

# Gene Regulation of *CYP4F11* in Human Keratinocyte HaCaT Cells<sup>S</sup>

Ying Wang, Jordan C. Bell, Diane S. Keeney, and Henry W. Strobel

Department of Biochemistry and Molecular Biology, the University of Texas-Houston Medical School, Houston, Texas (Y.W., J.C.B., H.W.S.); and Departments of Biochemistry and Medicine, Vanderbilt University, Nashville, Tennessee (D.S.K.)

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## ABSTRACT:

Mechanisms regulating *CYP4F* genes remain under investigation, although characterization of *CYP4F* regulatory modalities would facilitate the discovery of new drug targets. This present study shows that all-*trans*- and 9-*cis*-retinoic acids can inhibit *CYP4F11* expression in human keratinocyte-derived HaCaT cells. Transrepression of many genes by retinoic acids is mediated by interactions between retinoid receptors and the activator protein 1 (AP-1) complex. Proinflammatory cytokines tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 1 $\beta$ , which can activate the AP-1 complex, induce *CYP4F11* transcription in HaCaT cells. The c-Jun N-terminal kinase (JNK)-specific inhibitor 1,9-pyrazoloanthrone (SP600125) blocked the induction of *CYP4F11* by both cytokines, indicating involvement of the JNK pathway. Furthermore, TNF- $\alpha$  failed to

induce *CYP4F11* transcription when HaCaT cells were preincubated with retinoic acids. Retinoic acids are ligands for the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). The RXR agonist 6-(1(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cyclopropyl) nicotinic acid (LG268) greatly induced *CYP4F11* transcription, whereas the RAR agonist 4-(2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl)benzoic acid (TTNPB) markedly inhibited *CYP4F11* transcription, indicating that down-regulation of *CYP4F11* transcription by retinoic acid is mediated by RARs and may also be related to ligand competition for RXRs. Thus, the *CYP4F11* gene is positively regulated by multiple signaling pathways in HaCaT keratinocytes, including RXR and JNK signaling pathways.

Cytochrome P450 4F enzymes are involved in cellular protection and metabolism of numerous small molecules, including drugs, toxins, and eicosanoids. To date, studies have been focused on *CYP4F* functions rather than transcriptional regulatory mechanisms. This approach is partially because of the apparent complexity of *CYP4F* gene regulation and differences observed among the various model systems studied.

Despite the seeming complexity of *CYP4F* regulation, some research groups have made substantive contributions to this puzzle. Zhang and colleagues showed that retinoic acids and peroxisome proliferators can regulate *CYP4F2* gene activities in HepG2 cells, and that retinoid X receptor (RXR)  $\alpha$  heterodimers stimulated and retinoic acid receptor (RAR)  $\alpha$  repressed *CYP4F2* expression (Zhang and Hardwick, 2000; Zhang et al., 2000; Hsu et al., 2007) showed that statins induced *CYP4F2* in primary human hepatocytes and HepG2 cells. We recently showed that retinoic acids induced the expression of *CYP4F2*, *CYP4F3A*, *CYP4F3B*, and *CYP4F11* in primary human

epidermal keratinocytes, and that RXR (but not RAR) nuclear receptors mediate retinoic acid-induced up-regulation of *CYP4F2* and *CYP4F3A* (Kalsotra et al., 2008). In a preliminary study (data not shown) using a transformed immortal human keratinocyte cell line (HaCaT), a different story unfolded. Lovastatin did not affect HaCaT cell *CYP4F2* expression, which is in contrast to its up-regulatory effects on *CYP4F2* in human hepatic cells (Hsu et al., 2007). In addition, 9-*cis*-retinoic acid failed to induce *CYP4F11* in HaCaT cells; rather, it significantly decreased *CYP4F11* transcript levels.

Retinoic acids are derived from retinol, the animal form of vitamin A. Retinoic acids play a role in gene regulation via two groups of nuclear receptors (NRs), RARs and RXRs (Mangelsdorf et al., 1995), each of which consists of three isotypes:  $\alpha$ ,  $\beta$ , and  $\gamma$  (Mangelsdorf et al., 1990; Leid et al., 1993). Retinoic acids can up-regulate or down-regulate certain genes by three mechanisms: transactivation, transrepression, and competition with other NRs for heterodimerization with RXR. Transrepression by retinoic acids is the cross-talk between retinoid receptors and the activator protein 1 (AP-1) complex (Saatcioglu et al., 1994; Chen et al., 1995). Cytokines, oncogenes, and growth factors that activate protein kinase C modulate the activities of the AP-1 complex composed of c-Jun and c-Fos proteins (Zhang and Pfahl, 1993). Active RAR-RXR heterodimers interact with AP-1 complexes, thereby inhibiting the transcription of genes regulated by AP-1 (Fisher et al., 1998). Transrepression of AP-1 is a major mech-

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**ABBREVIATIONS:** RXR, retinoid X receptor; RAR, retinoic acid receptor; NR, nuclear receptor; AP-1, activator protein 1; TTNPB, 4-(2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl)benzoic acid; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; LG268, 6-(1(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cyclopropyl) nicotinic acid; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; IL-1 $\beta$ , interleukin 1 $\beta$ ; SP600125, 1,9-pyrazoloanthrone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; RT-PCR, reverse transcriptase-polymerase chain reaction; DMSO, dimethyl sulfoxide; qRT-PCR, quantitative real-time polymerase chain reaction; JNK, c-Jun N-terminal kinase.

anism of the pharmacological anti-inflammatory and antiproliferative effects of retinoids (Fisher and Voorhees, 1996).

RXR can form heterodimers with other NRs, including peroxisome proliferator-activated receptors (Green, 1995; Qi et al., 2000; Lazar, 2005), farnesoid X receptor (Laffitte et al., 2000; Desvergne, 2007), liver X receptors (Willy et al., 1995; Willy and Mangelsdorf, 1997; Desvergne, 2007), thyroid receptor (Apriletti et al., 1998; Barra et al., 2004), and NR-related 1 receptor (Perlmann and Jansson, 1995; Perlmann and Wallén-Mackenzie, 2004). RXRs can also form homodimers under certain conditions (Zhang and Pfahl, 1993). Thus, when an RAR-specific agonist stimulates the interaction of RXR with RARs, it can act to negatively regulate the activity of other NRs that partner with RXR in favor of RAR-mediated responses (Heinz Nau, 2007). The relative abundance of classic retinoid receptors and other RXR-interacting NRs, which have varying affinities for RXR, influences RXR dimerization and thereby gene regulation mediated by these NRs (Fisher and Voorhees, 1996). The aim of the current study is to clarify the mechanism and factors responsible for the down-regulation of HaCaT *CYP4F11* expression by retinoic acids.

### Materials and Methods

**Chemicals.** The 9-*cis*- and all-*trans*-retinoic acids, 4-(2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl)benzoic acid (TTNPB), and 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) were from Sigma-Aldrich (St. Louis, MO). 6-(1(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl) cyclopropyl) nicotinic acid (LG268) was a gift from Dr. Peter J. A. Davies (University of Texas-Houston). Human tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 1 $\beta$  (IL-1 $\beta$ ) were from Invitrogen (Carlsbad, CA). SP600125 and SB203580 were produced by Calbiochem (San Diego, CA). Optimal concentrations of drugs were chosen according to literature citations. Polyclonal anti-CYP4F11 antibody was from Proteintech Group, Inc. (Chicago, IL). Polyclonal antibodies against human RARs and RXRs were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal c-Jun and phosphor-c-Jun antibodies were from Cell Signaling Technology Inc. (Danvers, MA). All of the other chemicals used were of reagent grade quality or higher.

**Cell Culture.** The HaCaT cell line was from CLS-Cell Line Services (Heidelberg, Germany) (Boukamp et al., 1988). Cells were grown at 37°C in a humidified incubator with 5% CO<sub>2</sub> in high glucose Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), 1% L-glutamine (Invitrogen), 1% sodium pyruvate (Invitrogen), and 1% antibiotic-antimycotic (Invitrogen). Cells were passaged at a 1:10 ratio after they had been 100% confluent for 6 to 7 days and used for experiments at 70 to 80% confluency.

**Protein Isolation and Western Blot Analyses.** Cells were washed once with cold phosphate-buffered saline and then quickly detached by gently scraping. Cell pellets were collected by centrifugation for 2 min at 4500 rpm and stored at -80°C. Whole protein lysate buffer was prepared using 50 mM Tris-HCl buffer, pH 6.8, with 2% SDS. Cell pellets were dissolved in the lysate buffer by pipetting the suspension up and down and then boiled at 95°C for 5 min. The cell lysate was cleared by centrifugation at 13,000 rpm for 2 min. Protein concentrations of the clear supernatant fractions were measured using the bicinchoninic acid assay kit (Pierce, Rockford, IL) and subjected to Western blot assays.

Protein samples were boiled in Laemmli buffer and resolved electrophoretically on 4 to 15% gradient Tris-glycine-SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred to polyvinylidene fluoride membranes (wet transfer apparatus; Bio-Rad Laboratories). Membranes were blocked for 1 h at room temperature with 5% dried nonfat milk followed by incubation for 3 h at room temperature with antibodies raised against CYP4F11 (1:1000 dilution) or c-Jun (1:500 dilution). Membranes were then washed and incubated at room temperature with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) for 1 h. Immunoreactivity was visualized using a horseradish peroxidase chemiluminescence system (Pierce).

**Quantitative Real-Time Polymerase Chain Reaction.** Cells were washed with phosphate-buffered saline, and total RNA was isolated using TRIzol reagent (Invitrogen). All the samples were treated with DNase I (Invitrogen). Aliquots (200 ng) of total RNA were reverse-transcribed by SuperScript II Reverse Tran-

scriptase (Invitrogen) in triplicate, including a reverse transcription blank to evaluate presence of contaminating genomic DNA. Amplification was performed using *Taq* DNA Polymerase (Invitrogen) with an ABI Prism 7700 (Applied Biosystems, Foster City, CA) at 95°C for 1 min, followed by 40 cycles at 95°C for 12 s and 60°C for 30 s. CYP4F11 mRNA levels were measured using standard curves generated by plotting Ct versus the log of the amount of purified amplicon for *CYP4F11* (custom synthesis by Integrated DNA Technologies, Inc., Coralville, IA) (200 ag-2 pg). Abundance of human 18S RNA was used as the internal control. Primers and fluorescent probe sequences for *CYP4F11* and 18S RNA were reported (Kalsotra et al., 2008).

**Reverse Transcriptase-Polymerase Chain Reaction.** Aliquots (200 ng) of total RNA were reverse-transcribed and amplified using Superscript One-Step reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Invitrogen) and transcript-specific oligonucleotides designed using Primer3 (version 0.4.0; <http://frodo.wi.mit.edu/primer3/>) (Table 1). For RAR and RXR, the reverse-transcription reaction (50°C, 30 min) was followed by 40 cycles of amplification (94°C, 15 s; 60°C, 30 s; and 72°C, 1 min). For CYP4F11, the reverse-transcription reaction (60°C, 30 min) was followed by 40 cycles of amplification (94°C, 1 min and 65°C, 1.5 min). Products were resolved on 1.5% agarose gels and visualized with ethidium bromide.

**Plasmids.** To construct pGL3-CYP4F11 plasmid, *CYP4F11* promoter was cloned by PCR using human genomic DNA as a template with the forward primer 5'-TCT TAC GCG TGC TAG CTA CCC AGC ACC CAG AGT AGG-3' and the reverse primer 5'-CCG GAA TGC CAA GCT TGC TCC AAG GAC AGT GGA AAG-3'. The resulting 1729-base pair product was the promoter region of CYP4F11 (GenBank accession no. NG\_0008335) including exon 1 as the coding start site in exon 2. The PCR product was then cloned into HindIII- and NheI-digested linear pGL3 Basic luciferase vector (Promega, Madison, WI) using the infusion Advantage PCR Cloning Kit (Clontech, Mountain View, CA).

**Transfection and Luciferase Assays.** Cells were transfected by Attractene Transfection Reagent (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Cells were seeded onto six-well plates (3 × 10<sup>5</sup> cells/well). Immediately after the seeding, cells were transfected with 4.5  $\mu$ l of Attractene/well and 1.2  $\mu$ g of DNA/well. Transfection efficiency was monitored by simultaneous cotransfection with a  $\beta$ -galactosidase reporter construct at the concentration of 20 ng/well. The culture medium was changed at 6 h post-transfection. Twenty-four hours after transfection, cells were treated with agonists for RARs or RXRs with or without TNF- $\alpha$ . After the treatment, cells were lysed in passive lysis buffer (Promega). Luciferase activity was measured for 10 s in a luminometer.  $\beta$ -Galactosidase activity was measured to normalize for variations in transfection efficiency. Promoter activity of each construct was expressed as firefly luciferase/ $\beta$ -galactosidase activity. Each experiment was independently performed on three separate occasions with at least triplicate wells in each experiment.

**Statistical Analysis.** Data are presented as mean  $\pm$  S.E. One-way analysis of variance followed by Tukey's multiple comparison test was used for the statistical analysis. Statistical differences were considered significant if  $P < 0.05$ .

TABLE 1  
Sequences of primers for RARs, RXRs, and CYP4F11 for RT-PCR

Transcript	Oligonucleotide Sequences (5'-3')	Product Size/Annealing Temperature
RAR- $\alpha$	Forward GGCATGTCCAAGGAGTCTGT Reverse GTCCGAGAAGGTCATGGTGT	420 bp/60°C
RAR- $\beta$	Forward GAGAGGTGGCATTGATCCAGG Reverse GGCTGGGCCAGCCTGACCTC	435 bp/62°C
RAR- $\gamma$	Forward TCGAGATGCTGAGCCCTAGCTTCC Reverse CATGCCCACTTCAAGCACTTCTGT	351 bp/58°C
RXR- $\alpha$	Forward AATGAGGTGGAGTGCACCAG Reverse TCAGCACCTGTCAAAGATG	400 bp/60°C
RXR- $\beta$	Forward GGACAGAAGCTCAGGCCAAAC Reverse AATGGATCGGTGTGAGAAGG	540 bp/60°C
RXR- $\gamma$	Forward TGTGGTCAACAGTGTCAAG Reverse TCTGCACAGCTTCCCTCT	392 bp/60°C
CYP4F11	Forward GCCTCAGGATCCCACCCTCCAT Reverse ATGTGGTCCACAGCTGGGTCAATGT	290 bp/65°C

bp, base pair.

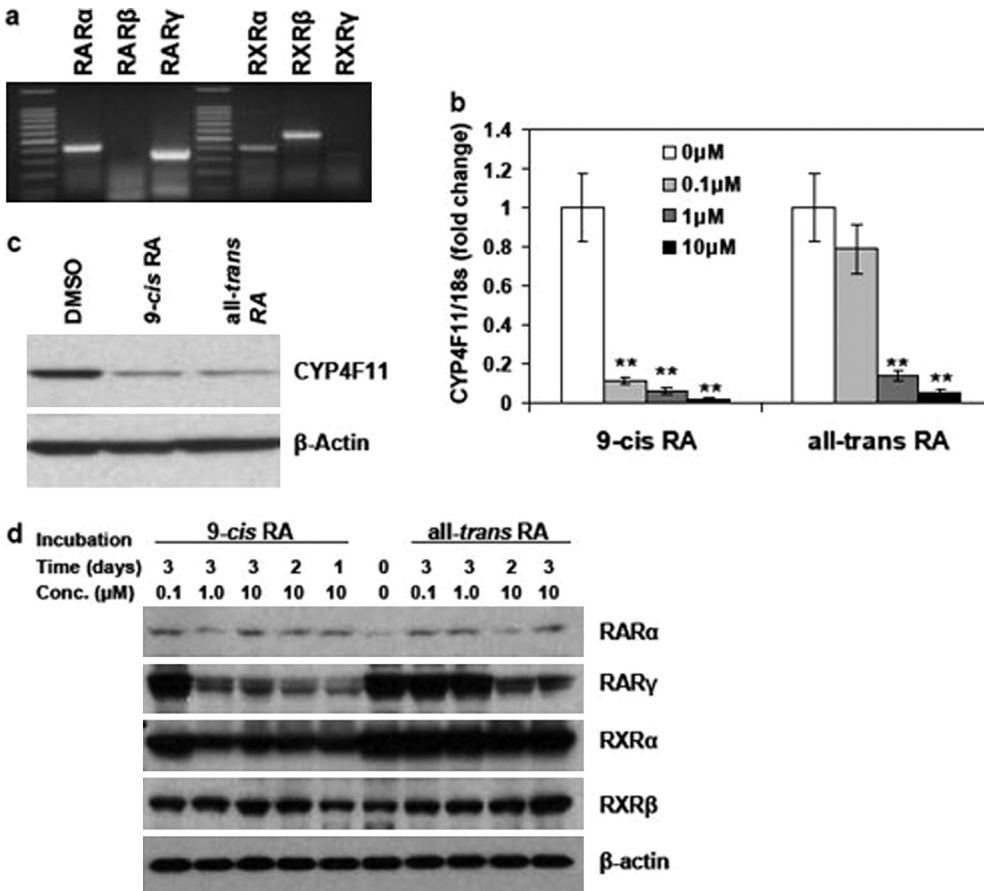
**Database Sequence Analysis.** The TRANSFAC database (BIOBASE, Beverly, MA) was searched using AliBaba 2.1 (BIOBASE) for putative AP-1 binding sites and hormone responsive elements on the 5' flanking sequence (2000 base pairs from the ATG start codon) of *CYP4F11* gene.

**Results**

**Expression of Retinoid Receptors in HaCaT Cells and Inhibitory Effects of Retinoic Acids on *CYP4F11* Expression.** We first determined which RXR and RAR isoforms are expressed in HaCaT cells. Results by RT-PCR show that HaCaT cells express RAR $\alpha$ , RAR $\gamma$ , RXR $\alpha$ , and RXR $\beta$ , whereas neither RAR $\beta$  nor RXR $\gamma$  was detectable (Fig. 1a). To elucidate mechanisms by which retinoic acids regulate *CYP4F11* expression, we treated HaCaT cells with 9-*cis*- and all-*trans*-retinoic acids for 3 days at 0.1, 1, and 10  $\mu$ M concentrations or vehicle [dimethyl sulfoxide (DMSO)] alone. Both retinoic acids resulted in dose-dependent suppression of *CYP4F11* transcript levels in HaCaT cells, measured by quantitative real-time polymerase chain reaction (qRT-PCR) (Fig. 1b). The reductions in *CYP4F11* mRNA at the 0.1, 1, and 10  $\mu$ M dose levels were 89.1, 93.9, and 98.3% for 9-*cis*-retinoic acid and 21.1, 86.5, and 94.8% for all-*trans*-retinoic acid, respectively. Data from luciferase assay indicated that at the concentration of 1  $\mu$ M, 9-*cis*- and all-*trans*-retinoic acids were able to decrease *CYP4F11* promoter activity by 83.0 and 70.9%, respectively (Supplemental Fig. 1). Furthermore, Western blot analyses showed the down-regulation of *CYP4F11* protein by both retinoic acids at 10  $\mu$ M (Fig. 1c). Western blots against RAR $\alpha$ , RAR $\gamma$ , RXR $\alpha$ , and RXR $\beta$  were performed to study the potential effects of retinoic acids on the expression levels of retinoid receptors. As shown in Fig. 1d, cells treated with high concentrations (1 and 10  $\mu$ M) of 9-*cis*-retinoic acid had reduced levels of RAR $\gamma$  and RXR $\alpha$ . Cells treated with 10

$\mu$ M all-*trans*-retinoic acid showed a lower level of RAR $\gamma$ . In contrast, 3-day treatment of 0.1  $\mu$ M 9-*cis*-retinoic acid, 0.1  $\mu$ M all-*trans*-retinoic acid, or 1  $\mu$ M all-*trans*-retinoic acid did not affect the protein level of RAR $\gamma$  and RXR $\alpha$ . The data also showed that retinoic acid treatments could not decrease the protein levels of RAR $\alpha$  and RXR $\beta$ .

**TNF- $\alpha$  and IL-1 $\beta$ , but Not TPA, Can Induce *CYP4F11* mRNA Levels in HaCaT Cells through the c-Jun N-Terminal Kinase Pathway.** We investigated the ability of TNF- $\alpha$  and IL-1 $\beta$  or TPA to induce *CYP4F* transcription in HaCaT cells through mechanisms involving AP-1, as reported for other cell systems (Lamph et al., 1988; Sassone-Corsi et al., 1990; Chedid et al., 1991; Kyriakis, 1999; Yu et al., 2008). We treated HaCaT cells with TNF- $\alpha$  (10 ng/ml) and IL-1 $\beta$  (10 ng/ml) or TPA (100 ng/ml) for 24 h. Both cytokines induced *CYP4F11* mRNA levels significantly, whereas TPA had no effect compared with the vehicle control (Fig. 2a). Luciferase assay also indicated the induction of *CYP4F11* promoter activity by the treatment of 10 ng/ml TNF- $\alpha$  for 24 h (Supplemental Fig. 1). Cytokines and many other factors activate the AP-1 complex through multiple signaling pathways such as the c-Jun N-terminal kinase (JNK), P38, and extracellular signal-regulated kinase pathways (Whitmarsh and Davis, 1996). Hence, we preincubated HaCaT cells with the JNK inhibitor SP600125 or the p38 inhibitor SB203580 for 4 h before administering TNF- $\alpha$  or IL-1 $\beta$ . Inhibiting JNK completely blocked the cytokine-induced *CYP4F11* transcription (Fig. 2, b and c). Inhibiting p38 had no effect on *CYP4F11* expression. After administering TNF- $\alpha$ , phosphorylated c-Jun was observed within 15 min; it was and remained elevated for at least 3 h (Fig. 2, e and f). Inhibiting JNK by SP600125 pretreatment markedly blocked the phosphorylation of c-Jun protein in HaCaT cells (Fig. 2, e and f). In contrast, TPA (100 ng/ml) had no effect on c-Jun phosphorylation (Fig. 2d). Cells were also preincu-



**Fig. 1.** Expression of RARs and RXRs in HaCaT cells and inhibition of *CYP4F11* expression by retinoic acids. a, expression of RARs and RXRs in HaCaT cells. Total RNA was isolated from HaCaT cells, and an aliquot of 200 ng of the total RNA was used to run RT-PCR assay with primers specific for each RAR and RXR isoform. The size of each PCR product is indicated in Table 1. b, inhibition of *CYP4F11* transcripts by retinoic acids. Cells were treated with 0.1, 1, or 10  $\mu$ M 9-*cis*- or all-*trans*-retinoic acid for 3 days. Cells treated with 0.1% DMSO were used as the vehicle control. The expression of *CYP4F11* was quantitated by qRT-PCR. Each data point represents  $n = 5$ . \*\*,  $P < 0.01$  compared with the relative control group. c, inhibition of *CYP4F11* protein expression by retinoic acids. Cells were treated with 10  $\mu$ M 9-*cis*- or all-*trans*-retinoic acid for 3 days. Whole cell lysates were isolated, and totally 30  $\mu$ g of protein from each sample was subjected to Western blot assay using *CYP4F11* antibody. The amount of  $\beta$ -actin expression from each sample was used as the loading control. d, time course and dose effects of retinoic acids on the protein expression of retinoid receptors. Cells were treated with 0.1, 1, or 10  $\mu$ M 9-*cis*- or all-*trans*-retinoic acid and collected at different time points as indicated in the figure. Cells treated with 0.1% DMSO were used as the vehicle control. Whole cell lysates were isolated, and totally 20  $\mu$ g of protein from each sample was subjected to Western blot assay using antibodies specific for RAR $\alpha$ , RAR $\gamma$ , RXR $\alpha$ , and RXR $\beta$ , respectively. The amount of  $\beta$ -actin expression from each sample was used as the loading control.



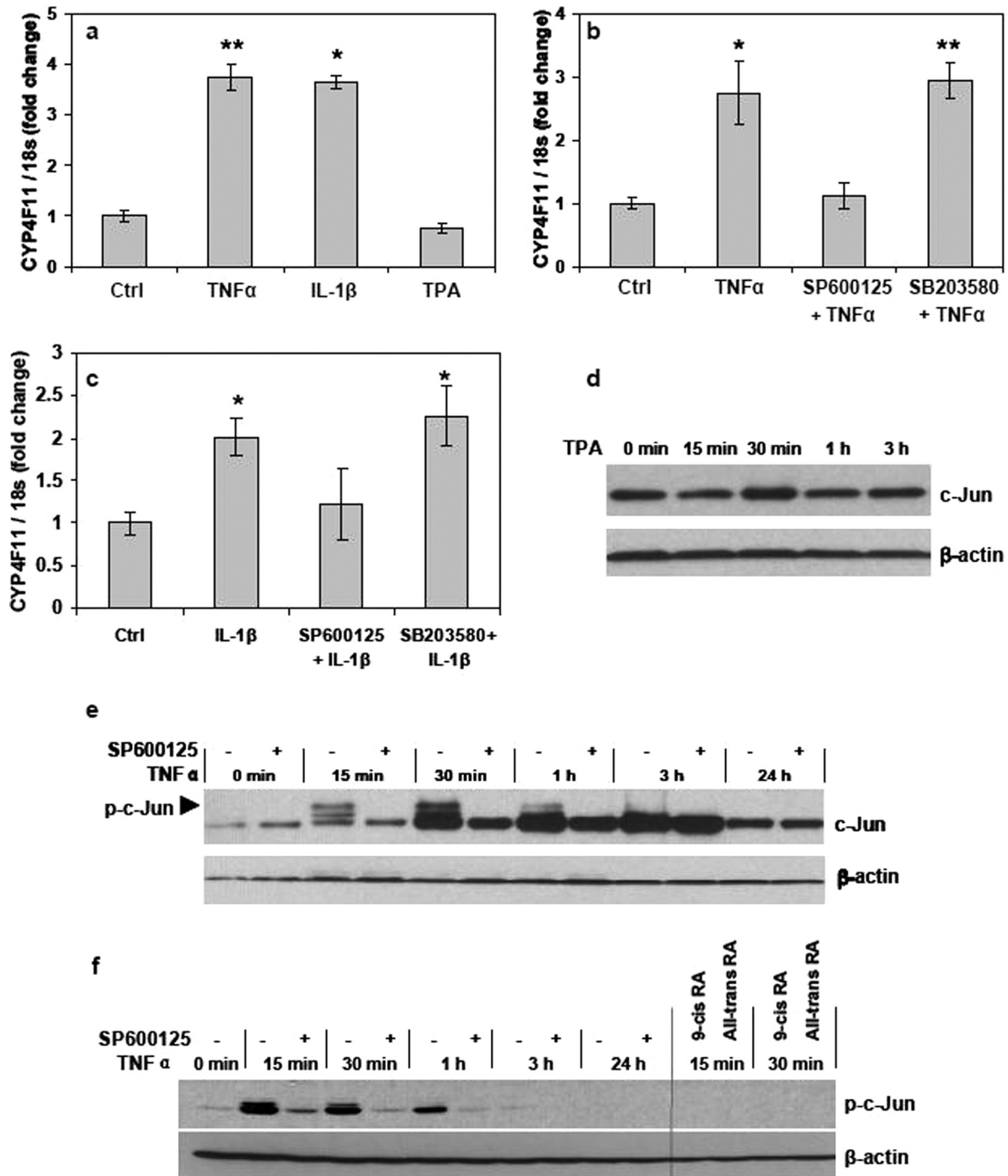


FIG. 2. Induction of *CYP4F11* by cytokines through the JNK pathway. a, effects of cytokines and TPA on *CYP4F11* transcripts in HaCaT cells. Cells were treated with 10 ng/ml TNF- $\alpha$ , 10 ng/ml IL-1 $\beta$ , or 100 ng/ml TPA for 24 h. Cells treated with 0.1% DMSO or 0.1% bovine serum albumin were used as the vehicle control. The expression of *CYP4F11* was quantitated by qRT-PCR. Each data point represents  $n = 5$ . \*,  $P < 0.05$ , \*\*,  $P < 0.01$  compared with the control group. b and c, effects of pathway inhibitors on the induction of *CYP4F11* by cytokines. HaCaT cells preincubated with or without SP600125 (JNK inhibitor) or SB203580 (p38 inhibitor) were treated with 10 ng/ml TNF- $\alpha$  (b) or 10 ng/ml IL-1 $\beta$  (c). At 24 h post-treatment, total RNA was isolated, and the expression of *CYP4F11* was quantitated by qRT-PCR. Cells preincubated with 0.1% DMSO and then treated with 0.1% bovine serum albumin were used as the vehicle control. Each data point represents  $n = 5$ . \*\*,  $P < 0.01$  compared with the relative control group. d, TPA had no effect on c-Jun phosphorylation in HaCaT cells. Cells were treated with 100 ng/ml TPA and collected at different time points as indicated in the figure. Whole cell lysate was isolated from each sample, and 20  $\mu$ g of proteins from each sample was loaded for Western blot assay.  $\beta$ -Actin was used as the loading control. e, effects of TNF- $\alpha$  on c-Jun phosphorylation in HaCaT cells. Cells preincubated with or without 20  $\mu$ M SP600125 were treated with 10 ng/ml TNF- $\alpha$  and collected at different time points as indicated in the figure. Whole cell lysate was isolated from each sample, and 15  $\mu$ g of proteins from each sample was loaded for Western blot assay.  $\beta$ -Actin was used as the loading control. f, inhibition effects of SP600125, 9-*cis*-retinoic acid, and all-*trans*-retinoic acid on TNF- $\alpha$ -induced c-Jun phosphorylation in HaCaT cells. Cells preincubated with or without 20  $\mu$ M SP600125, 1  $\mu$ M 9-*cis*-retinoic acid, or 1  $\mu$ M all-*trans*-retinoic acid were treated with 10 ng/ml TNF- $\alpha$  and collected at different time points as indicated in the figure. Whole cell lysate was isolated from each sample, and 15  $\mu$ g of proteins from each sample was loaded for Western blot assay.  $\beta$ -Actin was used as the loading control.

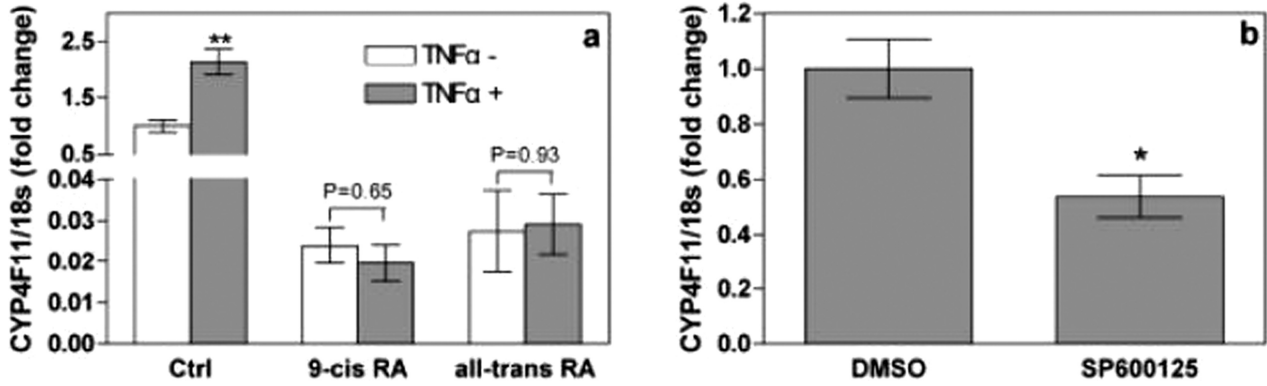


FIG. 3. JNK pathway is not the sole mechanism mediating *CYP4F11* regulation. a, effects of TNF- $\alpha$  on cells treated with retinoic acids. Cells preincubated with or without 10  $\mu$ M 9-*cis*- or all-*trans*-retinoic acid were treated with 10 ng/ml TNF- $\alpha$  for 24 h. The expression of *CYP4F11* was quantitated by qRT-PCR. Each data point represents  $n = 5$ . \*,  $P < 0.05$  compared with the negative control. b, effects of JNK inhibitor SP600125 on *CYP4F11* transcription in HaCaT cells. Cells were treated with 0.1% DMSO (vehicle control) or 20  $\mu$ M SP600125 for 24 h. The expression of *CYP4F11* was quantitated by qRT-PCR. Each data point represents  $n = 6$ . \*,  $P < 0.05$  compared with the control group.

bated with 1  $\mu$ M 9-*cis*- or all-*trans*-retinoic acid for 24 h and then treated with TNF- $\alpha$ . As shown in Fig. 2f, both retinoic acids completely blocked the phosphorylation of c-Jun at 15 and 30 min post-TNF- $\alpha$  administration.

**JNK Pathway Is Not the Sole Mechanism Mediating *CYP4F11* Regulation.** To determine whether the JNK pathway is the primary mechanism regulating *CYP4F11* expression, we treated HaCaT cells with cytokine TNF- $\alpha$  (10 ng/ml) for 24 h after a 3-day pretreatment with 9-*cis*- or all-*trans*-retinoic acid at 10  $\mu$ M concentration. TNF- $\alpha$  alone increased *CYP4F11* mRNA levels, and pretreatment with either retinoic acid blocked this induction (Fig. 3a). Hence, TNF- $\alpha$  could not reverse the inhibitory effects of retinoic acids on *CYP4F11* transcription. When we treated HaCaT cells with the JNK inhibitor SP600125 alone (20  $\mu$ M for 24 h), *CYP4F11* mRNA levels decreased by only 50% (Fig. 3b). At this concentration, SP600125 completely blocked the TNF- $\alpha$ -induced *CYP4F11* transcription (Fig. 2b). Inhibiting the JNK pathway did not totally suppress the transcription of *CYP4F11*, indicating other signaling pathways positively regulate *CYP4F11* expression in HaCaT cells.

**Opposing Effects of RAR and RXR Agonists on *CYP4F11* Expression in HaCaT Cells.** Because retinoic acids activate both RARs and RXRs, we investigated which NR(s) mediate the inhibitory

effects of retinoic acids on *CYP4F11* expression. We treated HaCaT cells for 2 or 3 days with the pan agonists for RXR (1  $\mu$ M LG268, also known as LG100268) and RAR (10  $\mu$ M TTNPB). The RXR pan agonist LG268 increased *CYP4F11* mRNA levels 12-fold versus vehicle (Fig. 4a). We confirmed this induction by visualizing RT-PCR products that were amplified using different primer sequences (Fig. 4b) and by Western blot analyses (Fig. 4c). *CYP4F11* protein levels were greater in cells treated with LG268 for 2 and 3 days compared with vehicle control. In HaCaT cells treated with the RAR pan agonist TTNPB, *CYP4F11* mRNA levels were greatly decreased versus vehicle, and combined treatment with both pan agonists for 3 days decreased *CYP4F11* mRNA levels even further (Fig. 5a). Western blot analyses confirmed the markedly decreased *CYP4F11* protein levels elicited by TTNPB treatment with or without LG268 (Fig. 5b).

**Discussion**

*CYP4Fs* catalyze the metabolism of both endogenous and exogenous molecules (Kalsotra and Strobel, 2006). For example, they inactivate the leukotriene and prostaglandin prompts for the inflammation cascade playing an anti-inflammatory role (Kikuta et al., 2002), and they also catalyze the metabolism of many drugs (Hashi-

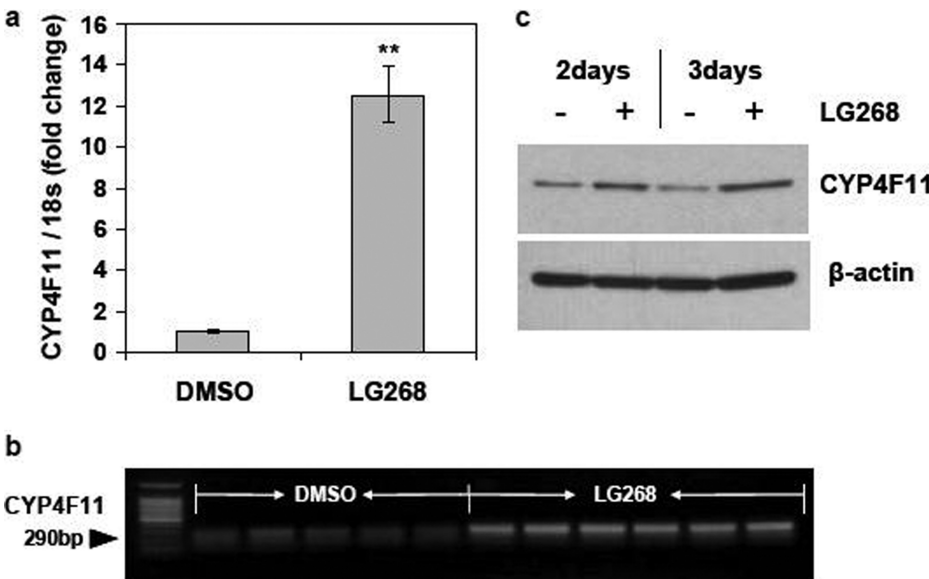


FIG. 4. Effects of LG268 on *CYP4F11* expression in HaCaT cells. a and b, effects of LG268 on the transcription of *CYP4F11*. HaCaT cells were treated with DMSO (vehicle control,  $n = 5$ ) or 1  $\mu$ M LG268 ( $n = 6$ ) for 3 days. The *CYP4F11* mRNA level was quantitated by qRT-PCR (a). \*\*,  $P < 0.01$  compared with the control group. Aliquots of 200 ng of total RNA from each sample were used to run RT-PCR (b) with primers specific for *CYP4F11*. c, effects of LG268 on expression of *CYP4F11* protein. HaCaT cells were treated with or without 1  $\mu$ M LG268 for 2 or 3 days. Cells treated with 0.1% DMSO were used as the negative control. Whole cell lysate was isolated, and 20  $\mu$ g of total protein from each sample was loaded to run Western blot assay.  $\beta$ -Actin was used as the loading control.

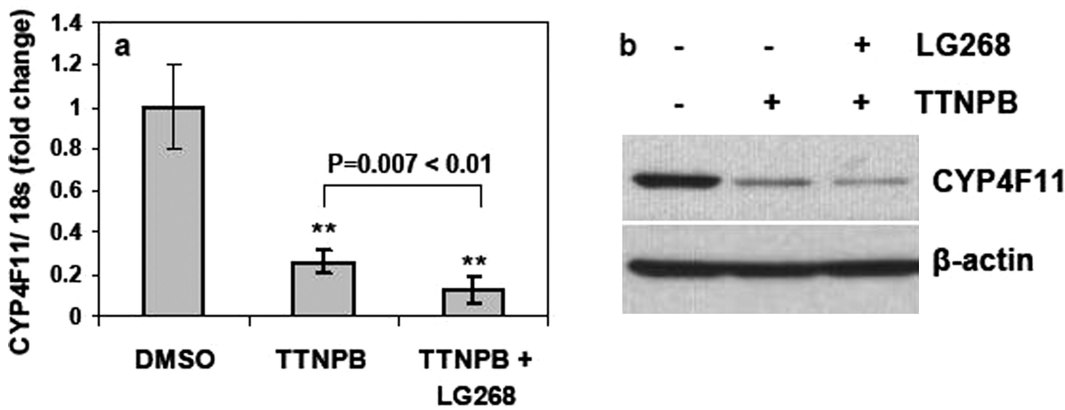


FIG. 5. Effects of TTNPB on *CYP4F11* expression in HaCaT cells. HaCaT cells were treated with 0.1% DMSO, 1  $\mu$ M TTNPB, or 1  $\mu$ M TTNPB plus 1  $\mu$ M LG268 for 3 days. a, the *CYP4F11* mRNA level was quantitated by qRT-PCR. Each data point represents  $n = 6$ . \*\*,  $P < 0.01$  compared with the control group. b, whole cell lysate was isolated, and 30  $\mu$ g of total protein from each sample was loaded to run Western blot assay.  $\beta$ -Actin was used as the loading control.

zume et al., 2002; Kalsotra et al., 2004). Among the human CYP4F enzymes, CYP4F11 is most active in metabolizing therapeutic drugs. To elucidate the role of CYP4F11, it is necessary to decipher the mechanism of *CYP4F11* gene regulation under various physiological and pathological conditions. The results of our present study have shown that several distinct but interacting pathways of gene regulation impinge on the regulatory region of the *CYP4F11* gene in HaCaT cells. We report for the first time that two possible pathways, the JNK/AP-1 pathway and the RXR-mediated pathway, are involved in the regulation of *CYP4F11* in HaCaT cells. These pathways and their interactions are depicted in the scheme as shown in Fig. 6b.

Our present study shows that retinoids down-regulate *CYP4F11* expression in HaCaT cells. The anti-AP-1 activity of retinoids supports the positive regulation of *CYP4F11* through the AP-1 complex by inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$ . Moreover, this induction of *CYP4F11* was shown to be mediated by the JNK pathway as indicated by the loss of cytokine induction of *CYP4F11*, as well as c-Jun phosphorylation in the presence of JNK inhibitor SP600125. TPA had no effect on *CYP4F11* mRNA levels in HaCaT, which is consistent with previous findings that TPA did not induce JNK activity in HaCaT cells (Zhou et al., 2000). *CYP4F11* expression was strongly induced by addition of the cytokines TNF- $\alpha$  and IL-1 $\beta$  but not TPA, indicating the efficacy of AP-1 sites in regulation of *CYP4F11* mRNA expression. The blockage of TNF- $\alpha$ -induced *CYP4F11* expression and c-Jun phosphorylation by 9-*cis*- or all-*trans*-retinoic acid confirms the role of AP-1 transrepression in down-regulating *CYP4F11*. Furthermore, five AP-1 sites were found using AliBaba2.1 program (BIOBASE) within the first 2000 bases upstream of the start site for *CYP4F11* (Fig. 6a). These results support transrepression of AP-1 complexes as a mechanism whereby retinoic acids suppress *CYP4F11* transcription, as shown for other gene systems (Saatcioglu et al., 1994; Chen et al., 1995).

The transrepression mechanism proposed here is not adequate to explain the findings that 9-*cis*- or all-*trans*-retinoic acid almost completely eliminated *CYP4F11* expression, whereas SP600125 alone only showed moderate inhibition, although SP600125 preincubation could completely block the induction of *CYP4F11* by TNF- $\alpha$ . It appears that the inducible regulation is different from the basal regulation through JNK activity. As also proposed in the scheme of Fig. 6b, retinoic acids bind to retinoid receptors and activate the formation of RAR/RXR heterodimers. RXRs, in conjunction with one or more other NRs, are proposed to stimulate *CYP4F11* transcription by acting through the hormone responsive element. On the other hand, the formation of RAR/RXR heterodimers reduces the number of RXRs available for interaction with NRs, thereby suppressing the transcription of *CYP4F11* through competition for RXRs. Thus, retinoid treatment of proliferating HaCaT cells leads to reduced expression of

*CYP4F11* through transrepression of the AP-1 pathway and/or by competition for RXRs. RAR/RXR heterodimers lead to down-regulation, whereas NR/RXR heterodimers would lead to up-regulation of *CYP4F11* expression.

Our results indicate that simultaneous activation of RARs and RXRs intensifies the inhibition of *CYP4F11* in HaCaT, which is consistent with data in Fig. 1b, given the fact that 9-*cis*-retinoic acid is able to activate both RARs and RXRs, whereas all-*trans*-retinoic acid only activates RARs (Leid et al., 1993; Fisher and Voorhees, 1996). It is worth noting that retinoids are able to activate retinoic acid 4-hydroxylase within hours (Marikar et al., 1998). Retinoic acid 4-hydroxylase hydroxylates all-*trans*- but not 9-*cis*-retinoic acid. Therefore, the activation of retinoic acid 4-hydroxylase may result in the reduced level of all-*trans*-retinoic acid, which may make all-*trans*-retinoic acid less effective than 9-*cis*-retinoic acid in the inhibition of *CYP4F11*. However, the distinct effects of TTNPB and LG268 on *CYP4F11* expression increase the possibility of a competition mechanism and allow for the potential existence of an NR that can directly activate *CYP4F11* transcription. It seems unlikely that RXR homodimers are involved in the induction of *CYP4F11* because 9-*cis*-retinoic acid, which is able to promote the formation of RXR homodimers (Zhang and Pfahl, 1993), inhibited *CYP4F11* to a greater extent than the specific RAR ligand all-*trans*-retinoic acid did. In addition, it has been reported that endogenous RAR/RXR heterodimers are the major functional forms regulating retinoid-responsive elements in human keratinocytes (Xiao et al., 1995), which further seems to exclude RXR homodimers as primary regulator for *CYP4F11* expression in HaCaT cells. Therefore, the partner of RXR in the up-regulation of *CYP4F11* could be other NRs that were not tested in this study or some unknown orphan NRs whose interaction with RXRs has not yet been clarified. It is worth noting that data from the luciferase assay (Supplemental Fig. 1) showed the down-regulation of *CYP4F11* promoter activity by retinoic acids and RAR agonist TTNPB, whereas RXR agonist LG268 was not able to induce the promoter activity. This promoter may be ascribed to the limited length of the promoter sequence we used in this assay. Two putative RXR- $\beta$  binding sites were found in this promoter region (Fig. 6a). However, it is possible that other untested or unknown RXR binding sites beyond the current sequence are essential for the regulation of *CYP4F11* by RXR agonists.

It is also worth noting that differences in cell type and cell status may greatly affect the regulation pattern of *CYP4F11*. We have previously reported that retinoid treatment of differentiated human primary keratinocytes induced the expression of *CYP4F11* and other *CYP4F* subfamily members, and this induction response seems to be mediated by RAR/RXR heterodimers (Kalsotra et al., 2008). These differences in response to retinoid treatments are likely attributable to

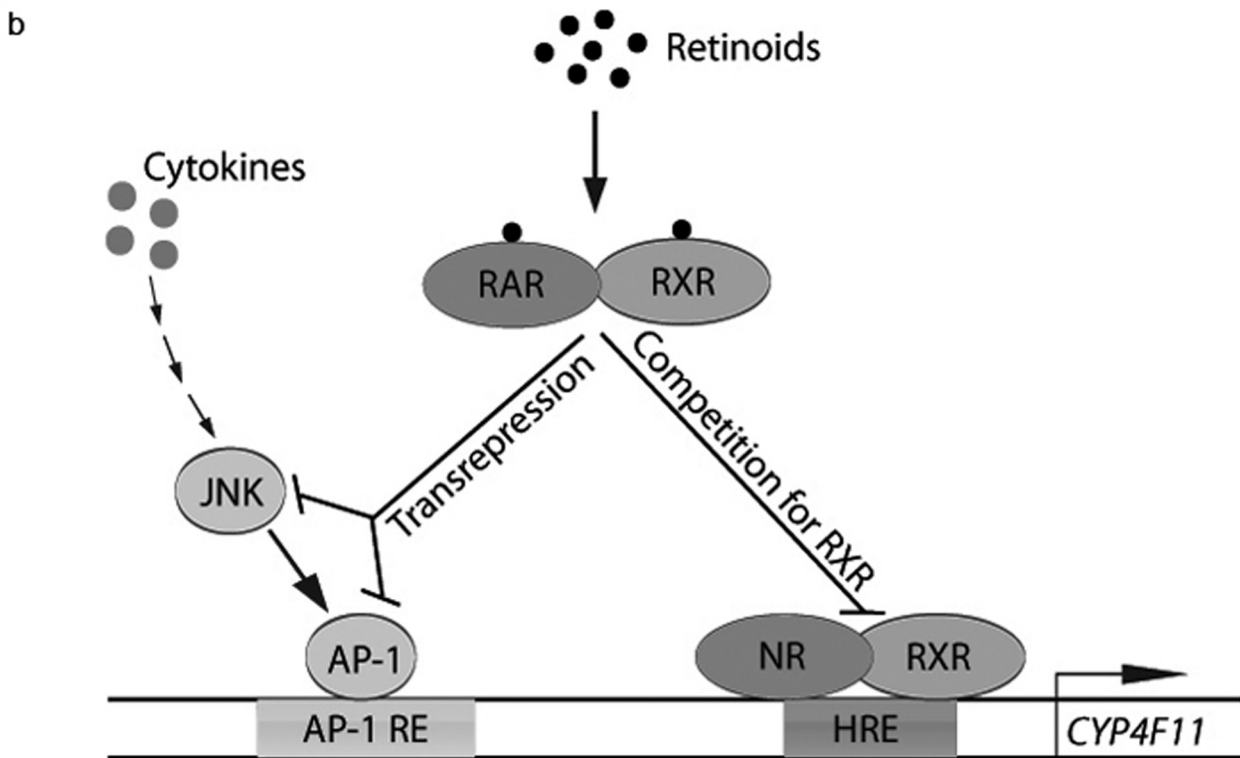
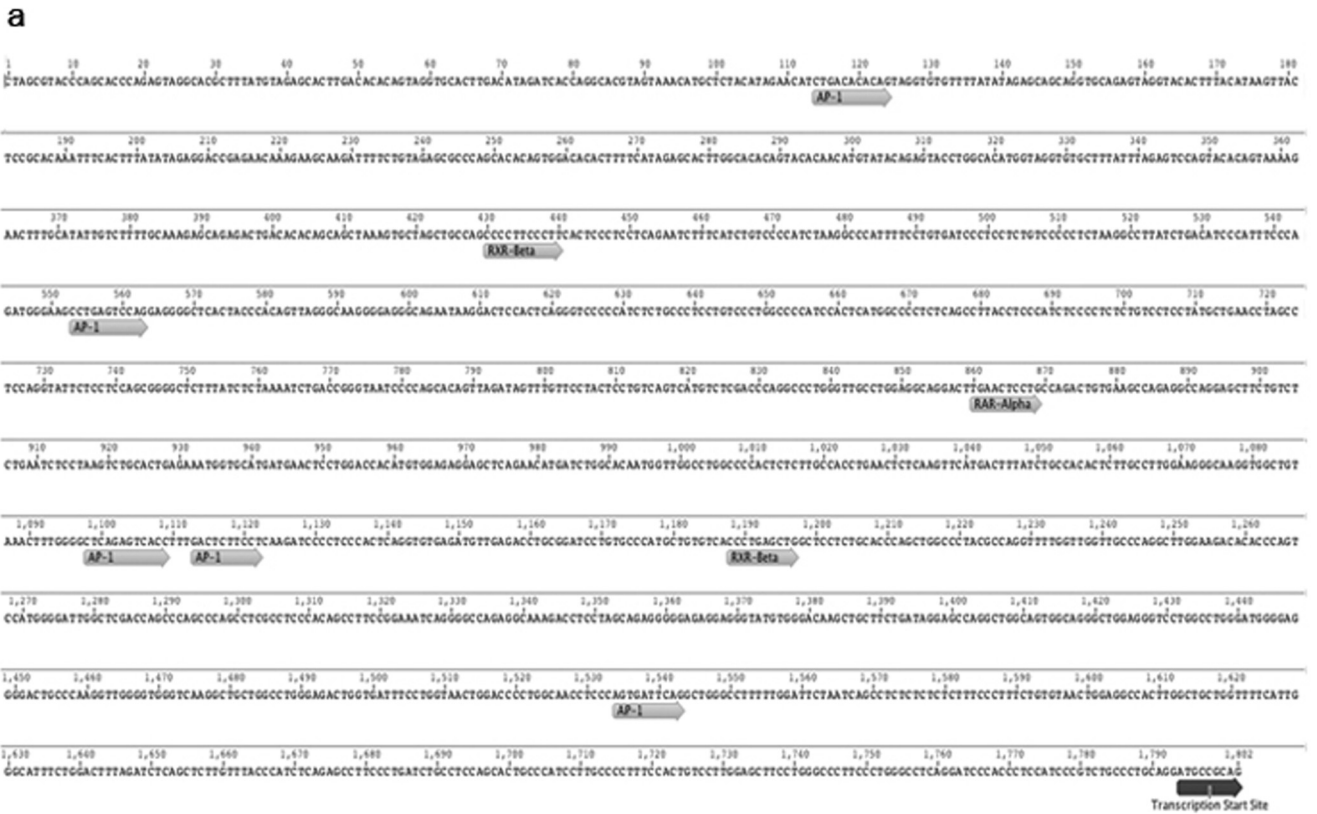


FIG. 6. Scheme for *CYP4F11* regulation. a, putative hormone responsive element and AP-1 binding sites in the promoter region of *CYP4F11* gene. Sites shown were predicted using AliBaba2.1 (BIOBASE). Figure was generated using Biomatters Geneious Pro 4.5.3 software (<http://www.biomatters.com/>). b, *CYP4F11* regulation model. The JNK/AP-1 pathway and the RAR/RXR pathway modulate the transcription of *CYP4F11* in HaCaT cells. Adobe (San Jose, CA) Illustrator CS4 was used to draw this model.



the fact that HaCaT cells are a spontaneously arising cell line derived from keratinocytes. Cultures state whether proliferating or differentiated plays a significant role in the type of response to retinoid treatment, although the expression pattern of retinoid receptors in HaCaT cells (Fig. 1a) appears to be the same as primary human keratinocytes (Elder et al., 1992). Such differences are also seen in the comparison of expression levels of CYP4Fs among various tissues. For example, in rat lung CYP4F1 is absent and CYP4F6 comprises 95% of CYP4F expression, whereas in kidney CYP4F1 expression comprises 90% of CYP4F expression and CYP4F6 expression accounts for only 8% (Kalsotra and Strobel, 2006).

Retinoic acids are important for skin development, and they are also therapeutic agents against many skin diseases such as inflammation and cancer. Therefore, the regulation of CYP4F11 by retinoic acids may result in the drug-drug interaction between retinoic acids and CYP4F11-targeted drugs. In addition, inflammation and cancer, which stimulate JNK/AP-1 pathway, may also affect CYP4F11 drug-metabolizing activity. Although CYP4F11 is the most active drug metabolizer in the CYP4F subfamily, no primary endogenous substrate for CYP4F11 has been uncovered. As the functions of CYP4F11 in normal physiological conditions and under disease conditions are clarified, CYP4F11 regulatory mechanisms will be known more completely, and their significance will become clearer.

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**Address correspondence to:** Henry W. Strobel, The University of Texas-Houston Medical School, 6431 Fannin Street, Houston, TX 77225. E-mail: henry.w.strobel@uth.tmc.edu

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