Systematic screening and identification of novel psoriasis-specific genes from the transcriptome of psoriasis-like keratinocytes

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Abstract. Psoriasis is a chronic inflammatory skin disease. Keratinocytes (KCs), as skin-specific cells, serve an important role in the immunopathogenesis of psoriasis. In the present study, transcriptome data derived from psoriasis-like KCs were used together with the reported transcriptome data from the skin/epidermis of patient with psoriasis, excluding known psoriasis-associated genes that have been well described in the previous studies according to GeneCards database, to screen for novel psoriasis-associated genes. According to the human expressed sequence tag of UniGene dataset, six genes that are located near psoriasis-associated loci were highly expressed in skin. Among these six genes, four genes (epiregulin, NIPA like domain containing 4, serpin family B member 7 and WAP four-disulfide core domain 12) were highly expressed in normal mouse epidermis (mainly KCs) and mouse psoriatic epidermis cells, but not in psoriatic dermis cells, which further emphasized the specificity of these genes. Furthermore, in systemic inflammatory response syndrome (SIRS), *SERPINB7* showed no difference in expression in immune-activated tissues from SIRS and control mice. It was also found that the mRNA expression levels of *SERPINB* in lesional skin of patients with psoriasis were significantly higher than in non-lesional psoriatic skin from the same patients. *SERPINB7* may be a valuable candidate for further studies. In the present study, a method for identifying novel key pathogenic skin-specific molecules is presented, which may be used for investigating and treating psoriasis.

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Introduction

Skin functions as an important natural barrier between an organism and its external environment, thus it has a unique biological structure and specific immune functions (1). Skin is composed of two distinct regions, the epidermis and dermis. The predominant cell type of the epidermis is keratinocytes (KCs), whereas the dermis contains cells of the immune system, including dendritic cells (DCs), T helper cells, γδT cells, natural killer T cells, macrophages and fibroblasts (2). A skin immune system imbalance can cause several immune-mediated skin diseases, such as psoriasis (1). Therefore, in-depth analysis of the skin immune system would be of great clinical significance for the treatment of these diseases.

Psoriasis is a common chronic inflammatory skin disease triggered by a dysregulated immune response, which typically manifests as plaques with adherent silvery scales and has a great impact on the physical and mental health of patients (3,4). This disease is characterized by excessive growth and aberrant differentiation of KCs. In psoriasis, the pathological changes in KCs include hyperkeratosis, parakeratosis, a loss of the normal granular layer and acanthosis with elongation of the epidermal rete ridges (5). Thus, as the skin specific cells, KCs are the main cell type affected by psoriasis.

The immunopathogenesis of psoriasis outlines the important role KCs serve in the induction and amplification of psoriatic inflammation. KCs respond to different signals and release the cathelicidin antimicrobial peptide (LL-37), which activates plasmacytoid DCs (pDCs) via Toll-like receptors (5-7). Myeloid DCs are activated by interferon (IFN)-α/β from the pDCs, stimulating psoriatic T cells that produce interleukin (IL)-17, IFN-γ and IL-22. KCs also produce chemokines and antimicrobial peptides, which attract myeloid DCs and T cells that produce IL-17. The cytokines released by these cells further stimulate the KCs, and the immune circuit is further amplified by feedback cytokines produced by the KCs (1,5,8). Thus, KCs have a central role in the immune circuits of psoriatic skin.

Several immunosuppressants are currently available for psoriasis treatment; their targets include cytokines, signaling molecules and receptors in the immune system (7). However, as with any drug designed to suppress the general immune system, the main concern is the risk of serious side effects.

Efalizumab, a monoclonal antibody targeting cluster of differentiation (CD)11a, has inhibitory effects against broad T cell subsets, resulting in systemic immune suppression, which can lead to serious infection, cancer and other severe complications (9,10). As a result, it was withdrawn 6 years after approval (FDA Issues Boxed Warning for Efalizumab, 2008) (11). The clinical development of briakinumab, an antibody targeting IL-23/P40, was halted after a series of major cardiovascular events occurred during clinical trials (9). In clinical application, other psoriasis drugs, including infliximab, [a tumor necrosis factor (TNF)-α blocker], corticosteroids, antibiotics and vitamins, can also cause different degrees of toxicity (12). In addition, IL-17-targeting antibodies and small molecule drugs targeting Janus kinases also have limitations in the treatment of psoriasis. The most fundamental reason is that these drugs do not have specific targets or cannot regulate the cutaneous immunity (13,14). Therefore, identifying novel psoriasis-specific molecules will help treatment.

In the present study, novel psoriatic skin-specific genes were screened from the transcriptome of psoriasis-like KCs. An integrative approach, combining psoriatic transcriptome data, psoriasis-associated genes information, genetic loci linked to psoriasis and human tissue expression pattern, was used to screen novel psoriasis-associated genes that were highly expressed in the skin/epidermis. The present study provides a novel way of identifying novel skin-specific genes for cutaneous diseases.

Materials and methods

Human skin samples. The present study was performed in accordance with the principles of the Helsinki Declaration and approved by the Ethics Committee of the West China Hospital, Sichuan University (Chengdu, Sichuan, China). Written informed consent was obtained from all study participants prior to the study. All patients were diagnosed based on the clinically apparent symptoms (fairly easily diagnosed as characteristic red colored plaques with well-defined borders and silvery-white dry scale), and histopathological criteria (abnormal proliferation and differentiation of the epidermis, hyperkeratosis and parakeratosis of keratinocytes) (5). All patients were assessed according to the Psoriasis Area and Severity Index (15). Skin samples ~0.5-1.0 cm were collected from eight patients with psoriasis (four females and four males; age, 21-63 years) at the West China Hospital, Sichuan University between March 2016 and November 2017. The lesional and non-lesional psoriatic skin (~0.5-1.0 cm) were taken from each patient, one was obtained from lesional skin of patients and the other from non-lesional skin of the same patients. The fresh skin samples were snap-frozen in liquid nitrogen and stored at -80˚C. All participants had not been treated with systemic therapy including investigational agents for at least 4 weeks prior to the study entry. Patients with a history of other autoimmune diseases, immunologic deficiency diseases or tumors were excluded.

Cell culture. The human HaCaT KCs were obtained from the China Center for Type Culture Collection (Wuhan, China; CCTCC no. 0106). HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin G and 0.1 mg/ml streptomycin sulfate (Thermo Fisher Scientific, Inc.). The conditions of cell culture were 37˚C and 5% CO_2. All cells were demonstrated to be free from mycoplasma contamination.

Induction of psoriatic keratinocytes model in vitro. The five proinflammatory cytokines (termed M5), including IL-17A, oncostatin-M, TNF-α, IL-22, and IL-1a (ProSpec Bio, East Brunswick, NJ, USA) were used to induce psoriasis-like KCs inflammation that recapitulates the features of psoriasis (16), as described in a previous study (17). Briefly, KCs were cultured to 80% confluency and then starved for 24 h in DMEM without serum prior to stimulation. The cells were then stimulated with 10 ng/ml recombinant IL-17A, 10 ng/ml recombinant oncostatin-M, 10 ng/ml recombinant TNF-α, 10 ng/ml recombinant IL-22 and 10 ng/ml recombinant IL-1α in combination or untreated for 24 h prior to the whole transcriptome gene expression analysis.

Microarray expression profiling. Gene array analysis was performed using Human Expr 12x135K Arr Del (Roche-NimbleGen; Roche Diagnostics, Basel, Switzerland) by KangChen Bio-tech, Inc. (Shanghai, China). In brief, total mRNA was isolated from KCs at 24 h post-stimulation with M5 using PureYield™ RNA Midiprep System (Promega Corporation, Madison, WI, USA). Total RNA was quantified by the NanoDrop ND-1000A, and RNA integrity and gDNA contamination was assessed by standard denaturing 1% agarose gel electrophoresis. Total mRNA of each sample was used for labeling and array hybridization were performed according to the manufacturers' protocols: Reverse transcription using SuperScript Double-Stranded cDNA Synthesis kit (Thermo Fisher Scientific, Inc.); ds-cDNA labeling with NimbleGen one-color DNA labeling kit (Roche Diagnostics, Mannheim, Germany); array hybridization using the NimbleGen Hybridization System followed by washing with the NimbleGen wash buffer kit (Roche Diagnostics); array scanning using the Axon GenePix 4000B microarray scanner (Molecular Devices LLC, Sunnyvale, CA, USA). Raw signal intensities were extracted and normalized using the Robust Multichip Average (RMA) method by NimbleScan v2.5 software (Roche NimbleGen Inc., Madison, WI), and low intensity (<100.0) genes were filtered. Further data analysis was performed using Agilent GeneSpring GX 11.5.1 software (Agilent Technologies, Inc., Santa Clara, CA, USA). Two biological replicates were used for each sample, expression values were normalized based on the mean expression value for each probe set, differently expressed probe sets were identified based on Student's t-test for paired samples' normalized expression values using the following cutoff: Absolute fold change (FC) >3 and a $P<0.01$, false discovery rate <0.05. In addition, 3,577 differentially expressed genes (DEGs) were obtained from psoriatic lesional and normal skin in the study by Li *et al* (18), and 1,446 DEGs in psoriatic lesional epidermis compared with non-lesional psoriatic epidermis in the study by Mitsui *et al* (19). To compare the present keratinocytes microarray data with previously published reports data, these

two transcriptome data sets and the transcriptome dataset in the psoriasis-like KCs model were further analyzed for enriched Gene Ontology (GO) terms using Gorilla (cbl-gorilla.cs.technion.ac.il/). Biological terms that have many genes in common can be grouped into a module of associated terms and genes, with a significance threshold of 0.001.

Gene expression datasets. GEO DataSets (GSE40263) were obtain of peripheral blood mononuclear cells (PBMCs) of psoriasis (n=5) and healthy controls (n=5) from National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40263). The ID_REF of *EREG, NIPAL4, SERPINB7* and *WFDC12* were retrieved in website of Affymetrix, Inc (https://www.affymetrix.com/analysis/netaffx/index.affx). Based on the gene of ID_REF, RMA signal intensity, which is a form of quantile normalization applicable to gene expression (microarray) experiments, was searched in the data table of psoriasis and healthy controls. The PBMCs expression levels of these four genes in the psoriasis and healthy controls were analyzed.

Sources of genetic loci linked to psoriasis. Genetic loci associated with psoriasis were obtained from a knowledgebase of Human Genes and Genetic Disorders [Online Mendelian Inheritance in Man (OMIM); search, 'psoriasis'; entries with 'gene map locus'; retrieve, 'gene map'; www.omim.org/search?index=entry&search=psoriasis&filter=gm_exists%3Atrue&sort=chromosome_number+asc%2C+chromosome_sort+asc&start=1&limit=100&retrieve=geneMap; accessed February 2018], and previous research reports prior to February 2018; these included 1p36, 1q21.3, 2p16, 5q15, 5q31.1, 5q33.3, 5q33.3, 8p23.2, 9q34.13, 13q12.11, 13q13.3, 14q13.2, 16p11.2, 17q11.2, 14q32.13, 18q21.2, 18q21.33, 18q22.1, 19p13, 19q13.41 and 20q13 (20-29).

Psoriasis-associated genes in GeneCards database. Using the GeneCards database (www.genecards.org/Search/Keyword?queryString=PSORIASIS; accessed February 2018), psoriasis-associated genes were obtained and the approximate degree of correlation was inferred from the score. A score ≥1 was considered to be a psoriasis-associated gene.

Expressed sequence tag (EST) sources. National Center for Biotechnology Information Unigene (www.ncbi.nlm.nih.gov/unigene/) was used to search the indicated homo candidate genes, their EST profiles were entered and the approximate gene expression of various tissues was inferred from the transcripts per million (TPM). Indicated genes with relatively high specific expression in skin tissue were selected by screening the indicated gene EST profiles inferred from TPM value.

Ethics statement. Wild-type C57BL/6 female mice 8-12 weeks old, weight, 17-18 g, n=180, were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The animals were housed under the following controlled conditions: 12 h light-dark cycle at a steady temperature of 25±1˚C, with free access to water and food. The animal protocols were approved by the Committee on the Ethics of Animal Experiments of Sichuan University. The experimental procedures were conducted according to the ethical Guidelines For The Care And Use Of Laboratory Animals of the National Institutes of Health (https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf) and the International Association for the Study of Pain (IASP). Every effort was made to decrease the number of animals used and to reduce animal suffering.

Tissue dissection. Following the sacrifice of the mice, various tissues (large intestine, lung, liver, testis, ovary, brain, spleen, and kidney, small intestine and heart) were collected as previous described (30). The backs of mice were shaved, the skin was wiped with alcohol prior to its removal, then subcutaneous fat was removed and was cut into small pieces convenient for digestion and separation, and the samples (~0.3 cm) were then incubated in dispase II (2.5 U/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 4˚C overnight followed by immersion in DMEM containing 50% (v/v) FBS to inactivate the dispase II. The epidermis and dermis were then separated at the epidermal-dermal interface under magnification with a dissecting microscope. Only pieces that consisted entirely of epidermis or dermis were used.

Imiquimod (IMQ)-induced psoriasis-like skin inflammation. The psoriasis animal model used in the present study was the IMQ-induced psoriasis-like skin inflammation. The IMQ mouse model of psoriasis-like skin inflammation was induced as previously described (31). Briefly, the day before induction, the backs of the mice were shaved. Subsequently, the backs of the mice were treated with Aldara cream (Sichuan MingXin Pharmaceutical Co., LTD., Sichuan, China) containing 5% IMQ (55 mg) once daily for 1-6 days.

Lipopolysaccharide (LPS)-induced systemic inflammatory response syndrome (SIRS). SIRS was induced by intraperitoneal injection of dose of 2.5 mg/kg of LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich; Merck KGaA). Groups of animals were sacrificed 3 h after a single injection of LPS (n=5) or normal saline (control group, n=5), three independent experiments were performed. Various tissues (large intestine, lung, liver, testis, ovary, brain, spleen, and kidney, small intestine, heart and skin) were collected as previous described (30). The systemic expression of the proinflammatory cytokine TNF-α in various tissues (large intestine, lung, liver, testis, ovary, brain, spleen, kidney, small intestine and heart) were induced by LPS and the systemic inflammatory response was activated.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells or mouse tissues (large intestine, lung, liver, testis, ovary, brain, spleen, and kidney, small intestine, heart and skin) were obtained and TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract the total RNA, according to the manufacturer's protocol. Gel electrophoresis was performed to detect the integrity of the total RNA extracted. Total RNA (2 μg) was reverse transcribed into cDNA, PrimeScript RT reagent kit with gDNA Eraser (Takara Bio, Inc., Otsu, Japan) was used for RT to produce cDNA at 42˚C for 50 min and at 85˚C for 5 min, according to the manufacturer's protocol. cDNA (20 ng) was subjected

to qPCR analysis with TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus; Takara Bio, Inc.) according to the manufacturer's protocol. PCR was run under the following conditions: An initial denaturation at 95˚C for 30 sec, 35 cycles of 95˚C for 5 sec, annealing and extension at 60˚C for 30 sec, and final extension at 72˚C for 5 min. *β-actin* was used as the internal control. All primers were obtained from Chengdu Qing Ke Zi Xi Biotechnology Co. (Chengdu, China). Human primers included *C-X-C motif chemokine ligand 1* (*CXCL1* forward, 5'-GCCAGTGCTTGCAGACCCT-3' and reverse, 5'-GGC TATGACTTCGGTTTGGG-3'), *CXCL2* (forward, 5'-CAA ACCGAAGTCATAGCCAC-3' and reverse, 5'-TCTGGT CAGTTGGATTTGCC-3'), *CXCL8* (forward, 5'-TCTGTC TGGACCCCAAGGAA-3' and reverse, 5'-GCATCTGGC AACCCTACAACA-3'), *C-C motif chemokine ligand 20* (*CCL20* forward, 5'-TGACTGCTGTCTTGGATACAC AGA-3' and reverse, 5'-TGATAGCATTGATGTCACAGC CT-3'), *CCL27* (forward, 5'-AGCACTGCCTGCTGTACT CA-3' and reverse, 5'-TCTTGGTGCTCAAACCACTG-3'), *S100 calcium binding protein A7* (*S100A7* forward, 5'-CCT TAGTGCCTGTGACAA-3' and reverse, 5'-CTGCTTGTG GTAGTCTGT-3'), *S100A8* (forward, 5'-AGTGTCCTCAGT ATATCA-3' and reverse, 5'-CATCTTTATCACCAGAAT G-3'), *S100A9* (forward, 5'-CAACACCTTCCACCAATA C-3' and reverse, 5'-TCATTCTTATTCTCCTTCTTGAG-3'), *S100A12* (forward, 5'-CAATACTCAGTTCGGAAGG-3' and reverse, 5'-CTTTGATATTCTTGATGGTGTTT-3'), *LL-37* (forward, 5'-GATAACAAGAGATTTGCCCTGCTG-3' and reverse, 5'-TTTCTCAGAGCCCAGAAGCCTG-3'), *β-defensin 2* [*BD2* (forward, 5'-TTCTCGTTCCTCTTCATA-3' and reverse, 5'-ATATGGCTCCACTCTTAA-3')], *serpin family B member 7* (*SERPINB7* forward, 5'-TTGGTGAAGGTG GCATAA-3' and reverse, 5'-CAGAGCACTTGGGAGATT-3'), *β-actin* (forward, 5'-CCACGAAACTACCTTCAACTCC-3' and reverse, 5'-GTGATCTCCTTCTGCATCCTGT-3'). Mouse primer sequences including *epiregulin* (*EREG* forward, 5'-ACC GCCTTAGTTCAGATG-3' and reverse, 5'-ATGTCC ACCAGGTAGATG-3'), *NIPA like domain containing* 4 (*NIPAL4* forward, 5'-GCACCCTGTCTGGCTTCGT-3' and reverse, 5'-AGTTTAATGACTGTGGGCTCTGG-3'), *phospholipase A2 group IVE* (*PLA2G4E* forward, 5'-GAT GGTGACAGACTCCTT-3' and reverse, 5'-GCAGCAAAG CCTAAAGTTA-3'), *SERPINB7* (forward, 5'-AATAAT CAGCCAGGACTTC-3' and reverse, 5'-CACACTCAA TGTAGTTCTTATG-3'), *solute carrier family 1 member 6* (*SLC1A6* forward, 5'-GGCATCATCATCTGGTATG-3' and reverse, 5'-GGTGACGAGGAAGTAGATA-3'), *WAP four-disulfide core domain 12* (*WFDC12* forward, 5'-GAC AACAGTGAAGAACAGAT-3' and reverse, 5'-GGAGTCCAA GATCAAGGT-3'), *β-actin* (forward, 5'-CCTCTATGCCAA CACAGTGC-3' and reverse, 5'-ACATCTGCTGGAAGGTGG AC-3'). Relative mRNA expression changes were calculated using the $2^{-\Delta\Delta Cq}$ method (32).

Statistical analysis. The data are expressed as the mean ± standard deviation. All statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Prism Inc., La Jolla, CA, USA). Comparison between two groups was performed by unpaired Student's t-test. Comparison among multiple groups was performed by one-way analysis of variance followed by a Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of differential expression genes in psoriatic KCs. To induce a psoriasis-like KCs model *in vitro*, KCs were stimulated with M5 combination (containing IL-1a, IL-17A, IL-22, oncostatin-M and TNF-α). The mRNA levels of the chemokines (CXCL1, CXCL2, CXCL8, CCL20 and CCL27) and antimicrobial peptides (S100A7, S100A8, S100A9, S100A12, LL-37 and BD2) were significantly increased by M5 stimulation of KCs (Fig. 1A). The result suggested that an *in vitro* model of psoriasis-like KCs was established (16). Subsequently, using a human gene expression microarray, a transcriptomic profile of the psoriasis-like KCs was generated. Different colors represent gene expression levels. Scatter plots provided a profile of psoriasis-like KC mRNAs that were upregulated, downregulated or unaffected compared with the control (Fig. 1B). These data identified 2,957 DEGs with a FC>3 ($P<0.01$) as a cutoff, of which 1,735 were upregulated and 1,222 were downregulated (Fig. 1B).

Comparative analysis of DEGs in psoriatic KCs, human psoriatic skin and human psoriatic epidermis. In previous studies, the DEGs between psoriatic lesional and normal skin samples reported by Li *et al* (18), and DEGs between lesional and non-lesional epidermis samples reported by Mitsui *et al* (19), were analyzed further. In the present study, these two data sets were analyzed and compared with the transcriptome dataset in the current psoriasis-like KCs model. GO enrichment analysis was performed on these three transcriptome data sets. The result produced a similar distribution pattern of biological processes and similar genes enrichment among the three datasets (Fig. 1C), indicating that the changes in the biological process observed in psoriasis-like KCs were also presents in patients with psoriasis.

The most significantly enriched biological processes among the upregulated genes included 'cellular response to biotic stimulus', 'cellular response to molecule of bacterial stimulus', 'cytokine-mediated signaling pathway', 'defense response', 'epithelial cell differentiation', 'immune effector process', 'immune response', 'immune system process' and 'innate immune response' (Fig. 1C). By contrast, the most significantly enriched biological processes among the downregulated genes included 'biological adhesion', 'cell adhesion' and 'extracellular matrix organization' (Fig. 1C). All these biological processes have been implicated in psoriasis. These results suggested that the psoriasis-like KCs model is an appropriate model for with similar changes observed as in clinical samples.

Screening of key novel psoriasis-associated genes from differential expression genes that are identified in psoriatic keratinocytes. The human gene expression microarray identified 2,957 DEGs in the induced psoriasis-like KCs model *in vitro* (Fig. 2, step 1). DEGs in the induced psoriasis-like KCs model (n=2,957, psoriasis-like vs. normal KCs) were integrated with the DEGs identified in previous studies [n=3,577, psoriatic lesions vs. normal skin] (18); and n=1,446, psoriatic lesional

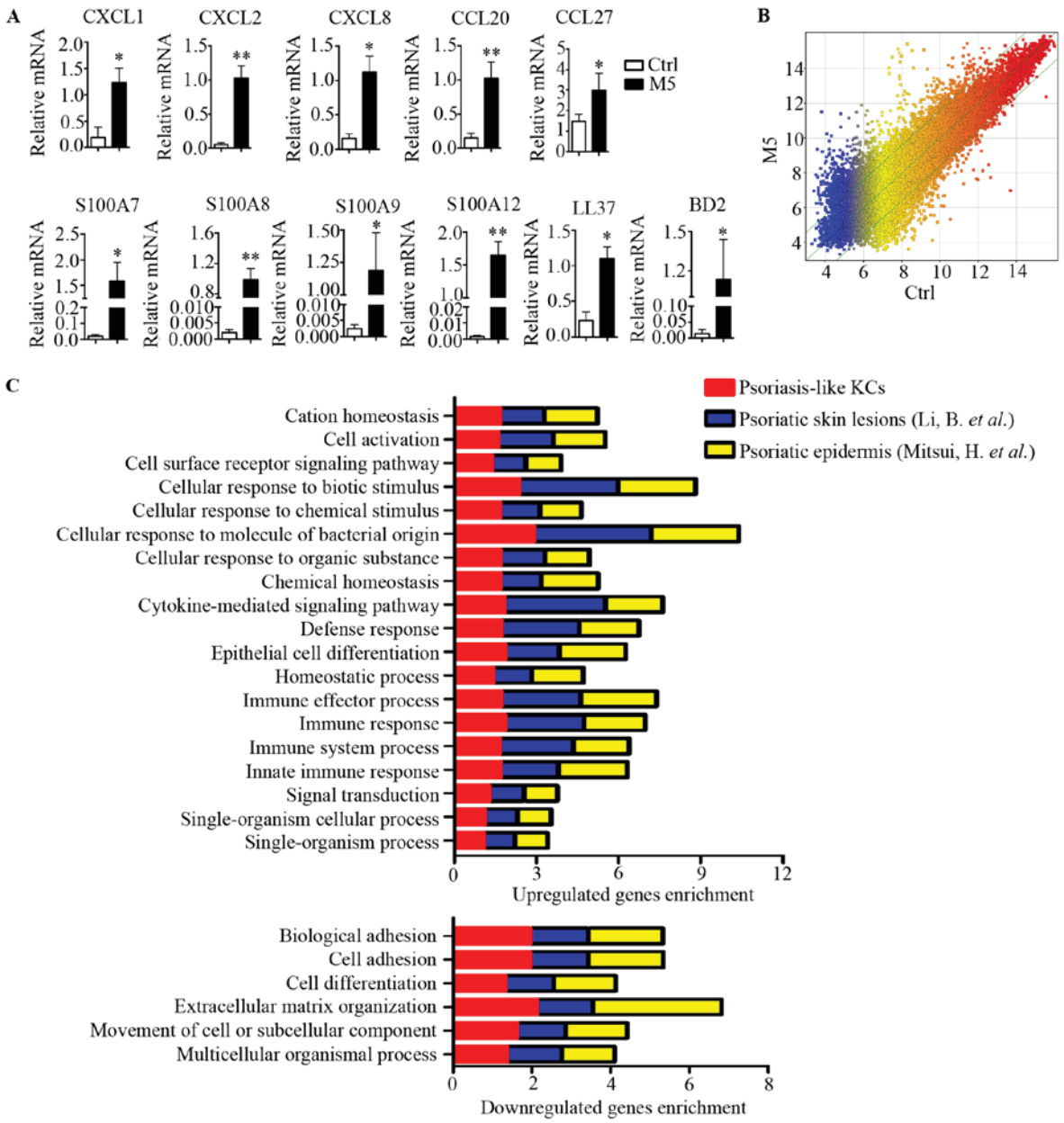


Figure 1. Comparison of transcriptomes in psoriasis-like KCs and patients with psoriasis. (A) KCs were stimulated with M5 for 24 h, reverse transcription-quantitative polymerase chain reaction analysis was performed for each indicated gene. (B) Whole transcriptome gene expression analysis visualizing the variation in the expression between M5 and control groups. Different colors represent gene expression levels. The values on the X and Y axes in the scatter plot are the averaged log2-scaled signal values of the groups. The green lines showed a fold change of 3.0. The levels of the mRNAs above the top green line and below the bottom green line were >3.0-fold different between the two groups. (C) DEGs of psoriasis-like KCs, reported DEGs of psoriatic skin lesions by Li *et al* (18), and reported DEGs of psoriatic epidermis by Mitsui *et al* (19), were separately sorted according to biological process. Enrichment analysis was performed using Gorilla and P<0.001 was the enrichment significance threshold. Bars represent different biological processes. Data are presented as the mean ± standard deviation, n=3, *P<0.05, **P<0.01 vs. Ctrl group. DEGs, differentially expressed genes; CXCL, C-X-C motif chemokine ligand; CCL, C-C motif chemokine ligand; Ctrl, control; BD, β-defensin; KCs, keratinocytes.

epidermis vs. non-lesional psoriatic epidermis (19)]. A core set of 506 overlapping genes (329+100+77) were identified, which were not only differently expressed in psoriasis-like KCs, but also in psoriatic lesions or lesional epidermis of patients with psoriasis (Fig. 2, step 2). Thus, 506 psoriasis disease-associated genes were obtained.

In order to screen for novel psoriasis-associated genes, an additional screening was performed to overlap between the 506 and the known psoriasis-associated genes from the GeneCards database. In the 506 psoriasis disease-associated genes, 73 psoriasis-associated genes that had been well described in previous studies according to GeneCards database were excluded (Fig. 2, step 3), and the remaining 433 genes (Fig. 2, step 3, marking i) were selected as novel psoriasis disease-associated genes.

If the candidate genes were present in the psoriasis susceptibility region, which genetic loci were also linked to psoriasis as described in the 'Materials and methods' section, these genes were likely to serve an important role in psoriasis. More than 100 genetic loci linked to psoriasis were obtained from OMIM (www.omim.org/search?index=entry&search=psoriasis&filter=gm_exists%3Atr ue&

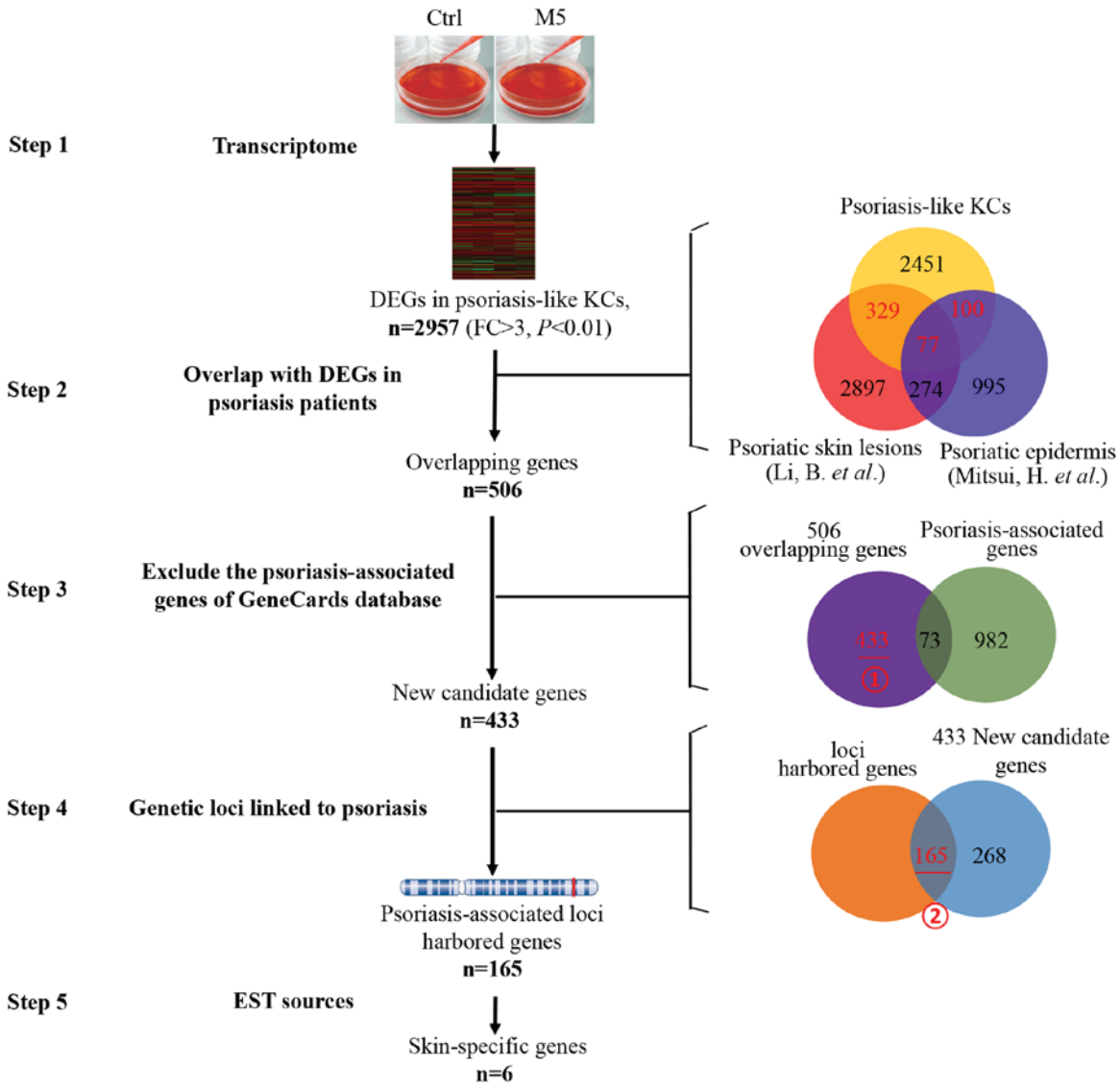


Figure 2. Screening of key skin-specific candidate genes from transcriptome of psoriasis-like KCs. Step 1: 2,957 DEGs were identified in psoriasis-like KCs. Step 2: Using the overlap between the 2,957 DEGs in psoriasis-like KCs and the reported DEGs in psoriasis patients [DEGs from Li *et al* (18) and by Mitsui *et al* (19)], and 506 overlapping genes were identified. Red number represents selected overlapping genes. Step 3: Further screening was performed to overlap between the 506 genes and the psoriasis-associated genes from GeneCards database excluding 73 psoriasis-associated genes. Step 4: In an additional screening was performed by genetic locus retrieval, and 165 genes were located near summary genetic loci linked to psoriasis. Step 5: These 165 genes were identified by analyses expression patterns in multiple human tissues inferred from EST sources. Finally, six genes were identified as skin-specific candidate genes. Ctrl, control; DEGs, differentially expressed genes; KCs, keratinocytes; FC, fold change; EST, expressed sequence tag.

sort=chromosome_number+asc%2C+chromosome_sort+asc &start=1&limit=100 &retrieve=geneMap) and from previous studies (20-29). These 433 novel psoriasis-associated genes were further screened by genetic locus retrieval, and it was revealed that 165 genes were located near the summary genetic loci linked to psoriasis (Fig. 2, step 4, marking ii), and these were identified as key novel psoriasis-associated genes.

Screening of skin-specific candidate genes associated with psoriasis. To screen skin-specific genes from the 433 novel psoriasis-associated genes, an additional screening was performed to assess expression patterns of 165 genes in normal human tissue by referring to the EST resources. Based on the TPM value of indicted gene in various tissues, six genes (*EREG*, *NIPAL4*, *PLA2G4E*, *SERPINB7*, *SLC1A6*, *WFDC12*) that were relatively tissue-specific and highly expressed in human skin were selected (Fig. 2, step 5). The tissue expression pattern of these six genes was demonstrated by their EST profile and the heat map analysis of log2-FC of TPM (Fig. 3).

As shown in Fig. 2, an integrated approach was used, combining psoriasis transcriptome data derived from the GeneCards database, psoriasis-associated locus and EST resources. Through the above screening process, six significantly differentially expressed genes (*EREG*, *NIPAL4*, *PLA2G4E*, *SERPINB7*, *SLC1A6 and WFDC12*) in psoriatic KCs and lesional skin/epidermis of patients with psoriasis were identified. As novel psoriasis-associated candidate genes, these six genes were located near the psoriasis-associated locus, and they were relatively tissue-specific and highly expressed in human skin tissue.

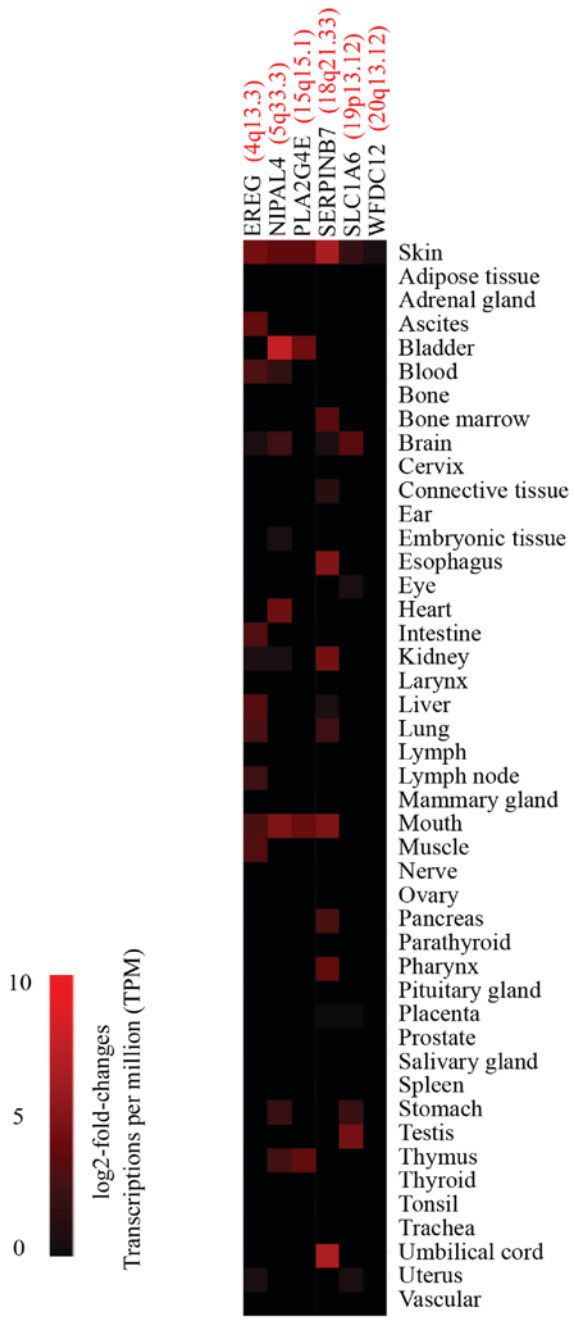


Figure 3. EST profiles of screened skin-specific candidate genes. Heat map analysis of the differential expression of six skin-specific candidate genes in normal human tissues. Black, no expression; red, high expression. Color gradient, log2-fold-changes of transcriptions per million from EST sources. Red indicates the genetic loci linked to psoriasis. EST, expressed sequence tag; EREG, epiregulin; NIPAL4, NIPA like domain containing 4; PLA2G4E, phospholipase A2 group IVE; SERPINB7, serpin family B member 7; SLC1A6, solute carrier family 1 member 6; WFDC12, WAP four-disulfide core domain 12.

Identification of candidate genes specifically and highly expressed in mouse skin epidermis. The tissue expression profile of the six novel candidate genes in the mouse was confirmed by RT-qPCR, and the results showed that four genes (*EREG*, *NIPAL4*, *SERPINB7* and *WFDC12*) were highly expressed in the skin, but their expression levels were lower in other major organs and tissues, including the large intestine, lung, liver, testis, ovary, brain, spleen, and kidney, small intestine and heart (Fig. 4A). This result was consistent with their EST profiles (Fig. 3). However, *PLA2G4E* and *SLC1A6* exhibited no such skin specificity (Fig. 4A).

The epidermis is composed primarily of KCs that are key skin-specific immune cells. The dermis is primarily composed of other immunocyte cell types, such as fibroblasts, dendritic cells (DCs), T helper cells, γδT cells (1,2). *EREG*, *NIPAL4*, *SERPINB7* and *WFDC12* exhibited higher expression levels in mouse epidermis than in dermis (Fig. 4B). This result suggested that these genes exhibited higher expression levels in the key skin-specific immune cells (KCs) than in other dermis immunocyte cell types, indicating their skin specificity.

Identification of candidate genes that are specifically increased in psoriasis-like skin epidermis. In order to define whether the candidate genes were differentially regulated in psoriasis-like skin, mRNA from IMQ-treated or untreated dorsal skin (day 0) was isolated and analyzed by RT-qPCR. Upon treatment with IMQ, the four candidate genes (*EREG*, *NIPAL4*, *SERPINB7* and *WFDC12*) were strongly induced on day 4 (*EREG*, *NIPAL4*, *SERPINB7*) and day 6 (WFDC12) (Fig. 5A). This result suggested that these four candidate molecules may be involved in local immune responses in psoriatic skin tissue.

Subsequently, in order to evaluate the psoriatic skin specificity of the four candidate genes, mRNA expression levels in dissected epidermis and dermis of IMQ-treated dorsal skin or normal dorsal skin was analyzed by RT-qPCR. In normal skin and IMQ-treated skin, the four genes were all highly expressed in epidermis compared with the dermis (Fig. 5B). Furthermore, the mRNA expression levels of *EREG*, *NIPAL4* and *SERPINB7* after 4 days, and *WFDC12* after 6 days of IMQ treatment were dramatically increased in the epidermis of dorsal skin. However, in dermis, there was no significant difference in the expression levels of these four genes between normal and IMQ-treated skin. As immunocytes are recruited to the dermis in psoriasis, this result indicated that the expressions levels of these molecules were differentially regulated in psoriatic epidermis (which is predominantly KCs), but not in immunocytes that infiltrate the psoriatic dermis. In addition, as many important immune molecules may exhibit expression alteration in peripheral blood immunocytes, published microarray data (GSE40263) of peripheral blood mononuclear cells (PBMCs) from patients with psoriasis, was used in the current study. There was no significant difference in the PBMCs expression levels of these four genes in the psoriasis and healthy control groups (Fig. 5C). Combined with differences in expression of these four genes induced by M5 in the psoriasis-like KC model (Fig. 5C), these results suggested that the expression of *EREG*, *NIPAL4*, *SERPINB7* and *WFDC12* were specifically regulated and highly expressed in psoriatic KCs (psoriatic skin-specific immune cells), but not in the dermis or PBMCs involved in the local immune response to psoriasis. Thus, these genes also were novel psoriatic skin-specific genes, and may serve a unique role in the pathogenesis of psoriasis, but this needs to be investigated further.

Differential expression pattern of candidate genes in SIRS. In order to confirm whether the candidate genes were involved in local immune responses in the skin, the expression levels of the five genes (*TNF-α*, *EREG*, *NIPAL4*, *SERPINB7* and

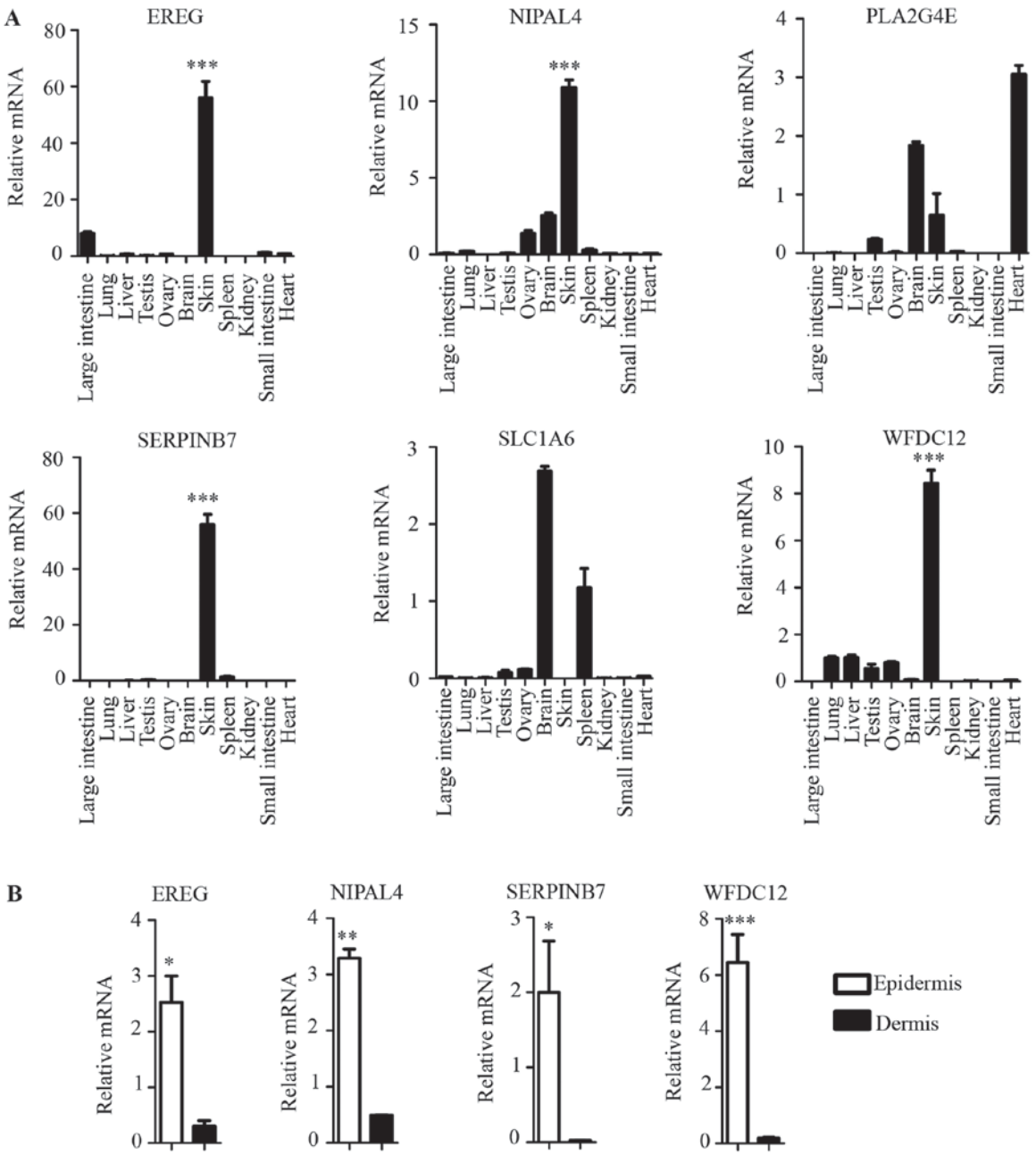


Figure 4. Identification of key skin-specific candidate genes. (A) RT-qPCR analysis was performed for each indicated gene from C57BL/6 WT mice different tissues. Data presented the mean ± standard deviation of three independent experiments, n=5 mice, ***P<0.001 skin vs. the other major organs and tissues (large intestine, lung, liver, testis, ovary, brain, spleen, and kidney, small intestine and heart). (B) RT-qPCR analysis was performed for each indicated gene from dissected mice epidermis and dermis. Data presented the mean ± standard deviation of three independent experiments, n=5 mice, *P<0.05, **P<0.01, ***P<0.001 vs. dermis. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; EREG, epiregulin; NIPAL4, NIPA like domain containing 4; PLA2G4E, phospholipase A2 group IVE; SERPINB7, serpin family B member 7; SLC1A6, solute carrier family 1 member 6; WFDC12, WAP four-disulfide core domain 12.

WFDC12) were determined by RT-qPCR in the LPS-induced SIRS and normal saline group. There was no difference in expression of the five genes in skin tissue between the two groups, but significantly higher levels of TNF-α were detected in the large intestine, lung, liver, testis, ovary, brain, spleen, kidney, small intestine, and heart of the SIRS group compared with the saline-treated control. These results suggested that LPS can induce inflammatory response of numerous tissues, but not in the skin in the SIRS model. Furthermore, it was found that the expression levels of *EREG* in lung, kidney, small intestine and heart of the SIRS group were higher compared with those of normal saline group. The expression levels of *NIPAL4* in lung and small intestine were significantly higher in the SIRS model compared with the control mice. *WFDC12* exhibited higher expression in lung, liver and heart of the SIRS model compared with those of the control mice. By contrast, there was no significant difference in the *SERPINB7* expression levels in all tissues between the two groups. This result suggested that *SERPINB7* may not participate in the local immune responses in the various tissues (large intestine, lung, liver, testis, ovary, brain, spleen, kidney, small intestine and heart) of the SIRS model examined (Fig. 6).

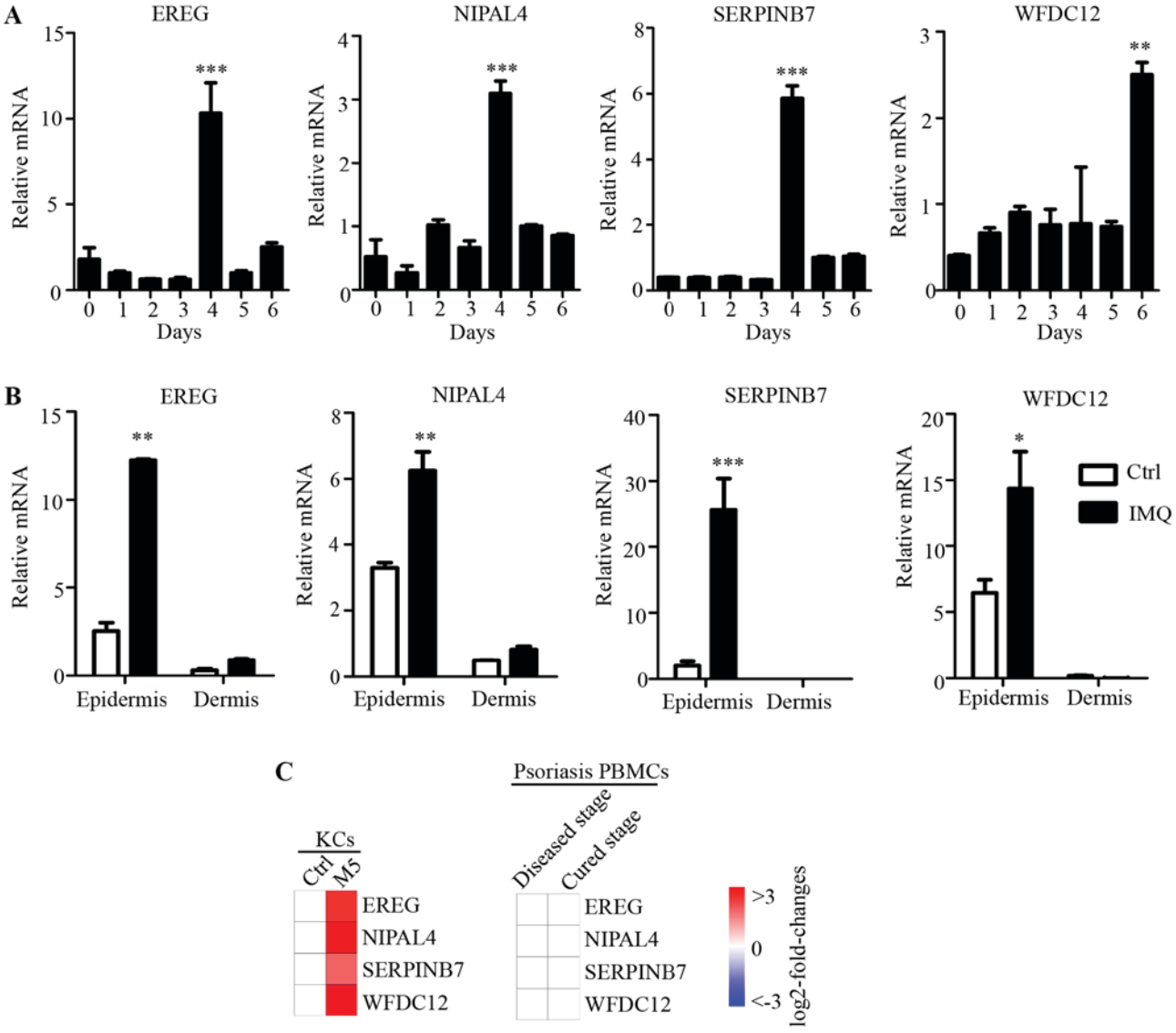


Figure 5. Expression of key candidate genes specifically increased in psoriatic epidermis. (A) The dorsal skin of C57BL/6 (n=5) mice was treated daily with IMQ (55 mg) for 6 days, RT-qPCR analysis was performed for each indicated gene at day 0, 1, 2, 3, 4, 5 and 6. Data are presented the mean ± standard deviation of three independent experiments, ***P<0.001, 4 day vs. other groups; **P<0.01, 6 day vs. other groups. (B) The dorsal skin of the C57BL/6 (n=5) mice were treated daily with IMQ (55 mg) for 6 days, then the dermis and epidermis were obtained by digested separation. RT-qPCR analysis of *EREG*, *NIPAL4* and *SERPINB7* at 4 days after IMQ treatment, *WFDC12* at 6 days after IMQ treatment. Data are presented the mean ± standard deviation of three independent experiments, *P<0.05, **P<0.01, ***P<0.001 vs. Ctrl. (C) Heat map of four key candidate genes in psoriasis-like KCs and psoriasis PBMCs from published microarray data (GSE40263). White, no change; green, downregulated; red, upregulated. Color gradient, log2-fold-changes. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; EREG, epiregulin; NIPAL4, NIPA like domain containing 4; SERPINB7, serpin family B member 7; WFDC12, WAP four-disulfide core domain 12; SLC1A6, solute carrier family 1 member 6; Ctrl, control; IMQ, imiquimod; KC, keratinocytes; PBMCs, peripheral blood mononuclear cells.

SERPINB7 mRNA expression levels in lesional and non-lesional psoriatic skin of patients with psoriasis. mRNA from lesional and non-lesional psoriatic skin of patients with psoriasis was isolated and was examined by RT-qPCR. The mRNA expression level of *SERPINB7* (selected as it was involved in the activated immune response of the skin, not other tissues) in lesional psoriatic skin of patients with psoriasis was significantly higher than in non-lesional psoriatic skin of the same patients (Fig. 7).

Discussion

Psoriasis is a common skin disease affecting 2% of the population worldwide and is characterized by increased proliferation and abnormal differentiation of KCs (33). There are not many effective psoriatic skin-specific targeted drugs to control the symptoms. In the present study, DEGs were detected from KCs (a skin-specific immune cell type), derived from psoriatic and non-psoriatic human and mice tissues, from a SIRS model, and skin-specific genes were identified. The present study introduced a cutaneous tissue-specific target for skin-related diseases treatment and at the same time provided a novel method for the exploration of unknown cutaneous tissue-specific targets for disease treatment.

Many DEGs identified in previous studies were localized to defined lesional psoriatic skin, or the epidermis and dermis of psoriatic lesions (18,19), whereas expression pattern analysis

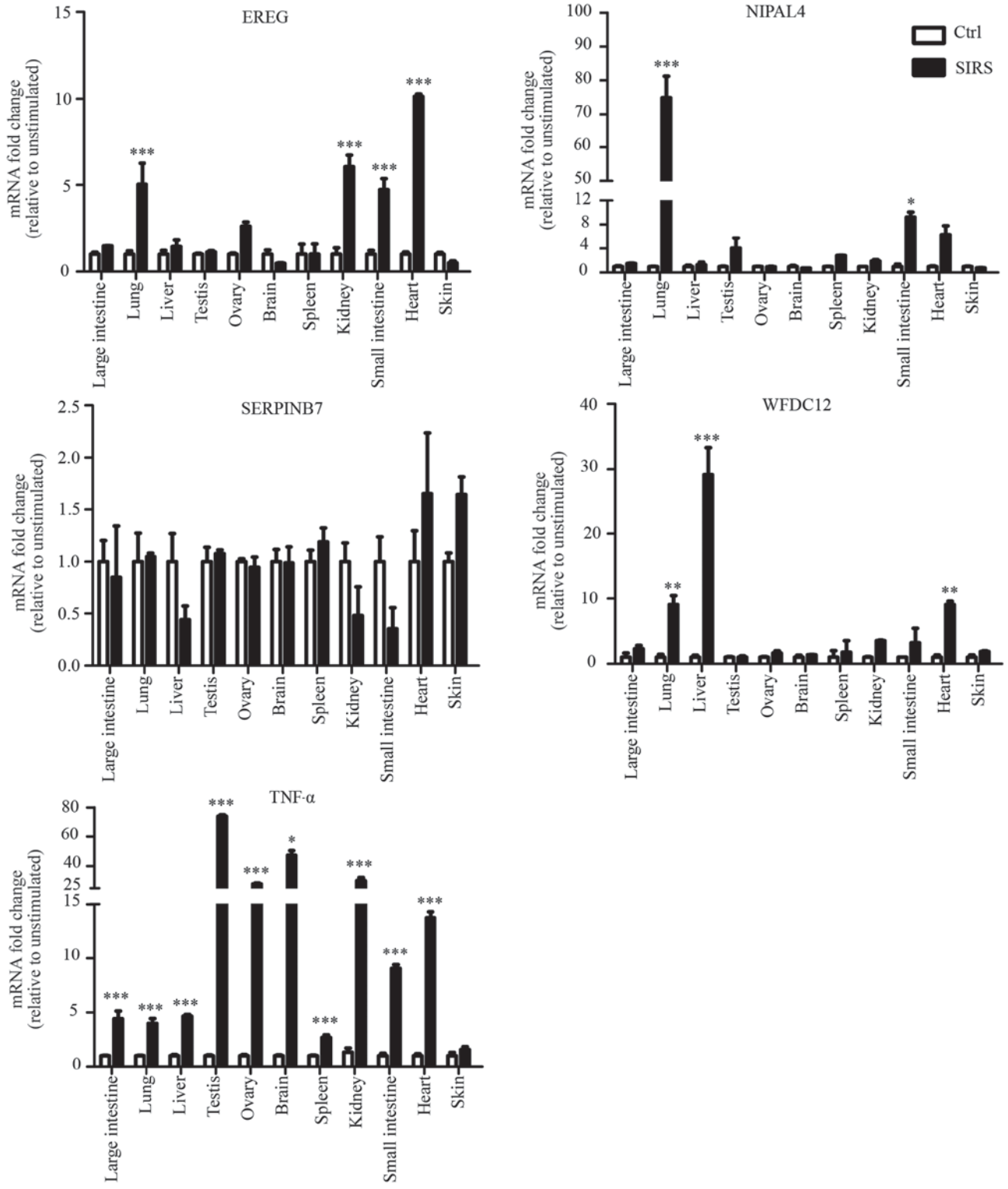


Figure 6. Differential expression patterns of candidate genes in SIRS. Tissue expression patterns of psoriatic-specific candidate genes in lipopolysaccharide-induced SIRS and normal saline group (n=5, each). Reverse transcription-quantitative polymerase chain reaction analysis of the indicated genes in various tissues was performed, and data presented the mean ± standard deviation of three independent experiments, *P<0.05, **P<0.01, ***P<0.001 vs. the Ctrl group. EREG, epiregulin; NIPAL4, NIPA like domain containing 4; Ctrl, control; SIRS, systemic inflammatory response syndrome; SERPINB7, serpin family B member 7; WFDC12, WAP four-disulfide core domain 12; TNFα, tumor necrosis factor-α.

in a psoriasis-like KCs model has not been reported frequently. In the present study, the gene expression profile was produced using a microarray analysis of psoriasis-like KCs. Although there have been data sets produced single cytokine-induced gene expression changes in cultured KCs (34,35), it appears that single cytokine stimulation has a limited effect on KCs, namely, a limited number and a limited modulated expression of targeted genes, reflecting only partial features of psoriasis (16). In previously published transcriptional profiling experiments, gene sets for KC responses to cytokines involved

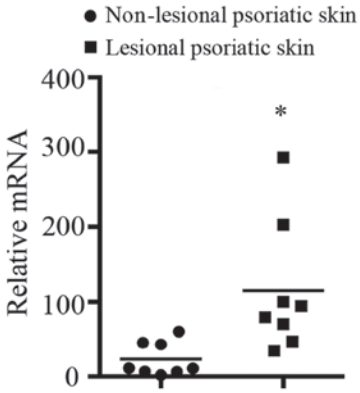


Figure 7. *SERPINB7* mRNA expression levels in lesional and non-lesional psoriatic skin of patients with psoriasis. Reverse transcription-quantitative polymerase chain reaction analysis was performed for *SERPINB7* in lesional and non-lesional psoriatic skin of patients with psoriasis. Data are presented as the mean ± standard deviation, n=8, *P<0.05 vs. non-lesional psoriatic skin. SERPINB7, serpin family B member 7.

in psoriasis were curated, including IL-17, TNF-α, and IL-22, alone and as a combination (36-39). In the present study, a psoriasis-like KCs model was established by treatment with IL-17, IL-1α, IL-22, TNF-α and oncostatin-M, which produced a strong transcriptional effect on KCs chemokines, cytokines, and antimicrobial peptide production, and these cells exhibit a psoriasis-like profile (16,40). In the present study, by comparing transcriptomes of psoriasis-like KCs and previously published data sets of lesional psoriatic skin/epidermis, it was revealed that enriched functions of DEGs had highly similar patterns in the current study and the previously published data. Thus, psoriatic skin-specific genes were identified by analysis of DEGs in psoriasis-like KCs.

Psoriasis is a complex multifactorial disease and the development of this disease remains largely unexplored. Recent research identified psoriasis susceptibility loci and genes that are closely associated with the pathogenesis of psoriasis (5). Genome-wide linkage scans and high throughput studies have been used to identify genes responsible for familial psoriasis and several susceptibility loci (41). In the present study, an integrated approach was used, which may be helpful for exploring the contributory factors involved in the initiation of a complex multifactorial disease such as psoriasis.

ESTs derived from different cDNA libraries can be prepared from different tissues, organs or cell types. It provides a rapid and efficient approach for deciphering gene expression levels in different tissues and screening tissue-specific molecules (42,43). Previous studies have used EST data to identify tissue-specific genes in the human prostate (44), heart (45), retina (46) and in cancer tissue (47). These studies highlight the advantages of using this approach. In the present study, integrated analysis of human ESTs provided a robust platform for psoriasis-like KCs transcriptome screening. Four genes with skin-specific expression were identified, indicating that EST assessment was highly accurate.

Gerber *et al* (48), using the Body Index of Human Gene Expression database and comparing the ratio of mean gene expression in skin with other tissue/cell types, investigated eight genes out of the top 100 genes preferentially expressed in normal human skin. The expression profiles of these eight candidate genes (*mucin like 1, WFDC5, SERPINB7, chromosome 5 open reading frame 46, transmembrane protein 45A, G protein-coupled receptor 115, cadherin related family member 1 and G protein-coupled receptor 87*) were analyzed in five tissues (skin, spleen, kidney, brain, liver) and four cell types (keratinocytes, fibroblasts, PBMCs, endothelial cells). The expression levels in cytokine-stimulated keratinocytes and in biopsies of skin diseases were analyzed (48). In the present study, novel candidate genes were investigated in another manner. Initially, based on gene description and publications from the human gene database (GeneCards), 73 genes that were closely associated with psoriasis were excluded. Subsequently, based on the summary of genetic loci linked to psoriasis, 165 candidate genes were identified. Finally, as skin specific cells, KCs constitute the majority of cells in the skin's epidermis; therefore, the expression of the six candidate genes (*EREG*, *NIPAL4*, *PLA2GE*, *SERPINB7*, *SLC1A6* and *WFDC12*) was examined in epidermis and dermis samples form mice.

The skin is composed of two distinct regions, the epidermis and dermis, it contains a variety of immune cells (1,2). The KCs are the predominant cell type in the epidermis, it is highly specialized epithelial cells designed to perform a very specific function. The dermis contains cells of the immune system including T cells and DCs (1,2,4). In the normal condition, four candidate genes (*EREG*, *NIPAL4*, *SERPINB7* and *WFDC12*) were highly expressed in skin compared with other tissues. In skin tissue, the expression of these genes was higher in the epidermis than in the dermis. This suggested that the four candidate genes were highly expressed in KCs. KCs exhibit hyperproliferation and abnormal differentiation in psoriatic epidermis, and a large number of inflammatory cells infiltrate into the dermal lesions (5,6). The expression levels of the four candidate genes were increased in psoriasis-like skin lesion. Furthermore, it was revealed that the expression levels of these candidate genes were increased in psoriatic epidermis compared with normal controls; however, the levels were not different in psoriatic dermis compared with normal controls. Furthermore, in psoriasis-like KCs, the expression levels of these genes were increased compared with untreated KCs. This suggested that the four candidate genes were highly expressed in psoriasis-like KCs, and they may be involved in the local immune response of psoriatic KCs, suggesting that they have psoriatic skin-specific roles.

The pathophysiology of SIRS involves a systemic immune response that affects pulmonary, gastrointestinal and renal function. As LPS induced the systemic expression of the proinflammatory cytokine TNF-α in various tissues (large intestine, lung, liver, testis, ovary, brain, spleen, kidney, small intestine and heart), the systemic inflammatory response was activated. However, TNF-α and the four other detected genes (*EREG*, *NIPAL4*, *SERPINB7* and *WFDC12*) exhibited no difference in expression the skin between the SIRS and control group. This suggested that LPS may have not induced an inflammatory response in skin tissue in the SIRS model. *EREG*, *NIPAL4*, and *WFDC12* were upregulated in certain tissues in the SIRS model compared with the normal control. There was no significant difference in *SERPINB7* expression between the two groups in the all tissues analyzed. These results suggested that *SERPINB7* was not involved in the

activated immune response of various tissues, except for the skin. *SERPINB7*, a serpin peptidase inhibitor, has critical roles in the immune system; it can increase mesangial cell proliferation and extracellular matrix (ECM) deposition and markedly suppress cell motility and invasion (49). *SERPINB7* appears to be involved in maintaining tissue integrity by preserving ECM homeostasis, and loss of expression may lead to loss of cell adhesion and tissue integrity (49). *SERPINB7* exhibits substantial expression variation in skin disorders, such as palmoplantar keratosis (50). Additionally, the mRNA levels of *SERPINB* were significantly higher in lesional psoriatic skin than in non-lesional psoriatic skin of patients with psoriasis. Therefore, *SERPINB7* requires further investigation to clarify its potential role in the pathogenesis of psoriasis.

As novel psoriatic skin-specific genes, *EREG*, *NIPAL4* and *WFDC12* may also be valuable candidates for further exploration. It was previously reported that *EREG* and *WFDC12* serve the critical immuno-regulatory roles in skin. *EREG* encodes epiregulin, which is a secreted peptide hormone and member of the epidermal growth factor family of proteins, is overexpressed in psoriatic epidermis (51). Functions associated with this gene include growth factor activity and epidermal growth factor receptor binding. Secreted epiregulin induces downregulation of inflammatory cytokine IL-18 mRNA expression in KCs (52) and can also stimulate proliferation of KCs. WFDC12 is one of an 18-member family of secreted proteins reported as protease inhibitors, and is an antimicrobial peptide also reported to participate in inflammation and host defense (53). However, the expression of *EREG* and *WFDC12* in psoriatic skin lesions is currently unknown. In addition, there are few studies on the role of *NIPAL4* in the skin. *NIPAL4*, also known as ichthyin, is composed of several transmembrane domains. It is associated with keratins and desmosomes in KCs and is involved in lipid metabolism (54,55). Mutations in this gene have been associated with autosomal recessive congenital ichthyosis (56).

The present data strongly suggests that these genes (*EREG*, *NIPAL4*, *SERPINB7* and *WFDC12*) were specifically expressed in psoriatic skin, and they may be involved in the local immune response of psoriatic skin. Further studies are required to gain further insights into their regulation and potential role in skin disorders.

In the present study, a pathological tissue-specific molecular screening method was established. This method used psoriatic transcriptome data, psoriasis-associated gene information, genetic loci associated with psoriasis and normal/pathological tissue expression patterns. Through the screening procedure, candidate genes with genetic loci associated with psoriasis that were specific expressed in normal skin and exhibited high expression in psoriatic KCs, rather than in other inflammatory cells or tissues, were identified. The present study identified the potential key pathogenic tissue-specific molecules for diseases, and the psoriatic skin-specific genes (*EREG*, *NIPAL4*, *SERPINB7* and *WFDC12*) may represent potential biomarkers or drug targets for the development of future diagnostics/therapeutics to treat psoriasis.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZW and JL designed the experiments and the present study. ZW, HPZ, HZ, NH, XW and JZ collected data and did experiments. ZW, XL, XT, ZH, XZ and WL analyzed the data. ZW, HPZ, HZ, XW and JZ contributed to critical revisions of the text.

Ethics approval and consent to participate

The present study was performed in accordance with the principles of the Helsinki Declaration and approved by the Ethics Committee of the West China Hospital, Sichuan University. Written informed consent was obtained from all study participants prior to the present study. The animal protocols were approved by the Committee on the Ethics of Animal Experiments of the Sichuan University (Chengdu, China). The experimental procedures were conducted according to the ethical guidelines for the care and use of laboratory animals of the National Institutes of Health and the IASP.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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