、siRNA-NC组及Blank组相比，均P<0.05)。STAT3 siRNA促进KCs晚期凋亡且尤以LUS组为著，其晚期凋亡百分比最高，为22.87% ± 1.28% (与L 组、siRNA-NC组及Blank组相比，均P<0.05)。STAT3 siRNA诱发KCs $[Ca^{2+}]_i$升高，LUS组钙离子荧光强度均值最高，为1213.67 ± 60.51 (与L 组、siRNA-NC组及Blank组相比，均P<0.05)。 STAT3 siRNA下调KCs cyclin D1和Bcl-xL的表达且尤以LUS组为著，其cyclin D1和Bcl-xL在mRNA 和蛋白水平表达比率分别为51.81% ± 9.58%、70.17% ± 4.22%和37.58% ± 4.92%、64.06% ± 7.78% (与L 组、siRNA-NC组及Blank组相比，均P<0.05)。

结论：STAT3 siRNA抑制银屑病KCs增殖并诱导其凋亡，可能部分通过改变细胞周期进程、升高$[Ca^{2+}]_i$、下调cyclin D1和Bcl-xL的表达。联合超声辐照和声诺维微泡的siRNA沉默银屑病皮损KCs靶基因STAT3，将有助于开创一种新的银屑病治疗方法。