Liver X Receptor-Retinoid X Receptor (LXR-RXR) Heterodimer Cistrome Reveals Coordination of LXR and AP1 Signaling in Keratinocytes

Qi Shen, Yuchen Bai, Ken C. N. Chang, Yongjun Wang, Thomas P. Burris, Leonard P. Freedman, Catherine C. Thompson, and Sunil Nagpal

From the Department of Women’s Health and Musculoskeletal Biology, Nuclear Receptors and Dermatology, Wyeth Research, Collegeville, Pennsylvania 19426 and The Scripps Research Institute, Jupiter, Florida 33458

Liver X receptors (LXRs) play a critical role in regulating lipid synthesis and transport in numerous tissues. In the skin, activation of LXR induces keratinocyte differentiation and improves epidermal permeability barrier homeostasis. To elucidate the mechanism of LXR action in skin, we mapped its cistrome by identifying LXRβ-RXRα binding sites using ChIP-on-chip in normal human epidermal keratinocytes (NHEKs). The cistrome was integrated with transcription data to obtain a global view of LXR action in keratinocyte biology. Here, we identify 2035 LXRβ-RXRα binding sites containing 4794 LXR response elements in NHEKs and show the presence of consensus heterodimer active regions in genes involved in keratinocyte lipid transport/synthesis and terminal differentiation. Bioinformatics analysis of the cistrome revealed an enrichment of AP1 cis-regulatory motifs in the vicinity of the LXRβ-RXRα binding sites. Importantly, we have demonstrated a direct interaction between LXR and Jun/Fos, indicating that the cooperation between LXR and AP1 may orchestrate keratinocyte differentiation. Finally, we corroborated these results by genome-wide mapping of the c-Fos and c-Jun cistromes in NHEKs, demonstrating that 77% of all the LXRβ-RXRα binding regions show the presence of AP1 motifs at adjacent locations. Our findings provide new insight into the mechanism of LXR action in keratinocyte differentiation, lipid production and barrier formation, further strengthening the validation of LXR as a potential therapeutic target for skin disorders including skin aging, psoriasis, and atopic dermatitis.

Liver X receptors (LXα1/NR1H3 and LXα2/NR1H2) are nuclear receptors that play critical roles in lipid metabolism and transport. Both LXR isoforms are also expressed in the skin, including in epidermal keratinocytes and fibroblasts (1–3). An ordered process of keratinocyte differentiation and lipid production in the epidermis results in the formation of a water-impermeable barrier, which is crucial for all of the physiological functions of the skin. LXRs are important regulators of epidermal biology, because their activation by specific ligands leads to stimulation of keratinocyte differentiation, epidermal lipid synthesis, and anti-inflammatory responses in skin cells (1–3). LXR ligands stimulate keratinocyte differentiation by inducing the expression of genes involved in cornified envelope formation, namely transglutaminase 1 (TGM1), involucrin (IVL), loricrin (LOR), and filaggrin (FLG) (3, 4). Epidermally produced neutral lipids such as cholesterol, ceramides, and fatty acids are essential for skin barrier formation and maintenance. Accordingly, LXR agonists upon topical application augment epidermal lipid synthesis in murine skin (5), presumably by inducing expression of the ABC family of lipid transporters and lipid synthesis genes. LXR ligands induce the expression of lipid transporters (ABCA1, ABCG1, ABCA2, ABCA12, and ABCA13) and lipid synthesis genes (SCD and FASN) in keratinocytes (3, 6). ABCA1 and ABCG1 are known cholesterol transporters (1), and ABCA12 gene mutations in mice and humans severely affect epidermal barrier formation (7, 8). In addition, a synthetic LXR ligand also increases the expression of genes involved in ceramide synthesis (3). These studies indicate that an LXR ligand might be an important regulator of cutaneous physiology in normal and disease states. Accordingly, LXR ligands show efficacy in murine models of atopic dermatitis, irritant dermatitis, epidermal proliferation, and photoaging (2, 3). Therefore, LXR ligands have tremendous potential as therapeutic agents for the treatment of skin inflammatory indications including skin aging. However, the mechanism of LXR action in skin, particularly in epidermal keratinocytes, is poorly understood. A limited number of LXR-responsive genes have been identified in skin cells, and of these only six (SREBF1, FASN, SCD1, ABCA1, ABCG1, and ApoE) have been shown to harbor an LXR-responsive element (LXRE) in their regulatory sequences (9–13). In addition, oxysterol LXR ligands have been shown to enhance the expression of the IVL gene by inducing AP1 (Jun/Fos) family proteins (14). Therefore, LXR agonists also have the potential to increase the expression of AP1-responsive keratinocyte differentiation genes by enhancing the expression of AP1 family members.
In recent years, genome-wide cistrome identification studies have elucidated the mechanism of estrogen receptor (ERs), androgen receptor (AR), and peroxisome proliferator-activated receptor (PPARγ) action in breast cancer cells, prostate cancer cells, and adipocytes, respectively (15–18). These cistrome studies not only have resulted in the identification of previously unknown cis-regulatory motifs but also reveal the identity of interacting transcription factors that cooperate with these nuclear receptors (15–18). Taking a cue from these studies, we decided to explore LXR action in the epidermis by genome-wide identification of LXR-RXR heterodimer binding sites in normal human epidermal keratinocytes (NHEKs) using chromatin immunoprecipitation (ChIP) coupled with on-chip (ChIP-on-chip) detection of genomic fragments. The combination of LXR cistrome and transcriptome studies described herein delineates an elaborate role of this nuclear receptor in the processes of epidermal differentiation, lipid synthesis, and transport by identifying new LXR-responsive genes involved in these pathways. Notably, we have uncovered co-enrichment of LXR and AP1 motifs, indicating the intersection of these two major pathways in keratinocytes. Our studies validate LXR as a target for skin aging and atopic dermatitis and, for the first time, provide evidence that LXR may also be a novel target for the treatment of psoriasis.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Three copies of PADI LXRE (5'-GGATCGTTAAGGTCA-3') were cloned into pTAL-Luc (Clontech, Mountain View, CA) through MluI and BglII. LXRα was cloned into pCDNA3.1 vector (Invitrogen) to construct the PADI-LXRE-Luc reporter. pCDNA3.1-LacZ was from Invitrogen, and pGL4.73 was from Promega (Madison, WI). For the construction of a reporter containing the PADI4 upstream region (921 bp) reporter containing the LXRE (PADI4–921bp-Luc), a fragment (chromosome 1: 17616464–17617385) including the LXRE was cloned into pTAL-Luc vector (Clontech) through MluI and XhoI. The primers used in PCR were: AGGGCTGCGGAGGGCTATCAT (forward) and GCCCTTACTAAGGTGCTGGGGA (reverse). The mutant construct was made using QuickChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) through MluI and XhoI. The primers used in PCR were: ACCTGCCTTAGCCTC (reverse).

Cell Culture and Cotransfections—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C under 5% CO2. Cells were plated in 96-well plates at a density of 15 × 103 cells/well 24 h before transfection. Each transfection contained 100 ng of the PADI-LXRE-Luc reporter, 50 ng of the pGL4.73 reporter (Renilla luciferase internal control reporter), and 50 ng of the receptor or control vector. Eight hours post-transfection, the cells were treated with LXR ligand GW39665 or dimethyl sulfoxide. Twenty-four hours post-treatment, the luciferase activity was measured using the Dual-Glo luminometer assay system (Promega). The values indicated represent the means ± S.E. from three independently transfected wells. The experiments were repeated three times, and representative experiments are shown.

Skin Cells—NHEKs (Cambrex/Lanza, Walkersville, MD) were cultured as per the vendor’s recommendations. In general, cells were trypsinized and seeded on day 0 and treated with T1317 (1 μM) on day 1. The cells were harvested on day 2 with lysis buffer (Applied Biosystems/Ambion, Foster City, CA) added directly to the cultured cells after a PBS wash. NHEKs were either used for RNA purification using Qiagen RNeasy RNA purification column (Qiagen, Hilden, Germany) as per the vendor’s protocol or processed directly to cDNA using “cell-to-cDNA” lysis buffer (Ambion).

LXβ WT and KO Skin and Keratinocytes—LXβ KO mice were obtained from Deltan (San Carlos, CA) in the 129 strain and backcrossed for seven generations into black C57BL/6J mice. LXβ KO was accomplished with LXβ gene sequence deletion from base 226 to 395 by using a homologous recombination vector (Deltan). Skins from newborn mice (2–3 days old) were isolated, floated on 2.5 mg/ml Dispase (Invitrogen) overnight at 4 °C, and then separated into epidermal and dermal layers using small forceps. The epidermal layer was minced, and keratinocytes were isolated as described previously (19). Keratinocytes were cultured in Eagle’s minimal essential medium containing fetal bovine serum (8%) in 24-well culture plates (day 0). Cells were treated with vehicle or T1317 on day 2 followed by isolation and purification of RNA on day 3 using RNeasy column (Qiagen). Gene expression profiles were analyzed by TaqMan qPCR gene assays (Applied Biosystems). The skin from LXβ KO and C57BL/6J mice obtained as described above was rinsed with PBS, harvested, and stored at −80 °C. Total RNA was isolated from the tissue and purified using a Qiagen RNeasy RNA purification column. Hairless mice (SKH1, Charles River Laboratories) were treated with T1317 (10 mM) applied topically twice a day for 3 days, and skin was harvested as described above. RNA was isolated and analyzed by real-time PCR.

Chromatin Immunoprecipitation—NHEKs were fixed with 1% formaldehyde for 15 min and quenched with 0.125 M glycine. Chromatin was isolated by adding lysis buffer followed by disruption with a Dounce homogenizer. Lysates were sonicated and the DNA sheared to an average length of 300–500 bp. Genomic DNA (input) was prepared by treating aliquots of chromatin with RNase and proteinase K, heating the samples for de-cross-linking and ethanol precipitation. Pellets were resuspended, and the resulting DNA was quantified using a NanoDrop spectrophotometer. Extrapolation to the original chromatin volume allowed quantitation of the total chromatin yield. An aliquot of chromatin (30 μg) was precleared with protein A/G-agarose beads with (Invitrogen). Factor-bound DNA sequences were isolated using antibodies against LXβ (sc-1000, sc-13068, sc-1001, and sc-34343), RXRα (sc-553), c-Fos (sc-7202), or c-Jun (sc-1694) (Santa Cruz Biotechnology). After incubation at 4 °C overnight, protein A/G-agarose beads with isolated immune complexes were washed, eluted with SDS buffer, and subjected to RNase and proteinase K treatments. Cross-links were reversed by incubation overnight at 65 °C, and ChIP DNA was purified by phenol-chloroform extraction and ethanol precipitation. Quantitative PCR (qPCR) reactions were
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carried out in triplicate on specific genomic regions using SYBR Green Supermix (Bio-Rad). The resulting signals were normalized for primer efficiency by carrying out qPCR for each primer pair using input DNA.

**ChIP-on-chip Analysis**—ChIP and input DNAs were amplified by whole genome amplification using the GenomePlex WGA kit (Sigma). The resulting amplified DNA was purified, quantified, and tested by qPCR at the same specific genomic regions as the original ChIP DNA to assess the quality of the amplification reactions. Amplified DNA was fragmented and labeled using the DNA terminal labeling kit (Affymetrix) and then hybridized to Affymetrix GeneChip Human Tiling 2.0R array sets at 45 °C overnight. Arrays were washed and scanned, and the resulting CEL files were analyzed with tiling analysis software (TAS; Affymetrix) or microarray analysis tool (MAT) software (20). The Affymetrix TAS analysis was conducted by GenPathway Inc. Briefly a two-sample analysis was performed comparing each CEL file of the ChIP samples against the CEL file from the input DNA array. The resulting BAR (binary analysis results) files contain the signal values for all probes on the arrays. Signal values are “estimates of fold enrichment” of ChIP DNA, which in essence are ratios (in linear scale) between the intensity of the probes on the ChIP DNA array divided by the intensity of the corresponding probe on the input DNA array. To make the values more significant, however, these ratios are computed by applying averaging and ranking steps to a set of probes within a 400-bp sliding window. An “interval” is a discrete genomic region as defined by the chromosome number and a start and end coordinate that represents the locations of signal peaks. For each BAR file, intervals are calculated and compiled into BED files. The three TAS parameters that determine the intervals are: threshold = 2.5; MaxGap = 300; and MinRun = 180. Overlapping intervals among different samples are grouped into active regions. MAT 2.09 was used with probes remapped to the hg18 Human Genome Assembly. The threshold cutoff for binding regions was FDR ≤ 1%.

LXR, RXR, and AP1 binding sites were considered overlapping when there was at least 1 bp in common between the binding regions. Screen shots of LXR and RXR binding regions relative to individual RefSeq genes in there native chromosomal locations were obtained from the UCSC (University of California-Santa Cruz) Genome Browser. An active region within 10 kb upstream or downstream of a gene is counted as being associated with that gene.

**Sequence Conservation and Enriched Motif Analysis**—The LXR, RXR, and AP1 ChIP active regions were aligned at their centers and uniformly expanded to 3 kb in each direction, and phastCons scores were retrieved and averaged at each position. The cis-regulatory element annotation system (CEAS) (21) was used to analyze the transcription factor motifs that are located relative to individual RefSeq genes in there native chromosomal locations.

**Global Map of LXRβ-RXRα Heterodimer Binding Sites in Keratinocytes**—We postulated that an overlay of LXRβ and RXRα target binding sites would be the best possible way to identify accurately LXRβ and ultimately heterodimer binding sites resulting from the endogenous complement of nuclear receptors. To identify potential binding sites in relevant epidermal cells, we used NHEKs. ChIPs for endogenous LXRβ and RXRα were performed using specific antibodies to these nuclear receptors. Four LXRβ antibodies were compared by ChIP-qPCR on previously characterized LXREs present in ABCA1 and SREBF1 genes (11, 12) in vehicle- or synthetic LXR ligand T1317 (23)-treated NHEKs. Two LXRβ antibodies provided the best signals on the ABCA1 and SREBF1 LXREs relative to a negative control sequence, and one of these (sc-1001) was used for genome-wide ChIP-on-chip (supplemental Fig. S1A). Note that the ligand treatment did not result in any increased enrichment of LXRE target sites, thus indicating that the ligand treatment did not affect LXRβ occupancy of the LXREs. The RXRα antibody used herein showed specific enrichment of ABCA1 and CYP24A1 LXRE and VDRE target sites, respectively, compared with the control DNA (supplemental Fig. S1B). To detect LXRβ-RXRα heterodimer binding sites on a genome-wide scale in an unbiased manner, ChIP-on-chip was employed on NHEKs. Keratinocytes were subjected to LXRβ and RXRα ChIPs followed by hybridization to Affymetrix whole genome arrays. Enriched binding sites were identified by analysis with TAS (GenPathway, Inc.) and MAT (20) tiling array algorithms using a threshold cutoff of =1% false discovery rate for LXR and ≤5% for RXR. This analysis identified a total of 4907 and 16,192 active regions bound by LXRβ and RXRα, respectively (Fig. 1A). Among these, 2035 (41% of the LXRβ binding regions) were shared by LXRβ and RXRα ChIP-on-chip experiments and, therefore, represented LXRβ-RXRα heterodimer binding sites. The 2035 unique regions of ~500 bp in length included known LXREs present in ABCA1, ABCG1, SREBF1, APOE, and SCD1 genes and contained 4794 potential LXREs when scanned by TransFac motif analysis (24). Eighty-two percent of the LXRβ-RXRα binding regions identified herein (1666 of 2035) showed the presence of at least one LXRE motif when scanned against TransFac LXRE matrices M00647 and M00766 (Fig. 1B). The LXRE matrix created by only the highly scored (92.3% match of TransFac LXRE matrix M00766) identified LXREs (total, 142 putative LXREs) (Fig. 1C, left panel), as well as that created by all of the 4794 potential LXREs present in the 2035 binding regions, is also presented (Fig. 1C, right panel).

**Identification and Validation of Novel LXRβ-RXRα Heterodimer Binding Sites**—Apart from the known LXR-RXR binding sites present in the ABCA1, ABCG1, SREBF1, APOE,
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and SCD1 genes, all other heterodimer binding sites identified in these and other annotated genes represent newly identified (hereto after referred to as "novel") LXREs. To validate the robustness of the heterodimer binding sites found with ChIP-on-chip, 16 of the target sites including 14 novel LXRβ-RXRα active regions were also tested by ChIP-qPCR using LXRβ and RXRα antibodies. The sequences of primer pairs used for qPCR analysis are presented in supplemental Table S1. As shown in Fig. 2A, the LXRβ and RXRα antibodies recognized not only the known LXREs present in ABCA1 and ABCG1 but also 14 other novel LXREs present in other genes. Therefore, by employing antibodies to two heterodimeric partners we can predict with 100% confidence that the sites identified herein are indeed true LXREs that are occupied in situ by LXRβ and RXRα. However, we do not rule out the possibility that we might have missed some binding regions, e.g. the LXRE present in FASN was not picked by our ChIP-on-chip in NHEKs. We employed the CEAS to map the novel LXRβ and RXRα binding sites relative to annotated genes in the human genome (21). A comparison of sequences from all of the LXRβ and RXRα binding sites among the genomes of various vertebrates revealed high conservation within the binding sites but not in the surrounding regions (Fig. 2B). CEAS analysis revealed that the majority of the LXRβ-RXRα binding sites (62.5%) were in the distal intergenic regions or within introns (29.5%), and only 2.2% were located at the proximal promoter regions (Fig. 2C).

Lipid Transport and Synthesis Genes Contain Novel LXRβ-RXRα Active Regions—The ABC family of lipid transporters (ABCA1, ABCG1, and ABCA12) are LXR-responsive genes in keratinocytes (3), and LXREs have been identified in the ABCA1 and ABCG1 genes (10, 11). Here, we demonstrate that these genes contain multiple LXRβ-RXRα heterodimer binding sites (Fig. 3A). Therefore, most of the binding sites identified in the classical and most studied LXR-responsive genes (ABCA1 and ABCG1) are novel. ABCA1 contains a known LXRE in the proximal promoter region, and we found two additional LXRβ-RXRα heterodimer active regions. ABCG1 is known to contain one LXRE, which is present in the proximal promoter region (Fig. 3A). Here we have identified two LXRβ-RXRα binding regions that are present in the proximal promoter and also 50 kb upstream of the start site (Fig. 3A). LXR-RXR binding regions were also identified upstream of the ABCA12 gene, an ABC family lipid transporter LXR-responsive gene in keratinocytes. Notably, mutations in ABCA12 in humans cause harlequin ichthyosis, a disorder characterized by extremely dry and scaly skin as a consequence of perturbed barrier function (7). These results also imply that an LXR ligand could be used for the treatment of certain forms of ichthyosis. Known keratinocyte LXR-responsive genes that are involved in fatty acid synthesis, namely SREBF1 and SCD1, also contain two LXRβ-RXRα heterodimer active regions (Fig. 3A). In addition, transcriptional profiling analysis identified fatty acid desaturases Fads1 (Δ5 desaturase) and Fads2 (Δ6 desaturase), enzymes involved in the biosynthesis of polyunsaturated fatty acids, as LXR-responsive genes, because their expression was up-regulated by T1317 in mouse skin (Fig. 3B). Both FADS1 and FADS2 showed the presence of a newly identified LXRβ-RXRα binding site in their upstream regions (Fig. 3A).

ACSL family members play a key role in higher order lipid synthesis and long chain fatty acid degradation. Here, we demonstrate that Acsl3 is a newly identified LXR-responsive gene,
because its expression was up-regulated by T1317 in wild type murine primary keratinocytes and fibroblasts and not in LXRβ null skin cells (Fig. 3C). As expected, ChIP-on-chip identified the presence of two LXR-RXR active regions, one in the proximal promoter and another in the distal intergenic region in the human ACSL3 gene (Fig. 3A). The above results on the LXR-RXR cistrome, in conjunction with transcriptional profiling, not only revealed novel heterodimer binding regions but also identified or substantiated the LXR responsiveness of a number of genes involved in keratinocyte lipid transport, lipid synthesis, fatty acid synthesis, and fatty acid transport/binding pathways.

**LXR Cistrome, Transcriptome, and Keratinocyte Differentiation**—Transcriptional profiling of LXR wild type versus knock-out murine skin also revealed novel pathways regulated by LXR in the skin. This analysis showed LXR-dependent regulation of peptidylarginine deiminase (Padi) family members (Padi1, Padi3, and Padi4), as their expression was down-regulated in LXRβ knock-out mouse skin (Fig. 4A). Furthermore, qPCR analysis revealed that the topological application of the synthetic LXR ligand T1317 induced the expression of Padi1, Padi3, and Padi4 in murine skin (Fig. 4B). PADI is a family of Ca2+-dependent enzymes that catalyze the post-translational deamination of arginine residues to citrullines. The terminally differentiated cornified layer of epidermis contains deaminated keratins (K1, K10, K14, and K5) and filaggrin (25, 26), thus suggesting a role for protein deimination during the final stages of epidermal differentiation. By modifying arginine residues to citrulline, the PADI family of enzymes is proposed to regulate the unfolding of epidermal proteins, an important function because disordered structure of cornified envelope component proteins is required for optimal interaction with intermediary filaments and TGase I (27). There are five clustered PADI genes (1p35–6) in humans, namely PADI1–PADI5, and of these PADI1 and PADI3 are expressed in the epidermis (28, 29). Interestingly, the PADI locus was found to contain one LXRβ-RXRα binding site, which is present downstream of the PADI3 and upstream of PADI4 genes (Fig. 4C). A ChIP assay in the absence or presence of T1317 did not reveal any significant differences in the LXR occupancy of this LXRE (data not shown).

We next examined the ability of the PADI LXRE to mediate transcriptional activation through LXR. Three copies of the PADI LXRE were cloned upstream of a luciferase reporter to construct PADI-LXRE-Luc reporter. The PADI-LXRE-Luc reporter was transfected into HEK293 cells that were treated with vehicle or a synthetic LXR ligand, GW3965 (2). As shown in Fig. 4D, the synthetic LXR ligand GW3965 induced expression of the PADI-LXRE-Luc-reporter by 2.4 fold when compared with vehicle treatment. These results demonstrate that the PADI LXRE is capable of mediating transcriptional activation by ligand-occupied LXR. To further confirm the functionality of PADI LXRE, we cloned 921-bp upstream fragment of PADI4 containing the LXRE motif in a luciferase reporter (PADI4–921bp-Luc; WT). In addition, we also constructed another luciferase reporter containing the same 921-bp region but with a mutated LXRE. As shown in Fig. 4E, the LXR ligand
GW3965 induced the expression of WT PADI-LXRE reporter (bar 2) compared with the vehicle-treated control cells (bar 1) in an LXR-dependent manner. In contrast, GW3965 failed to induce the expression of mutant PADI4-LXRE luciferase reporter (Fig. 4E, bar 4). These results confirm that the PADI LXRE is functional in the context of its native upstream region.

In addition to the proposed regulation of PADI expression by LXR/H9252–RXR/H9251, the expression of this locus has been found to be under the control of an AP1 motif located within the PADI1–2 intergenic region (28). Oxysterol and LXR ligands also increase the expression of early (IVL) as well as late keratinocyte differentiation genes (LOR, FLG2, and TGM1) in human keratinocytes (3). The LXR ligand presumably induces the expression of these genes by increased AP1 activity, as it augmented the expression of IVL via its upstream AP1 motif (14). Surprisingly, none of these genes showed any LXR/RXR binding within 300 kb upstream or downstream of these genes. However, LXR/RXR binding was observed in the distal enhancer region of the FOS gene (Fig. 4C). Interestingly, c-Fos expression was down-regulated in LXR/RXR knock-out murine skin (Fig. 4F), and the LXR ligand T1317 induced the expression of FOS and FOSL1 (Fra-1) in NHEKs (Fig. 4G). FOSL1 did not show any LXR-RXR binding regions but has been reported to be 12-O-tetradecanoylphorbol-13-acetate inducible and to show regulation via its upstream AP1 motif (30). Because AP1 activity is required for keratinocyte terminal differentiation, these results provide the first indication of a plausible orchestration of keratinocyte differentiation by LXR-AP1 cross-talk.

**LXR-RXR Cistrome Reveals an Enrichment of AP1 Motifs**

To identify transcription factors that potentially cooperate with LXR to regulate gene expression in keratinocytes, we searched for the presence and enrichment of DNA motifs near the LXR/RXR binding sites. This analysis revealed the presence of 1735 AP1 motifs adjacent to 2035 heterodimer binding regions. Therefore, 85.3% of the LXR/RXR heterodimer binding sites contained AP1 motifs in their vicinity (Fig. 5A), thus strengthening the notion that these sites may cross-talk with LXREs. To further confirm LXR-RXR and AP1 association, we next performed genome-wide profiling of AP1 motifs in NHEKs using c-Fos and c-Jun antibodies. The c-Fos/c-Jun ChIP-on-chip experiments identified 18,581 c-Fos, 20,795 c-Jun, and 14,244 c-Jun/c-Fos heterodimer (AP1) binding regions. Interestingly, LXR-RXR and c-Jun/c-Fos ChIP-on-chip data analysis revealed that 1573 of the 2035 LXR/RXR active regions (77.3%) overlapped with the c-Fos/c-Jun binding sites (Fig. 5B). The presence of AP1 motifs near the majority of the LXR/RXR heterodimer binding sites indicated that AP1 members and LXR may interact with each other to form enhancers. Because nuclear receptor family members have been shown to interact directly with AP1 proteins, we next tested the possibility of a direct protein-protein interaction between LXRβ and c-Jun/c-Fos. Lysates from c-Myc-tagged LXRβ,

**FIGURE 3. Identification of novel LXR-RXR heterodimer binding regions in human primary keratinocytes.** A, LXR and RXR binding sites detected by ChIP-on-chip frequently overlap and cluster around target genes. All target genes are shown in their native chromosomal location according to 2006 Human Genome Assembly (hg18) in the UCSC Genome Browser. The red and blue blocks represent regions of enriched LXR and RXR binding signals, respectively. Vertical lines within the genes represent exons, horizontal lines represent introns, and the arrowheads represent the direction of transcription. B, LXR-dependent regulation of newly identified responsive genes. The relative expression level of genes Fads1 and Fads2 was compared by real-time PCR in mouse skin after topical treatment with vehicle or T1317 (10 μM). C, the relative expression level of Acsl3 was compared by TaqMan real-time PCR in wild type or LXRβ KO mouse primary epidermal keratinocytes and dermal fibroblasts treated with vehicle (blue bars) or T1317 (red bars).
c-Jun- and c-Fos-expressing HEK293 cells were subjected to immunoprecipitation with anti-LXR\(^\beta\) antibody and Western blotting with anti-Myc antibody. The results presented in Fig. 5C demonstrate that LXR\(^\beta\) interacts with both AP1 members, as the LXR antibody co-immunoprecipitated both c-Jun and c-Fos in the absence of ligand.

Interestingly, the c-Fos/c-Jun ChIP-on-chip showed that \(PADI1\), \(PADI3\), and \(PADI4\) genes contained two AP1 motifs, each located 28–38 kb downstream of \(PADI1\) and \(PADI3\) and 10 kb upstream of the \(PADI4\) start site (supplemental Table S2). Similarly, \(ABCA1\), \(ABCG1\), \(ABCA12\), \(ABCA13\), \(SCD1\), \(FADS1\), \(FADS2\), \(ACSL3\), and \(TGM6\) showed multiple c-Fos/c-Jun binding regions ranging from 2–11 for each of these genes (supplemental Table S2). Taken together, these results indicate extensive cross-talk between LXR and AP1 for the regulation of a number of LXR-responsive genes in skin.

DISCUSSION

Epidermal homeostasis is critical for survival of an organism, and any changes in the skin barrier function through alterations in keratinocyte differentiation and/or lipid synthesis/transport may predispose individuals to cutaneous inflammation. To avoid severe consequences of epidermal barrier perturbations and to swiftly adjust epidermal homeostasis, the skin system appears to employ lipid-sensing nuclear receptors, namely LXRs and PPARs. The natural ligands of these receptors present in skin regulate and fine-tune gene expression programs that modulate keratinocyte differentiation, lipid synthesis, and transport, which are physiologically important processes essential for epidermal barrier formation and maintenance. A limited number of genes involved in keratinocyte differentiation (\(TGM1\), \(IVL\), \(LOR\), and \(FLG2\)) and lipid synthesis (\(SREBF1\) and \(SCD1\)) and transport (\(ABCA1\), \(ABCG1\), and \(ABCA12\)) have been identified as LXR-responsive genes based upon their regulation by natural and/or synthetic LXR agonists in keratinocytes (3, 6, 31). However, the mechanism of their regulation and whether keratinocyte differentiation genes and \(ABCA12\) are direct LXR target genes is not clearly understood.

To elucidate the mechanism of LXR action in skin, we employed an unbiased approach to identify LXR\(^\beta\)-RXR\(\alpha\) het-
erodimer binding regions in human keratinocytes. As observed with PPARγ (18), most of the LXRβ-RXRα binding sites were located in the distal intergenic regions and introns, and very few sites were found in the proximal promoter regions (Fig. 2C). The distally located binding sites may interact with the basal transcription machinery by DNA looping, as suggested for estrogen and androgen receptors (15, 17). The de novo motif search of the heterodimer binding regions against TransFac LXREs yielded DR4 as the predominant cis-acting element (Fig. 1C). As part of the LXR-RXR cistrome analysis, we discovered that 85% of the LXR-RXR heterodimer binding regions contained at least one AP1 motif (Fig. 5A). These results were confirmed by the identification of genome-wide ChiP-on-chip of c-Fos/c-Jun (AP1) binding regions in NHEKs, which revealed that about 80% of the LXR-RXR heterodimer binding regions also contained AP1 binding sites (Fig. 5B). Our observation of specific enrichment of AP1 motifs in LXR-RXR binding regions suggests that these transcription factors may orchestrate the process of keratinocyte differentiation, as AP1 is required for the expression of a large number of keratinocyte differentiation genes. This point was further corroborated by the observation that the LXR-RXR cistrome analysis did not show the presence of LXR binding regions in classical keratinocyte differentiation genes (TGM1, IVL, LOR, and FLG). These four major genes that facilitate keratinocyte differentiation are AP1-responsive, and the increased expression of IVL by oxysterol LXR ligands has been attributed to its AP1 motif (3, 14, 32–36). LXR ligands also increased AP1 activity and induced the expression of FOSL1 and FOS in NHEKs (Fig. 4G) (14). Therefore, LXR ligands could induce AP1-dependent expression of keratinocyte differentiation genes either directly by AP1-LXR cross-talk or indirectly by inducing AP1 activity via increased expression of Jun/Fos family members. The cistrome analysis showed the presence of an LXR-RXR motif upstream of the FOS gene, whereas the expression of both FOS and FOSL1 was increased by the synthetic LXR ligand T1317 in NHEKs (Fig. 4G), thus indicating that FOS and not FOSL1 may be a direct LXR target. Like other keratinocyte differentiation markers, FOSL1 is an AP1-responsive gene; therefore, LXR agonist may increase its expression by inducing AP1 activity and/or by a plausible mechanism involving long range regulation by DNA looping and co-occupancy of distant located LXR-RXR and AP1 motifs.

Importantly, through both LXR knock-out and pharmacological studies using a specific synthetic ligand, we have demonstrated for the first time that PADI family members (PADI1, PADI3, and PADI4) are LXR-responsive genes (Fig. 4, A and B), and the expression of the PADI locus might well be under the control of an LXRE located in the PADI3 and PADI4 intergenic region (Fig. 4, C–E). Interestingly, PADI3 has been identified as an AP1-responsive gene, for which expression is controlled by a long range enhancer (AP1 site present 86 kb upstream from the PADI3 promoter) by a mechanism involving chromatin looping (28). PADI1, PADI3, and PADI4 were also found to contain two AP1 motifs each (supplemental Table S2), thus pointing to a plausible AP1-LXR cross-talk for ligand-dependent regulation of this newly identified family of LXR-responsive genes. Further, TGM6, expression of which was regulated by LXR in keratinocytes and in mice (3), also contains two AP1 binding motifs (supplemental Table S2).

Inflammatory skin disorders like atopic dermatitis and psoriasis exhibit defects in keratinocyte terminal differentiation and epidermal barrier formation (37–39). Loss-of-function mutations in FLG strongly predispose individuals to atopic dermatitis (40). Similarly, psoriasis exhibits abnormal or psoriatic keratinocyte differentiation, which is characterized by decreased expression of LOR and FLG (41). Activation of LXR stimulates keratinocyte differentiation and lipid synthesis, thus leading to epidermal barrier formation (2–5). The novel mode of regulation of AP1-mediated gene expression by LXR assumes potential therapeutic significance because AP1 activity is reduced in psoriatic lesional skin, and in addition, deletion of its components JunB and c-Jun leads to psoriasis-like plaques with inflammatory phenotype in double mutant mice (42). On the basis of our results and previously reported pharmacology of LXR, we identified this nuclear receptor as a novel first-in-class target for psoriasis, a dermal inflammatory indication characterized by epidermal hyperproliferation, abnormal keratinocyte differentiation, and cutaneous inflammation. LXR ligand inhibits epidermal proliferation in vivo, induces keratinocyte differentiation (Fig. 4, A and B), and inhibits cutaneous inflammation in murine models of atopic and irritant dermatitis (2–4).

Reduced epidermal neutral lipid synthesis (sphingolipids, cholesterol, and fatty acids) leading to defective barrier repair is a hallmark of atopic dermatitis, a chronic inflammatory condition of the skin that afflicts ~15–20% of the children. Moreover, xerosis or dry skin because of reduced keratinocyte lipid

![FIGURE 5. LXR-AP1 association and interaction.](image-url)
synthesis is a major manifestation of chronological skin aging, which is recognized as a low-grade chronic cutaneous inflammatory condition. Therefore, enhancement of epidermal barrier function via increased keratinocyte differentiation and lipid production may result in amelioration of inflammation and contribute to the efficacy of LXR ligand in skin aging, psoriasis, and atopic dermatitis. T1317 induced the expression of (a) enzymes involved in sphingolipid and ceramide biosynthetic pathways, (b) proteins required for fatty acid synthesis, (c) cholesterol/lipid transporters, and (d) lipid-binding proteins (3).

We have demonstrated that the genes involved in these processes contain multiple LXR-RXR heterodimer binding regions (Fig. 3A). Our observation that ABCA12 has two heterodimer binding regions with LXREs indicates that it is a direct LXR target (Fig. 3A) and suggests that LXR ligands could be used for certain forms of ichthyosis (keratinization disorders) and also for dryness and scaliness associated with skin aging and psoriasis. The studies presented herein demonstrating that ACSL3, FAD31, and FAD32 are newly identified LXR-responsive genes (Fig. 3B and C) further strengthen the notion that LXR is a major regulator of epidermal lipid synthesis. Accordingly, LXR ligands improved epidermal barrier function in vivo and showed efficacy in models of contact and irritant dermatitis (2).

Over the years nuclear receptors have proven to be ligands of choice for dermal inflammatory indications. Natural and synthetic ligands of glucocorticoid receptors, vitamin D receptor, and retinoic acid receptor are currently in clinical practice for the treatment of psoriasis, dermatitis, and atopic dermatitis. Glucocorticoids are prescribed extensively for the treatment of psoriasis and atopic dermatitis. T1317 induced the expression of (2).

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**LXR-RXR Cistrome in Keratinocytes**

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