

Letter to the Editor

Toluene downregulates filaggrin expression via the extracellular signal-regulated kinase and signal transducer and activator of transcription-dependent pathways

To the Editor:

Volatile organic compounds (VOCs) can be an important environmental risk factor in the development and/or aggravation of atopic dermatitis (AD). A previous study reported that xylene and formaldehyde intensified allergic inflammation in an experimental mouse model.¹ In a prospective study to evaluate the clinical effects of air pollution including VOCs on skin symptoms in children with AD,² concentrations of total VOCs were higher on days when the patients had symptoms of AD than on days without symptoms. A significant increase in transepidermal water loss after exposure to VOCs was also reported.³ Although these accumulated data show an association between exposure to VOCs and aggravation of AD, the molecular effects of VOCs on the aggravation of AD are still unclear.

Filaggrin is a key regulator in epidermal barrier function. The pathogenesis of AD may be proposed as an epidermal barrier defect that leads to diminished epidermal defense mechanisms.³ In a cohort study, filaggrin mutations were associated with the predilection sites of AD that are exposed areas of the body such as hands and cheeks, whereas there were no predilection sites in persons with the wild-type gene.⁴ These sites are typically

exposed to environmental factors including irritants, air pollution, and chemicals. This suggests that persons with mutations in genes encoding proteins of the skin barrier are susceptible to AD when exposed to environmental triggers. On the basis of these reports, we investigated the effects of toluene, a typical VOC that usually occurs in indoor air from the use of common household products (paints, paint thinners, adhesives, synthetic fragrances, and nail polish) and cigarette smoke, on filaggrin-mediated epidermal barrier dysfunction.

After toluene exposure, the expression of filaggrin mRNA and protein decreased (Fig 1, A and B). Furthermore, to determine whether toluene affects the expression of other keratinocyte differentiation-associated markers (eg, involucrin, loricrin, keratin 10, and keratin 5), we measured the mRNA levels of each of these molecules after toluene treatment. The present study demonstrated that toluene did not significantly affect the expression of involucrin, loricrin, keratin 10, and keratin 5 (data not shown). Fluorescence microscopy analysis also showed that filaggrin levels were lower in toluene-treated human skin equivalent models than in untreated human skin equivalent models (Fig 1, C). In addition, we evaluated the filaggrin breakdown products such as natural moisturizing factor (NMF) associated with transepidermal water loss. Pyrrolidone carboxylic acid and urocanic acid are known as the main components of NMF. As a result, levels of NMF components (pyrrolidone carboxylic acid/urocanic acid) were decreased by toluene treatment (see Fig E1 in this article's Online Repository at www.jacionline.org).

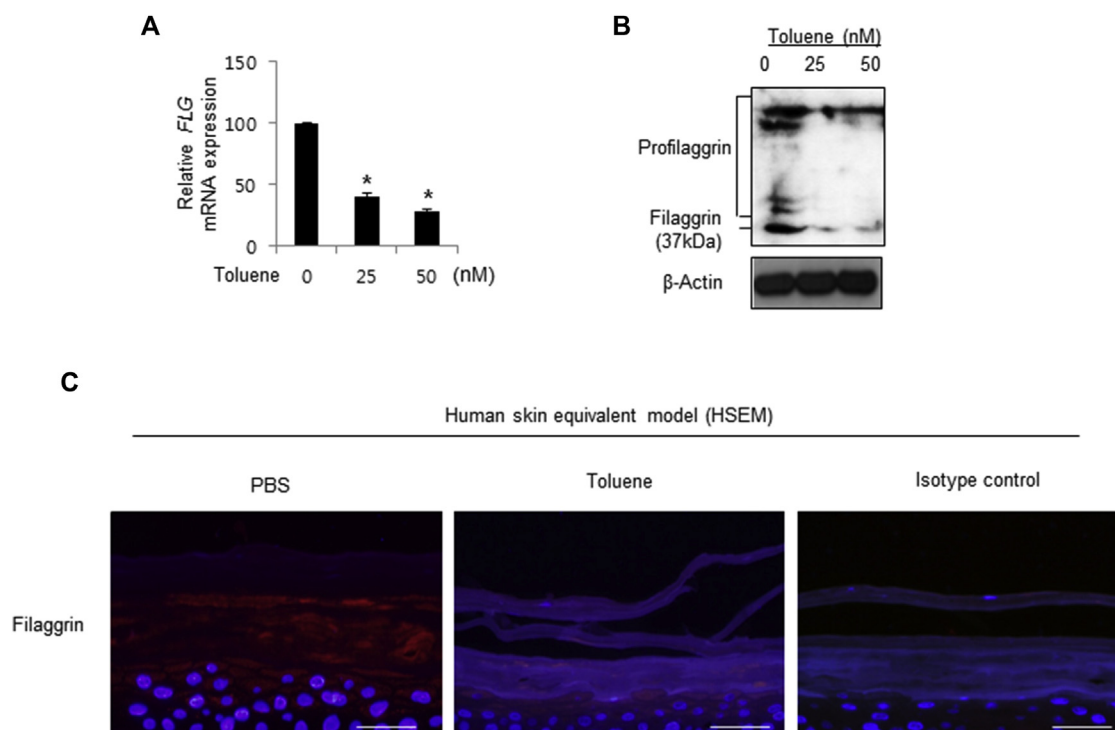


FIG 1. Toluene inhibits filaggrin expression in keratinocytes in a dose-dependent manner. Filaggrin expression was decreased by toluene exposure at the mRNA (**A**) and protein level (**B**) in keratinocytes. Downregulated filaggrin protein expression was detected by immunofluorescence staining in HSEMs (**C**). HSEM, Human skin equivalent model. Columns and error bars represent means $\pm$ SEMs from 3 independent experiments. * $P < .05$. Bar = 50 μ m.

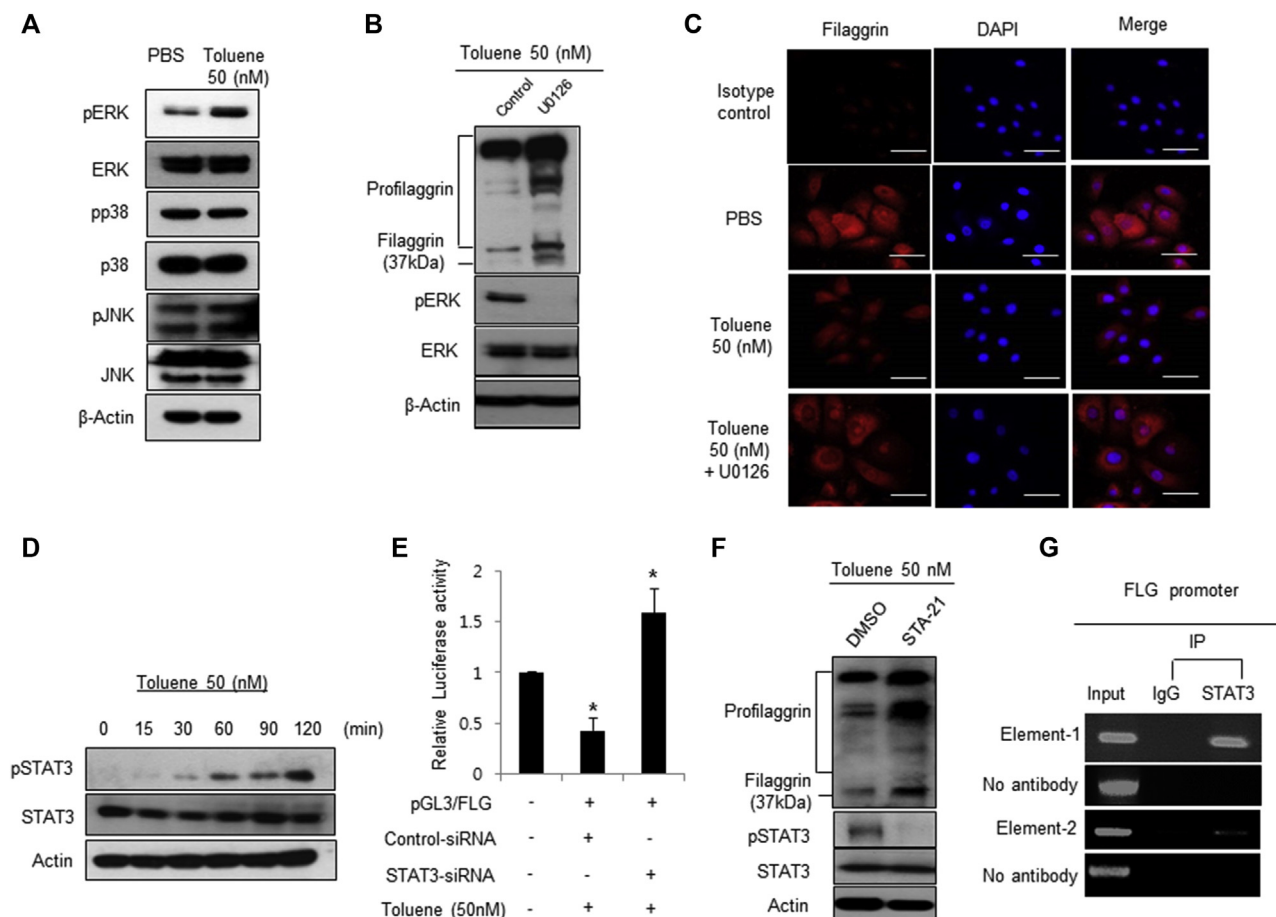


FIG 2. Toluene induces the downregulation of filaggrin expression by ERK/STAT-3-dependent mechanism. Toluene increased the ERK phosphorylation in keratinocytes (**A**). Inhibition of ERK restored the toluene-mediated downregulation of filaggrin expression shown by Western blot (**B**) and immunofluorescence assay (**C**). Phosphorylated STAT-3 protein expression was increased after toluene exposure (**D**). Downregulated filaggrin expression was upregulated by STAT3 siRNA at the promoter activity (**E**), protein level (**F**), and after toluene exposure. Results of chromatin immunoprecipitation assay showed that the STAT-3 binds directly to the putative STAT-3 binding sequence of the filaggrin promoter (**G**). DAPI, 4'-6-Diamidino-2-phenylindole, dihydrochloride; DMSO, dimethyl sulfoxide. Columns and error bars represent means $\pm$ SEMs from 3 independent experiments. * $P < .05$. Bar = 100 μ m.

The involvement of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase in cells under stress due to exposure to benzene, toluene, and formaldehyde has been reported.⁵ To understand the regulatory mechanisms of toluene-induced filaggrin downregulation, our study investigated whether the mitogen-activated protein kinase signaling pathway is induced in keratinocytes after toluene exposure. Toluene increased the activation of ERK phosphorylation in keratinocytes. However, it did not induce noticeable phosphorylation of p38 and JNK (Fig 2, A). Pretreatment with the ERK inhibitor U0126 markedly restored filaggrin expression suppressed by toluene exposure (Fig 2, B and C). But suppression of p38 with SB239063 or JNK with SP600125 did not alter filaggrin expression (see Fig E2 in this article's Online Repository at www.jacionline.org). To further establish the role of ERK1/2 activation, we used siRNA-mediated incapacitating approach and assessed the filaggrin expression. Treatment with siRNA effectively attenuated ERK1/2 and resulted in restoration of filaggrin expression (see Fig E3, A, in this article's Online Repository at www.jacionline.org). These results suggest that

the activation of ERK is required for toluene-induced filaggrin downregulation.

It is known that ERK can be involved in the regulation of signal transducer and activator of transcription 3 (STAT3) expression in cells. A recent study has shown that STATs may be important mediators of environmental factor, toluene-induced inflammatory disease.⁶ Previously, we reported that thymic stromal lymphopoietin suppression of filaggrin expression is STAT3 dependent.⁷ Therefore, we assumed that STAT3 may regulate toluene-induced filaggrin downregulation in keratinocytes. After toluene treatment, STAT3 protein levels peaked after 2 hours (Fig 2, D). After treatment with 50 nM of toluene, filaggrin promoter activity was higher in STAT3 siRNA-transfected keratinocytes than in nontargeting siRNA-transfected keratinocytes (Fig 2, E). To confirm the STAT3 dependency of filaggrin reduction, we analyzed the effect of STAT3 on filaggrin expression by STAT3-specific inhibitors (STA-21). Compared with control, in the keratinocytes treated with STA-21, filaggrin expression increased at the protein levels (Fig 2, F). Moreover, treatment with siRNA effectively attenuated STAT3 and resulted in

restoration of filaggrin expression (see Fig E3, B, in this article's Online Repository at www.jacionline.org). These results demonstrate that STAT3 contributes to the regulation of filaggrin expression by toluene in keratinocytes.

To investigate whether STAT3 suppresses filaggrin expression by binding directly to its promoter, keratinocytes were exposed to toluene and incubated for 24 hours. We then detected the specific amplification of the filaggrin promoter region after a chromatin immunoprecipitation assay using antiphospho-STAT-3-specific antibody. To exclude the nonspecific binding of antibodies, chromatin complexes were also immunoprecipitated with the anti-IgG antibody as a negative control (Fig 2, G). The arrows in Fig E3, C (see this article's Online Repository at www.jacionline.org), show the positions and directions of the PCR primers used for detecting the products of the chromatin immunoprecipitation assay.

Recent studies suggest the possibility of VOC-induced oxidative stress and redox imbalance affecting the development of AD.⁸ Moreover, Kim et al⁹ showed that a 1-ppb increase in total toluene concentration was related to a 12.7% increase in AD symptoms. It was reported that the typical indoor levels of toluene are less than 5 ppb.⁹ The concentration of toluene in the present study was approximately 5 ppb. Further studies are needed to confirm the threshold levels for eliciting skin symptoms in AD. Nevertheless, this study is the first to demonstrate the inhibition of filaggrin synthesis in keratinocytes by toluene.

The present study demonstrated that the downregulation of filaggrin by toluene is mediated by ERK/STAT3-dependent pathways. Collectively, these results suggest that toluene and the related signaling might disrupt the epidermal barrier in keratinocytes.

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REFERENCES

1. Bonisch U, Bohme A, Kohajda T, Mogel I, Schutze N, von Bergen M, et al. Volatile organic compounds enhance allergic airway inflammation in an experimental mouse model. *PLoS One* 2012;7:e39817.
2. Ahn K. The role of air pollutants in atopic dermatitis. *J Allergy Clin Immunol* 2014;134:993-9, discussion 1000.
3. Huss-Marp J, Eberlein-Konig B, Breuer K, Mair S, Ansel A, Darsow U, et al. Influence of short-term exposure to airborne Der p 1 and volatile organic compounds on skin barrier function and dermal blood flow in patients with atopic eczema and healthy individuals. *Clin Exp Allergy* 2006;36:338-45.
4. Carson CG, Rasmussen MA, Thyssen JP, Menne T, Bisgaard H. Clinical presentation of atopic dermatitis by filaggrin gene mutation status during the first 7 years of life in a prospective cohort study. *PLoS One* 2012;7:e48678.
5. Lecureur V, Arzel M, Ameziane S, Houlbert N, Le Vee M, Jouneau S, et al. MAPK- and PKC/CREB-dependent induction of interleukin-11 by the environmental contaminant formaldehyde in human bronchial epithelial cells. *Toxicology* 2012;292:13-22.
6. Fujimaki H, Win-Shwe TT, Yoshida Y, Kunugita N, Arashidani K. Dysregulation of immune responses in an allergic mouse model following low-level toluene exposure. *Toxicology* 2011;286:28-35.
7. Kim JH, Bae HC, Ko NY, Lee SH, Jeong SH, Lee H, et al. Thymic stromal lymphopoietin downregulates filaggrin expression by signal transducer and activator of transcription 3 (STAT3) and extracellular signal-regulated kinase (ERK) phosphorylation in keratinocytes. *J Allergy Clin Immunol* 2015;136:205-8.e9.
8. Bowler RP, Crapo JD. Oxidative stress in allergic respiratory diseases. *J Allergy Clin Immunol* 2002;110:349-56.
9. Kim EH, Kim S, Lee JH, Kim J, Han Y, Kim YM, et al. Indoor air pollution aggravates symptoms of atopic dermatitis in children. *PLoS One* 2015;10:e0119501.

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METHODS

Chemical reagents

SB239063, SP600125, U0126, STA-21, dimethyl sulfoxide (DMSO), toluene, 2-pyrrolidone-5-carboxylic acid (PCA), and urocanic acid (UCA) were purchased from Sigma-Aldrich (St Louis, Mo).

Preparation of toluene

Cells were exposed to various concentrations of toluene by adding toluene to the cell culture medium (internal medium). Toluene stock solution (concentration, 100 mM/mL) was prepared in DMSO.

Cell culture

Normal human epithelial keratinocytes were purchased from Millipore (Billerica, Mass) and were cultured using human epithelial keratinocyte complete media kit (EpiGRO; Millipore). The cells were grown at 37°C in a humidified atmosphere of 5% CO₂.

3D epidermis

Human-reconstituted 3D epidermis model EpiDerm (EPI-200), which was used as a human skin equivalent model (HSEM), was purchased from MatTek Corporation (Ashland, Mass). HSEMs resemble normal human epidermis and consist of multilayered differentiated tissue, including basal, spinous, granular, and cornified layers. HSEMs were incubated in assay medium at 37°C in 5% CO₂.

Cell viability assay

Cytotoxicity was assessed by performing thiazolyl blue tetrazolium bromide (3-[4,5]dimethylthiazol-2,5 diphenyltetrazolium bromide [MTT]) cell viability assay that is based on the reduction of soluble yellow MTT tetrazolium salt to its insoluble blue MTT formazan product by mitochondrial succinic dehydrogenase. Before toluene treatment, HaCaT cells (density, 5×10^4 cells/well) were maintained for 12 hours in 24-well plates containing culture medium lacking FBS. After toluene treatment, the cells were cultured for 4 hours. MTT (0.5 mg/mL) was then added, and the cells were incubated for 4 hours at 37°C. After incubation, the supernatant was removed and 700 μ L/well DMSO was added to dissolve the formazan product. Absorbance was measured at 540 nm by using a microplate reader (SpectraMax M2e; Bucher Biotec, Basel, Switzerland).

RT-PCR

For RT-PCR, total cellular RNA was isolated from toluene-treated human keratinocytes by using TRIzol reagent (Invitrogen, Carlsbad, Calif), according to the manufacturer's protocol. RT was performed using 1 μ g of the total RNA. PCR was performed using the following primers: FLG, 5'-GGT CTG GAC GTT CAG GGT CT-3' (forward) and 5'-GGA TGT GGT GTG GCT GTG AT-3' (reverse); β -actin: 5'-ACAGGAAGTCCCTTGCCATC-3' (sense) and 5'-AGGGAGACAAAAGCCTTCA-3' (antisense). Amplification was performed using MyCycler Thermal Cycler (Bio-Rad, Hercules, Calif).

Western blotting

Expression of filaggrin and phosphorylation of mitogen-activated protein kinases in toluene-treated keratinocytes were analyzed by performing Western blotting. Proteins were extracted by lysing the cells in lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 100 mg/mL phenylmethanesulfonyl fluoride, and protease inhibitors). Protein concentrations were determined using bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill), with BSA as the standard. Protein lysates (50 μ g) were resolved on 10% to 12% sodium dodecyl sulfate-polyacrylamide gel. Western blotting was performed on polyvinylidene difluoride membranes (Millipore) by using specific mAbs against filaggrin (sc-66192, 1:5000; Santa Cruz Biotechnology, Santa Cruz, Calif) and phosphorylated ERK, phosphorylated p38 mitogen-activated protein kinase, phosphorylated JNK, phosphorylated STAT3, and β -actin (1:1000 dilution; Cell Signaling Technology, Beverly, Mass). Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

DNA construction

The FLG promoter (−916/+1 bp) linked to the luciferase gene was cloned by performing PCR with human genomic DNA as the template and primers 5'-ctagccgggctcgag GGAGATGCAATCTGCTCAACATAAC-3' and 5'-gatgcagatctcgag ACATTCAGATTTCCACCTTGGT-3'. PCR fragments were digested with XhoI/XhoI and were ligated to pGL3.0 luciferase reporter vector (Promega, Madison, Wis).

Luciferase assay

Human keratinocytes were cultured for 16 hours in 12-well plates (cell density, 4×10^4 cells/mL) containing Dulbecco modified Eagle medium supplemented with 5% FBS. The cells were then cotransfected with the pGL3.0/filaggrin-Luc plasmid and control vector pRL-TK (Promega) by using Lipofectamine 2000 (Invitrogen). The pGL3.0/filaggrin-Luc plasmid was constructed as reported previously. Variations in transfection efficiency were normalized using Renilla luciferase-encoding plasmid pRL-TK as the control. After 5 hours of transfection, the medium was replaced with Dulbecco modified Eagle medium containing 0.5% FBS and incubated for 16 hours, followed by cell exposure to toluene.

Immunofluorescence

Keratinocytes were fixed with acetone and were blocked with 20% goat serum. The blocked slides were incubated with a polyclonal antibody against filaggrin (Abcam ab81468). Unconjugated antibodies were detected using CY3-labeled goat anti-rabbit antibody (KPL, Gaithersburg, Md). Nuclei were visualized using 4',6-diamidino-2-phenylindole. The samples were photographed using FV-1000/ES confocal microscope (Olympus, Tokyo, Japan).

Gene silencing

STAT3 siRNA (GenePharma, Shanghai, China) was used for silencing STAT3, and scrambled siRNA was used as a control. Cells were cultured in 60-mm cell culture dishes until they reached 40% confluence, after which they were transfected with 20 nM of STAT3 or scrambled siRNA by using Lipofectamine 2000, according to the manufacturer's instructions. The cells were allowed to stabilize for 24 hours before using them for further experiments.

Chromatin immunoprecipitation assay

Cells were incubated for 24 hours after treatment with toluene and then treated with 1% formaldehyde for 10 minutes at 37°C. The cells were harvested, and chromatin immunoprecipitation was performed using the chromatin immunoprecipitation assay kit from Upstate Biotechnology (Temecula, Calif), according to the manufacturer's instructions. After immunoprecipitation, the captured genomic fragments were then recovered by phenol-chloroform extraction. Identification of the filaggrin promoter fragments was performed by PCR analysis, using promoter-specific primers. PCR was performed in 35 cycles, and the amplified products were analyzed on a 2% agarose gel. The oligonucleotide sequences used for the PCR amplification of the filaggrin promoters were as follows: element 1: 5'-GGCATTTCATCT CATGGCAAG-3' (forward) and 5'-GGCTTGACAGGTGCTTGGTA-3' (reverse); element 2: 5'-CAAGCCAAAGTGGGGTTACA-3' (forward) and 5'-GAAGGCTGAGATAATGGCCC-3' (reverse).

HPLC analysis of NMF

For the HPLC analysis, 500 μ L KOH solution, 0.1 mol, was added to HSEM, followed by 2 hours of continuous shaking. The alkaline extracts were neutralized with 3 μ L perchloric acid, 12 mol, and filtered through a 0.2- μ m membrane filter. The concentrations of NMF (PCA/UCA) were determined by HPLC using the HP1100 system (Hewlett Packard, Palo Alto, Calif). An aliquot of 10 μ L was introduced into the HPLC system containing a 250 $\times$ 4.6 mm reverse-phase Prevail HPLC column (Kinetex C18, Phenomenex, Torrance, Calif) with a flow rate of 0.8 mL/min. For the analysis of PCA and UCA, isocratic elution was performed with 20 mM ammonium formate containing 1.5 mM tetrabutylammonium hydroxide and 1% acetonitrile at pH 7.3. The effluent was monitored at 210 nm for PCA and at 270 nm for UCA.

Statistical analysis

Analysis was performed in triplicate and independently repeated at least 3 times. Statistical significance of the obtained data was determined using 2-way

ANOVA with GraphPad PRISM version 4.02 for Windows (GraphPad Software, San Diego, Calif). The results are expressed as mean $\pm$ SD, and the level of significance was set at *P* less than .05.

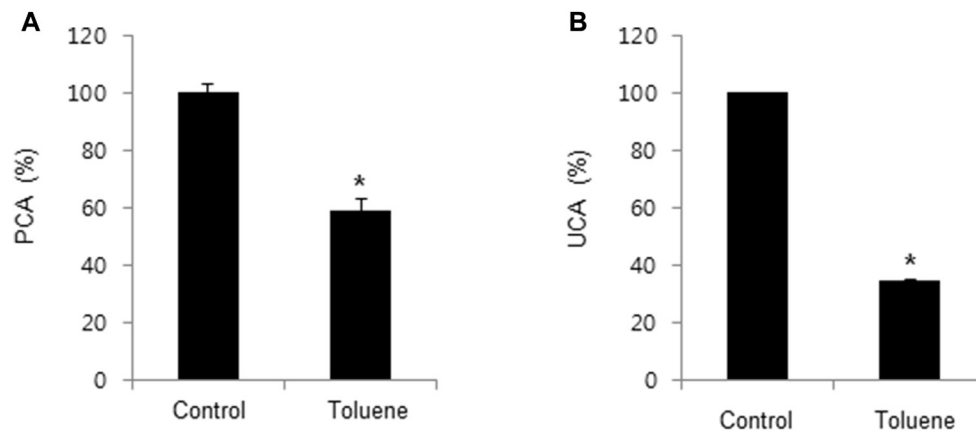


FIG E1. NMF: relationship with toluene exposure. Toluene-treated HSEMs were lysed in 0.1 mol KOH and subjected to HPLC. Exposure to toluene resulted in a 40% and 60% reduction in the PCA and UCA content, respectively. The PCA levels were 42.3 ± 3.43 and 26.6 ± 4.41 $\mu\text{g/mL}$ for control and toluene, respectively (**A**). The respective UCA levels were 4.1 ± 0.05 and 1.4 ± 0.03 $\mu\text{g/mL}$ for control and toluene, respectively (**B**). * $P < .001$.

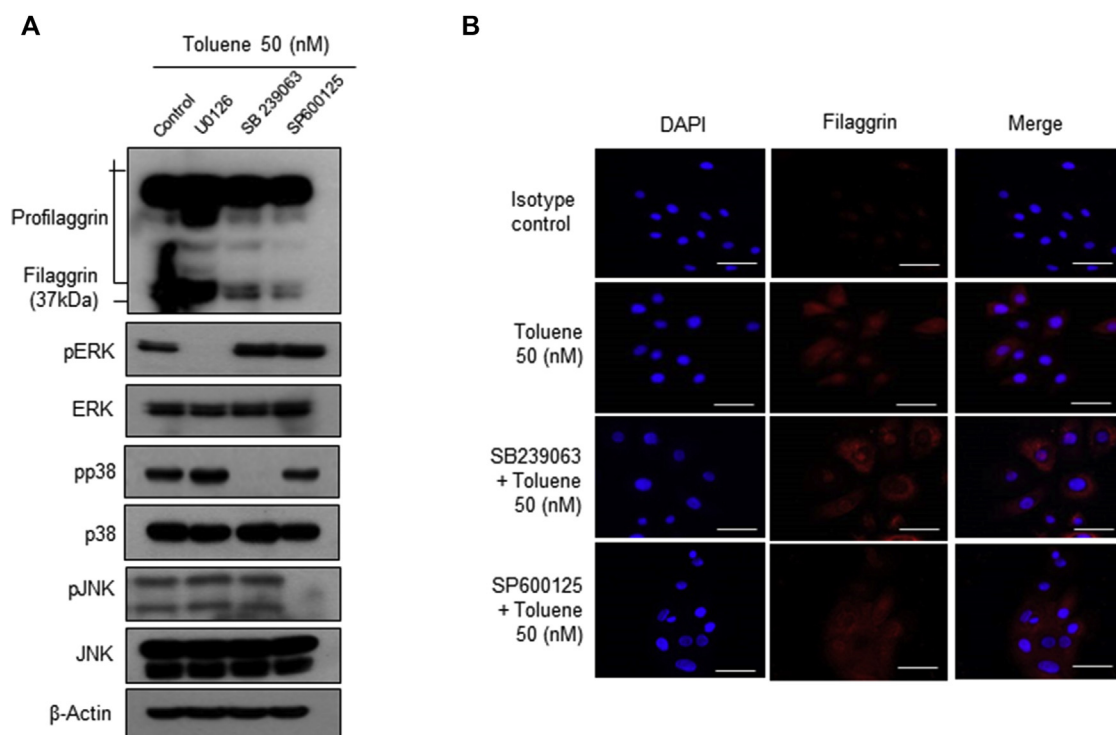


FIG E2. Suppression of p38 with SB239063 or JNK with SP600125 does not change filaggrin expression. Toluene did not increase p38 phosphorylation and JNK phosphorylation in keratinocytes. Keratinocytes were preincubated with the specific inhibitors against ERK (U0126), p38 (SB239063), and JNK (SP600125) before stimulation with toluene. Western blot analysis characterized the expression of total ERK, pERK (Thr202/Tyr204), total p38 MAPK, pp38 MAPK (Thr180/Tyr182), total JNK, pJNK (Thr183/Tyr185), and filaggrin (**A**). Also, the expression of filaggrin was visualized by IF staining (**B**). Data are representative of 3 independent experiments. Bar = 100 μ m. DAPI, 4'-6-Diamidino-2-phenylindole, dihydrochloride; IF, immunofluorescence; MAPK, mitogen-activated protein kinase.

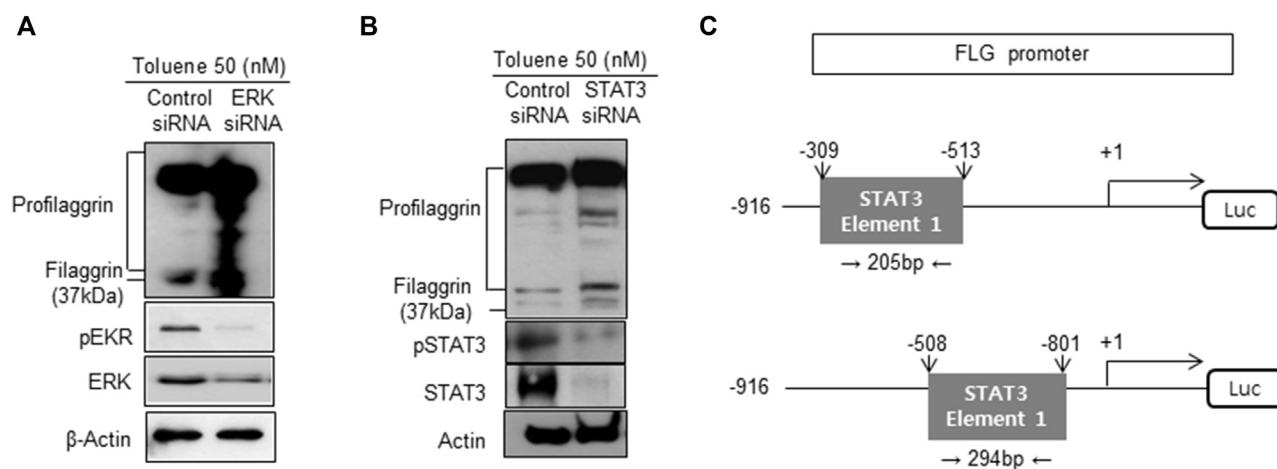


FIG E3. ERK and STAT3 siRNA block the toluene-mediated downregulation of filaggrin expression. Keratinocytes were transfected with ERK siRNA (**A**) and STAT3 siRNA (**B**) and then exposed to toluene. STAT3 and ERK siRNA block the toluene-mediated downregulation of filaggrin expression. Schematic diagram shows the position and directions of PCR primers used for detecting the products of the chromatin immunoprecipitation (ChIP) assay (**C**).