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ISG15 promotes M5-induced hacat cell proliferation through Wnt signaling in psoriasis

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Abstract

Objective: Psoriasis is a common chronic, recurrent, immune-mediated inflammatory skin disease, which tends to occur in cold areas. Its pathogenesis is currently unclear. This study aims to screen differentially expressed genes in the psoriasis dataset, identify the central genes, detect the expression of central genes in psoriasis lesions of patients in the cold regions and then conduct further research. **Methods:** Differential genes associated with psoriasis in the GEO database were analyzed, and functional enrichment analysis and protein-protein interaction network analysis. The expression results of the identified genes were validated in psoriasis cell models. The *ISG15* gene, which showed the most significant difference in expression, was further studied. The expression level of *ISG15* protein in psoriasis was examined. Then, we knocked out *ISG15* in psoriasis cell models and detected keratinocyte proliferation by MTT, Real-Time PCR and Western Blot. Western Blot showed the expression of β-catenin after *ISG15* gene knockout. **Results:** We detected the protein expression of ISG15 in the cold area of Northeast China, and found that the expression of ISG15 increased in patients with psoriasis, and the proliferation of keratinocytes and the expression of β-catenin decreased in psoriasis cell model after ISG15 was knocked down. *ISG15* regulates keratinocyte proliferation through Wnt signaling pathway in psoriasis. **Conclusions:** *ISG15* expression is increased in psoriatic cells and skin lesions of patients with psoriasis. In psoriasis, *ISG15* promotes keratinocyte proliferation through the Wnt signaling pathway.

Keywords

psoriasis; keratinocyte proliferation; *ISG15*; Wnt signaling pathway

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1 Introduction

Psoriasis is a common chronic, recurrent, immune-mediated inflammatory skin disease[1]. It is not only a skin disease but also involves multiple systems[2]. The etiology of psoriasis is related to various factors such as heredity, immunity, and environment. The disease tends to worsen in winter and improve in summer, with a higher incidence rate in cold, high latitudes areas is higher compared to warm, low latitude areas. Clinically, psoriasis is characterized by local or widespread erythema and scales and is often associated with other systemic abnormalities[3]. Biologics have played an active role in the management of psoriasis; however, achieving clinical remission remains a challenge for most patients, with relapse posing as a persistent issue in its treatment.

The pathogenesis of psoriasis involves an immune response mediated by T lymphocytes, with the participation of various immune cells[4]. Both the innate immune and adaptive immune systems are involved in this process, with myelocytes, T cells, and keratinocytes (KCs) playing key roles[5-6]. The dendritic cell (DC)/IL-23/T17/IL-17/KCs axis forms a key positive feedback loop that promotes the progression of psoriasis[7].

ISG15 (Interferon-Stimulated Gene 15) is a member of the ubiquitin-like protein (UBLS) family, produced under the stimulation of interferon. *ISG15* is expressed at low levels in normal cells and under physiological conditions[8]. However, type I interferon and virus infection can induce its expression[9]. While *ISG15* plays an important role in resisting viral infection, its expression level is abnormally increased in many inflammatory reactions and diseases. Raposo *et al.*[10]

conducted RNA high-throughput sequencing on psoriatic skin lesions and found that *ISG15* was significantly overexpressed in psoriatic skin lesions. In contrast, there was no significant difference in peripheral AVP expression in psoriasis patients. Gao *et al.*[11] discovered high *ISG15* expression in psoriasis through bioinformatics data analysis and the detection of psoriasis cell model of human keratinocytes stimulated by TNF-α. Lu *et al.*[12] found that *ISG15* expression level in psoriatic lesions significantly decreased after three months of treatment, with biological agents more effectively reducing *ISG15* expression compared to other drugs. These findings suggest that *ISG15* may be closely related to the severity of psoriasis and activity of psoriatic disease.

However, there has not been any study on whether *ISG15* plays a role in the pathogenesis of psoriasis. In this study, differentially expressed genes in the psoriasis dataset in the GEO database were first screened. Central genes were then identified through GO and KEGG enrichment analyses and protein-protein interaction (PPI) network analysis. The expression of *ISG15* was detected in psoriasis lesions of patients in the cold regions of Northeast China. The effect of *ISG15* on the proliferation of the psoriatic cell model was validated by knocking down *ISG15 in vitro*. The results suggest that *ISG15* may be involved in the pathogenesis of psoriasis by activating the Wnt signaling pathway to promote keratinocyte proliferation.

2 Methods

2.1 Data sources

Gene expression profile datasets GSE13355 and GSE30999 were downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). The GSE13355 dataset includes 58 psoriasis lesion samples and 58 non-lesion samples, while the GSE30999 dataset comprises 85 psoriasis lesion samples and 85 non-lesion samples. All patients included in this study were diagnosed with psoriasis vulgaris.

2.2 Data analysis

The original data, platform annotation files, and other relevant information were downloaded from the GEO database. The data were standardised again using the normalizeBetweenArrays function of the limma analysis method. The differences between the two groups were analyzed using the limma method, and the results were visualized using a volcano plot.

The samples were divided into two groups: psoriasis patients and healthy controls. The limma analysis method was used to identify the differentially expressed genes between the two groups. The criteria for screening differentially expressed genes were set as $|\log2FC| > 2$ and P value < 0.05. The DEGs results from the two datasets were merged to obtain the common differentially expressed genes.

2.3 Functional enrichment analysis

GO and KEGG analyses were performed using DAVID online data analysis software. GO analysis describes the molecular biological function of genes from three aspects: biological process (BP), cell component (CC) and molecular function (MF).

2.4 PPI network analysis

STRING protein database collects, evaluates, and integrates information about PPI, and can also predict and supplement relevant information using computational methods. In this study, the selected differentially expressed genes were imported into the STRING protein database (https://cn.string-db.org/) to obtain the PPI network related to these genes. MCODE is a plug-in in Cytoscape software used to identify key genes within the PPI network.

2.5 Clinical sample collection

The clinical study subjects were recruited from patients with psoriasis vulgaris in the Department of Dermatology, the Second Affiliated Hospital of Harbin Medical University, located in the cold region of Northeast China. All patients provided signed informed consent, and the study was reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (No. 2023-145).

Patient screening criteria: (1) Patients were aged between 20 and 60 years, regardless of sex. (2) Inclusion criteria were patients diagnosed of psoriasis vulgaris and a baseline PASI score ≥ 10. (3) Patients had not received topical treatment for at least 3 weeks and systemic treatment for at least 5 weeks prior to recruitment.

Control samples were collected surgically from skin tissue of healthy patients at the Department of Plastic Surgery, Second Affiliated Hospital of Harbin Medical University, also located in the cold northeastern region of China. All skin samples were immediately stored in liquid nitrogen containers after collection.

2.6 HaCaT cell culture and establishment of a psoriasis cell model

HaCaT cells were initially harvested and resuspended in a 37°C water bath. The cells were cultured in DMEM medium supplemented with 10% FBS, and their growth and passage were monitored regularly. To establish the psoriasis inflammation model, cells were subjected to the following protocol: (1) cells were starved in serum-free medium for 24 h; (2) subsequently, cells were stimulated with a combination of 5 cytokines: 10 ng/mL each of human recombinant proteins IL-17A, TNF-α, IL-1α, IL-22, and oncostatin-M (designated as M5); (3) experiments were conducted 24 h after cytokine stimulation.

2.7 MTT assay

Cells (5 × 10^3) were seeded in 96-well plates and incubated at 37°C for 3 days. Subsequently, 20 μL of MTT solution (5 mg/mL diluted in PBS) was added to each well and then incubated at 37°C for 4 h. After incubation, the violet-blue MTT formazan crystals were solubilized in 200 μL DMSO. The optical density (OD) was measured at 570 nm using a microplate reader. The percentage of viable cells was calculated using the following formula: Percent viability (%) = OD of sample/OD of control × 100, where OD of sample represents the optical density of the experimental wells, and OD of control represents the optical density of the control wells (cells without treatment).

2.8 Cell transfection

Based on the human *ISG15* gene sequence and RNA interference sequences available in GenBank, siRNA sequences targeting *ISG15* were designed and synthesized by Generic BioAnhui. The sequences used were as follows:

For si*ISG15*: Forward: 5'-GCGAACACACUGAGCAUTT-3'
Reverse: 5'-UCUCUGGUGAUGUCGCTT-3'
For negative control NC-siRNAs:
Forward: 5'-UUCUCCGAGGUCACGUTT-3'
Reverse: 5'-ACGUGACACGUUCGGGAGGA-3'

HaCaT cells treated with the M5 cytokine combination were transfected with either *ISG15* siRNA or NC siRNA. After 24 h of transfection, cells were harvested, and protein blotting was performed to assess the efficiency of *ISG15* knockdown.

2.9 Immunofluorescence staining

Tissue sections were deparaffinised, hydrated, and subjected to a antigen retrieval using antigen repair buffer. Subsequently, sections were blocked and incubated with specific antibodies. DAPI staining was performed, followed by sealing the slides with a fluorescence quencher. The slides were then observed and photographed under a microscope to capture staining results. Finally, ImageJ software was used for analysis and quantification of the staining.

2.10 Real time PCR

Total RNA from skin tissues and cells was extracted with TRIpure reagent, followed by reverse transcription into cDNA using BeyoRT II M-MLV reverse transcriptase. The synthesized cDNA served as a template for real-time PCR. Quantitative fluorescence analysis was performed using the ExyclerTM 96 Fluorescence Quantitative Analyzer, and the relative mRNA expression levels were calculated using the 2-ΔΔCt method. The sequences of the primer pairs used are as follows:

ISG15: Forward: 5'-CAGCGAACTCATCTTGCCAG-3' and Reverse: 5'-GACACCTCGGAATTCGTTGC-3';
RSAD2: Forward: 5'-TTGGACATTCGCTATCTCCT-3' and Reverse: 5'-AGTGCTTTGATCTGTTCCGT-3';
LTF: Forward: 5'-CGCGATCCCACCACTGC-3' and Reverse: 5'-AGTGCAGGGTCCGAGGTATT-3';
β-actin: Forward: 5'-GGCACCCAGCACAATGAA-3' and Reverse: 5'-TAGAAGCATTTGCGGTGG-3'.

2.11 Western blot

Cellular proteins were extracted using a PMSF solution, separated by SDS/PAGE gel electrophoresis, and transferred to a PVDF membrane. The membranes were then incubated overnight at 4°C with specific primary antibodies against anti-*ISG15* and β-actin (1 ∶ 1000 dilution). Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies (1 ∶ 500 dilution) at 37°C for 1 h. Finally, the membranes were analyzed and quantified using ImageJ software.

2.12 Statistical analysis

Statistical analyses and graph preparation were performed using SPSS 26.0, GraphPad Prism 9.5 and Adobe Illustrator 2022 software. A *t*-test was used for comparisons between two groups, one-way ANOVA for statistical analyses involving multiple groups, and the chi-squared test for count data. Measurement data are presented as mean ± standard deviation. A significance level of $P < 0.05$ was considered statistically significant statistically significant.

3 Results

3.1 Analysis of expression and functional enrichment of differential genes in psoriasis from the GEO database

Eighty and 376 differentially expressed genes were identified in datasets *GSE13355* and *GSE30999*, respectively ($P < 0.05$ and $|\log FC| > 2$) (Fig. 1A). Integration of differential expression

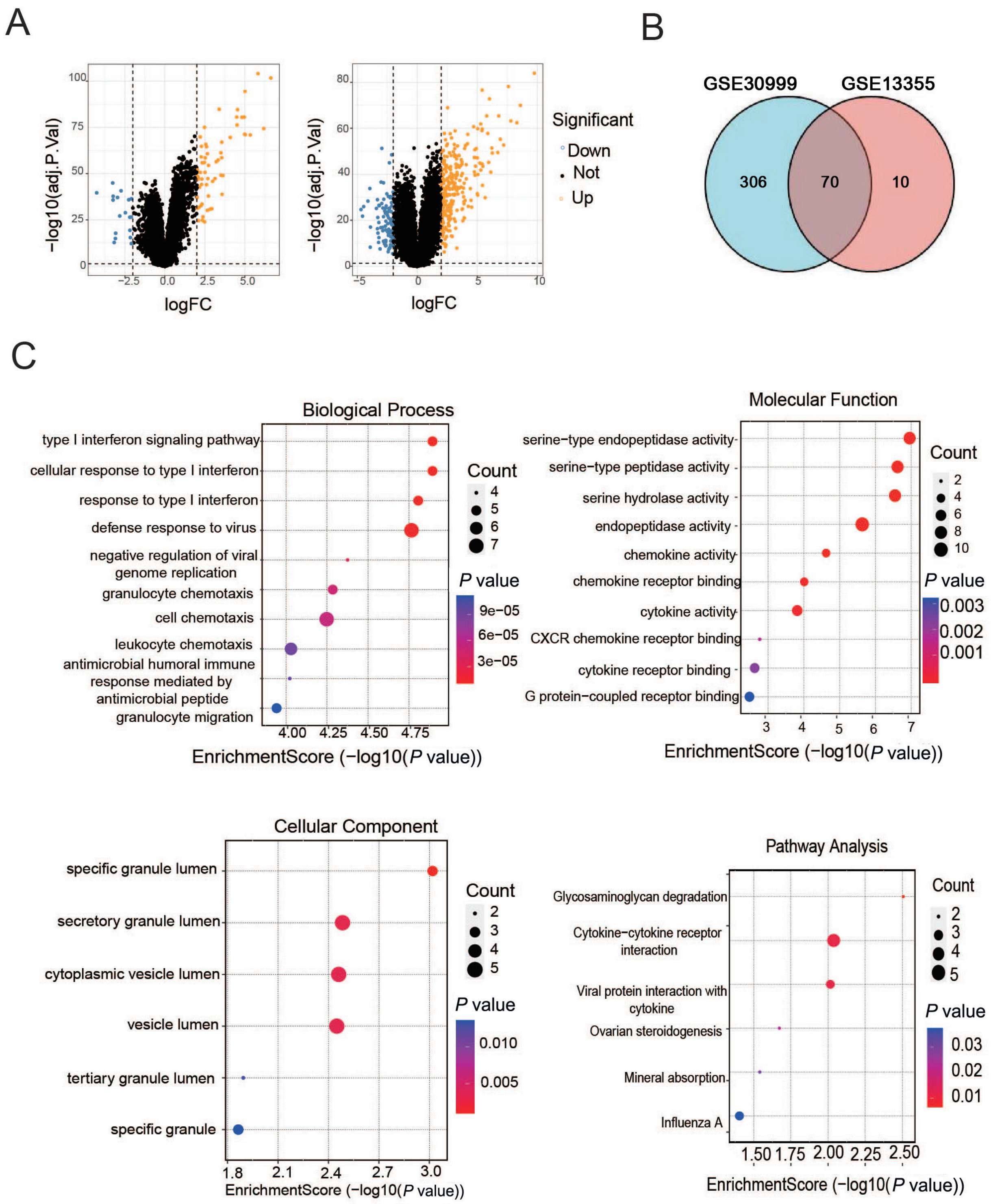

Fig. 1 GO and KEGG enrichment analyses of differentially expressed genes derived fromGSE13355 and GSE30999 datasets

(A) Volcano map showing differentially expressed genes in GSE13355 and GSE30999 datasets. Orange color indicates high expression and blue low expression. (B) Venn diagram of the common differentially expressed genes (cross area) of GSE13355 and GSE30999 datasets. (C) Bubble map illustrating GO analysis (including biological process, cell component, and molecular function) and KEGG enrichment analysis of differentially expressed genes.

analysis results from both datasets identified 70 common differentially expressed genes (Fig. 1B). GO and KEGG pathway enrichment analyses were performed on the 70 differentially expressed genes. In the GO analysis, the differentially expressed genes were primarily enriched in BP such as the type I interferon signaling pathway, defense response to viruses, and immune response to antimicrobial peptides (Fig. 1C). KEGG pathway enrichment analysis revealed enrichment in pathways such as cytokine-cytokine receptor interaction, viral protein-cytokine interaction, and cytokine receptors (Fig. 1C).

3.2 PPI network analysis of differentially expressed genes and validation of gene expression in psoriasis cell models

To identify central genes, 70 differentially expressed genes were analyzed in the PPI network using the STRING database, and interactions were visualized (Fig. 2A). Genes with a degree > 4 were screened, resulting in the identification of 10 central genes involved in the pathogenesis of psoriasis: *CXCL9*, *CCL20*, *RSAD2*, *ISG15*, *OASL*, *SAMD9*, *SERPINB4*, *LTF*, *CXCL13*, and *S100A12*. Previously investigated genes *CXCL13*, *S100A12*, *SERPINB4*, *CXCL9*, *SAMD9*, *CCL20*, and *OASL* were excluded. Based on GO/KEGG enrichment analysis, *RSAD2*, *ISG15*, and *LTF* genes related to keratinocytes, antimicrobial peptides, and antiviral genes were selected for further study.

Real-time PCR was performed to measure mRNA expression levels of *ISG15*, *LTF*, and *RSAD2* genes in the psoriasis cell model established by M5-treated HaCat cells and in the blank control group. The results showed that *ISG15* mRNA expression was significantly increased in psoriasis cell models compared to controls, with a statistically significant difference ($P < 0.001$) (Fig. 2B). *RSAD2* mRNA expression was also increased significantly in psoriasis cell models compared to controls ($P < 0.001$) (Fig. 2B). There was no significant difference in the mRNA expression of *LTF* between psoriasis cell models and controls (Fig. 2B). *ISG15*, which exhibited the most significant upregulation, was selected for further study.

3.3 Upregulated expression of *ISG15* protein in psoriasis patients

The expression of *ISG15* protein was assessed by immunofluorescence staining in 20 psoriasis patients and 15 normal controls from cold areas of northeast China. The results demonstrated a significant upregulation of *ISG15* expression in the skin lesions of psoriasis patients compared to the control group (Fig. 3A). Statistical analysis confirmed a significant difference (Fig. 3B). In the immunofluorescence images, the dotted white line delineates the boundary between the epidermis and the dermis. The upper part of the picture is the epidermis, the lower part is the dermis, and *ISG15* is highly expressed in the epidermis of psoriasis patients. *ISG15* was observed to be highly expressed in the epidermis of psoriasis patients, indicating significant upregulation in both psoriasis cell models and psoriasis patients.

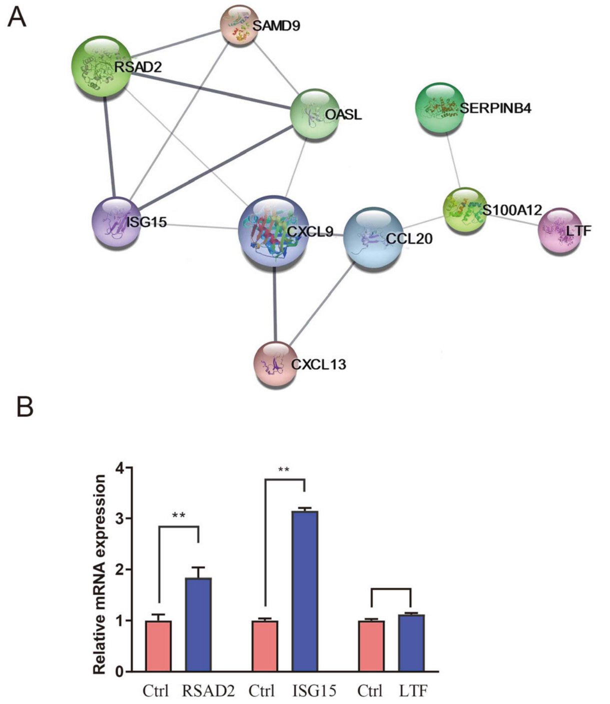

Fig. 2 Protein-protein interaction (PPI) networks of differentially expressed genes and real-time PCR quantification of expression levels of key genes

(A) PPI networks of differentially expressed genes. The size of the circle is proportional to the value. (B) Real time-PCR quantification of the expression levels of *ISG15*, *LTF*, and *RSAD2* genes in psoriasis patients. $^{**}P < 0.01$.

3.4 *ISG15* knockdown inhibits keratinocyte proliferation in psoriasis

ISG15 expression was knocked down in M5-induced HaCaT cells using siRNA. Western blot analysis confirmed significant knockdown in the si*ISG15*# group (Fig. 4A), with statistical significance (Fig. 4B), establishing it for subsequent experiments. The role of *ISG15* in psoriatic cell proliferation was assessed using the MTT assay. In psoriasis cell models, cell proliferation was significantly reduced in the *ISG15* knockdown group (si*ISG15* + M5) compared to the control group (NC-siRNA + M5) ($P < 0.001$) (Fig. 4C). This indicates that *ISG15* knockdown can inhibit M5-induced keratinocyte proliferation in psoriasis.

3.5 *ISG15* silencing in psoriasis reduces Wnt signalling

Previous studies indicate that the Wnt signalling pathway plays

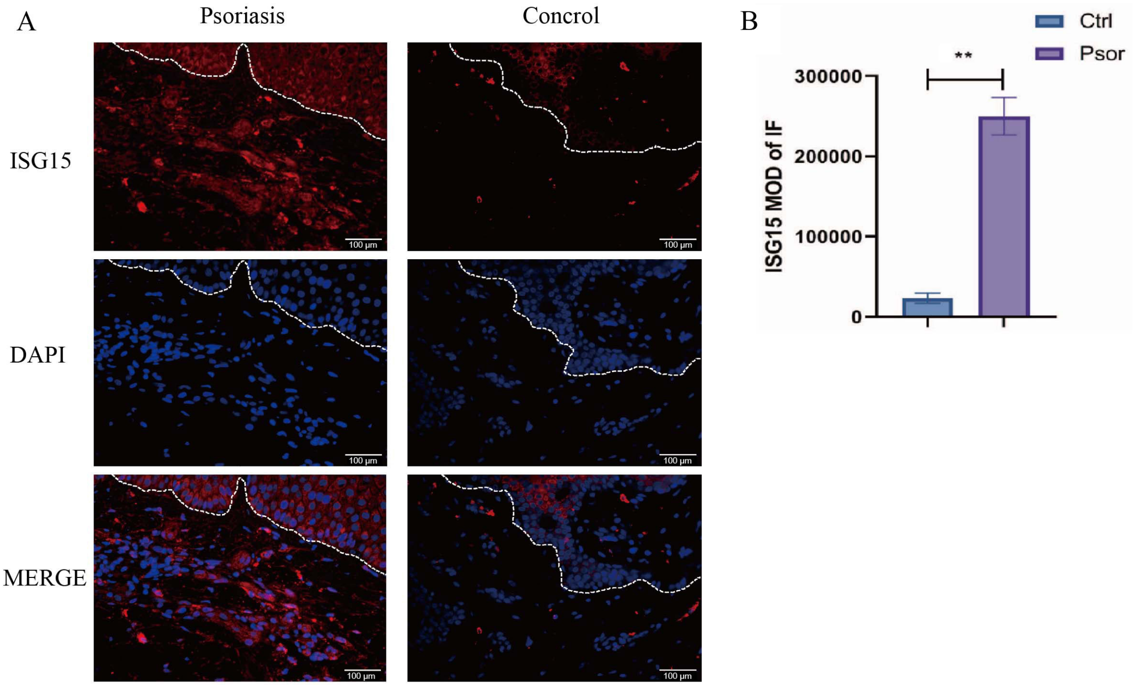

Fig. 3 Immunofluorescence staining of expression and distribution of *ISG15* in psoriasis lesions

(A) Immunofluorescence (IF) was used to visualize the expression and distribution of *ISG15* in psoriatic lesions. (B) Statistical data of immunofluorescent intensity. $^{**}P < 0.01$.

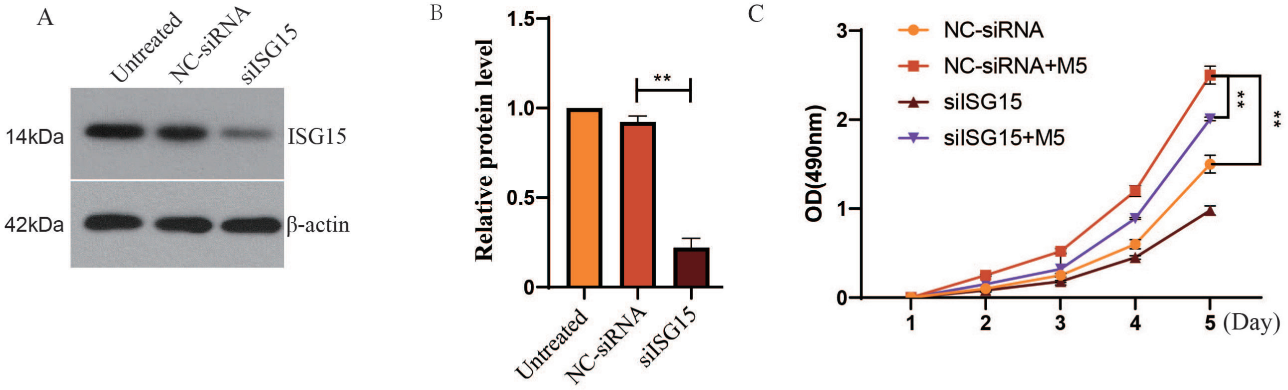

Fig. 4 Silencing *ISG15* in a psoriatic cell model and verifying its phenotypic proliferation efficiency

(A) Western blot images showing *ISG15* protein expression after transfection with NC-siRNA or si*ISG15*. (B) Statistical data of western blot bands. Note that silencing *ISG15* reduced psoriatic cell proliferation. (C) Psoriatic cell proliferation rate following *ISG15* knockdown determined by the MTT assays conducted on 1, 2, 3, 4 and 5 days after si*ISG15* transfection. $^{**}P < 0.01$.

an important role in the pathogenesis of psoriasis. Elevated Wnt pathway activity correlates positively with psoriasis severity, and increased β-catenin expression may enhance IL-6/Th-17 cell expression. T17 cells, IL-17, and keratinocytes (KCs) are pivotal in psoriasis pathogenesis, prompting our focus on the Wnt pathway. Western blot analysis demonstrated a significant decrease in β-catenin expression, a key protein in the Wnt pathway, in the *ISG15* knockout group (si*ISG15* + M5) compared to the control (NC-siRNA + M5) group (Fig. 5A). Statistical analysis confirmed the significant difference (Fig. 5B) ($P < 0.001$). These results suggest that silencing *ISG15* in psoriasis reduces the expression of the Wnt signalling pathway, implicating *ISG15* in the regulation of Wnt pathway activity in psoriatic conditions.

3.6 *ISG15* regulates keratinocyte proliferation through the Wnt signalling pathway in psoriasis

Based on our findings, we hypothesised that *ISG15* may

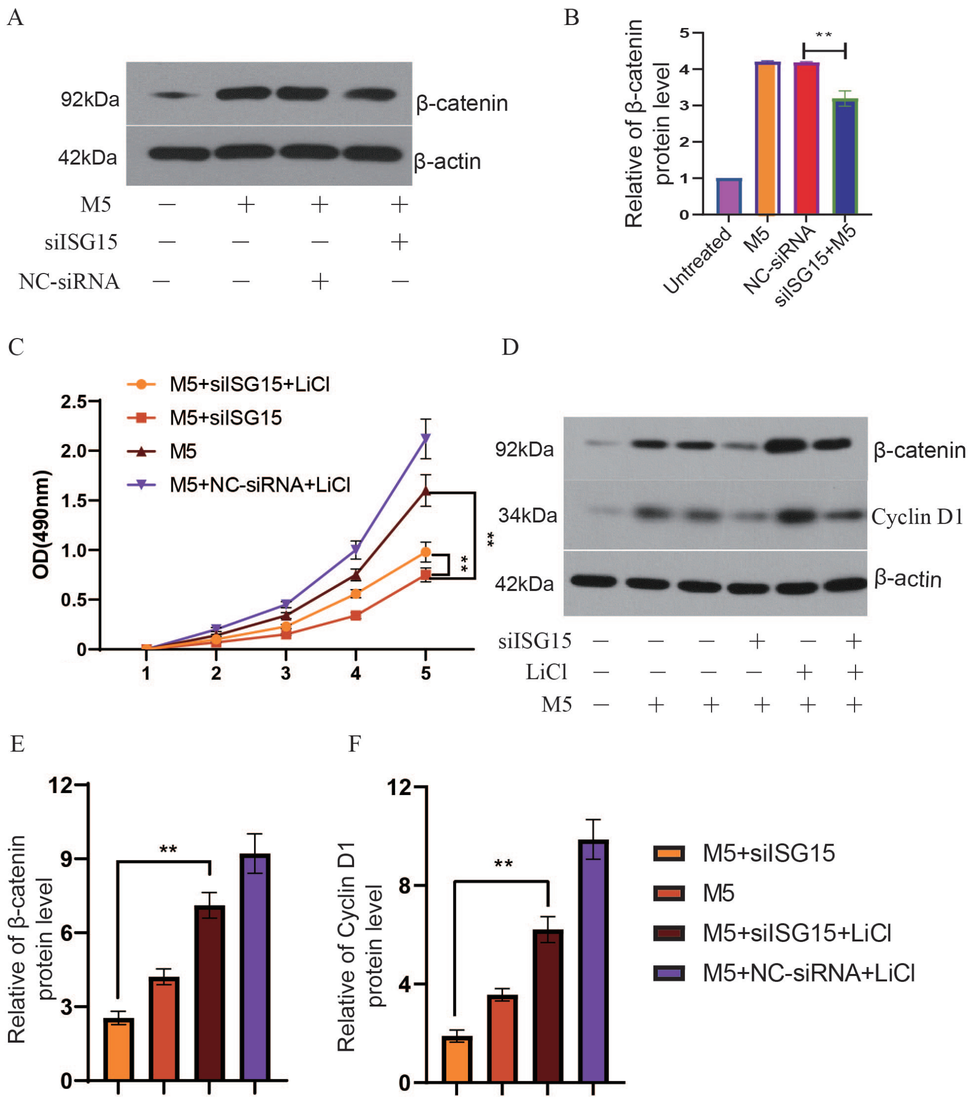

Fig. 5 Silencing *ISG15* reduces the expression of Wnt signaling and regulates keratinocyte proliferation in psoriasis

(A) Western blot images depicting the changes of protein levels of β-catenin post-*ISG15* silence. (B) Statistical data of Western blot bands for β-catenin. (C) Psoriatic cell proliferation rate with *ISG15* knockdown was measured by MTT assay at 1, 2, 3, 4 and 5 days. (D) Western blot images showing the alterations of protein levels of β-catenin and Cyclin D1, a key regulator of cell cycle. (E-F) Statistics of Western blot bands. $^{**}P < 0.01$.

influence psoriasis proliferation through the Wnt signalling pathway. We used siRNA transfection to reduce *ISG15* expression in psoriatic cell models while concurrently activating Wnt signalling using LiCl. Cell proliferation levels were assessed using the MTT assay, revealing a significant increase in keratinocyte proliferation in the dual treatment group compared to the *ISG15* knockdown alone (Fig. 5C). Western blotting was employed to assess β-catenin and cyclin D1 expression levels (Fig. 5D). Compared to the *ISG15* knockdown group, the dual treatment group exhibited elevated levels of β-catenin and cyclin D1 expression, with statistically significant differences (Fig. 5E-F). These findings suggest that *ISG15*'s role in promoting cell proliferation may be mediated through modulation of the Wnt signalling pathway.

4 Discussion

Psoriasis, a chronic inflammatory disease known for its recurring nature, was investigated in this study through analysis of differential genes from the GEO database. Functional enrichment and PPI network analyses highlighted the *ISG15* gene, which exhibited significant upregulation in psoriatic lesions and cell models. *ISG15* was found to enhance keratinocyte proliferation *via* the Wnt signaling pathway. Inhibition of *ISG15* effectively reduced keratinocyte proliferation by decreasing β-catenin expression levels. In this study, differentially expressed genes were identified from the analysis of GSE13355 and GSE30999 datasets obtained from the GEO public database. GO/KEGG enrichment analysis and PPI network analyses were conducted to identify central genes, focusing on antiviral proteins (AVPs) relevant to psoriatic keratinocytes, including *ISG15*, *LTF*, and *RSAD2*. Recent studies have highlighted the role of AVPs in the immune pathogenesis of psoriasis[13], yet their specific mechanisms remain unclear. Real-time PCR confirmed the elevated expression of the *ISG15* gene in psoriasis, consistent with previous findings on AVP overexpression in this condition[14]. Immunofluorescence further validated increased *ISG15* protein expression in psoriatic epidermal lesions. Given *ISG15*'s role as a significant AVP, further exploration of *ISG15* in psoriasis promises to elucidate its pathogenic mechanisms and potentially identify more precise therapeutic targets for patients. In this study, siRNA was used to silence *ISG15* expression, and the MTT assay demonstrated a reduction in keratinocyte proliferation following *ISG15* knockdown. Additionally, Lu *et al.*[12] found a positive correlation between *ISG15* expression levels and PASI scores during treatment with biological agents, methotrexate, and tolfatinib, underscoring *ISG15*'s association with psoriasis severity.

The regulation of keratinocyte proliferation and the expression of inflammatory factors involve a complex network system. The Wnt signaling pathway, in particular, plays a significant role in regulating keratinocytes under various stimulatory factors[15]. However, the potential of the Wnt signaling pathway as a therapeutic target for psoriasis remains uncertain. Previous studies comparing healthy and psoriatic skin biopsies found decreased mRNA and protein levels of Wnt inhibitory factor 1 (WIF1), a Wnt signal inhibitor, in psoriatic skin. Additionally, the injection of recombinant WIF1 improved the imiquimod (IMQ)-induced mouse model of psoriasis[16]. The Wnt signaling pathway is crucial in regulating the proliferation and differentiation of epidermal stem cells, with increased cytoplasmic β-catenin expression leading to over-proliferation of these cells. The question arises: does *ISG15* affect keratinocyte proliferation through the Wnt signaling pathway in psoriasis? To investigate this, Western blot analysis was applied to detecting β-catenin expression, a key protein in the Wnt pathway, following *ISG15* knockdown. The results indicated a significant decrease in β-catenin levels. Subsequently, LiCl, a Wnt pathway activator, was applied alongside *ISG15* knockdown. The MTT assay revealed that cell proliferation in the dual-treatment group was significantly higher than that in the *ISG15* knockdown-only group. Western blot analysis further demonstrated increased Cyclin D1 expression in the dual-treatment group. These findings suggest that *ISG15* may regulate keratinocyte proliferation *via* the Wnt signaling pathway.

Our findings shed light on the correlation between *ISG15* and the continuous proliferation of psoriatic keratinocytes. The limitations of this study are that there are few kinds of psoriasis mouse models and it is not proved that ISG15 can directly regulate the proliferation of psoriasis through Wnt signal. In the future, we will employ advanced techniques such as co-immunoprecipitation (CO-IP) to uncover the direct mechanisms underlying *ISG15*-mediated functions. By using these methods to pinpoint the precise mechanisms of *ISG15*, we aim to identify more accurate and effective therapeutic targets for psoriasis.

5 Conclusions

ISG15 promotes keratinocyte proliferation through the Wnt signaling pathway. Its expression is increased in the epidermal tissue of patients with psoriasis. Knocking down *ISG15* inhibits the proliferation of psoriatic cells by suppressing β-catenin, providing a theoretical basis for targeting the pathogenesis of psoriasis.

Author contributions

Conceived and designed the experiments: Sun X Q; data acquisition: Yu H W, Lin J Y and Wang C; data analysis and interpretation: Sun X Q, Li Y Z and Liu Q L; manuscript writing and critical revision: Sun X Q and Bai B X.

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Ethical approval

The study adhered to the Declaration of Helsinki and was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (No. 2023-145).

Informed consent

All patients provided signed informed consent.

Conflict of interest

All authors have no competing interests.

Data availability statement

All data in this study are available from the corresponding author upon request.

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