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Original Article

Gene set enrichment analysis and ingenuity pathway analysis to verify the impact of Wnt signaling in psoriasis treated with Taodan granules

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Abstract: Background: Taodan granules (TDGs), traditional Chinese herbals, have effectiveness in relieving skin erythema, scales, and other symptoms of psoriasis. Yet mechanisms of TDGs remain indistinct. Objective: To indicate the molecular mechanisms of TDGs in treating psoriasis. Materials and methods: Primarily, transcriptional profiling was applied to identify differentially expressed genes (DEGs), proceeding with Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. Gene Set Enrichment Analysis (GSEA) and Ingenuity Pathway Analysis (IPA) analysis were used for functional enrichment analysis. Subsequently, levels of selected genes were validated by RT-PCR and western blotting. Results: The GSEA results revealed TDGs could down-regulate the Wnt signaling pathway to ameliorate skin lesions of imiquimod (IMQ)-induced psoriatic models mice. IPA core network associated with Wnt signaling pathways in TDGs for psoriasis was established. Thereinto zeste homolog 2 (EZH2), CTNNB1, tumor protein p63 (TP63), and WD repeat domain 5 (WDR5) were considered as upstream genes in the Wnt signaling pathway. Experimental verification indicated TDGs could down-regulate EZH2, CTNNB1, and WDR5 at the mRNA and protein levels, along with up-regulate TP63 levels. Moreover, TDGs were confirmed to reduce RAC2 and WNT5A at mRNA and protein levels of the Wnt signaling pathway. Conclusions: TDGs may improve psoriasis through the regulation for upstream genes (down-regulating levels of EZH2, CTNNB1, and WDR5; up-regulating TP63 levels) of Wnt signaling pathway, thus reducing levels of RAC2 and WNT5A in the Wnt signaling pathway.

Keywords: Psoriasis, Taodan granules, Chinese herbs, Wnt signaling pathway, gene set enrichment analysis, ingenuity pathway analysis

Introduction

Psoriasis is a common chronic and intractable dermatosis. The pathogenesis, diagnosis, and treatment of psoriasis remain currently one of the major research topics in dermatology. With high prevalence, psoriasis is not merely implicated in the skin, accompanied by a variety of comorbidities (cardiovascular disease, hepatic disease, nephropathy, and etc.) [1, 2], affecting the quality of life of patients [3], and causes a heavy social and economic burden [4]. Biologic therapies are recognized as the most effective therapeutic care in the clinic up till now for moderate to severe psoriasis [5], while numerous adverse reactions and contraindications occur with high relapse of 94.7% after 18 months of discontinuation [6]. Therefore, exploration of more effective therapeutic approaches is imperative.

Chinese herbal medicines have been widely used in clinical treatment of psoriasis. Chinese medicine (CM) theory has shown that herbal medicines promote blood circulation and remove blood stasis with therapeutic effect on psoriasis: *Salvia miltiorrhiza* Bunge can effectively treat psoriasis, with inner mechanism

mainly through modulating STAT3 [7]. *Ligusticum striatum* DC. is a classic psoriatic drug, and tetramethylpyrazine (the active ingredient of *Ligusticum striatum* DC.) can alleviate psoriasis-like inflammation through inhibiting TRAF6/c-JUN/NFκB signaling pathway in keratinocytes (KCs) [8]. Taodan granules (TDGs) were formulated from Chinese medicine (CM) theory of promoting blood circulation and removing blood stasis, composed of *Salvia miltiorrhiza* Bunge, *Curcuma aeruginosa* Roxb., *Astragalus mongholicus* Bunge, *Glycyrrhiza inflata* Batalin, *Angelica sinensis* (Oliv.) Diels, *Ligusticum striatum* DC., *Prunus persica (L.)* Batsch, *Cyathula officinalis* K. C. Kuan, and *Smilax china* L. (the plant name has been checked with http://www.theplantlist.org). Our preliminary studies indicated the improvement of psoriasis areas and severity index (PASI) score in psoriatic patients treated with TDGs was 76.64%, while TDGs could reduce the expression of interleukin (IL)-2, IL-4, IL-6, secretion of neuropeptides, and other psoriatic phenotypes in psoriatic patients [9-11]. For another, TDGs have been proven to significantly alleviate erythema, scales, and thickening of typical skin lesions, along with decrease KC proliferation with TDGs in an imiquimod (IMQ)-induced psoriasis-like mouse model, preliminarily substantiating the possible route involving up-regulation of metabolic signaling pathways, such as Gly-Ser-Thr axis, down-regulating immune and inflammatory pathways, accompanied by reduction of RAC2 and ARHGDIB concentrations [12]. Pharmacology-based approaches are used to identify hub genes and kernel pathways of TDGs treated psoriasis, confirming that TDGs down-regulated the mRNA expression of *Mmp2*, *Il-6*, *Tnf*, *Ccl2*, *Cxcl2*, *Il-1b*, and *Jun*, while up-regulated *Il-10* expression. Besides, TDGs could regulate IL-17 signaling pathway and TNF signaling pathway in psoriatic mouse models [13]. However, the regulatory mechanisms of TDGs remain obscure and need further investigation.

Recently, transcriptome sequencing has become a routine method for identifying numerous genes regulated by specific medications. Nevertheless, in view of the multi-target characteristics of CM compounds, extracting meaningful biological insights from transcriptome sequencing nonetheless remains challenging. Recently, Ingenuity Pathway Analysis (IPA) and Gene Set Enrichment Analysis (GSEA) have gained popularity for omics data of functional enrichment analysis. Omics data are resolved via IPA to further investigate the regulatory relationship between genes and bioinformatics application, referred functional analysis, integration, and further understanding [14]. GSEA can analyze and interpret changes of the coordinate path levels in transcriptomics experiments. Both IPA and GSEA have unique advantages in analyzing data. IPA predicts the upstream regulators and mechanism networks for genes, while the algorithm of GSEA is calculated according to the overall trend of actual data. GSEA can make up valuable information easily overlooked by general differential analysis, including biological characteristics in important genes, relationships between gene regulatory networks, and the functions with significant genes [15]. The combination of IPA and GSEA can better explain the mechanism of drug action.

In this work, Liquid Chromatograph Mass Spectrometer/Mass Spectrometer (LC-MS/MS) analysis was widely applied in the identification and quantification of compounds in TDGs. Differentially expressed genes (DEGs) before and after treatment with TDGs were identified via transcription profiling, proceeding Gene ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. On this basis, GSEA was used to discover that Wnt signaling pathway was the core process in TDGs treated psoriasis. Subsequently, IPA core networks related to the Wnt signaling pathway was established. Verified by RT-PCR and western blotting, TDGs acted on upstream genes (down-regulating levels of zeste homolog 2 (EZH2), CTNNB1, and WD repeat domain 5 (WDR5); up-regulating tumor protein p63 (TP63) levels) of Wnt signaling pathway, and reduced RAC2 and WNT5A levels in the Wnt signaling pathway to treat psoriasis.

Materials and methods

TDG materials preparation

The TDGs were made up of nine Chinese herbs (Table S1), authenticated as per standard protocols by a pharmacognosist of the Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of

Traditional Chinese Medicine. The preparation method of TDG water decoction was the same as that reported in our previous studies [12, 13].

LC-MS/MS analysis was used for quantitative control of TDGs, conducted using Waters ACQUITY UPLC I-Class coupled with a 5500 QTRAP mass spectrometer (SCIEX), with 35°C Column temperature. The mobile phase was delivered at 0.3 ml/min with a mixture of 0.1% formic acid aqueous solution and 0.1% formic acid acetonitrile solution, as the gradient elution: 0-3 min (5%-25% B), 3-8.5 min (25%-45% B), 8.5-12 min (45%-95% B), 12-15 min (95%-98% B), 15-15.2 min (98%-5% B), 15.2-18.2 min (5% B).

Animals

Specific pathogen-free (SPF)-grade male BALB/c mice, weighted 25±3 g, were housed in a germ-free environment (temperature of 23±2°C). Ethical approval was obtained by the Ethics Committee of Yueyang Hospital affiliated to Shanghai University of Traditional Chinese Medicine (Supplementary File 1).

Model establishment and interventions

In a nutshell, after adaptive feeding, the hair on the back of mice was removed (2 × 2 cm^2). Mice were divided into three groups: control group, psoriatic model group (IMQ group), and psoriatic model with TDGs treated group (IMQ + TDG group) (n = 4). Establishment of psoriatic modeling was treated with 62.5 mg of 5% IMQ cream for 6 h on ears and back skin, while mice in the control group were applied isodose petroleum jelly. Mice in the IMQ + TDG group were followed by intragastric administration of 1.8 g/kg TDGs (the optimal concentration of TDGs for applying in the subsequent experiments shown in Figure S1), and mice in the IMQ group were administered with intragastric administration of 0.9% NaCl solution.

Transcriptome sequencing

On day 12, mice were euthanized. The full-thickness back skin (1 × 1 cm^2) was extracted for Transcriptome sequencing. Illumina HiSeq™ 2500 was used for sequencing, and the transcriptome analysis was performed by Shanghai OE Biotechnology Co., Ltd. Standardization disposal was carried out using DESeq software (version 1.18.0) to standardize the gene count for each sample. The DEGs were screened according to the results of |log2FoldChange| > 1 and p-value < 0.05.

GO and KEGG analysis

DAVID (https://david.ncifcrf.gov/) was used to analyze the significance of GO and KEGG analysis for DEGs. A p-value of < 0.05 was considered as statistically significant.

IPA

DEGs with |log2FoldChange| > 2 as well as p-value < 0.05, pathway analysis, and the construction of protein-protein interaction (PPI) network of the expression data were executed with IPA (QIAGEN, Redwood City, CA, USA). Results with |z-score| ≥ 2 and/or overlap p-value < 0.05 were considered as statistically significant. Upstream networks were described on the IPA database.

GSEA

GSEA (https://www.gsea-msigdb.org/gsea/index.jsp) with probes ranked by signal-to-noise ratio and statistical significance determined by 1,000 gene set permutations were used to investigate differences among groups for exploring the potential molecular mechanisms and functions of DEGs [16, 17]. A p-value of < 0.01 and false discovery rate (FDR) with a q-value of < 0.05 were considered as statistically significant.

RT-PCR

The mice were euthanized with CO_2 inhalation on day 12. The back skin of mice was extracted and preserved in TRIzol reagent kit. The Reverse Transcription System First Strand cDNA Synthesis Kit was utilized with 20.0 a reaction volume. Real-time fluorescent PCR was performed for RT-PCR. The specific experimental method was the same as the previous study [18]. The primer sequences were revealed in Table S2.

Western blotting

On day 12, the mice were euthanized with CO_2 inhalation. Then, the back skin of mice was

extracted for western blotting. The experimental procedure was the same as in our previous study [18]. The antibodies used for western blotting included Anti-EZH2 antibody (ab283270, diluted at 1:1000), Anti-CTNNB1 antibody (ab32572, diluted at 1:5000), Anti-WDR5 antibody (ab178410, diluted at 1:1000), Anti-RAC2 antibody (PA5-29681, diluted at 1:1000), Anti-WNT5A antibody (ab229200, diluted at 1:500), Anti-TP63 antibody (ab124762, diluted at 1:1000), and β-Actin (ab1801, diluted at 1:1000).

Statistical methods

SPSS 24.0 (IBM Corp., Armonk International Business Machines, New York, USA) was used for analyzing the data, described as mean ± standard deviation (SD). A t-test was used to compare the two groups, while a *p*-value of < 0.05 was considered as statistically significant.

Results

Transcriptional regulations of TDG treatment for psoriasis

Transcriptional profiles analysis of DEGs following TDG treatment: Firstly, to integrally control the quality of TDGs, LC-MS/MS analysis was used for identification and quantification of compounds in TDGs. There were 0.68 ug/ml Rutin, 14.92 ug/ml Caffeic acid, 3.56 ug/ml Cryptotanshinone, 2.77 ug/ml Tanshinone IIA, 2.95 ug/ml Formononetin, 95.87 ug/ml Liquiritin, and 82.11 ug/ml Danshensu possessed the highest content in TDGs. A total ion flow chromatogram of TDGs was shown in Figure S2. The structure of the major components was displayed in Table S3.

Afterwards, to further determine the effect of TDG treatment for psoriasis, the transcriptional profiles of skin lesions treated or nor treated with TDGs of psoriatic mouse models were detected and compared based on RNA sequencing [12]. The DEGs following TDG treatment were identified by RNA sequencing, and the data were stored in the Sequence Read Archive (SRA) repository (SRP292449). A total of 1233 DEGs were identified, with 539 up-regulated and 694 down-regulated genes (Figure S3A). The 20 most significantly up-regulated and down-regulated DEGs following TDGs were demonstrated in Table S4. Biological characteristics of the potential targets for TDGs were estimated by KEGG and GO analysis. KEGG analysis indicated the most up-regulated gene categories (Top 3) were for the neuroactive ligand-receptor interaction, estrogen signaling pathway, biosynthesis of unsaturated fatty acids, and glycine, serine, and threonine (Gly-Ser-Thr) metabolism; and the most down-regulated gene categories (Top 3) were for cytokine-cytokine receptor interaction, Staphylococcus aureus infection, osteoclast differentiation, and chemokine signaling pathway (Figure S3B). GO analysis revealed the most up-regulated gene categories (Top 3) were for the intermediate filaments, keratin filaments, cellular components, and structural molecule activity; and the most down-regulated gene categories (Top 3) were inflammatory response, immune system process, extracellular space, and innate immune response (Figure S3C). Therefore, the effect of TDGs for psoriasis was intimately involved in metabolism, inflammation, and immune regulations.

Upstream analysis of DEGs following TDG treatment: To further investigate the therapeutic pathway of TDGs for psoriasis, we analyzed the potential upstream regulatory functions of DEGs following TDG intervention. We selected DEGs with |log2FoldChange| > 2 and *p*-value < 0.05 based on the sequencing results to construct the core regulatory network in the IPA database (**Figure 1**). IPA upstream regulator analysis identified 30 significant upstream regulators with 21 down-regulation (**Figure 1A**) and 9 up-regulation (**Figure 1B**) following TDGs (|z-score| ≥ 2 and overlap *p*-value < 0.05). Among them, the most remarkable inhibited transcription regulators (Top 3) were Kruppel-like factor (KLF) 4 (overlap *p*-value = 3.11E-16), T-cell leukemia (TCL) 1A (overlap *p*-value = 2.04E-15), and CCAAT/enhancer binding protein epsilon (CEBPE) (overlap *p*-value = 1.95E-11), while the most distinct activated transcription regulators (Top 3) were PAX1 (overlap *p*-value = 1.43E-09), zinc finger protein (ZFP) 36 (overlap *p*-value = 4.98E-09), and SRY-box transcription factor (SOX) 7 (overlap *p*-value = 4.54E-08). Our above-mentioned results demonstrated these upstream transcription factors were central in the improvement of psoriasis following TDGs.

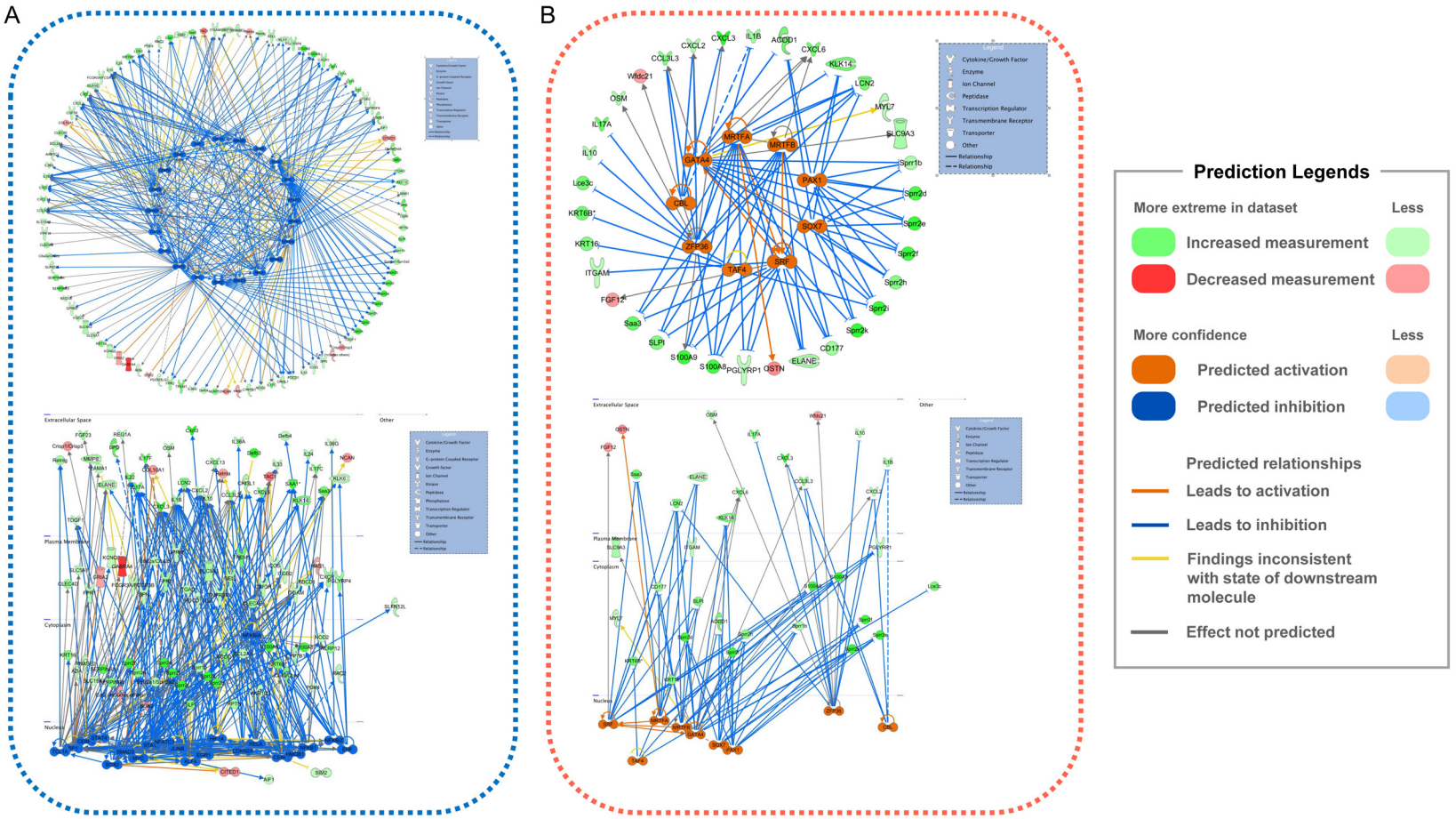

Figure 1. Ingenuity Pathway Analysis (IPA) upstream analysis of differentially expressed genes (DEGs) following Taodan granules (TDGs). A. 21 significantly down-regulated upstream regulators following TDGs. B. 9 significantly up-regulated upstream regulators following TDGs.

Transcriptional regulation of TDG treatment for psoriasis via Wnt signaling pathway

GSEA and IPA of critical GO following TDG treatment: We comprehensively analyzed the changed gene sets following TDG intervention, which wielded GSEA for critical GO. The top eight regulated gene sets incorporated intermediate filament (GO: 0005882), negative regulation of Wnt signaling pathway (GO: 0030178), collagen fibril organization (GO: 0030199), cell fate commitment (GO: 0045165), Wnt-activated receptor activity (GO: 0042813), ureteric bud development (GO: 0001657), hair follicle development (GO: 0001942), and negative regulation of canonical Wnt signaling pathway (GO: 0090090), ranked by normalized enrichment score (NES) (**Figure 2A**). Given the above confirmation, the therapeutic effect of TDGs on psoriasis might be closely implicated in the Wnt signaling pathway. Wnt signaling pathway was validated to be activated in the pathogenesis of inflammatory diseases (psoriasis vulgaris, atherosclerosis, rheumatoid arthritis, sepsis, etc.) [19]. The differentially expressed core enrichment mRNAs in three relevant pathways of Wnt were displayed in **Figure 2B**.

To further investigate the possible regulatory relationships in the three enriched Wnt related signaling pathways, IPA core network was constructed. These genes were associated with Wnt signaling pathway (GO: 0030178), consisted of complex, cytokines, enzymes, group/complex, growth factor, kinase, peptidase, transcription regulator, transmembrane receptor, and others, and were used for constructing the core regulatory network on the IPA database. Platelet derived growth factor (PDGF) BB, Wnt, extracellular signal-regulated kinase (ERK) 1/2, and phosphoinositide 3-kinase (PI3K) were shown to have the highest correlation in the regulations of other proteins in this pathway following TDG treatment (**Figure 2C**). The proteins encoded by these genes were associated with Wnt-activated receptor activity (GO: 0042813) including complex, G-protein coupled receptor, group, growth factor, kinase, transmembrane receptor, together with others were integrated into IPA core network. G protein-coupled receptors (Gpcr), epidermal growth factor (EGF), as well as protein kinase B (Akt) indicated higher levels of activity (**Figure 2D**). Furthermore, genes of complexs, cytokines, enzymes, G-protein coupled receptors, growth factors, transcription regulators, transmembrane receptors, and others were consolidated into the IPA core network associated with Wnt signaling pathway (GO: 0090090). Axis inhibition protein (AXIN) 1, Wnt, along with histone deacetylase (Hdac) showed significant activities in regulating other proteins (**Figure 2E**).

GSEA and IPA of critical KEGG pathways following TDG treatment: On the other hand, we also proceeded with GSEA for KEGG Pathways. Briefly, ranked by normalized enrichment scores, the top nine representative gene sets included basal cell carcinoma (mmu05217), hippo signaling pathway (mmu04390), melanogenesis (mmu04916), ECM-receptor interaction (mmu04512), breast cancer (mmu05224), biosynthesis of unsaturated fatty acids (mmu01040), protein digestion and absorption (mmu04974), cushing's syndrome (mmu04934), and Wnt signaling pathway (mmu04310) (**Figure 3A**). The differentially expressed core enrichment mRNAs of this pathway were displayed in **Figure 3B**.

Similarly, we constructed IPA core networks associated with the Wnt signaling pathway (mmu04310), with genes consisting of complexs, enzymes, kinases, ligand-dependent nuclear receptors, transcription regulators, and others. In this pathway, CTNNB1, mitogen activated protein kinase (MAPK) 8, together with casein kinase (Ck) 2 played key regulatory roles in the Wnt signaling pathway following TDG treatment in psoriasis (**Figure 3C**).

Upstream analysis of TDG treatment for psoriasis via Wnt signaling pathway and experimental validation

After the above preliminary confirmation that TDG treatment for psoriasis was closely related to the control of Wnt signaling pathway, we further predicted the upstream transcriptional regulations. Critical genes were identified as being enriched with significance using IPA database (overlap p-value < 0.05), while six transcription regulators, consisted of EZH2 (overlap p-value = 1.52E-15), CTNNB1 (overlap p-value = 2.83E-12), SRY-related HMG-box transcription factor (SOX) 11 (overlap p-value = 1.26E-11), FOS (overlap p-value = 1.49E-09), TP63 (overlap p-value = 9.28E-09), and WDR5 (over-

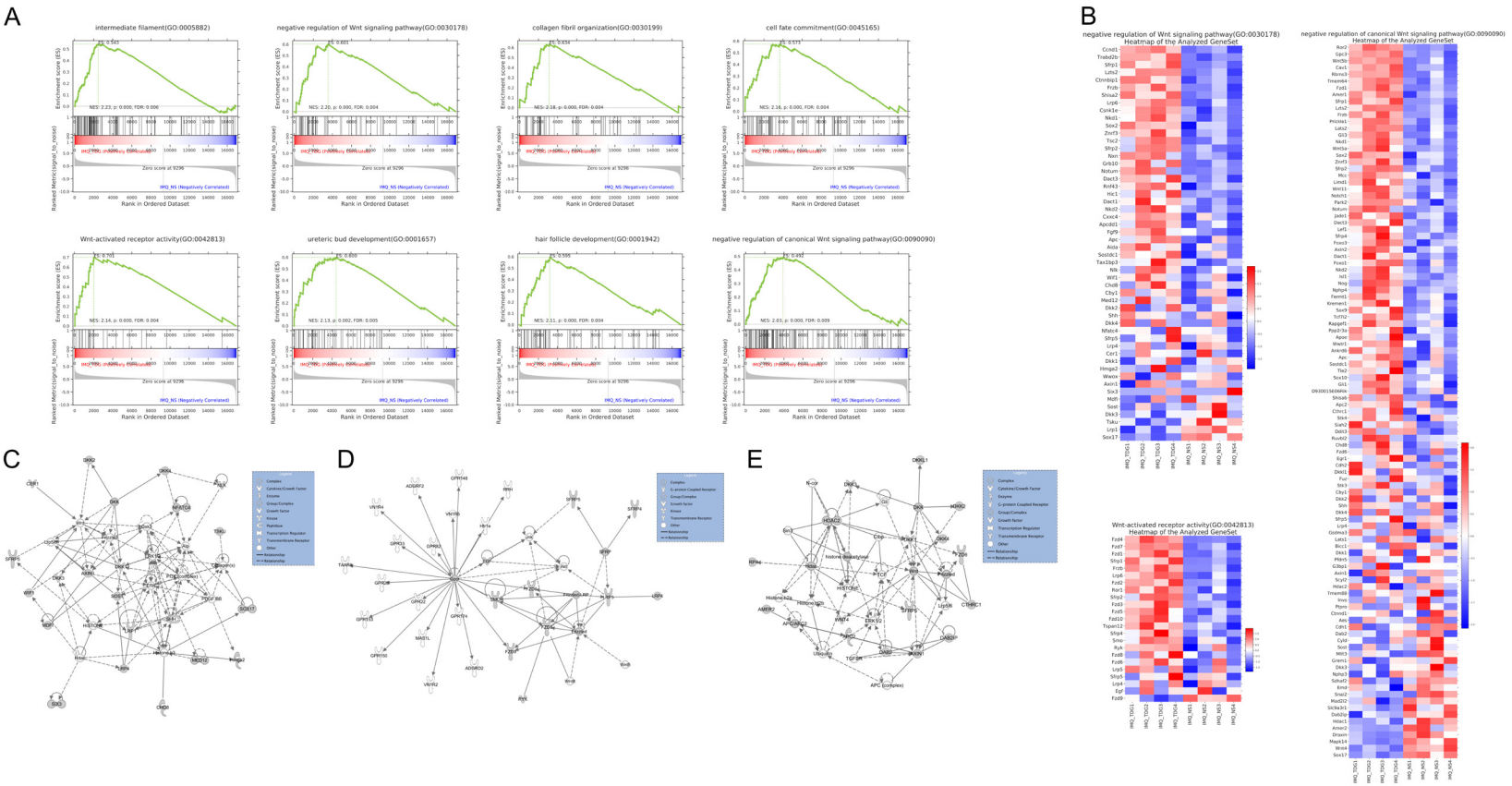

Figure 2. Gene Set Enrichment Analysis (GSEA) analysis and Ingenuity Pathway Analysis (IPA) analysis of Gene ontology (GO) items. A. GSEA of Taodan granules (TDGs) treated compared with disease group. B. Heatmap of corresponding genes in three Wnt signaling pathways. C-E. IPA network analysis of corresponding genes in three Wnt signaling pathways.

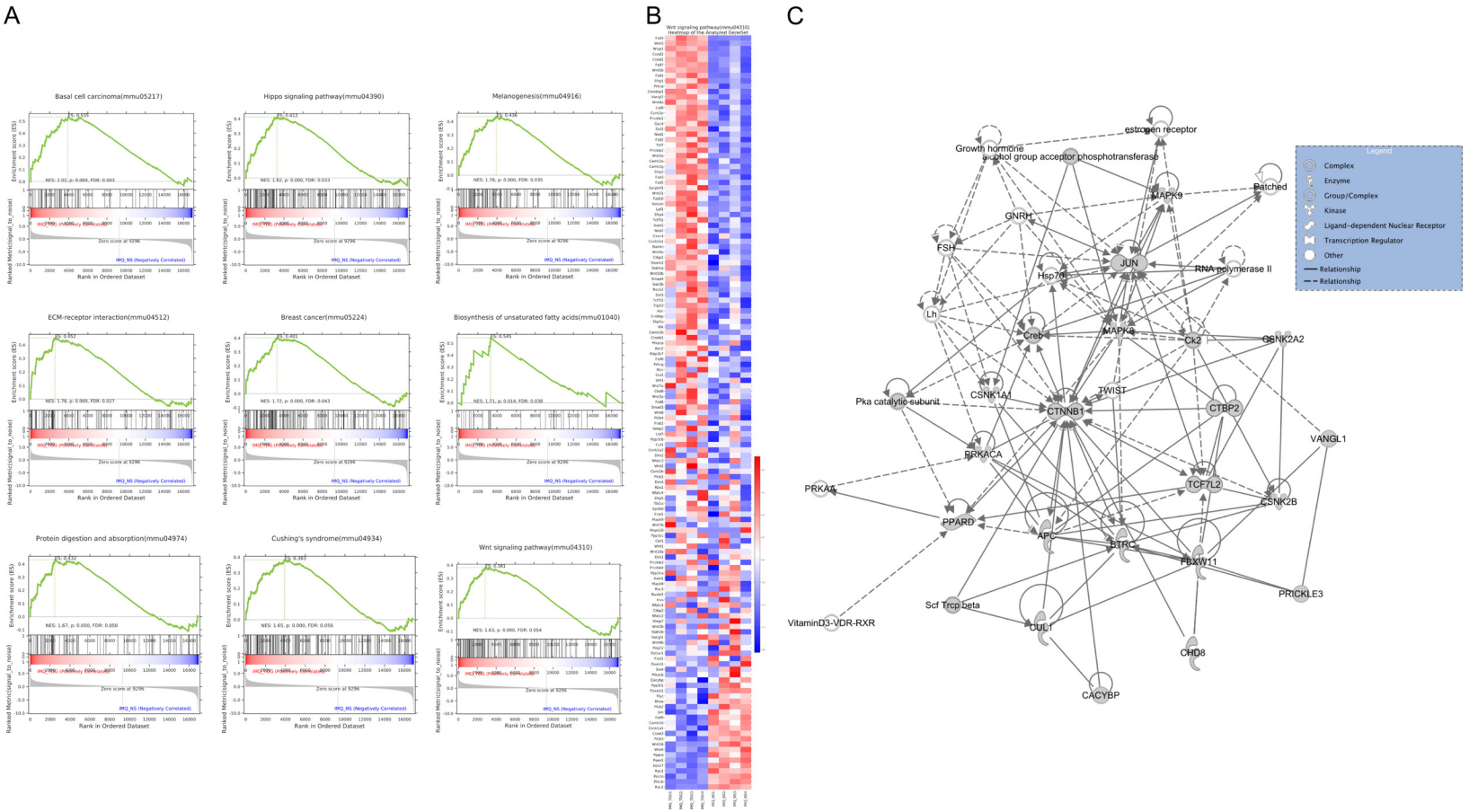

Figure 3. Gene Set Enrichment Analysis (GSEA) analysis and Ingenuity Pathway Analysis (IPA) analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) items. A. GSEA of Taodan granules (TDGs) treated compared with disease group. B. Heatmap of corresponding genes in the Wnt signaling pathway. C. IPA network analysis of corresponding genes in the Wnt signaling pathway.

lap *p*-value = 3.13E-08), were of great importance in the upstream of TDGs for psoriasis via Wnt signaling pathway, thus down-regulating RAC2 and WNT16 expressions (**Figure 4A**).

For verifying whether the six transcription regulators upstream had significant corresponding functions in the TDG treatment for psoriasis, we assessed mRNA expressions in the back skin lesions of IMQ-induced psoriatic models mice. Compared with normal skin, the *Ezh2*, *Ctnnb1* and *Wdr5* mRNA expressions of psoriatic back lesions were specifically elevated, while *Tp63* expressions were reduced. Following TDGs, the decline in mRNA expressions of *Ezh2*, *Ctnnb1* together with *Wdr5*, and the rise in mRNA expressions of *Tp63* emerged (**Figure 4B**). Besides we verified TDGs further down-regulated the mRNA expressions of *Rac2* and *Wnt5a* (a classical non-canonical Wnt ligand) in downstream Wnt signaling pathway (**Figure 4C**). Other than that, the protein levels of these genes were consistent with the trend of mRNA expressions (**Figure 4D**).

Discussion

We applied the LC-MS/MS analysis for quality control of TDGs. To further explore the potential mechanism of TDG treatment for psoriasis, transcription profiling was applied for identifying DEGs with TDGs, proceeding with KEGG and GO analysis. Further analysis of GSEA found that the Wnt signaling pathway was the core process in TDGs treated psoriasis, and IPA core network was established for upstream gene analysis. Combining experimental verification, results showed TDGs acted on upstream genes (down-regulating levels of EZH2, CTNNB1, and WDR5; up-regulating TP63 levels) of the Wnt signaling pathway, and down-regulated RAC2 and WNT5A of the Wnt signaling pathway in the psoriatic treatment.

A novel and significant finding of the present study was that the treatment mechanisms of TDGs might be closely relative with the down-regulation of Wnt signaling pathway. Previous studies have confirmed Wnt signaling pathway, a crucial pathway of proliferative signal transduction, plays a negative regulatory role in psoriasis. β-catenin, an important transcription factor of Wnt signaling pathway, is translocated in the skin granular cell layer in psoriasis [20]. WNT5A is a classical non-canonical Wnt ligand, proved to be highly expressed in psoriatic lesions, positively correlated with the severity of psoriasis, and as one of the immunohistochemical predictors of the severity of the disease [21, 22]. Inhibition of the β-catenin encoding gene CTNNB1 in HaCaT cells leads to the decrease of downstream factor expression, thereby inhibiting IL-22-induced cell proliferation [23]. After stimulating HaCaT cells, IWP-2 (Wnt inhibitor) can inhibit cell proliferation and secretion of pro-inflammatory factors, promoting cell differentiation [24]. Our experimental validation also confirmed the protein and mRNA levels of CTNNB1 and WNT5A in psoriatic lesions were down-regulated following TDG intervention.

In addition, this study used the construction of the IPA core network to clarify the upstream genes of TDG action for down-regulation of Wnt signaling pathway to achieve a therapeutic effect. For exploring the upstream regulation of Wnt signaling pathway following TDGs, we applied the upstream analysis of IPA to forecast upstream transcription factors, and carried out experimental verification. It turned out that critical transcription factors with prominent enrichment included EZH2, CTNNB1 and WDR5, which were remarkably down-regulated by TDGs. The methyltransferase EZH2 as a valid target for psoriasis therapy, consistent with our results, overexpresses in skin lesions of psoriatic mouse models and HaCaT cells. *In vivo*, GSK126, the inhibitor of EZH2, can ameliorate the IMQ-induced psoriatic lesions. EZH2 involved in the development of psoriasis by impairing miR-125a-5p inhibition of SFMBT1 and leading to inhibition of the TGFbeta/SMAD pathway [25-27]. The WD40 protein family member WDR5 in the Wnt signaling pathway has several functions on tumorigenesis and development of multiple organ tumors. Overexpression of WDR5 has been demonstrated to associate with poor prognosis in patients with esophageal squamous cell carcinoma (ESCC), while WDR5 may act as a potential novel prognostic biomarker for ESCC [28]. Knockdown of WDR5 in NHEKs (human epidermal keratinocytes) resulted in a significant decrease in proliferation [29]. However, the impact on psoriasis of WDR5 has not been elucidated. At the same time, the TP63 levels were significantly increased following TDGs by verification. TP63 can act as a transcription factor, activating or

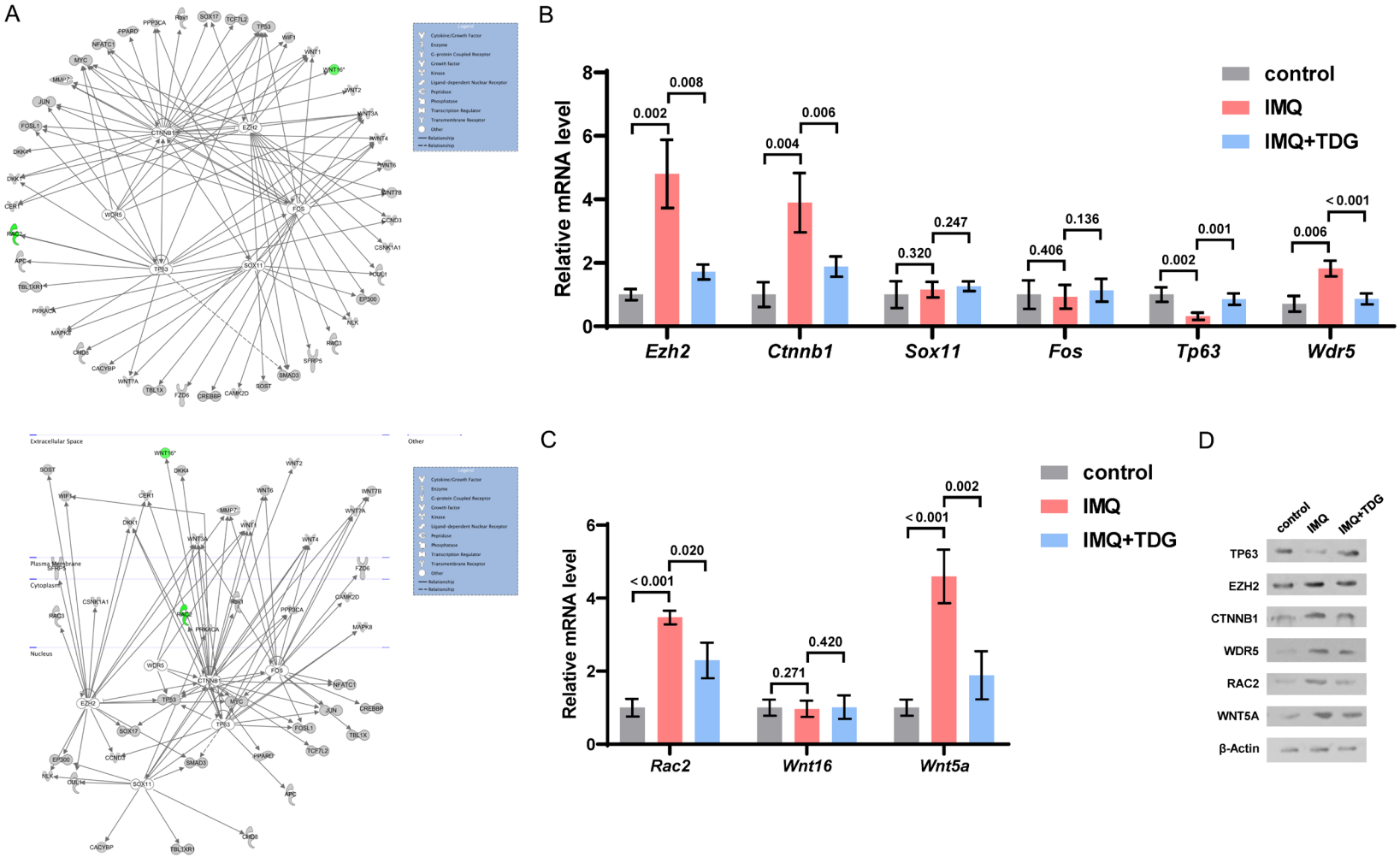

Figure 4. Experiment validation of Ingenuity Pathway Analysis (IPA) upstream analysis in Wnt signaling pathway following Taodan granules (TDGs). A. IPA upstream analysis in Wnt signaling pathway following TDGs. B-D. Experiment validation on IMQ-induced psoriatic mouse models of significant upstream regulators and genes in Wnt signaling pathway following TDGs. *p*-value as digitals was shown (n = 4).

repressing expression from a variety of gene promoters; it is believed to be crucial for normal development of ectodermal derived structures such as skin and oral mucosa. The down-regulation of *TP63* mRNA in psoriatic lesions was compared to both clinically normal skin from patients and matched healthy controls [30]. Gao et al. reported the expression of TP63 in psoriatic lesions was increased after treatment, which was consistent with our verification results [31]. In summary, we predicted and verified that TDGs alleviated psoriasis via regulating upstream transcription factors in the Wnt signaling pathway. On the other hand, IPA results also suggested TDGs down-regulated the expression of RAC2 in Wnt signaling pathway by regulating the above upstream genes. In our previous study, RAC2 has been confirmed to be highly expressed in the skin lesions of psoriatic mice, while significantly reduced following TDG intervention [12]. Notably, *Sox11* and *Fos* of the significant upstream transcription factors, as well as *Wnt16* of Wnt signal pathway enriched by IPA analysis revealed no obvious difference in mRNA levels in psoriatic lesions, normal skin, and lesions with TDG treatment. Although differences were not statistically significant, upstream subtle changes might lead to mutative expressions of downstream pathways.

To sum up, our data provides evidence that TDGs may improve psoriasis by acting on upstream genes (down-regulating levels of EZH2, CTNNB1, and WDR5; up-regulating TP63 levels) of the Wnt signaling pathway, thus down-regulating RAC2 and WNT5A in the Wnt signaling pathway. Further, the role of immune function in psoriasis has helped to manage this complex disease. The epidermal immune environment proved to be more significant in the dermis and coincided with the inflammation occurring during psoriasis. The epidermal immune microenvironment plays a dominant role in psoriasis [32]. To further find the possible mechanism by which TDGs may influence the functions of immune cells of psoriasis is a suitable application.

Conclusion

The present study proved that TDGs improved psoriasis by regulating the upstream genes (down-regulating levels of EZH2, CTNNB1, and WDR5; up-regulating TP63 levels) of Wnt signaling pathway, thus down-regulating RAC2 and WNT5A in Wnt signaling pathway. In the future, functional experiments, starting with immunization, as well as the phenotypes and mechanisms of the acquired monomers of TDGs in the treatment of psoriasis will be investigated.

Acknowledgements

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Disclosure of conflict of interest

None.

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Table S1. Prescription of Taodan granules (TDGs)

Botanical plant	English translation	Local name	Amount (g)	Lot No.	Commercial source
Salvia miltiorrhiza Bunge	Danshen Root	Danshen	30	2004201-1	Shanghai Wanshicheng National Pharmaceutical Products Co., LTD
Curcuma aeruginosa Roxb.	Zedoary	Ezhu	30	200309	Shanghai Hongqiao Traditional Chinese Medicine Decoction pieces Co., LTD
Astragalus mongholicus Bunge	Root	Huangqi	15	20200519-1	Shanghai Wanshicheng National Pharmaceutical Products Co., LTD
Glycyrrhiza inflata Batalin	Liquoric Root	Gancao	10	200426	Shanghai Hongqiao Traditional Chinese Medicine Decoction pieces Co., LTD
Angelica sinensis (Oliv.) Diels	Chinese Angelica	Danggui	15	2020011002	Shanghai Huapu Chinese Medicine Decoction pieces Co., LTD
Ligusticum striatum DC. (also named as *Conioselinum anthriscoides "Chuanxiong"*)	Szechuan Lovage Rhizome	Chuanxiong	10	200409	Shanghai Kangqiao Pharmaceutical Co., LTD
Prunus persica (L.) Batsch	Peach Seed	Taoren	10	2020021008	Shanghai Huapu Chinese Medicine Decoction pieces Co., LTD
Cyathula officinalis K.C.Kuan	Medicinal Cyathula Root	Chuanniuxi	15	200226	Shanghai Qingpu Traditional Chinese Medicine Yinpian Co., LTD
Smilax china L.	Chinaroot Greenbier Rhizome	Baqia	30	2020051201	Shangyao Yutiancheng (Shanghai) Pharmaceutical Co., LTD

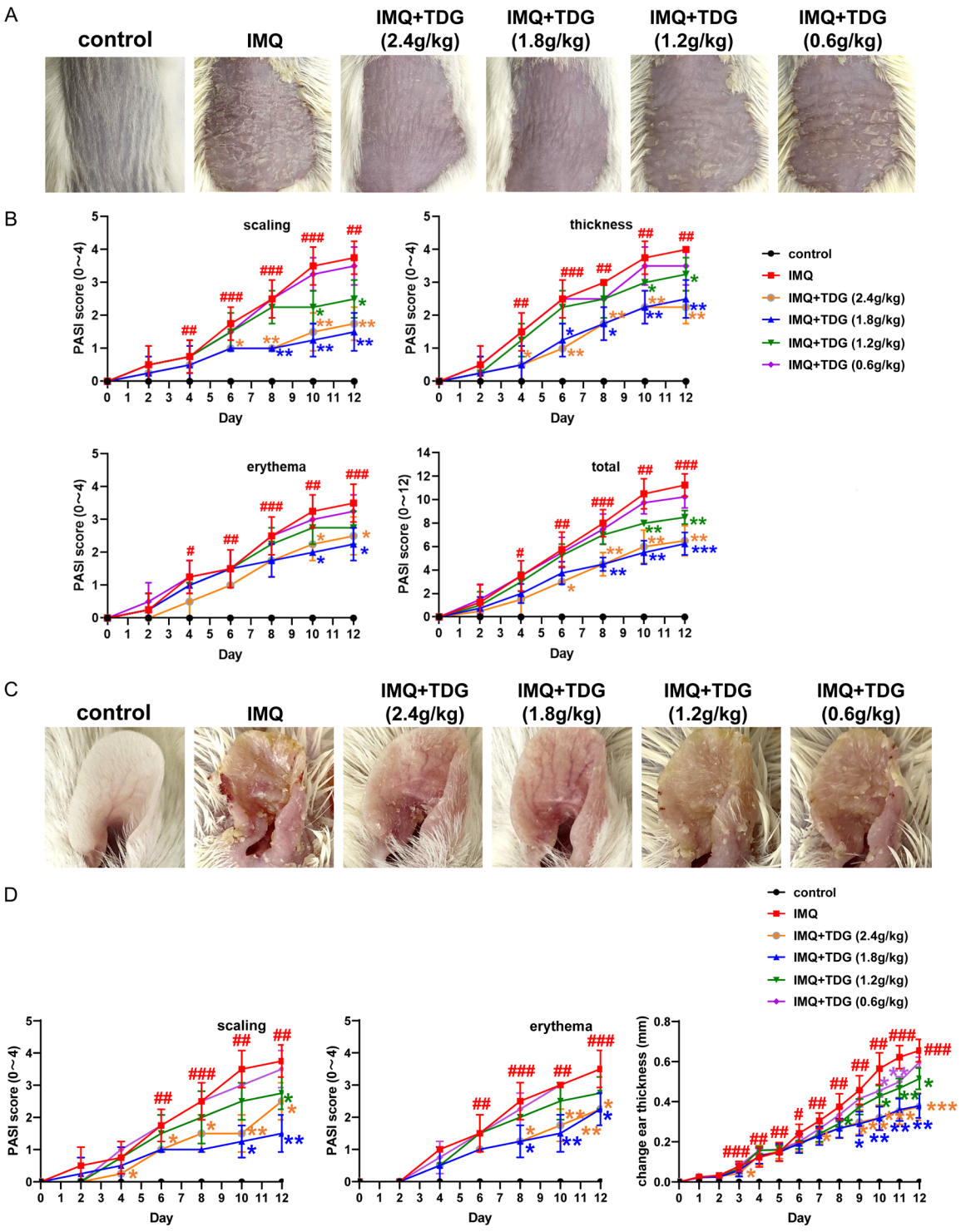


Figure S1. Comparison of different doses for Taodan granules (TDGs) alleviated the lesions in the imiquimod (IMQ)-induced psoriasis-like mice. A. The appearance of back lesions in each group on day 12. B. The psoriasis area severity index (PASI) score (0-4) with scales, thickness, erythema, and a total score. C. The appearance of ear lesions in each group on day 12. D. The PASI score (0-4) with scales, thickness, and erythema. The data are expressed as mean ± SD. Four skin lesions in each group were included for analysis. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with the control group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the IMQ group ($n = 4$).

Table S2. Primer sequences for RT-PCR

GENE NAME	F	R
Ezh2	GACATCGAAGGCAGTGGAGT	TTTTACACGCTTCCGCCAAC
Ctnnb1	CGCCGCTTATAAATCGCTCC	TTCACAGGACACGAGCTGAC
Sox11	GCCTTCATGGTGTGGTCCAA	GGGTCCGTCTTGGGCTTTTTG
Fos	TACTACCATTCCCCAGCCGA	GCTGTCACCGTGGGGATAAA
Tp63	GCTGAAAGGAAGAAACGCCC	GGGGTTTCTATGAAACGCTGG
Wdr5	CAGATCGTCTGCTCTCGC	TTAACTGGTGTGGGCTTGC
Rac2	CACGTCCTTCTCCCAACACA	AATGTCGGGGGAGCCATTTT
Wnt16	ATGTCCAGTACGGCATGTGG	CCAGCAGGTTTTCACAGCAC
Wnt5a	CTGACAATCAGGAGGCGTGA	GGGCGTGATTGTGCAAAAGA
β-actin	GAGCGCAAGTACTCTGTGTG	GGTGTAAAACGCAGCTCAGTAA

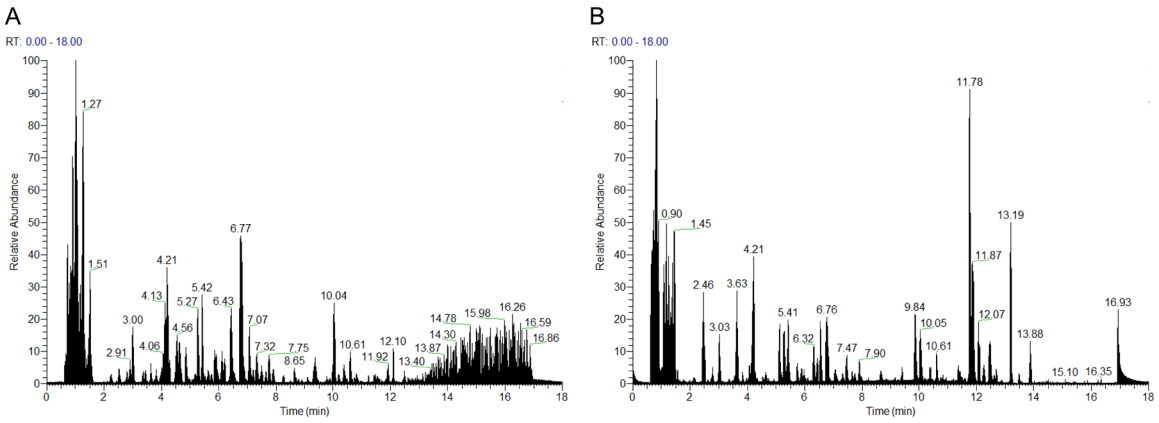
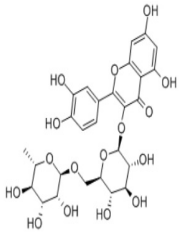
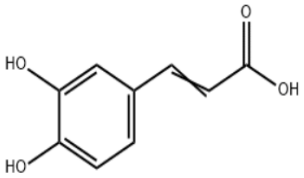
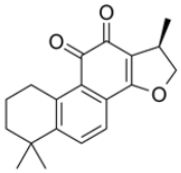
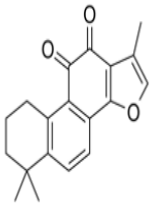
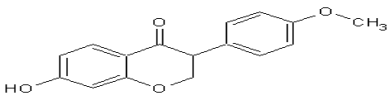
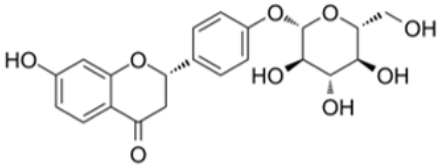
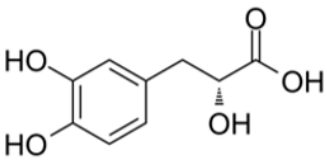


Figure S2. Total ion flow chromatogram of the Taodan granules (TDGs) analyzed by Liquid Chromatograph Mass Spectrometer/Mass Spectrometer (LC-MS/MS). A. Negative polarity. B. Positive polarity.

Table S3. Structure of the major components in Taodan granules (TDGs)

Compound	Molecular formula	Content of TDGs (ug/ml)
Rutin		0.68
Caffeic acid		14.92
Cryptotanshinone		3.56
Tanshinone IIA		2.77
Formononetin		2.95
Liquiritin		95.87
Danshensu		82.11

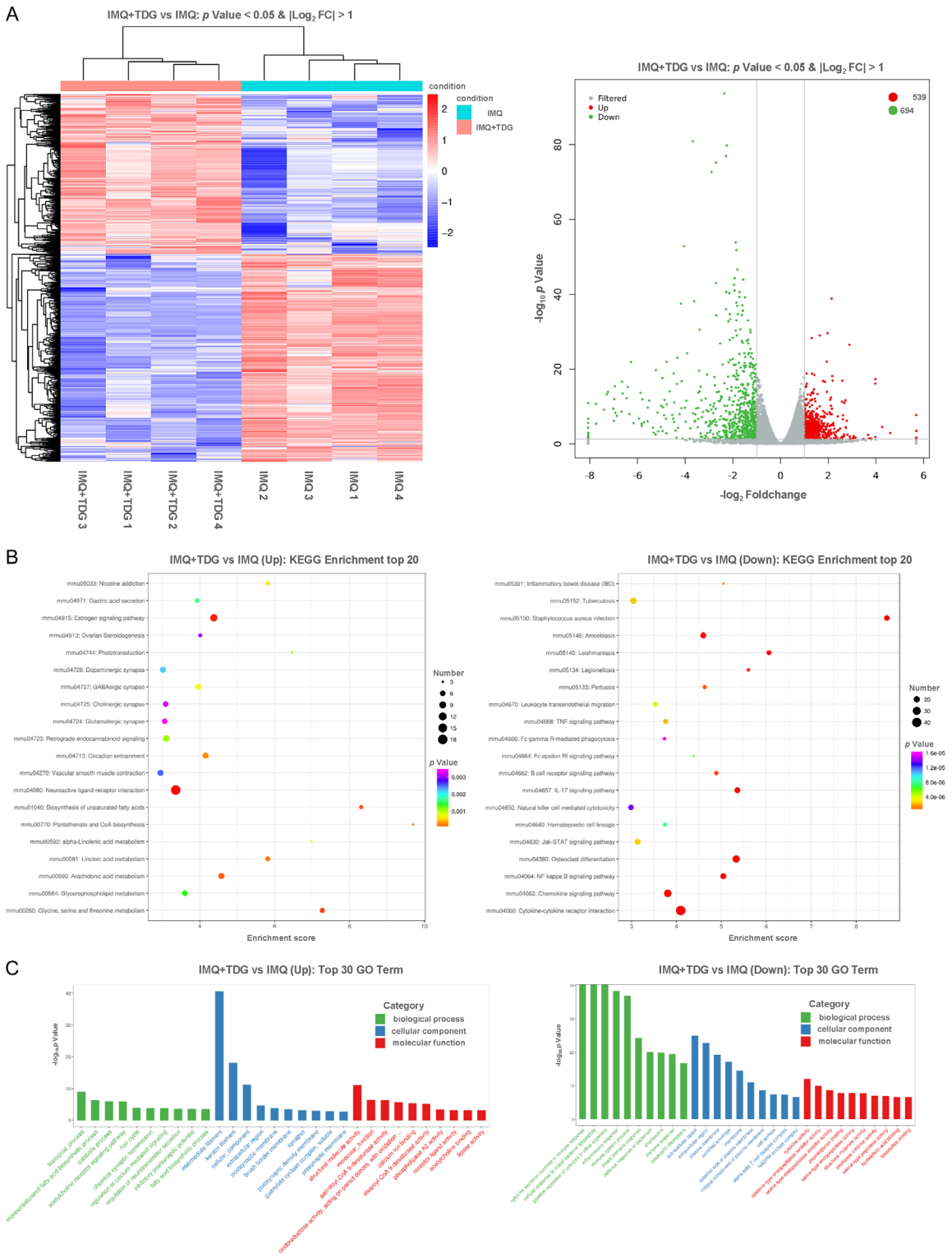


Figure S3. Differentially expressed genes (DEGs) induced following Taodan granules (TDGs). A. Cluster analysis of DEGs among samples and groups. The color of the heat map indicates the relative gene expression. The deeper red color indicates the higher gene expression, whereas the deeper blue color indicates the lower gene expression (Left). The volcano map suggests the overall DEGs in the IMQ + TDG group, compared with the IMQ group (Right). B. Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of up-(Left) and down-(Right) regulated DEGs. C. Enriched gene ontology analysis of up-(Left) and down-(Right) regulated DEGs.

Table S4. Top 20 up-regulated and down-regulated genes in the psoriatic skin of the mice models with or without Taodan granules (TDGs) (Genes with |log2FoldChange| > 1 and *p*-value < 0.05)

Gene ID	Gene Name	Gene Definition	LOG_2|FC|	*p*-value	*q*-value
Top 20 upregulated genes					
66107	Wfdc21	WAP four-disulfide core domain 21	2.141861603	1.34E-39	1.15E-36
432839	Gprin2	G protein regulated inducer of neurite outgrowth 2	1.969102658	2.22E-30	9.29E-28
108151	Sema3d	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	1.643954426	8.96E-30	3.66E-27
16008	Igfbp2	insulin-like growth factor binding protein 2	1.311271087	4.79E-29	1.83E-26
406220	Krt77	keratin 77	2.889566113	2.87E-27	9.37E-25
230613	Skint10	selection and upkeep of intraepithelial T-cells 10	1.940699804	1.00E-22	2.61E-20
14622	Gjb5	gap junction protein, beta 5	1.0953902	1.35E-19	2.34E-17
79362	Bhlhe41	basic helix-loop-helix family, member e41	1.320164851	1.48E-19	2.54E-17
73442	Hspa12a	heat shock protein 12A	1.420015579	3.70E-19	6.16E-17
66203	Lce1m	late cornified envelope 1M	2.169646121	6.75E-19	1.07E-16
435350	Serpinb6e	serine (or cysteine) peptidase inhibitor, clade B, member 6e	3.976679949	4.75E-18	6.83E-16
69117	Adh6a	alcohol dehydrogenase 6A (class V)	2.187395639	1.07E-17	1.48E-15
238395	Serpina3j	serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3J	2.590357248	1.13E-17	1.56E-15
107585	Dio3	deiodinase, iodothyronine type III	1.781280765	1.45E-17	1.95E-15
11519	Add2	adducin 2 (beta)	1.861512402	2.43E-17	3.14E-15
271047	Serpina3b	serine (or cysteine) peptidase inhibitor, clade A, member 3B	3.973024928	7.68E-17	9.65E-15
12411	Cbs	cystathionine beta-synthase	1.100796697	8.20E-17	1.02E-14
68659	Fam198b	family with sequence similarity 198, member B	1.064341173	3.47E-16	3.95E-14
192166	Sardh	sarcosine dehydrogenase	1.064351251	5.48E-16	6.04E-14
18162	Npr3	natriuretic peptide receptor 3	1.39877771	2.50E-15	2.59E-13
Top 20 downregulated genes					
16409	Itgam	integrin alpha M	-2.359917295	1.99E-94	3.57E-90
170677	Cdhr1	cadherin-related family member 1	-3.677378859	1.32E-81	1.19E-77
16414	Itgb2	integrin beta 2	-2.253358932	1.70E-80	1.02E-76
19354	Rac2	RAS-related C3 botulinum substrate 2	-2.283072327	1.24E-77	5.58E-74
83382	Siglece	sialic acid binding Ig-like lectin E	-2.712092471	6.31E-76	2.27E-72
217306	Cd300e	CD300E molecule	-2.896226408	2.08E-73	6.24E-70
73656	Ms4a6c	membrane-spanning 4-domains, subfamily A, member 6C	-1.87982465	1.47E-54	3.76E-51
100042514	Sprr2a3	small proline-rich protein 2A3	-4.039888592	1.45E-53	3.25E-50
83490	Pik3ap1	phosphoinositide-3-kinase adaptor protein 1	-1.852487496	1.62E-52	3.23E-49
15163	Hcls1	hematopoietic cell specific Lyn substrate 1	-1.807265374	2.60E-47	4.68E-44
23880	Fyb	FYN binding protein	-1.927183842	5.81E-45	9.50E-42
72042	Cotl1	coactosin-like 1 (Dictyostelium)	-1.563171219	1.30E-44	1.94E-41

246256	Fcgr4	Fc receptor, IgG, low affinity IV	-2.70846219	1.04E-43	1.44E-40
18173	Slc11a1	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	-1.951701945	3.15E-43	4.05E-40
26888	Clec4a2	C-type lectin domain family 4, member a2	-1.930263987	1.02E-41	1.22E-38
65221	Slc15a3	solute carrier family 15, member 3	-2.280078606	2.26E-41	2.54E-38
14127	Fcer1g	Fc receptor, IgE, high affinity I, gamma polypeptide	-1.713529716	2.48E-41	2.62E-38
244233	Cd163l1	CD163 molecule-like 1	-2.148022202	4.31E-41	4.31E-38
20525	Slc2a1	solute carrier family 2 (facilitated glucose transporter), member 1	-1.727582584	4.31E-40	4.08E-37
11433	Acp5	acid phosphatase 5, tartrate resistant	-1.490765474	6.27E-40	5.63E-37

q-value: A Benjamini value (an adjusted *p*-value).