

# ***BCL11B* Is Involved in Stress-Induced Differentiation of Keratinocytes and Has A Potential Role in Psoriasis Pathogenesis**

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## Abstract

**Objective:** Psoriasis is a common, auto-immune skin disease characterized by abnormal proliferation and differentiation of keratinocytes. Studies revealed the role of stress stimulators in the pathogenesis of psoriasis. Oxidative stress and heat shock are two important stress factors tuning differentiation and proliferation of keratinocytes, regarding to psoriasis disease. *BCL11B* is a transcription factor with critical role in embryonic keratinocyte differentiation and proliferation. Given this, in keratinocytes we have investigated potential role of *BCL11B* in stress-induced differentiation. Furthermore, we searched for a potential intercommunication between *BCL11B* expression and psoriasis-related keratinocyte stress factors.

**Materials and Methods:** In this experimental study, data sets of psoriatic and healthy skin samples were downloaded in silico and *BCL11B* was chosen as a potential transcription factor to analyze. Next, a synchronized *in vitro* model was designed for keratinocyte proliferation and differentiation. Oxidative stress and heat shock treatments were employed on HaCaT keratinocytes in culture, and *BCL11B* expression level was measured. Cell proliferation rate and differentiation were analyzed by synchronized procedure test. Flow cytometry was done to analyze cell cycle alterations due to the oxidative stress.

**Results:** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) data revealed a significant upregulation of *BCL11B* expression in keratinocytes, by 24 hours after initiating differentiation. However, it was followed by a significant down-regulation in almost all the experiments, including the synchronized model. Flow cytometer data demonstrated a G1 cell cycle arrest in the treated cells.

**Conclusion:** Results indicated a remarkable role of *BCL11B* in differentiation and proliferation of HaCaT keratinocytes. This data along with the results of flow cytometer suggested a probable role for *BCL11B* in stress-induced differentiation, which is similar to what is happening during initiation and progression of normal differentiation.

**Keywords:** *BCL11B*, Keratinocyte, Psoriasis, Stress-Induced Differentiation, Transcription Factor

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## Introduction

Psoriasis is a chronic immune mediated skin disease significantly affecting quality of life, both physically and psychologically (1, 2). Psoriasis frequency affects approximately 2 to 3 percent of general population. It is characterized by epidermal hyper proliferation, abnormal differentiation of keratinocytes and inflammation along with infiltrated immune cells in the skin (3). There existed different factors with crucial roles in pathogenesis of psoriasis, including several subtypes of T cells, environmental factors and complex genetic background of patients. A combination of these factors,

most probably, results in abnormal proliferation and differentiation of epidermal keratinocytes in psoriasis patients (4, 5).

Stress is one of the environmental factors that interfere with homeostasis. It can also change molecular balance of different tissues. Skin is one the organs that responds to various stressors vigorously. Several different signals are received by the skin and responded based on their importance (6). It has been reported that some stressors trigger skin responses eventually leading to psoriasis.

Reactive oxygen species (ROS) is one of the skin

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stressors which involve in oxidative stress (7). ROS is produced from both endogenous sources and external pro-oxidant stimuli which expose the skin to oxidative stress. ROS is involved in several cellular processes including cell proliferation, apoptosis, immune responses and cell differentiation. Moreover, it is also generated within dysregulated signal transduction events, and bimolecular (lipids, proteins, DNA) damage processes. Amount of ROS determines its positive or destructive role in cellular mechanisms (8). It has been shown that the imbalance between ROS and antioxidant cause oxidative stress with a role in the pathogenesis of psoriasis (7).

Heat shock response in the skin is another stressor in the pathogenesis of psoriasis. Heat shock proteins are the most important components of heat shock response involved in different biological processes including maintenance of intracellular redox potential balance, stabilization of the cytoskeleton, as well as regulation of complicated processes of cellular proliferation, differentiation and apoptosis (9).

Gene expression alterations could ignite all of the aforementioned molecular and cellular mechanisms. Transcription factors are the most important mediators of gene expression regulation. Transcription factors may be constitutively expressed or could be activated during special conditions. *BCL11B*, a zinc finger transcription factor, is highly expressed in skin and immune system. This gene was identified as a bi-functional transcriptional regulator and acts as a repressor or a trans-activator (10, 11). A critical role for *BCL11B* in epidermal proliferation and keratinocytes terminal differentiation of adult skin cells has already been reported. These findings make the gene a novel candidate for potential treatment of psoriasis (12). Given these information, we investigated here a probable role of *BCL11B* in keratinocytes differentiation, under different stress conditions related to psoriasis.

## Materials and Methods

### Ethical statements

This experimental study did not involve human participants, use human samples or tissues. It neither did include experimenting on animals. The *in vitro* experiments on commercial cell lines and standard techniques of molecular genetics was approved for a Ph.D. thesis proposal, by Tarbiat Modares Research Ethics Committee (IR.MODARES.REC.1398.119).

### Bioinformatics analysis

All data sets were downloaded from the NCBI gene expression omnibus (GEO). A total of 170 microarray series, regarding the psoriasis research, were retrieved from GEO. After a careful review, three gene expression profiles (GSE166388, GSE68939, and GSE68937) were selected. GEO2R online analysis tool (<https://www.ncbi.nlm.nih.gov/geo/GEO2R/>) was used to

predict potential transcription factors of screened differentially expressed mRNAs of lesional psoriatic and healthy control skin biopsies. Transcription factors that met the cutoff criteria, adjusted  $P < 0.05$  and  $|\log_2 fc| \geq \pm 2.0$ , were considered as potential transcription factors. Statistical analysis was carried out for each dataset and common data was identified using the Venn diagram webtool (bioinformatics.psb.ugent.be/webtools/venn/).

Each transcription factor was then checked for tissue specificity through the human protein atlas portal (website: <http://www.proteinatlas.org/>), GTEX portal (website: <https://gtexportal.org>). *BCL11B* was then selected according to the cutoff criteria and tissues in which it was expressed.

### Cell culture

The spontaneously immortalized human keratinocyte (HaCaT) cells were kindly given by Dr. Majid Mojarrad, Mashhad University of Medical Sciences (Mashhad, Iran). HaCaT cells were cultured in dulbecco's modified eagle's medium, high glucose (DMEM, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA), penicillin (100 u/ml) and streptomycin (100 mg/ml, Bio basic, Canada), and maintained in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. The cells were sub-cultured every 2-3 days or when they reached 80-90% confluency.

### Cell viability

The HaCaT cells at logarithmic growth phase were seeded at  $1 \times 10^5$  cells/ml into each wells of a 96-well cell culture plate. Effect of H<sub>2</sub>O<sub>2</sub> on cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide assay (MTT, Sigma, USA). 100 µl medium containing H<sub>2</sub>O<sub>2</sub> (0 to 1000 µmol/l) was added to each well for 24 hours. 20 hours later incubation period, the medium was aspirated and 20 µl 5 mg/ml MTT solution was added. After 4 hours the supernatant was discarded, 150 µl dimethyl sulphoxide (DMSO) was added to dissolve the precipitate. Survival rate of the cells was then measured.

### Synchronization procedure

Synchronization procedure of HaCaT cells done by cultivation at high density in the absence of serum. Cells were grown at complete DMEM medium containing 10% FBS at 100% confluence for 2 days. The culture medium was then replaced with serum free DMEM and incubated for more 2 days. The synchronized cells were trypsinized and seeded into new 24 wells plate in 10% FBS high-glucose DMEM. The cells were counted and their viability determined by trypan blue staining. Total RNA was collected at 0, 16, 24, 36 and 48 hours, when the cells reached 100%

confluence in complete medium and/or in serum free medium.

### Oxidative stress

The HaCaT cells were cultured and then for the next passage were seeded in a 48 wells plate ( $7 \times 10^4$  cells/well). Later, when cultures reached 70-80% confluence, the medium was changed with complete DMEM containing  $300 \mu\text{mol/l H}_2\text{O}_2$ . The cells were incubated at a humidified atmosphere at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  for 2 days. Total RNA was collected 0, 1, 2, 4, 6, 8, 12, 24, 36 and 48 hours later, after oxidative stress.

### Heat shock stress

Heat shock assay was conducted culturing HaCaT cells in 6-well plate ( $3 \times 10^5$  cells/well) incubating at  $42^\circ\text{C}$  for 1 hour. The plate was then incubated in normal condition at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ . Total RNA was then collected in different time points including 0, 1, 2, 4, 8, 12 and 24 hours after heat shock treatment.

### Quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from HaCaT keratinocytes using trizol (Thermo Fisher Scientific, USA) in different time points following the manufacturer's instructions. RNA concentration was determined by measuring the 260 nm absorbance. In order to eliminate DNA contamination,  $1 \mu\text{g}$  of RNA was first treated with DNase I (Thermo Fisher Scientific, USA), then first-strand cDNA was synthesized using the cDNA synthesis kit (Takara Bio, Japan). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiments were performed to quantify the relative abundance of each interested mRNAs using biofact™ 2x real-time PCR master mix (Biofact, South Korea) through ABI StepOne real time PCR system (Thermo Fisher Scientific, USA). Specific primers were used for quantifying *BCL11B*-F: 5'CAAACAGGGCAACCCGCAGCA3' R: 5'ACAGGTGAGCAGGTCAGGGTC3'

Abundance of the gene of interest was normalized to the expression of beta 2-microglobulin (b2m) of each examined sample.

### Cell cycle characterization

Flow cytometry was employed to observe possible alterations in different phases of cell cycles, induced in oxidative stress. The keratinocyte cell line, HaCaT, was stimulated with  $300 \mu\text{mol/l H}_2\text{O}_2$  for 24 hours, washed with phosphate buffered saline (PBS, Aminsan, Iran) and then fixed with 4% paraformaldehyde solution for 15 minutes. Subsequently, the cells were stained with propidium iodide (pi), and distribution of the cells

in cell cycle phases was assessed by fluorescence-activated cell sorting (FACS) and flow cytometer (Becton Dickinson, USA).

### Statistical analysis

The  $2^{-(\Delta\text{Ct})}$  method was used for qRT-PCR data analysis and gene expression profiling. All experiments were analyzed using Graphpad prism (version 8.4; Graphpad software inc, La Jolla, CA, USA). P values were analyzed using the student t test and one-way ANOVA. Statistical significance was considered at a  $P < 0.05$ .

## Results

### *BCL11B* transcription factor was down-regulated in skin tissues of psoriasis patients

Three gene expression profile (GSE166388, GSE68939, and GSE68937) were analyzed in this study. A total of 19 samples were acquired from 10 skin biopsies of lesional psoriatic skin and 9 healthy skin controls. Detailed information about each databases is presented in Table 1.

**Table 1:** Gene expression profiles analyzed in this study

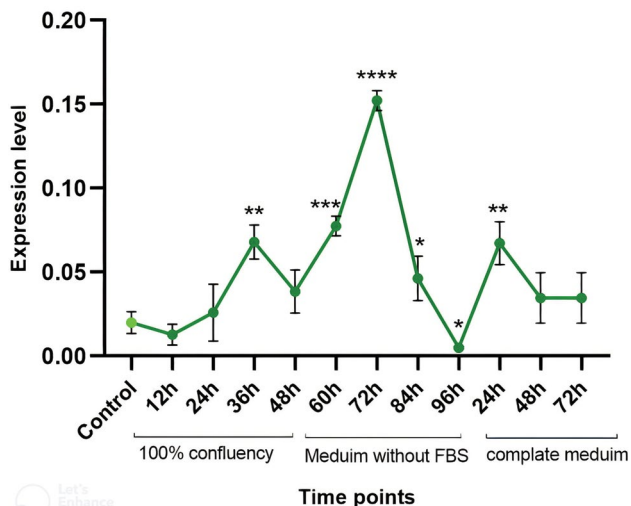
GEO	Sample size		Platform
Accession number	Controls	Patients	
GSE166388	4	4	Affymetrix human genome u133 plus 2.0 array
GSE68939	2	3	Agilent-028004 sureprint g3 Human ge 8x60k microarray
GSE68937	3	3	Agilent-028004 sureprint g3 Human ge 8x60k microarray

Based on the criteria of  $P < 0.05$  and  $|\log_2 \text{fc}| \geq 2$ , a number of transcription factors were short-listed. As we expected, data from the human protein atlas and GTEX portal indicated that among all nominated transcription factors, *BCL11B* mRNA expression level was relatively higher in skin and immune cells, which made this gene a potential candidate for the research. Expression changes of *BCL11B* was then plotted in the analyzed GEO dataset between psoriasis patients and healthy controls (Fig.1).

### *BCL11B* was up-regulated during proliferation and differentiation of HaCaT keratinocytes in synchronized procedure

Role of *BCL11B* was studied under different conditions linking to stress in keratinocytes. For this

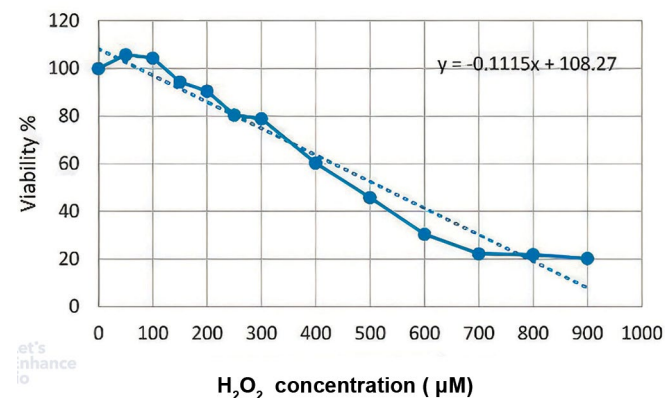




**Fig.2:** Kinetic analysis of *BCL11B* expression levels in synchronized HaCaT cells. HaCaT cells were grown in DMEM medium containing 10% FBS and 1% PS for 48 hours at 100% confluence. Confluent cells were serum starved for 48 hours by adding DMEM containing 1% PS without FBS. Cells then sub-cultured with complete fresh DMEM and followed for the next 72 hours. All experiments were repeated three times, each in triplicate statistical t test were used and \*, P<0.05, \*\*, P<0.01, \*\*\*, P<0.001, \*\*\*\*, P<0.0001, h; Hour, DMEM; Dulbecco's Modified Eagle Medium, PS; Penicillin streptomycin, and FBS; Fetal bovine serum.

### Cell cycle and BCL11B expression alteration in stress-induced differentiation

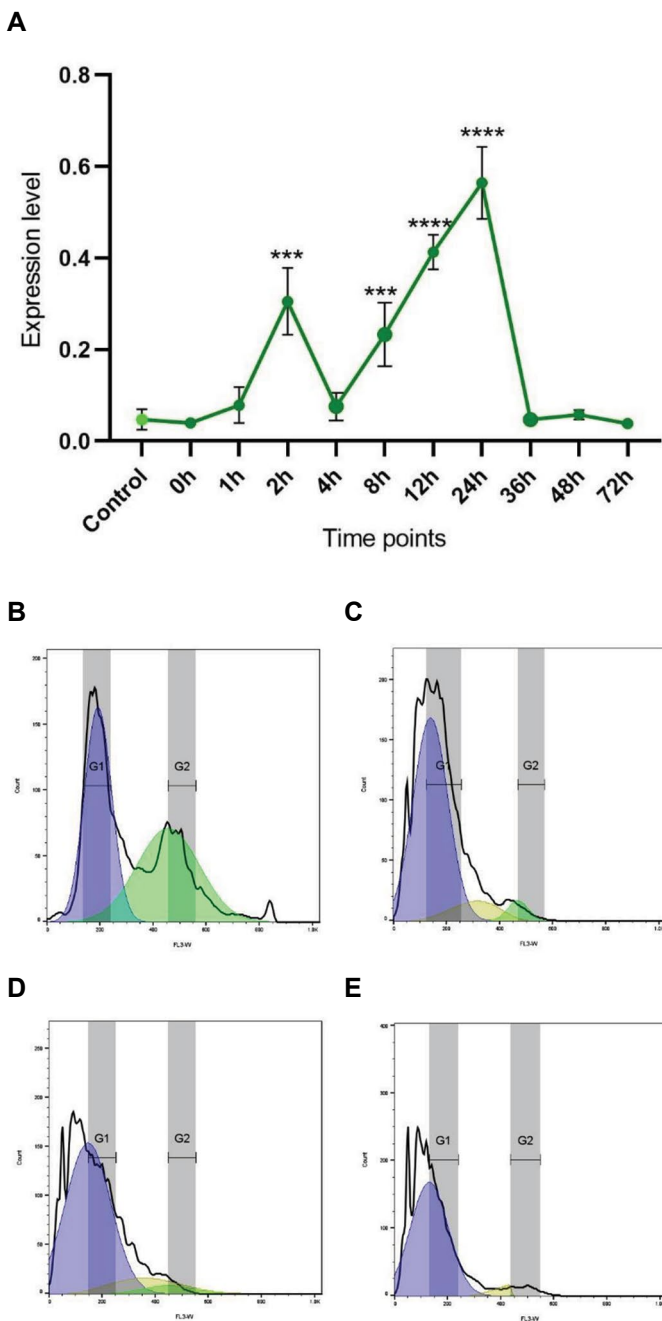
Cytotoxicity of H<sub>2</sub>O<sub>2</sub> on HaCaT cells was assayed by the MTT method. Results showed that LD50 value was 522 μmol/l H<sub>2</sub>O<sub>2</sub> for HaCaT cells. Concentration of 150 μmol/l H<sub>2</sub>O<sub>2</sub> was also for LD10 range (Fig.3).



**Fig.3:** Cytotoxicity assessments by MTT in HaCaT cells. HaCaT cells were cultured with different concentrations of H<sub>2</sub>O<sub>2</sub> (0 to 1000 μmol/l) for 24 hours. Four hours prior to the end of the incubation period, MTT assays was done to quantify metabolic activity.

Expression level of *BCL11B* was increased significantly 24 hours after using H<sub>2</sub>O<sub>2</sub>, as a component to increase oxidative stress in HaCaT cells. Then, there was a decrease in the expression level of *BCL11B* (Fig.4).

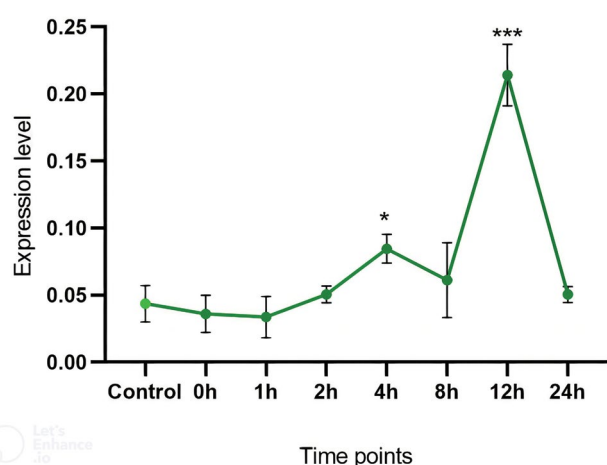
To get more detail of potential contribution of BCL11B in cell cycle during oxidative stress, flow cytometry was done in different time points following oxidative stress procedure. The results of flow cytometry analysis indicated a meaningful G1 arrest in oxidative stress especially at 24 hours after treatment by H<sub>2</sub>O<sub>2</sub> (Fig.4).



**Fig.4:** Cell cycle and *BCL11B* expression alteration in oxidative stress. **A.** HaCaT cells were cultured and 300 μM/ml H<sub>2</sub>O<sub>2</sub> were added to the medium of the cells at the confluency of 70%. Total RNA was extracted from the cells in different time points. Expression level of *BCL11B* was measured using Quantitative reverse transcription polymerase chain reaction (qRT-PCR). All experiments were repeated three times, each in triplicate statistical t test were used and \*\*\*, P<0.001, \*\*\*\*, P<0.0001. **B.** Normal control HaCaT cells with equal percentage of G1 and G2 (G1= 46%, G2=48%). **C.** H<sub>2</sub>O<sub>2</sub> treated HaCaT cells after 4 hours, percentages of G1 was increased compared to G2 (G1=85%, G2=5.05%). **D.** H<sub>2</sub>O<sub>2</sub> treated HaCaT cells after 12 hours, cells arrested more in G1 (G1=89%, G2=5%). **E.** H<sub>2</sub>O<sub>2</sub> treated HaCaT cells after 24 hours, highest level of g1 arrest (G1=91%, G2=0.16%).

### Expression level of *BCL11B* was increased significantly under heat shock treatment

Briefly, HaCaT cells were seeded at 6 wells plate and incubated at 42°C for 1 hour. One hour after heat shock, the cells were placed in the normal incubation condition of 37°C with 5% CO<sub>2</sub>. total RNA was collected in different time points after heat shock treatment between 0-24 hour(s). Results of heat shock treatment on HaCaT cells indicated that expression level of *BCL11B* was increased significantly 12 hours after treatment, followed by a decrease until 24 hours post-treatment (Fig.5).



**Fig.5:** Expression level of *BCL11B* in different time points of heat shock treatment. HaCaT cells were cultured at the confluency of 70%, the cells were incubated at 42°C for 60 minutes. The cells were then incubated at normal condition of 37°C and 5% CO<sub>2</sub>. Total RNA were then extracted from the cells in different time points. expression level of *BCL11B* were measured using quantitative reverse transcription polymerase chain reaction (qRT-PCR). All experiments were repeated three times, each in triplicate statistical t-test were used and \*, P<0.05 and \*\*\*, P<0.001.

### Discussion

*BCL11B* is a transcription factor which plays critical role in development and differentiation of the investigated organs, such as skin (13). Studies demonstrated that *BCL11B* acts as a regulator of keratinocytes proliferation and differentiation (11). Hyperproliferation and aberrant differentiation of keratinocytes are two most important characters of psoriasis (14). We recruited an *in vitro* model of keratinocyte proliferation and differentiation, using synchronized keratinocytes HaCaT cell line. In this model, both proliferation and differentiation stages were designed in order to explore expression pattern and probable role of the gene in each stages.

Our results demonstrated a notable up-regulation of *BCL11B* in differentiation stage in contact inhibition condition with serum free medium which indicated a potential role of *BCL11B* in keratinocytes differentiation. In addition, there was a remarkable increase of *BCL11B* expression in proliferation stage of the model system. The

results of both differentiation and proliferation stages were well matched with those of previous studies. It has been demonstrated that *BCL11B* could regulate proliferation and differentiation of epidermal cells, via EGFR and notch signaling pathways (15, 16).

Given that serum starvation makes a stressful condition for cells and psoriasis is well known to connect to stressful conditions for different kinds of cells, especially keratinocytes. For these reasons, we aimed to investigate expression pattern of *BCL11B* under different conditions related to the stress in psoriasis.

Oxidative stress, which arises due to the imbalance of ROS, is an important factor to induce psoriasis (17). There are different stressful stimuli which can produce ROS to cause oxidative stress and affect skin, including ultraviolet (UV) radiation of the sun and oxygen from the air. Oxidative stress can promote inflammation and ROS can attract and activate neutrophils in the skin which results in reduced antioxidative capacity and leads to psoriasis formation (18). Studies showed that cell cycle arrest at H<sub>2</sub>O<sub>2</sub> induced oxidative stress condition resulting in DNA damage and cell death (19, 20). Moreover, it was demonstrated that some kind of cells can arrest cycle in G<sub>0</sub>/G<sub>1</sub> and differentiate them (21). Our data demonstrated an induction of G<sub>1</sub> cell cycle arrest in H<sub>2</sub>O<sub>2</sub> treatment, 24 hours after treatment, which was accompanied with an increased level of *BCL11B* expression at the same time under this treatment. In addition, this results was well matched to the results of keratinocytes synchronization experiment which showed a significant increase in the expression level of *BCL11B* at 24 hours after serum starvation indicating keratinocyte differentiation.

Studies showed that there was a relationship between *BCL11B* and *STIR1*, in which *BCL11B*- mediated repression in the transcription involved recruitment of *STIR1* (22). It has been investigated that expression level of *STIR1* was down-regulated in psoriatic skin (17). Considering the role of *STIR1* in differentiation (23) and different cellular pathways, including NF-κB pathway, a possible role of *BCL11B* in stress-induced differentiation conditions, such as psoriasis, is suggested.

For further confirmation on the role of *BCL11B* in stress-related differentiation of the skin, we also analyzed the expression pattern of the genes under heat-shock stress condition. Several studies reported that expression level of heat-shock proteins was changed in some cells, during differentiation and injury with inflammatory mediators or any other stressful stimuli that affect the skin (24, 25). Our data revealed a significant increase followed by a significant decrease of *BCL11B* expression level in the keratinocytes, following the heat shock treatment, which indicated its role under heat stressful conditions of the cells.

### Conclusion

Given that *BCL11B* is a bi-functional transcription

factor, highly expressed in the skin, it is suggested that any change in the expression pattern of *BCL11B* could alter expression level of several other genes. We made a cellular monolayer *in vitro* model of differentiation and proliferation similar to psoriasis, using contact inhibition in the keratinocytes. Confirming the role of *BCL11B* in this *in vitro* model, we looked into expression alteration of *BCL11B* in different stressful conditions related to psoriasis including oxidative stress and heat shock treatment. During different stressful stimulation related to differentiation, similar pattern of *BCL11B* expression alteration (a significant increase, followed by a significant decrease), along with FACS results, suggested a potential role for *BCL11B* in the stress induced differentiation skin. It is suggested that *BCL11B* is needed for differentiation, and when the cells are going through complete or incomplete differentiation process, expression level of the gene will be decreased again. To better understand exact role of *BCL11B* in the skin stressful condition, more experiments are needed.

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## Authors' Contributions

S.P.; Contributed to the study conception and design, performed all of the experiments, data and statistical analysis, and interpreted the data. Z.-S.S., S.J.M.; Supervised the research and help with the study plan. H.A.O., E.B., P.M., E.L.; Advised the research and help with the research progression. All authors read and approved the final manuscript.

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