All-trans retinoic acid alters the expression of the tight junction proteins Claudin-1 and -4 and epidermal barrier function-associated genes in the epidermis

JING LI, QIANYING LI and SONGMEI GENG

Department of Dermatology, Northwest Hospital, Xi'an Jiaotong University, Xi'an, Shaanxi 710004, P.R. China

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Abstract. All-trans retinoic acid (ATRA) regulates skin cell proliferation and differentiation. ATRA is widely used in the treatment of skin diseases, but results in irritation, dryness and peeling, possibly due to an impaired skin barrier, although the exact mechanisms are unclear. The present study established an ATRA-associated dermatitis mouse model (n=32) in order to examine the molecular mechanisms of skin barrier impairment by ATRA. Changes in epidermal morphology and structure were observed using histological examination and transmission electron microscopy (TEM). Gene expression was analyzed by microarray chip assay. Histology and TEM demonstrated pronounced epidermal hyperproliferation and parakeratosis upon ATRA application. The stratum corneum layer displayed abnormal lipid droplets and cell-cell junctions, suggesting alterations in lipid metabolism and dysfunctional cell junctions. Gene expression profiling revealed that factors associated with epidermal barrier function were differentially expressed by ATRA, including those associated with tight junctions (TJs), cornified envelopes, lipids, proteases, protease inhibitors and transcription factors. In the mouse epidermis, Claudin-1 and -4 are proteins involved in TJs and have key roles in epidermal barrier function. ATRA reduced the expression and altered the localization of Claudin-1 in HaCaT immortalized keratinocytes and the mouse epidermis, which likely leads to the disruption of the epidermal barrier. By contrast, Claudin-4 was upregulated in HaCaT cells and the mouse epidermis following treatment with ATRA. In conclusion, ATRA exerts a dual effect on epidermal barrier genes: It downregulates the expression of Claudin-1 and upregulates the expression of Claudin-4. Claudin-4 upregulation may be a compensatory response for the disrupted barrier function caused by Claudin-1 downregulation.

Introduction

All-trans retinoic acid (ATRA) exerts essential roles in reproduction, embryogenesis, cell proliferation, differentiation and apoptosis (1,2). ATRA also regulates skin function and is widely used for the treatment of skin diseases such as acne, psoriasis, ichthyosis and skin cancer (3-5), but its clinical application is limited by adverse skin reactions, including erythema, scaling, dryness, desquamation and vessel dilation. These reactions are potentially associated with epidermal barrier dysfunction (6), but the mechanisms are largely unknown.

Tight junctions (TJs) in epithelial and endothelial tissues have been well studied, and a previous study suggested that the TJs of the stratum granulosum (SG) are responsible for the protective function of epithelial tissues (7). TJs consist of transmembrane Claudins, adherent junction (AJ) molecules, occludin, plaque proteins (e.g. zonula occludens-1, -2 and -3, and multiple PDZ domain protein), and cell polarity complex proteins [e.g. the protein kinase C ι-type/partitioning defective 3 homolog (Par3)/Par6 complex] (8). Claudins contain two extracellular loops (cytoplasmic c- and N-terminal) and four transmembrane domains (9,10). Claudin-1 and -4 are involved in the pathogenesis of skin lesions (18 -20). How Claudin-1 and -4 are regulated in response to ATRA is largely unknown.

To understand the molecular basis of ATRA-induced barrier dysfunction, a gene expression array was used to observe the differential gene expression in mouse skin and HaCaT cells following treatment with ATRA. Using a mouse model and a
gene expression array, it was demonstrated that ATRA does, in fact, alter the structure of TJ in mouse skin. Therefore, the hypothesis was that Claudins possibly exert an essential role in barrier dysfunction during ATRA-induced skin irritation. The present study aimed to investigate the molecular mechanisms of barrier dysfunction during ATRA-induced skin irritation.

Materials and methods

**Animals.** Male BALB/c mice (n=32; 8 weeks of age; weight, ~25 g) were obtained from Xi’an Jiaotong University Animal Center (Xi’an, China). The mice were fed standard chow and had access to water *ad libitum*, and were caged in a controlled environment (12 h light/dark cycle; temperature, 20-25°C; humidity, 45-55%). The mice were acclimatized for 3 days prior to any experiments. All experimental procedures were performed in accordance with the ‘Principles of Laboratory Animal Care’ (NIH) and with the approval of the laboratory animal care committee of Xi’an Jiaotong University (no. XJTULAC2017-733).

**Animal treatment.** The skin on the backs of the mice was shaved using an electric shaver. The mice were divided into two groups: i) Treated with topical 0.1% ATRA cream (Chongqing Winbond Pharmaceutical Co., Ltd., Chongqing, China) twice a day; and ii) treated with an oil/water cream (vehicle control) twice a day. The mice were divided into two groups: i) Treated with topical 0.1% ATRA cream (Chongqing Winbond Pharmaceutical Co., Ltd., Chongqing, China) twice a day; and ii) treated with an oil/water cream (vehicle control) twice a day. The mice were acclimatized for 3 days prior to any experiments.

After 5 days of treatment, the mice were anesthetized using pentobarbital at 50 mg/kg. The skin from the backs of the mice, including the dermis and subcutaneous tissues, was removed. The skin was washed with pre-cooled PBS, excess fat was removed, and the specimens were placed in liquid nitrogen for RNA extraction. Subsequently, the mice were sacrificed by cervical dislocation.

**Histological and ultrastructural analysis.** ATRA-treated and untreated mouse dorsal skin specimens (1x1.5 cm) were fixed in 4% paraformaldehyde for 24 h at 4°C, dehydrated and embedded in paraffin. The samples were sectioned at 7 µm and stained with hematoxylin for 5 min and eosin for 2 min at room temperature. The epidermal thickness, parakeratosis, spongiosis and degree of inflammation were evaluated. Spongiosis (presence of widened intracellular spaces between epidermal keratinocytes) and inflammation were evaluated on a scale of 0-4. Epidermal thickness and spongy edema were measured using the built-in measurement tool in the software ndp.view2 (Hamamatsu Photonics K.K., Hamamatsu, Japan; https://www.hamamatsu.com/jp/en/product/type/U12388-01.html; version 2.7). The expanded capillaries were counted at high magnification (x200) in 10 randomly selected fields under an Olympus BX51 light microscope equipped with a DP70 digital camera (Olympus Corporation, Tokyo, Japan). Incomplete keratinization was assessed as present or absent (22).

The specimens were cut into 1-cm blocks and dipped in ice-cold sodium cacodylate buffer solution containing 2.5% glutaraldehyde at 4°C for 24 h. The blocks were washed three times and treated with 1% osmium tetroxide at 4°C for 1 h. The samples were dehydrated using an ethanol series and embedded in Epon 812. Ultra-thin sections were stained with lead citrate for 10 min and uranyl acetate for 30 min at room temperature. Ultrastructural changes were observed by transmission electron microscopy (TEM) using a H7650 microscope (Hitachi, Ltd., Tokyo, Japan).

**Cell culture and treatment.** HaCaT cells (immortalized keratinocytes) were obtained from the Fourth Military Medical University (Shan’xi, China) and grown in RPMI-1640 (GE Healthcare, Chicago, IL, USA) with 10% fetal bovine serum (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin-streptomycin (GE Healthcare). Short tandem repeat profiling was performed by Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China) to validate the cell line. The cells were subcultured following dissociation with 0.25% trypsin/0.05% EDTA (1:1) and passaged at a ratio of 1:4 every 3 days.

HaCaT cells (5,000 cells/well) were incubated in 96-well plates for 48 h and treated by different concentrations (0.1, 0.5, 1, 5 and 10 µM) of ATRA at 37°C for a further 36 h. A volume of 20 µl MTT solution (5 mg/ml) was added to each well and the culture continued for 4 h. Subsequently, the medium was removed, and 150ul dimethyl sulfoxide (DMSO) was added to each well. The light absorption value of each well was measured at optical density 490 nm (570 nm) with a microplate reader. Following determination of the half-maximal inhibitory concentration using different concentrations of ATRA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), HaCaT cells were incubated with or without 1 µM ATRA for 36 h at 37°C, which was chosen for use in the subsequent experiments. Stock solutions of ATRA (0.01 M) were prepared in DMSO, stored in the dark at -20°C, and further diluted with RPMI-1640. The concentration of DMSO was 1%.

**Microarray analysis.** Microarray analysis was used primarily to identify candidates that were later confirmed via reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and/or immunohistochemistry. The gene expression profiles in the skin of mice and in HaCaT cells treated or untreated with ATRA were compared using NimbleGen Gene Expression analysis (Nimblegen Systems Inc., Madison, WI, USA). The ds-cDNA samples were washed and labeled according to the NimbleGen Gene Expression Analysis protocol (Nimblegen Systems Inc.). A NanoDrop ND-1000 was used to quantify the cDNA following purification. Cy3 labeling was conducted using the NimbleGen One-Color DNA labeling kit (NimbleGen Systems, Inc.), according to the manufacturer’s protocol. Subsequently, 100 U Klenow fragment (New England Biolabs, Inc., Ipswich, MA, USA) and 100 pmol deoxynucleoside triphosphates were added and incubated at 37°C for 2 h. One-tenth volume of 0.5 M EDTA was added to stop the reaction. The labeled ds-cDNA was purified using isopropanol/ethanol precipitation. The microarrays were immersed in the NimbleGen hybridization buffer/hybridization component A, which was supplemented with 4 µg
ds-cDNA with Cy3 labeling. The reaction system was kept in a hybridization chamber (Nimblegen Systems, Inc.) at 42˚C for 4 h. The NimbleGen Wash Buffer kit (Nimblegen Systems, Inc.) was used to wash the microarrays in an environment without ozone. The Axon GenePix 4000B microarray scanner was used to scan the microarrays.

**RT-qPCR.** The RNAeasy Midi kit (Qiagen AB, Sollentuna, Sweden) was used to isolate the total mRNA, according to the manufacturer's protocol. RNA (1 µg) was added in a 20-µl reaction system (PrimeScript RT Reagent Kit; Takara, Otsu, Japan) at 37˚C for 15 min and 85˚C for 5 sec for cDNA synthesis. The SYBR Premix Ex Taq II amplification kit (Perfect Real Time; Takara Biotechnology Co., Ltd., Dalian, China) was used for qPCR on a Bio-Rad IQ5 System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The conditions were: i) 95˚C denaturation for 10 min; and ii) 40 cycles at 95˚C for 10 sec and 60˚C for 30 sec. The primers are listed in Table I. β-actin was used for normalization. The results were analyzed using the IQ5 software (version 2.5). Gene expression levels were calculated using the 2-∆∆Cq method (23).

**Western blotting.** ATRA-treated and untreated HaCaT cells were lysed in radioimmunoprecipitation assay buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40 and protease inhibitor cocktail; Sigma-Aldrich; Merck KGaA] at 4˚C for 3 h. Protein concentration was measured using a bichinchonic acid protein assay kit (Beijer Institute of Biotechnology, Haimen, China). Equal amounts of proteins (30 µg) were separated on 12% SDS-PAGE gels and transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA). TBS buffer containing Tween-20 (TBST) and 5% non-fat milk was used to block non-specific binding at room temperature for 2 h. Following blocking, primary antibodies were incubated overnight at 4˚C: Rabbit anti-human Claudin-1 (cat. no. 13050-1-AP; ProteinTech Group, Inc., Chicago, IL, USA; 1:200), goat anti-human Claudin-4 (cat. no. 17664; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; 1:200), goat anti-human Claudin-4 (cat. no. 13050-1-AP; ProteinTech Group, Inc., Chicago, IL, USA; 1:200), goat anti-human Claudin-4 (cat. no. 13050-1-AP; ProteinTech Group, Inc., Chicago, IL, USA; 1:200), and mouse anti-human β-actin (cat. no. 47778; Santa Cruz Biotechnology, Inc.; 1:1,000). The membranes were washed three times with TBST for 5 min. Horseradish peroxidase (HRP)-conjugated AffiniPure Rabbit Anti-Goat IgG (H+L) (cat. no. SA00001-2; ProteinTech Group, Inc.; 1:3,000), HRP-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (cat. no. SA00001-1; ProteinTech Group, Inc.; 1:3,000), and HRP-conjugated AffiniPure Donkey Anti-Goat IgG (H+L) (cat. no. SA00001-3; ProteinTech Group, Inc.; 1:4,000) were incubated for 1 h at 37˚C prior to visualization with enhanced chemiluminescence (Amersham; GE Healthcare). Densitometry was used to quantify the signal intensities using Quantity One 4.5 software (Bio-Rad Laboratories, Inc.). All measurements were performed in triplicates from three independent experiments.

**Immunofluorescence.** HaCaT cells and cryostat sections (4 µm) of skin from ATRA-treated and untreated mice were fixed in ice-cold 4% paraformaldehyde in PBS for 30 min. Non-specific binding was blocked with 10% normal goat serum (OriGene Technologies, Inc., Beijing, China) for 20 min at 37˚C. Goat anti-human Claudin-4 (1:50) and rabbit anti-human Claudin-1 antibodies (1:50) were incubated overnight at 4˚C. PBS was used as a negative control. The sections were washed twice for 5 min in PBS. Alexa Fluor 594-labeled secondary antibodies (cat. nos. A32758 and A32740; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; 1:200) were incubated for 1 h at room temperature and revealed using DAPI (1:5,000 in PBS) for 2 min at room temperature. A laser scanning confocal microscope (LSM510; Zeiss AG, Oberkochen, Germany) was used to capture images of the cells (x200 magnification).

**Statistical analysis.** All statistical analyses were conducted using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. All measurements were performed in triplicate from three independent experiments. The distribution was tested for normality using the Kolmogorov-Smirnov test. Statistical significance was evaluated by independent sample t-test for normally distributed data and using the Mann-Whitney U test for non-normally distributed data. P<0.05 was considered to indicate a statistically significant difference.

For the microarray data, the NimbleScan software (version 2.5; NimbleGen Systems, Inc.) was used to conduct grid alignment and analyze the expression profiles. The Robust Multichip Average (RMA) algorithm and quantile normalization in NimbleScan software were applied to the normalized expression data. The gene levels were inputted into the Agilent GeneSpring software (version 12.0; Agilent Technologies, Inc., Santa Clara, CA, USA). Fold-change filtering was applied for the identification of genes with differential expression levels. The threshold was set at fold-change ≥2.0. The Agilent GeneSpring GX software (version 12.0; Agilent Technologies, Inc.) was used to conduct hierarchical clustering. The roles of the differentially expressed genes were determined using Gene Ontology (GO; www.geneontology.org) and pathway analyses (https://www.genome.jp/kegg) conducted on the basis of the standard enrichment computation method, and using Fisher's exact test to determine whether the amount of overlaps between the GO annotation list and the list of differentially

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**Table I. Primers for reverse transcription-quantitative polymerase chain reaction.**

| Gene symbol | Primers |
|-------------|---------|
| β-actin     | F-5’-AGCAGAGAATGGAAGACTAAA-3’ |
|             | R-5’-ATGCTGCTATCATGTTCGAT-3’ |
| CLDN4       | F-5’-TATGGGATGACCTGCTGTTG-3’ |
|             | R-5’-GATGATGTVGTAGTACGAG-3’ |
| CLDN1       | F-5’-GAAGTGTGATAGAAGTGCTTGG-3’ |
|             | R-5’-GGTCTAGGGTCATAGAAT-3’ |
| CLDN2       | F-5’-GTGAAGCGAGAGTAGAGAGG-3’ |
|             | R-5’-ATGGATTTGCGTCTTTTGG-3’ |
| FLG         | F-5’-AGACTGGAGGCAAGCTACAAC-3’ |
|             | R-5’-TGGTTTTGGAGGATTGGAT-3’ |

F, forward; R, reverse; CLDN, Claudin; FLG, filaggrin.
expressed genes was significant. $P \leq 0.05$ indicated that the GO term enrichment was statistically significant.

**Results**

*Topical ATRA induces histological alterations in the skin of treated mice.* After 5 days of treatment, no marked alterations were detected in the control skin. Circumscribed erythema occurred after 3 days of ATRA application and gradually expanded, presenting as fine flat scales covering the surface of the erythema and peaking at 5 days (Fig. 1). Hematoxylin and eosin staining of ATRA-treated skin demonstrated crust, focal parakeratosis, hyperproliferation and intercellular edema of the stratum spinosum (Fig. 2A). In the ATRA-treated dermis, large amounts of inflammatory cell infiltration and capillary dilation were observed (Fig. 2A). Epidermal thickness was 2.5 times higher following ATRA intervention compared with that of the control group ($P < 0.0001$), in addition to increases in parakeratosis ($P < 0.001$) and spongiosis ($P < 0.002$). In the dermis, perivascular lymphocytic infiltration ($P < 0.002$) and...
telangiectasia (P<0.002) were observed in the dermal papilla and in the superficial layer (Fig. 2B).

**ATRA treatment causes ultrastructural abnormalities in the epidermis.** Compared with control skin, keratohyalin granules were decreased in number in the SG (n=3; Fig. 3A). TEM also demonstrated that the keratinocyte cytoskeleton was damaged by ATRA and that the cytoskeletal network disappeared in the local upper stratum corneum (Fig. 3A). In the upper stratum corneum, multiple lipid droplets were observed in the cytoplasm of corneocytes in the ATRA-treated skin (Fig. 3B). In the epidermis of ATRA-treated skin, TEM revealed a disordered arrangement of stratum spinosum cells, in addition to the appearance of significantly larger nucleoli and wider intercellular spaces (Fig. 3B). Desmosomes were decreased and disordered in ATRA-treated skin (Fig. 3C). Quantitative analysis of keratohyalin granules, lipid droplets and desmosomes is presented in Fig. 3D.

**Gene expression profiling reveals dysregulation of epidermal barrier-associated genes in the mouse epidermis and HaCaT cells treated with ATRA.** With the aim of improving the
understanding of the molecular mechanisms of ATRA-induced barrier dysfunction, gene expression array analysis (n=1) was used to identify candidate genes for barrier function in the mouse skin and HaCaT cells. There were 897 upregulated and 1,087 downregulated genes following treatment with ATRA in the mouse epidermis. Similarly, there were 1,220 upregulated and 905 downregulated genes following treatment with ATRA in HaCaT cells. The genes involved in epidermal barrier function were revealed by gene expression analyses and are presented in Tables II-V. The downregulated epidermal barrier-associated genes following treatment with ATRA in the mouse skin included genes involved in ‘cornified envelope components’, ‘intermediate filament’, ‘lipid metabolic process’, ‘gap junction’, ‘tight junction’ and ‘desmosome’ (Table II). The upregulated epidermal barrier-associated genes following treatment with ATRA in mouse skin included genes involved
Table II. Downregulation of epidermal barrier-associated genes following treatment with all-\textit{trans} retinoic acid in mouse skin (n=1 per group; fold change >2; \(P<0.05\)).

### A. Cornified envelope components

| GenBank accession no. | Gene name | Gene full name             | Fold downregulation |
|-----------------------|-----------|----------------------------|---------------------|
| AF510860              | FLG       | Filagrin                   | 2.13                |
| BC108980              | FOXN1     | Forkhead box N1            | 2.42                |
| BC107019              | SPRR4     | Small proline-rich protein 4 | 2.67               |
| BC109181              | LCE1M     | Late cornified envelope 1M | 6.64                |
| BC031486              | PPHLN1    | Periphilin 1               | 2.40                |
| BC119192              | GPRC5D    | Protein-coupled receptor, class C, group 5, member D | 20.66               |

### B. Intermediate filament

| GenBank accession no. | Gene name | Gene full name             | Fold downregulation |
|-----------------------|-----------|----------------------------|---------------------|
| BC003472              | KRT23     | Keratin 23                 | 2.67                |
| BC129847              | KRT24     | Keratin 24                 | 2.66                |
| BC018391              | KRT25     | Keratin 25                 | 6.56                |
| BC116672              | KRT26     | Keratin 26                 | 7.78                |
| AB288231              | KRT28     | Keratin 28                 | 4.28                |
| BC117553              | KRT32     | Keratin 32                 | 6.04                |
| BC12542               | KRT34     | Keratin 34                 | 101.08              |
| BC100542              | KRT35     | Keratin 35                 | 6.86                |
| BC119521              | KRT40     | Keratin 40                 | 10.11               |
| BC125346              | KRT71     | Keratin 71                 | 2.90                |
| BC067067              | KRT73     | Keratin 73                 | 4.34                |
| BC107395              | KRT77     | Keratin 77                 | 9.12                |
| BC119366              | KRT80     | Keratin 80                 | 2.21                |
| AF312018              | KRT81     | Keratin 81                 | 30.65               |
| BC10897               | KRT82     | Keratin 82                 | 10.18               |
| BC152922              | KRT85     | Keratin 85                 | 19.46               |
| BC09257               | KRT33A    | Keratin associated protein 33A | 39.26          |
| NM_013570             | KRT33B    | Keratin associated protein 33B | 4.79            |
| NM_001085526          | KRTAP1-2  | Keratin associated protein 1-3 | 13.42          |
| XM_894811             | KRTAP3-1  | Keratin associated protein 3-1 | 29.03          |
| BC156698              | KRTAP4-2  | Keratin associated protein 4-2 | 19.09          |
| NM_026834             | KRTAP4-6  | Keratin associated protein 4-6 | 24.72          |
| BC115508              | KRTAP4-7  | Keratin associated protein 4-7 | 33.02          |
| NM_001085547          | KRTAP4-8  | Keratin associated protein 4-8 | 43.14          |
| NM_001085548          | KRTAP4-9  | Keratin associated protein 4-9 | 24.62          |
| BC016249              | KRTAP4-16 | Keratin associated protein 4-16 | 42.20         |
| NM_015809             | KRTAP5-4  | Keratin associated protein 5-4 | 19.54          |
| NM_027771             | KRTAP7-1  | Keratin associated protein 7-1 | 137.50         |
| AK133727              | KRTAP8-1  | Keratin associated protein 8-1 | 18.92          |
| BC116210              | KRTAP9-1  | Keratin associated protein 9-1 | 11.56          |
| BC156686              | KRTAP9-3  | Keratin associated protein 9-3 | 35.46          |
| NM_001085527          | KRTAP9-5  | Keratin associated protein 9-5 | 12.10          |
| BC116219              | KRTAP15   | Keratin associated protein 15 | 60.31          |
| BC116200              | KRTAP19-4 | Keratin associated protein 19-4 | 10.75          |
| BC115545              | KRTAP19-3 | Keratin associated protein 19-3 | 8.54           |
| BC132612              | KRTAP6-5  | Keratin associated protein 6-5 | 14.96          |
| BC132658              | KRTAP16-3 | Keratin associated protein 16-3 | 39.91          |
| BC128283              | KRTAP26-1 | Keratin associated protein 26-1 | 6.51           |
in ‘cornified envelope components’, ‘serine protease and protease inhibitors’, ‘lipid metabolic process’, ‘tight junction’ and ‘transcriptional factors’ (Table III). The downregulated epidermal barrier-associated genes following treatment with ATRA in HaCaT cells included genes involved in ‘intermediate filament’, ‘proteases and protease inhibitors’ and ‘tight junction’ (Table IV). The upregulated epidermal barrier-associated genes following treatment with ATRA in HaCaT cells included genes involved in ‘intermediate filament’, ‘proteases and protease inhibitors’ and ‘tight junction’ (Table V).

The pathway analyses demonstrated that differentially expressed genes were significantly associated with AJs, TJs and focal adhesion in ATRA-treated epidermal tissues. The top ten pathways of up- and downregulated differentially expressed genes are presented in Fig. 4A and B, respectively. The top ten three GO-fold enrichment of differentially expressed genes revealed significant downregulation of epidermal barrier function-associated processes, including ‘regulation of keratinocyte differentiation’, ‘keratin filament’ and ‘gap junction’ channel activity (Fig. 4C-H). In ATRA-treated epidermal tissues, a series of dysregulated genes associated with the epidermal differentiation complex (EDC) were observed, which included small proline-rich region proteins (SPRRs), filaggrin (FLG), loricrin (LOR) and the S100 gene family. These proteins are essential for the formation of the cell envelope during terminal differentiation. A number of these genes were members of
the SPRR family: SPRR4 (-2.67-fold), SPRR2J (+2.38-fold) and SPRR2G (+3.57-fold). The remainder were part of the S100 gene family: S100A8 (+3.31-fold), S100A9 (+3.81-fold) and FLG (-2.13-fold) (Tables II and III). During keratinocyte differentiation, the late cornified envelope protein (LCE) was induced and had similar functions to the functions of the SPRR family (cross-linking proteins) in the CE. LCE1M (6.64-fold) was downregulated, while LCE3B (3.17-fold) and LCE3A (2.74-fold) were upregulated (Tables II and III). Nevertheless, no notable abnormalities were observed in the above genes in HacaT cells. In ATRA-treated epidermal tissues and HacaT cells, marked dysregulation of numerous keratins and keratin-associated proteins was observed, suggesting abnormal terminal differentiation of keratinocytes upon ATRA treatment. Serine proteases and serine protease inhibitors are involved in the differentiation of keratinocytes.

Table III. Upregulation of epidermal barrier-associated genes following treatment with all-trans retinoic acid in the mouse skin (n=1 per group; -fold change >2; P<0.05).

| GenBank accession no. | Gene name | Gene full name | Fold upregulation |
|-----------------------|-----------|----------------|------------------|
| A, Cornified envelope components |
| BC115788 | LCE3B | Late cornified envelope 3B | 3.17 |
| BC119239 | LCE3C | Late cornified envelope 3C | 5.35 |
| BC125542 | SPRR2J | Small proline-rich protein 2J | 2.38 |
| BC130233 | SPRR2G | Small proline-rich protein 2G | 3.57 |
| BC078629 | S100A8 | S100 calcium binding protein A8 (calgranulin A) | 3.31 |
| AK143826 | S100A9 | S100 calcium binding protein A9 (calgranulin B) | 3.81 |
| B, Serine protease and protease inhibitors |
| BC031119 | KLK6 | Kallikrein related-peptidase 6 | 3.22 |
| BC002100 | KLK10 | Kallikrein related-peptidase 10 | 2.90 |
| XM_893506 | KLK12 | Kallikrein related-peptidase 12 | 2.50 |
| BC054091 | SERPINE1 | Serine (cysteine) proteinase inhibitor, clade E, member 1 | 2.15 |
| BC010675 | SERPINE2 | Serine (cysteine) proteinase inhibitor, clade E, member 2 | 2.98 |
| C, Lipid metabolic process |
| BC010829 | ACNAT2 | Acyl-coenzyme A amino acid N-acyltransferase 2 | 2.03 |
| DQ469311 | ACNAT1 | Acyl-coenzyme A amino acid N-acyltransferase 1 | 2.94 |
| AK171255 | ACOT11 | Acyl-CoA thioesterase 11 | 3.73 |
| BC050828 | UGCG | UDP-glucose ceramide glucosyltransferase | 2.21 |
| BC060600 | PLA2G4E | Phospholipase A2, group IVE | 2.39 |
| BC003470 | PLA1A | Phospholipase A1 member A | 2.06 |
| BC003943 | DPAGT1 | Dolichyl-phosphate (UDP-N-acetylgalcosamine) glycerol | 2.10 |
| D, Tight junction |
| BC015252 | CLDN2 | Claudin-2 | 2.43 |
| E, Transcriptional factors |
| BC070398 | PPARδ | Peroxisome proliferator activator receptor δ | 2.00 |
and desquamation (24). In ATRA-treated epidermal tissues and HaCaT cells, the differential expression of serine proteases and serine protease inhibitors was observed. Among them, it was observed that KLK6 and KLK10 were consistently expressed in the mouse chip and HaCaT cells, and all of them were upregulated.

Genes encoding enzymes associated with lipid metabolism were demonstrated to be induced by ATRA in the mouse epidermis. Compared with the cells, the mice exhibited significant downregulation of a subset of enzymes associated with ceramide, free fatty acids and phospholipid synthesis: i) Sphingomyelin phosphodiesterase 3 (SMPD3; -2.15-fold) and sphingomyelin phosphodiesterase 2 (SMPD2; -2.10-folds), which catalyze the conversion of sphingolipids to ceramides; ii) epidermal ceramide synthase 5 (LASS5/Cers5; -2.82-fold), which catalyzes the synthesis of ceramides; iii) lysophospholipase II (LYPLA; -2.00-fold), phospholipase A2 group IIE2 (PLA2G2E; -2.60-fold), and phospholipase C eta 2 (PLCH2; -2.13-fold), which catalyze free fatty acid formation from phospholipids; and iv) 1-acylglycerol-3-phosphate O-acyltransferase 4 (AGPAT4; -3.81-fold) and 1-acylglycerol-3-phosphate O-acyltransferase 5 (AGPAT5; -3.17-fold), which catalyze the acylation of lysosphatidic acid into phosphatidic acid. The latter is the primary precursor of all types of glycerolipids. Furthermore, arachidonate lipooxygenase (ALOX12E; a member of the epidermis-type LOX family that catalyzes the conversion of polyunsaturated fatty acids into oxygenated products) was downregulated by ATRA.

The dysregulation of numerous cell-cell junction-associated genes was observed in ATRA-treated mouse skin. Among these genes were components of TJs (TJAP1, MPP7, MARVELD2 and CLDN2), gap junctions (GJA, GJA and GJB4) and desmosomes (DSG2). Compared with the mouse skin specimens, only the TJ protein molecules Claudin-4 and occludin were upregulated in the cells.

**Table IV. Downregulation of epidermal barrier-associated genes following treatment with all-trans retinoic acid in HaCaT cells (n=1 per group; -fold change >2; P<0.05).**

**A. Intermediate filament**

| GenBank accession no. | Gene name | Gene full name               | Fold downregulation |
|-----------------------|-----------|------------------------------|---------------------|
| NM_181619             | KRTAP21-1 | Keratin associated protein 21-1 | 2.38                |
| NM_033448             | KRT71     | Keratin 71                   | 2.00                |
| BC024292              | KRT5      | Keratin 5                    | 2.22                |

**B. Proteases and protease inhibitors**

| GenBank accession no. | Gene name | Gene full name | Fold downregulation |
|-----------------------|-----------|----------------|---------------------|
| BC069417              | SERPINB7  | Serpin family B member 7 | 3.15          |
| NM_000185             | SERPIND1  | Serpin family D member 1   | 2.36          |
| NM_007173             | PRSS23    | Serine protease 23         | 4.13          |
| NM_022046             | KLK14     | Kallikrein related peptidase 10 | 2.29          |

**C. Tight junction**

| GenBank accession no. | Gene name | Gene full name | Fold downregulation |
|-----------------------|-----------|----------------|---------------------|
| AK128686              | PDZD2     | PDZ domain containing 2 | 2.15          |
Discussion

In the present study, the ATRA-associated dermatitis animal model presented erythema, scaling and dryness of the treated skin, similar to the irritation observed in ATRA-treated human skin (25,26), indicating that this animal model is a useful tool to evaluate the effectiveness and side effects of ATRA. Furthermore, treatment with ATRA altered the normal morphology and ultrastructure of the mouse epidermis. Microarray analysis was used, primarily to identify candidates that were later confirmed via RT-qPCR and/or immunohistochemistry.

In the mouse model of ATRA-stimulated dermatitis, the epidermis of the mice exhibited obvious scales, while histopathology revealed parakeratosis of the epidermis, suggesting that the epidermal differentiation was abnormal, and that the abnormal differentiation of keratinocytes led to an epidermal keratinization envelope. In the mouse gene expression profiles, it was observed that the majority of alterations occurred among EDC genes, including FLG, SPRR4, SPRR2J, SPRR2G, LCE3C, LCE3B, LCE1M, S100A8 and S100A9. The proteins encoded by those genes, together with LOR, involucrin, trichohyalin and hornerin, are associated with CE generation via crosslinking of insoluble membranous proteins (27). Keratinized proteins act as markers of differentiation in the epidermis. In the mice and cells, the abnormal expression of keratins, including KRT15, KRT17 and KRT14, and keratin-associated proteins, including KRT33A and KRTAP1-3, was widely observed, further confirming the abnormal terminal differentiation in the epidermis following ATRA treatment.

Proteases, together with their inhibitors and targets, serve an essential role in desquamation (24). In the ATRA-treated mouse epidermis and HaCaT cells, the upregulation of tissue kallikreins (KLK6, KLK10, KLK12 and KLK14) and protease inhibitors (SERPINE 1 and SERPINE 2) was demonstrated. It is noteworthy that KLK6 and KLK10 were upregulated in mice and cells following ATRA treatment, which may be

Table V. Upregulation of epidermal barrier-associated genes following treatment with all-trans retinoic acid in HaCaT cells (n=1 per group; fold change >2; P<0.05).

| GenBank accession no. | Gene name     | Gene full name                                      | Fold upregulation |
|-----------------------|---------------|-----------------------------------------------------|-------------------|
| NM_002275             | KRT15         | Keratin 15                                          | 2.07              |
| BC072018              | KRT17         | Keratin 17                                          | 2.68              |
| AB096945              | KRTAP19-4     | Keratin associated protein 19-4                    | 2.07              |
| BC101555              | KRTAP7-1      | Keratin associated protein 7-1 (gene/pseudogene)   | 2.05              |
| NM_032524             | KRTAP4-4      | Keratin associated protein 4-4                     | 2.16              |

B, Proteases and protease inhibitors

| GenBank accession no. | Gene name     | Gene full name                                      | Fold upregulation |
|-----------------------|---------------|-----------------------------------------------------|-------------------|
| NM_001085             | SERPINA3      | Serpin family A member 3                            | 12.30             |
| NM_000624             | SERPINA5      | Serpin family A member 5                            | 2.03              |
| BC034528              | SERPINB8      | Serpin family B member 8                            | 2.11              |
| NM_000934             | SERPINF2      | Serpin family F member 2                             | 2.06              |
| NM_002575             | SERPINB2      | Serpin family B member 2                             | 2.17              |
| BC009726              | PRSS22        | Serine protease 22                                   | 4.26              |
| AF335478              | KLK3          | Kallikrein related peptidase 3                       | 2.27              |
| NM_001012964          | KLK6          | Kallikrein related peptidase 6                       | 2.95              |
| NM_002776             | KLK10         | Kallikrein related peptidase 10                      | 2.44              |
| NM_006853             | KLK11         | Kallikrein related peptidase 11                      | 2.43              |
| NM_015596             | KLK13         | Kallikrein related peptidase 13                      | 2.80              |
| NM_017509             | KLK15         | Kallikrein related peptidase 15                      | 2.02              |

C, Tight junction

| GenBank accession no. | Gene name | Gene full name | Fold upregulation |
|-----------------------|-----------|----------------|-------------------|
| BC029886              | OCLN      | Occludin       | 2.63              |
| NM_001305             | CLDN4     | Claudin-4      | 2.02              |
Figure 4. Top ten pathways of up- and downregulated genes. The bar plots indicate the top ten (A) down- and (B) upregulated enrichment score $[-\log_{10}(P\text{-value})]$ values of the significantly enriched pathways (n=1 per group). The top ten (C) down- and (D) upregulated fold enrichment $[(\text{Count}/\text{Pop. Hits})/(\text{List. Total}/\text{Pop. Total})]$ values of the significant biological processes are presented. The bar plots indicated the top ten (E) down- and (F) upregulated fold enrichment $[(\text{Count}/\text{Pop. Hits})/(\text{List. Total}/\text{Pop. Total})]$ values of the significant cellular components. The top ten (G) down- and (H) upregulated fold enrichment $[(\text{Count}/\text{Pop. Hits})/(\text{List. Total}/\text{Pop. Total})]$ values of the significant molecular functions are presented. Count, the number of differentially expressed genes associated with the listed GO ID; Pop. Hits, the number of background population genes associated with the listed GO ID; List. Total, the total number of differentially expressed genes; and Pop. Total, the total number of background population genes. GO, Gene Ontology.
Figure 5. mRNA and protein expression levels of CLDN1 and CLDN4 in immortalized keratinocyte HaCaT cells treated with ATRA. HaCaT cells were incubated with or without 1 µM ATRA for 36 h. (A) In the cells, CLDN-4, TJP3 and JGB4 were upregulated, while CLDN1 was downregulated. (B) In mice, CLDN1 and FLG were downregulated, while CLDN4 and CLDN2 were upregulated. (C) CLDN1 and CLDN4 protein expression levels were determined by western blotting. β-actin was used as a loading control. Data are presented as the mean ± standard deviation from three independent experiments performed in triplicate (n=3). *P<0.05 vs. respective vehicle group. CLDN, Claudin; ATRA, all-trans retinoic acid; TJP3, tight junction protein 3; GJB4, gap junction protein β4; FLG, filaggrin.

Figure 6. Localization and expression of CLDN1 and CLDN4 in cultured HaCaT cells treated with ATRA. The localization of CLDN4 and CLDN1 (both red) was observed at the intercellular border in a string-like pattern in HaCaT cells treated with the vehicle. In ATRA-treated cells, CLDN4 immunofluorescence at the cell-cell contact sites appeared to be more intense compared with that in the vehicle group, while CLDN1 exhibited increased punctate localization in ATRA-treated cells. DAPI was used as a counterstain (blue). Scale bar, 20 µm. CLDN, Claudin; ATRA, all-trans retinoic acid.
closely associated with the appearance of scales on the mouse skin. Furthermore, alterations in the balance between proteases and protease inhibitors in the skin lead to inflammatory reactions, which causes itching, scaling, redness and other typical clinical symptoms (28).

Morphologically, the appearance of profilaggrin is consistent with the formation of keratohyalin granules. The newly-synthesized profilaggrin accumulates in keratohyalin granules with high phosphoric acid and histidine following phosphorylation. The present study demonstrated that the number of keratohyalin granules decreased significantly following ATRA treatment. Therefore, it was speculated that decreases in the number of keratohyalin granules may affect the phosphorylation and accumulation of newly-synthesized profilaggrin, affecting the production of FLG. In addition, a number of factors that are important in controlling FLG expression have been described, such as transcription factors of the API family (Jun and/or Fos), POU-domain proteins, transcription factor p63, and the peroxisome-proliferator-activated-receptor (PPAR) family (29). Aberrant profilaggrin processing has also been observed in 12R-lipoxygenase (12R-LOX)-deficient mice (30). In the present study, the gene chip results revealed that PPAR was upregulated and ALOX12E was downregulated following ATRA treatment. As expected, the gene chip analysis and RT-qPCR revealed that the expression of FLG was downregulated following ATRA treatment in the mouse skin, as supported by Sybert et al (31), who reported that patients with ichthyosis vulgaris displayed a reduction or absence of profilaggrin and FLG along with a morphological reduction in the amount of keratohyalin. Filaggrin is also involved in the production of primary natural moisturizing factors in the stratum corneum (29). Whether these alterations in expression may affect the expression of FLG and the specific regulatory mechanism requires further investigation.

Following ATRA treatment, alterations in the ultrastructure of the epidermis were observed under an electron microscope. The images revealed that there were numerous circular vacuolated structures in the stratum corneum, which were similar to the ultrastructure of lipid droplets. Ponec et al (32) studied the lipid and ultrastructure of reconstructed skin models by electron microscopy. Their results indicated that lipid droplets in keratinocytes appeared in all three skin models, but the
extent of the lipid droplets varied. The structure of the lipid droplets in their images was consistent with the structure of lipid droplets observed under electron microscopy in the present study. It was also thought that the different levels of lipid droplets in keratinocytes were associated with the hyper-proliferative state of reconstructed skin. On the other hand, the epidermal layers of the mice in the present study were increased following ATRA treatment, indicating that there was a degree of proliferation. Therefore, it was speculated that these structures may be lipid droplets. Nevertheless, this is a limitation of the present study.

The ATRA-treated mouse skin displayed a large number of lipid droplets in certain corneocytes [similar in appearance to the droplets observed by Ponce et al (32)], suggesting that lipid metabolism was abnormal. Gene expression analysis of skin samples from ATRA-treated and control skin demonstrated significant induction of functional proteins associated ceramide, non-esterified fatty acids and phospholipid synthesis. The present study revealed downregulation of SMPD3, SMPD2 and LASS5/Cers5, which catalyze the synthesis of ceramides, suggesting decreased ceramide synthesis in ATRA-treated mice. Conversely, it was observed that ATRA also increased the expression of UDP-glucose ceramide glucosyltransferase (UGCG). Moreover, Amen et al (33) reported that mice with deficiencies in UGCG display an ichthyosis-like skin phenotype and impairment in the differentiation of keratinocytes, which is associated with delayed wound healing.

Mao-Qiang et al (34) demonstrated that inhibiting PLA2 resulted in a defective extracellular lipid membrane and impaired homeostasis in the permeability barrier (34). In the present study, ATRA not only downregulated PLA2G2E and other phospholipases including PLCH2, but also upregulated PLA2G4E, LYPLA and DEG. Lu et al (35) demonstrated that the transcription levels of AGPAT-1, -2 and -3 were rapidly increased by ATRA. The present gene expression analysis of ATRA-treated skin revealed a marked reduction in the levels of AGPAT4 and AGPAT5.

Previously, lipoxygenases (LOXs) were reported to exert essential roles in regulating epithelial proliferation and/or differentiation, maintaining the permeability barrier, skin inflammation and wound healing (36). In the present study, no marked alterations in the protein levels of eLOX-3 and 12R-LOX in ATRA-treated skin were detected, but ATRA-treatment significantly downregulated ALOX12E, which may have a role in regulating keratinocyte differentiation (37).

TEM analysis of the mouse skin revealed an increase in the intercellular space, suggesting that the keratinocytes of the mice may have had abnormalities in the TJs. TJs function as a paracellular barrier beneath the stratum corneum (38). In the present study, Claudin-1 was decreased, but Claudin-4 was increased following ATRA-treatment in HaCaT cells and the mouse epidermis. Claudin-1-knockout mice exhibit increased epidermal permeability, severe water loss and skin wrinkling, and succumb within 24 h after birth (17). Downregulation of Claudin-1 results in decreased transepidermal electrical resistance (39). In addition, patients with atopic dermatitis exhibit low levels of Claudin-1 expression (40). Nevertheless, a previous in vitro study of cultured keratinocytes uncovered barrier defects resulting from knocking down Claudin-4 (41).

The present study revealed that the expression of Claudin-4 was increased following ATRA treatment in HaCaT cells and the mouse epidermis, as previously observed in the oral mucosa (14). Nevertheless, significant upregulation of Claudin-4 was observed in the non-lesional skin of patients with atopic dermatitis, which may indicate a compensatory response to disrupted barrier function caused by Claudin-1 downregulation that leads to the upregulation of other Claudins (18,42). Therefore, downregulated Claudin-1 and the alteration of its localization in HaCaT cells and the mouse epidermis was likely responsible, at least in part, for the observed disruption of TJ barrier function following ATRA treatment.

It was observed that Claudin-2 mRNA was upregulated 2.5-fold. Telgenhoff et al (43) studied the regulation of Claudin-2 mRNA and protein expression by ATRA in human keratinocytes, and discovered that ATRA increases the expression of Claudin-2 in keratinocytes in a dose-dependent manner. In addition, Claudin-2 is highly expressed in the TJs of mouse renal proximal tubules, which possess a leaky epithelium whose unique permeability properties underlie their high rate of NaCl reabsorption (43,44). On the other hand, the Cldn2-KO mice display a normal appearance, activity, growth and behavior, with no abnormalities in the epidermal barrier (44); therefore, it was not included in the present study as a candidate gene due to funding limitations, but it may be considered in future studies.

Notably, certain differences were observed between the in vivo and the in vitro experiments. The possible causes include the following: i) HaCaT cells are human-derived immortalized keratin-forming cells, while the skin tissues were derived from mice, thus the two are different in species; ii) cells growing in vitro and in vivo are not completely analogous; and iii) the doses of ATRA in vivo and in vitro were different. In addition, the present study is limited since it focused primarily on Claudin-1 and -4, and additional studies are required to assess the exact contribution to ATRA-induced dermatitis of all the identified differentially expressed genes. The microarray experiment would ideally have been performed in multiple samples, but research funding was very limited; thus, there was only one sample per group, which is a limitation. Nevertheless, the microarray experiment only provides an idea of the potential genes that may be examined in future studies. Subsequent experiments in vivo and in vitro will be performed to confirm the results.

In conclusion, the results suggest that ATRA disrupts the normal morphology and ultrastructure of the mouse epidermis and exerts an essential role in the function of the epidermal barrier. Gene expression analyses revealed numerous dysregulated genes associated with the synthesis/generation of transcription factors, protease inhibitors, proteases, junctional proteins, lipids, corneocytes and cornified envelopes. ATRA not only alters the expression of Claudin-1 and -4, but also alters their localization in HaCaT cells and the murine epidermis. ATRA exerts a dual effect on epidermal barrier genes: It downregulates the expression of Claudin-1 and upregulates the expression of Claudin-4. Claudin-4 upregulation may be a compensatory response for the disrupted barrier function caused by Claudin-1 downregulation.
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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the GEO repository (GSE124183): https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE124183.

Authors’ contributions

SG and JL designed the experiments. JL conducted the experiments, performed the statistical analysis and drafted the manuscript. QL performed the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were performed in accordance with the ‘Principles of Laboratory Animal Care’ (National Institutes of Health) and the guidelines of the laboratory animal care committee of Xi’an Jiaotong University (no. XJTULAC2017-733).

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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