Regulation of ABCA1 expression in human keratinocytes and murine epidermis

Yan J. Jiang,* Biao Lu,* Peggy Kim,* Peter M. Elias,† and Kenneth R. Feingold[1,*]

Metabolism Section* and Dermatology Service,† Veterans Affairs Medical Center, Northern California Institute for Research and Education, University of California at San Francisco, San Francisco, CA 94121

Abstract **Keratinocytes require abundant cholesterol for cutaneous permeability barrier function; hence, the regulation of cholesterol homeostasis is of great importance. ABCA1 is a membrane transporter responsible for cholesterol efflux and plays a pivotal role in regulating cellular cholesterol levels. We demonstrate that ABCA1 is expressed in cultured human keratinocytes (CHKs) and murine epidermis. Liver X receptor (LXR) activation markedly stimulates ABCA1 mRNA and protein levels in CHKs and mouse epidermis. In addition to LXR, activators of peroxisome proliferator-activated receptor (PPAR)α, PPARβ/δ, and retinoid X receptor (RXR), but neither PPARγ nor retinoic acid receptor, also increase ABCA1 expression in CHKs. Increases in cholesterol supply induced by LDL or mevalonate stimulate ABCA1 expression, whereas inhibiting cholesterol synthesis with statins or cholesterol sulfate decreases ABCA1 expression in CHKs. After acute permeability barrier disruption by either tape-stripping or acetone treatment, ABCA1 expression declines, and this attenuates cellular cholesterol efflux, making more cholesterol available for regeneration of the barrier. In addition, during fetal epidermal development, ABCA1 expression decreases at days 18–22 of gestation (term = 22 days), leaving more cholesterol available during the critical period of barrier formation. Together, our results show that ABCA1 is expressed in keratinocytes, where it is negatively regulated by a decrease in cellular cholesterol levels or altered permeability barrier requirements and positively regulated by activators of LXR, PPARs, and RXR or increases in cellular cholesterol levels.**—Jiang, Y. J., B. Lu, P. Kim, P. M. Elias, and K. R. Feingold. **Regulation of ABCA1 expression in human keratinocytes and murine epidermis.** ***J. Lipid Res.*** **2006.** 47: **2248–2258.**

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Cholesterol levels are tightly regulated in cells, and the maintenance of cholesterol homeostasis is of particular importance in keratinocytes/epidermis. In addition to being an essential component of all cell membranes, cholesterol is also required in keratinocytes to form lamellar bodies (1). Secretion of these unique organelles then delivers lipids, including cholesterol, to the extracellular spaces of the stratum corneum, which mediate permeability barrier function (1). The ability to limit the transcutaneous movement of water and electrolytes is required for terrestrial life. Although cholesterol synthesis rates are high under basal conditions, after permeability barrier disruption cholesterol synthesis increases (2), as do the levels of receptors that enhance the uptake of cholesterol into the cell, such as the LDL receptor and scavenger receptor class B type I (3, 4). Inhibition of cholesterol synthesis perturbs permeability barrier function (5), and a selective deficiency in cholesterologenesis largely accounts for the barrier abnormality in chronologically aged epidermis (6, 7). Cholesterol is also the precursor of an important bioregulatory molecule in keratinocytes, cholesterol sulfate (CS), which regulates corneocyte desquamation and cohesion (8, 9) and keratinocyte differentiation (10–12).

Cholesterol homeostasis is regulated by a balance between the de novo synthesis of cholesterol, the uptake of cholesterol from lipoproteins, and cholesterol efflux. The efflux of cholesterol from cells is mediated predominantly by ABCA1, which is a member of the ATP binding cassette transporter superfamily that contains 12 putative transmembrane domains and two ABCs (13, 14). The importance of ABCA1 is shown by Tangier disease, in which ABCA1 deficiency results in the accumulation of cholesteryl esters in various tissues and clinically shows markedly enlarged yellow tonsils and very low HDL cholesterol levels (15–18). ABCA1 promotes the transport of cholesterol and phospholipids across cell membranes, where these lipids then complex with acceptor particles, such as apolipoprotein A-I, leading to HDL formation (14), thereby reg-

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Abbreviations: ATRA, all-*trans*-retinoic acid; 8-Br-cAMP, 8-bromoadenosine-3′,5′-cyclic monophosphate; CHK, cultured human keratinocyte; 9-*cis*-RA, 9-*cis*-retinoic acid; CS, cholesterol sulfate; LXR, liver X receptor; 25(OH), 25-hydroxycholesterol; PPAR, peroxisome proliferator-activated receptor; 22R, 22(*R*)-hydroxycholesterol; RXR, retinoid X receptor; TO, TO901317.

[1] To whom correspondence should be addressed.
e-mail: kfngld@itsa.ucsf.edu

ulating cellular cholesterol homeostasis (19). Activation of liver X receptor (LXR) by oxysterols and other pharmacologic ligands increases ABCA1 gene expression (20–22). As levels of cellular cholesterol increase, the levels of oxysterols also increase, leading to the activation of LXR and an increase in cholesterol efflux (22). In macrophages, the activation of peroxisome proliferator-activated receptors (PPARs)γ and β/δ has also been shown to increase LXR expression, indirectly affecting ABCA1 expression and cholesterol efflux (23, 24). Finally, three ABCA1 alternative transcripts have been identified in liver and macrophages, and expression of these alternative transcripts is regulated differentially in response to environmental stimuli (25, 26).

Previously, we and others have demonstrated that the nuclear hormone receptors LXR-α, and -β as well as PPARs α, β/δ, and γ are expressed in epidermis/keratinocytes (27–30). The activation of PPARs and LXRs stimulates keratinocyte differentiation, accelerates the development of the epidermis in fetal rats, increases the expression of Sult2B1b, which catalyzes the synthesis of CS, and stimulates epidermal lipid synthesis, thereby improving epidermal permeability barrier homeostasis (28, 31–35). Whether the activation of LXRs and PPARs regulates ABCA1 in keratinocytes remains unknown.

In this study, we explored the regulation of ABCA1 in keratinocytes and the epidermis. We hypothesized that when the epidermal keratinocytes require cholesterol for the formation of the permeability barrier, the expression of ABCA1 would decrease, allowing the cell to retain cholesterol for permeability barrier homeostasis.

MATERIALS AND METHODS

Materials

Hairless female mice (hr/hr), 6–8 weeks old, were purchased from Charles River Laboratories (Wilmington, MA). Timed pregnant Sprague-Dawley rats (plug date = day 0), estimated gestational age days 17–22, were obtained from Simonsen Laboratories (Gilroy, CA). 22(*R*)-Hydroxycholesterol (22R), 25-hydroxycholesterol [25(OH)], clofibrate, WY14643, all-*trans*-retinoic acid (ATRA), 9-*cis*-retinoic acid (9-*cis*-RA), CS, lovastatin, 8-bromoadenosine-3′,5′-cyclic monophosphate (8-Br-cAMP), and mevalonate were purchased from Sigma (St. Louis, MO). Ciglitazone and TO901317 (TO) were purchased from Cayman Chemical Co. (Ann Arbor, MI). The PPARδ activator GW 610742X was a generous gift from Dr. Tim Willson (GlaxoSmithKline). Human LDL was from Intracel Resources, LLC (Frederick, MD). LPS (*Escherichia coli* 55:B5) was obtained from Difco and freshly diluted to the desired concentration in pyrogen-free 0.9% saline. Cytokines (human tumor necrosis factor-α and interleukin-6) were from R&D Systems, Inc. (Minneapolis, MN). Molecular-grade chemicals such as TRI reagent were obtained from either Sigma or Fisher Scientific (Fairlawn, NJ). [α-^{32}P]dCTP (3,000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). The Advantage RT-for-PCR kit for first-strand cDNA synthesis was purchased from BD Biosciences (Palo Alto, CA). All reagents and supplies for real-time PCR were purchased from Applied Biosystems (Foster City, CA). Primary monoclonal and polyclonal antibodies against human ABCA1 were purchased from Abcam, Inc. (Cambridge, MA), and Novus Biologicals, Inc. (Littleton, CO), respectively. Polyclonal antibodies against human ABCA1 also cross-react with mouse tissue. All other reagents for Western blot (NuPAGE® Novex precast gels, 3–8% Tris-acetate), buffers, protein standards, and detection kits were purchased from Invitrogen (Carlsbad, CA).

Keratinocyte culture

Human foreskin keratinocytes, second passage, were seeded and maintained in 0.07 mM Ca^{2+} KGM (serum-free keratinocytes growth medium; Clonetics, San Diego, CA). Once the cells attached, the culture medium was switched to either 0.03 mM Ca^{2+} (low Ca^{2+}) or 1.2 mM Ca^{2+} (high Ca^{2+}). In the presence of high Ca^{2+}, keratinocytes are induced to differentiate. In a typical experiment, cells were treated with each reagent at its optimized concentration at preconfluence (60–70%) in either low or high calcium conditions and were harvested at 80–100% confluence. Control keratinocytes were treated with vehicle (0.05% ethanol or DMSO).

Animal treatment

Adult mouse skin was treated twice per day for 3.5 days with either vehicle (100% acetone) or the LXR activator TO (15 mM). At the end of treatment, the dorsal skin was collected and epidermis was isolated as described previously (4).

Acute barrier disruption model

Adult mouse skin was treated either by gently applying acetone-soaked cotton balls for 5–10 min or by sequential applications of cellophane tape as described previously (2, 36). For acetone treatment, each group contained five mice; controls for acetone perturbation of the barrier were treated with cotton balls soaked in 0.9% (w/v) sodium chloride. For the tape-stripping model, each group contained six mice; untreated animals served as controls for tape stripping. The transepidermal water loss was measured immediately after treatment using a Meeco electrolytic water analyzer as described previously (2). Animals with a transepidermal water loss rate of $\geq$4 mg/cm^2/h (normal $<$ 0.3 mg/cm^2/h) after barrier disruption were included in this study.

Epidermis preparation

The whole skin from each estimated gestational age group of rat fetus was collected as described (37). For epidermis isolation, adult mouse or fetal rat skin was placed dermis side down in 10 mM EDTA in calcium-magnesium-free PBS (pH 7.4) for 35–45 min at 37°C (4). The skin was blotted dry, and the epidermis was separated from dermis by scraping with a scalpel blade. Alternatively, full-thickness mouse skin was incubated in 10 mM DTT in PBS (pH 7.4) for 40 min at 37°C. Epidermal fractions (outer and lower layers) were removed, snap-frozen in liquid nitrogen, and stored at −80°C until processed (38). For the fetal rat study, four samples were analyzed for each time point. For day 19, 20, 21, and 22 rats, epidermis was isolated from a single fetus for each sample. For day 17 and 18 rats, to obtain enough samples for RNA analysis, epidermis was pooled from two to three fetuses for each data point. Experiments were repeated at least once to ensure reproducibility.

Total RNA isolation, cDNA probes, and Northern blotting

Total RNA was isolated using TRI reagent according to the manufacturer's protocol, and Northern blotting was performed as described previously (35). Briefly, Northern blots were prepared and the uniformity of sample applications was checked by ultraviolet light visualization of the acridine orange-stained gel before transfer to Nytran membranes. Blots were then hybridized

with ^{32}P-labeled ABCA1 probe overnight at 65°C and washed. Human ABCA1 probe was prepared using PCR, and the PCR primer pairs used were ABCA1 forward (5′-TCTCTGCTATCTC-CAACCTCATC-3′) and ABCA1 reverse (5′-ACGTCTTCAC-CAGGTAATCTGAA-3′). Subsequently, the blots were exposed to X-ray films for various durations to ensure that measurements were performed on the linear portion of the curve, and the target bands were quantitated by densitometry (Bio-Rad Laboratories, Hercules, CA). The same blot was probed with cyclophilin, and the densitometry quantitation of the target gene was adjusted for cyclophilin or 18S level and expressed as a percentage of control, with the control as 100%.

Western blot analysis

Western blot analysis was carried out according to the manufacturer's protocol. Briefly, the whole cell extract was prepared from either cultured human keratinocyte (CHK) or mouse epidermis, and 40–50 μg of proteins was fractionated on precast gradient gels (3–8%) and transferred to polyvinylidene difluoride (0.2 μM pore size) membranes overnight (4°C). The proteins on the membrane were subsequently probed with either monoclonal or polyclonal primary antibodies against human ABCA1 (1:1,000). The antibodies were visualized with horseradish peroxidase-conjugated anti-rabbit secondary antibody (polyclonal antibodies, 1:20,000) or anti-mouse antibody (monoclonal antibody, 1:500) using the ECL Western Blotting Detection System Kit. Membranes were then exposed to CL-XPosure film. An identical blot was probed with anti-GAPDH antibody to verify equal loading of the protein.

Quantitative real-time PCR

First-strand cDNA for PCR was synthesized using the Advantage RT-for-PCR kit according to the manufacturer's protocol. Briefly, cDNA was synthesized from 1–2 μg of total RNA using Moloney murine leukemia virus reverse transcriptase with random hexamer primer at 42°C for 60 min. Relative mRNA levels of target genes (mouse and rat ABCA1) and an invariant transcript, 36B4, were determined using a Mx3000P™ Real-Time PCR System (110 V) with a notebook computer (Stratagene, La Jolla, CA). The primer sequences for PCR were as follows: human ABCA1 sense (5′-GCAAGGCTACCAGTTACATTTG-3′) and antisense (5′-GTCAGAAACATCACCTCCTG-3′); mouse/rat ABCA1 sense (5′-GCAAGGCTACCAATTACATTTG-3′) and antisense (5′-GGTCAGAAACATCACCTCCTG-3′). Primer sequences for three human ABCA1 alternative transcripts (exons 1b, 1c, and 1d) were used as described (26). Individual PCRs were carried out in a mixture of 20 μl containing 30 ng of cDNA, 600 nM forward or reverse primers, and 10 μl of 2× SYBR Green Q-PCR Master Mix (Applied Biosystems). The reaction was performed at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of amplification of melting at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s on Mx3000P 96-well plates (Stratagene). PCR was performed in triplicate and replicated in two independent experiments. Gel electrophoresis and melting-curve analyses were performed to confirm correct PCR product sizes and the absence of nonspecific bands. The expression level of each gene was normalized against cyclophilin (human) or 36B4 (mouse, rat) using the comparative threshold cycle method according to the manufacturer's protocols.

Statistical analysis

All data are expressed as means ± SEM. Comparisons between two groups were undertaken using two-tailed and unpaired *t*-tests. Differences in values are considered significant at $P < 0.05$.

RESULTS

ABCA1 is expressed in CHKs but is not affected by changes in differentiation

We initially assessed whether ABCA1 is present in CHKs and regulated by keratinocyte differentiation. As shown in **Fig. 1**, a single band corresponding to ABCA1 mRNA was detected by Northern blot analysis in undifferentiated keratinocytes. When these cells were differentiated by increasing the calcium content of the medium, there was no change in ABCA1 mRNA levels, indicating that the expression of ABCA1 in CHKs is not regulated by changes in differentiation alone. These results confirm previous studies by Schmitz's group (39) demonstrating that ABCA1 is expressed in CHKs but does not change with differentiation.

Activation of LXR, PPARs, and RXR increases ABCA1 mRNA expression in CHKs

We next assessed whether ABCA1 is regulated by the activation of LXR, PPARs, and retinoid X receptor (RXR) in CHKs. Before our formal experiments, the optimized dose for each activator was determined. As shown in **Fig. 2A**, ABCA1 mRNA level increased markedly by LXR activation [22R, 5-fold; 25(OH), 2-fold; TO, 4.5-fold]. Activators of both PPARα (clofibrate) and PPARβ/δ (GW501516) significantly, but to a lesser extent, also increased ABCA1 mRNA by 34% and 48%, respectively (Fig. 2B) ($P < 0.05$).

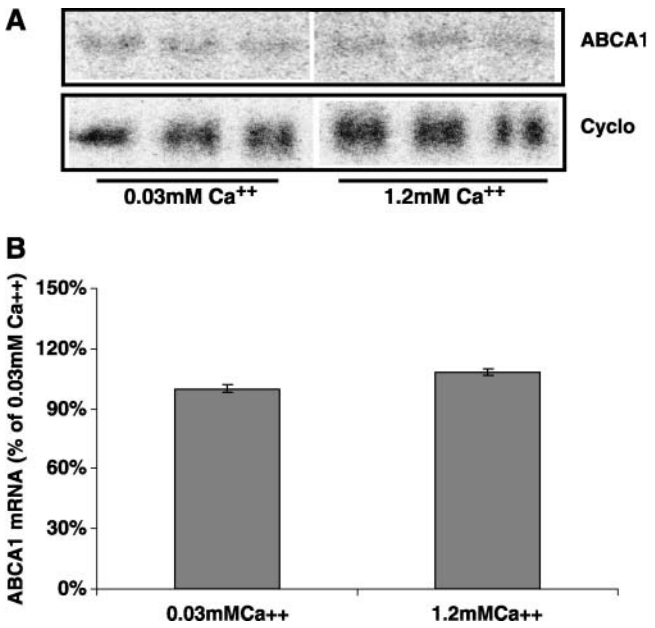

Fig. 1. ABCA1 is expressed in cultured human keratinocytes (CHKs) but not affected by changes in differentiation. A: Human keratinocytes were grown either in low-calcium (0.03 mM) or high-calcium (1.2 mM) serum-free keratinocytes growth medium for 24 h. Northern blots were probed for human ABCA1 mRNA as described in Materials and Methods. The densitometry values from a typical Northern blot were verified and normalized by cyclophilin (Cyclo). A representative blot is shown. B: Data are expressed as percentages of control, plotted, and presented as means ± SEM (n = 3–4 in each group). Similar results were obtained when the experiment was repeated with a different batch of cells.

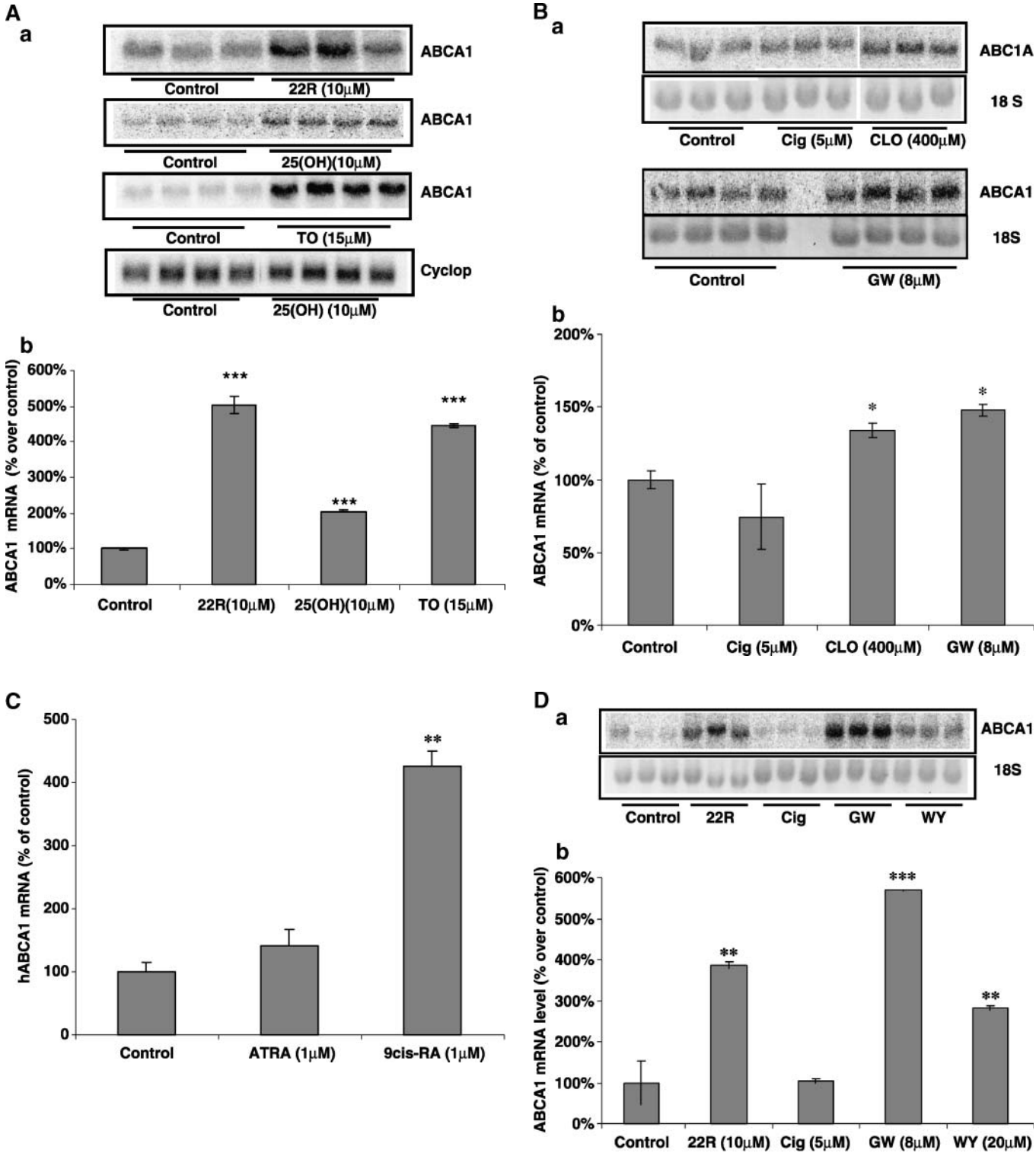

Fig. 2. Activation of liver X receptor (LXR), peroxisome proliferator-activated receptors (PPARs), and retinoid X receptor (RXR) increases ABCA1 mRNA expression in CHKs. A–C: Human keratinocytes were incubated with either vehicle control or LXR activators [A: 10 μM 22(*R*)-hydroxycholesterol (22R), 10 μM 25-hydroxycholesterol {25(OH)}, or 15 μM TO901317 (TO)]; PPAR activators [B: 5 μM ciglitazone (Cig), 400 μM clofibrate (CLO), or 8 μM GW501516 (GW)]; or retinoic acid receptor or RXR activator [C: 1 μM 9-*cis*-retinoic acid (9-*cis*-RA) or 1 μM all-*trans*-retinoic acid (ATRA)] in low-calcium medium for 24 h. Total RNA was isolated and subjected to Northern blot (A, B) or real-time PCR (C) analyses to determine mRNA levels of ABCA1 as described. The densitometry value from a typical Northern blot was verified and normalized by cyclophlin/18S (A, B, D). D: Alternatively, cells were incubated with vehicle control, 22R, ciglitazone, GW501516, or WY14643 (WY) for 24 h in high-calcium medium. Representative Northern blots are shown in the top panels. Data from Northern blots are expressed as percentages of control, plotted, and presented as means ± SEM (n = 3–4 in each group) in the bottom panels. Similar results were obtained when the experiment was repeated with a different batch of cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

In contrast, activation of PPARγ (ciglitazone) had no effect (Fig. 2B). Finally, an activator of RXR (9-*cis*-RA), but not the activator of retinoic acid receptor (ATRA), also increased ABCA1 mRNA level by 4.3-fold (Fig. 2C). We next assessed whether LXR and PPAR activators would increase ABCA1 mRNA levels in differentiated CHKs. As shown in Fig. 2D, ligand activation of LXR, PPARβ/δ, and PPARα increased ABCA1 mRNA levels by 2.9-, 4.7-, and 1.8-fold, respectively. Consistent with the observations in undifferentiated CHKs, activation of PPARγ had no effect on ABCA1 mRNA levels (Fig. 2D).

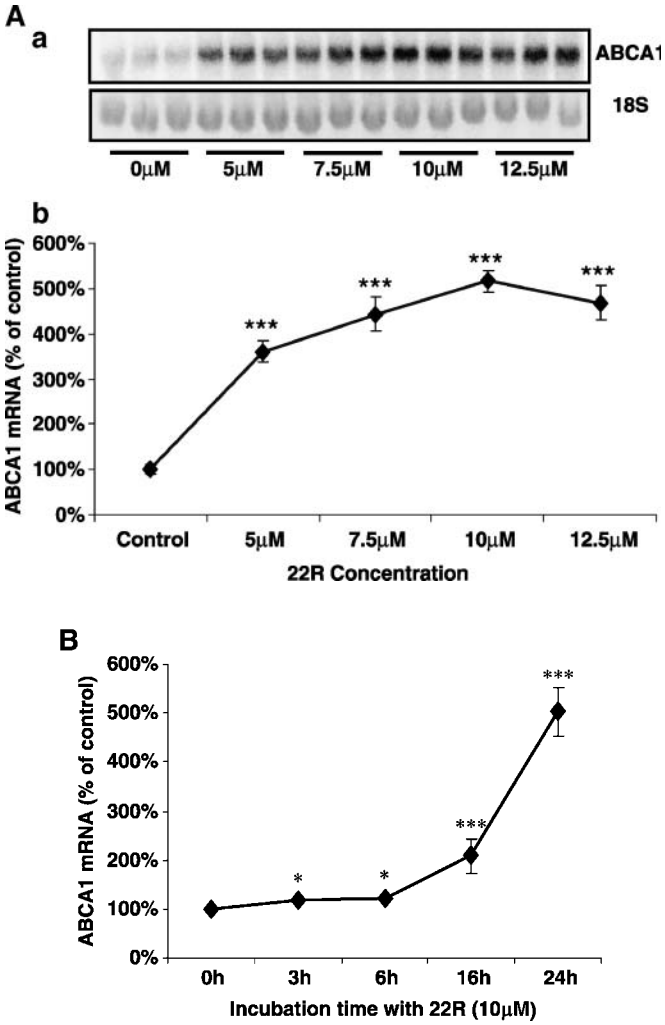

Fig. 3. LXR activation increases ABCA1 mRNA expression in a dose- and time-dependent manner. A: Human keratinocytes were incubated with either vehicle control or 22R at various concentrations (5.0, 7.5, 10, and 12.5 μM) in low-calcium medium for 24 h. B: Alternatively, cells were incubated with 10 μM 22R for various periods of time (0, 3, 6, 16, and 24 h) in the same medium. Total RNA was isolated and subjected to Northern blot analyses to determine ABCA1 mRNA levels. The densitometry value from a typical Northern blot was verified and normalized by 18S. A representative blot is shown in the top panel for A. Data are expressed as percentages of control, plotted, and presented as means ± SEM (n = 3 in each group at each time or dose point) in the bottom panels. Similar results were obtained when the experiment was repeated with a different batch of cells. * $P < 0.01$, *** $P < 0.001$.

LXR activation increases ABCA1 mRNA expression in both a dose- and time-dependent manner

We next determined the dose response and time course for stimulation of ABCA1 expression by the LXR activator 22R in undifferentiated keratinocytes. As shown in **Fig. 3A**, ABCA1 mRNA levels increased significantly at 5.0 μM 22R (3.6-fold), peaked at 10 μM (5.2-fold), and decreased slightly at 12.5 μM (4.7-fold). Furthermore, as early as 3 h, ABCA1 levels increased by 37% ($P < 0.01$) and continued to increase up to 24 h (5-fold) (Fig. 3B). Thus, the LXR-induced increase in ABCA1 mRNA levels occurs rapidly and is sustained for an extended period of time.

CHKs express alternative transcripts of ABCA1 that are upregulated by LXR activators

Three alternative transcripts of ABCA1 have been identified recently in both macrophages and liver (25, 26). To investigate which of these transcripts is expressed in CHKs, we used quantitative real-time PCR to examine the mRNA levels of exons 1b, 1c, and 1d of human ABCA1. As shown in **Fig. 4**, all three transcripts were expressed at moderate levels in undifferentiated CHKs, and interestingly, activation of LXR by 22R increased mRNA levels of all three transcripts (by 4.4-, 4.8-, and 5.1-fold for exons 1b, 1c, and 1d, respectively) (Fig. 4). Similar results were obtained when cells were incubated with another LXR activator, 25(OH) (data not shown). Thus, all of the alternative variants of ABCA1 are expressed in CHKs, and these three transcripts seem to contribute equally to the increase in ABCA1 mRNA that occurs in response to LXR activation.

LXR activation increases ABCA1 protein expression

As shown in **Fig. 5**, activation of LXR by either 22R or 25(OH) markedly increased ABCA1 protein levels. To

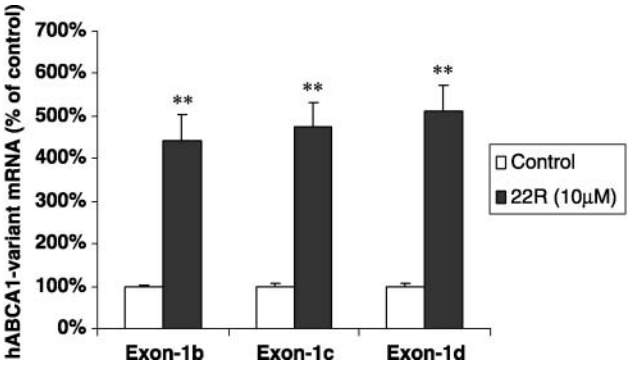

Fig. 4. Alternative transcripts of ABCA1 are expressed in CHKs and upregulated by LXR activators. Human keratinocytes were incubated with either vehicle control or 10 μM 22R in low-calcium medium for 24 h. Total RNA was isolated and subjected to real-time PCR analysis as described in Materials and Methods. Under our experimental conditions, the absolute threshold cycle values for the basal levels of these three ABCA1 transcripts (exons 1b, 1c, and 1d) in CHKs were 25.2, 25.5, and 26.0, respectively. Data are expressed as percentages of control (100%), plotted, and presented as means ± SEM (n = 6 in each group). ** $P < 0.01$.

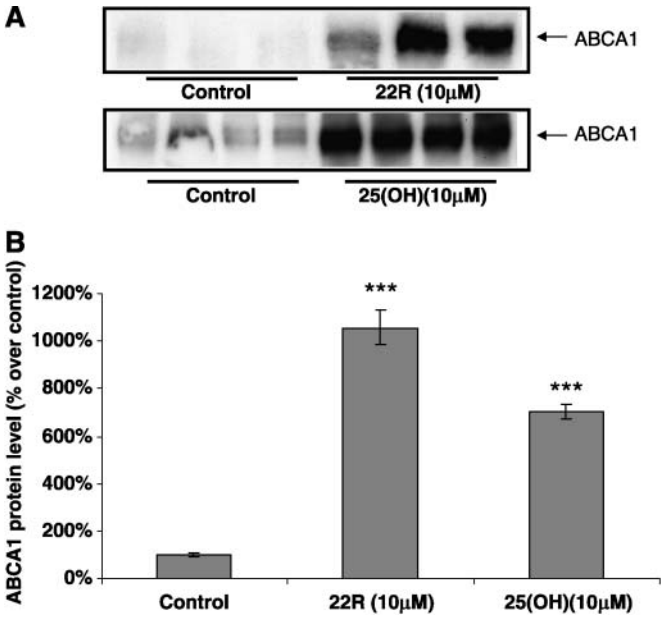

Fig. 5. LXR activation increases ABCA1 protein expression. Human keratinocytes were incubated with either vehicle control or LXR activators [10 μM 22R or 25(OH)] in low-calcium medium for 24 h. The whole cell extract was prepared and subjected to Western blot analyses to determine protein levels of ABCA1 as described in Materials and Methods. A: A representative blot is shown. B: The densitometry values from a typical Western blot are expressed as percentages of control, plotted, and presented as means ± SEM (n = 3–4 in each group). Similar results were obtained when the experiment was repeated with a different batch of cells. *** $P < 0.001$.

ensure the accuracy of these results, we used two different ABCA1 antibodies in our protocol and observed similar results (data not shown). Thus, LXR activation enhances not only mRNA but also protein levels of ABCA1.

Changes in cellular sterol levels regulate ABCA1 mRNA expression in CHKs

To examine whether ABCA1 expression is regulated by changes in cellular sterol levels, we first treated keratinocytes with exogenous LDL (100 μg/ml) to increase cellular cholesterol levels. The addition of LDL increased ABCA1 mRNA levels by ~140% (24 h) and ~200% (48 h) in both undifferentiated and differentiated cells (**Fig. 6A**). To confirm this observation, keratinocytes were treated with various doses (200–500 μM) of mevalonate, a precursor of cholesterol, and again, a dose-dependent increase in ABCA1 mRNA level was observed (Fig. 6B). Conversely, inhibition of cholesterol synthesis in CHKs, by incubating cells with either lovastatin or CS, markedly decreased ABCA1 expression (Fig. 6C). Together, these results demonstrate that ABCA1 mRNA levels are regulated by cell sterol levels, with increases in cell cholesterol (induced by increased cholesterol synthesis or uptake) leading to an increase in ABCA1 expression, whereas decreases in cell cholesterol (induced by decreased cholesterol synthesis) leading to a decrease in ABCA1 expression.

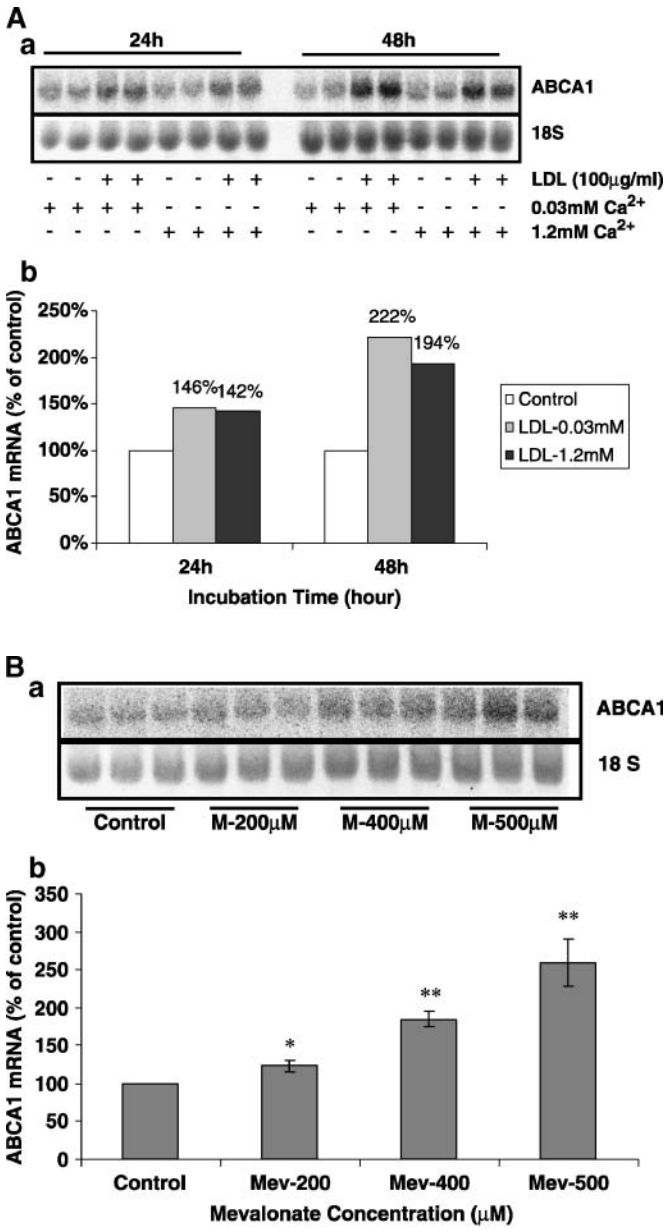

Fig. 6. Cellular cholesterol levels regulate ABCA1 mRNA expression in CHKs. A: Human keratinocytes were incubated with either vehicle control or LDL (100 μg/ml) in 0.03 or 1.2 mM calcium medium for 24 or 48 h (n = 2). B, C: Alternatively, cells were incubated with mevalonate (M or Mev) at various concentrations (200, 400, or 500 μM) (B; n = 3) or 10 μM lovastatin (L) or 40 μM cholesterol sulfate (CS) (C; n = 3) in low-calcium medium for 24 h. The mRNA level of ABCA1 was determined by Northern blot analysis as described. The densitometry values from a typical Northern blot were verified and normalized by 18S. Representative blots are shown in the top panels (a). Data are expressed as percentages of control, plotted, and presented as means ± SEM in the bottom panels (b). Similar results were obtained when the experiment was repeated with a different batch of cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

LXR activators also increase ABCA1 expression in vivo

We next determined whether ABCA1 is also expressed and regulated in vivo. As shown in **Fig. 7**, ABCA1 mRNA was present in murine epidermis and distributed equally

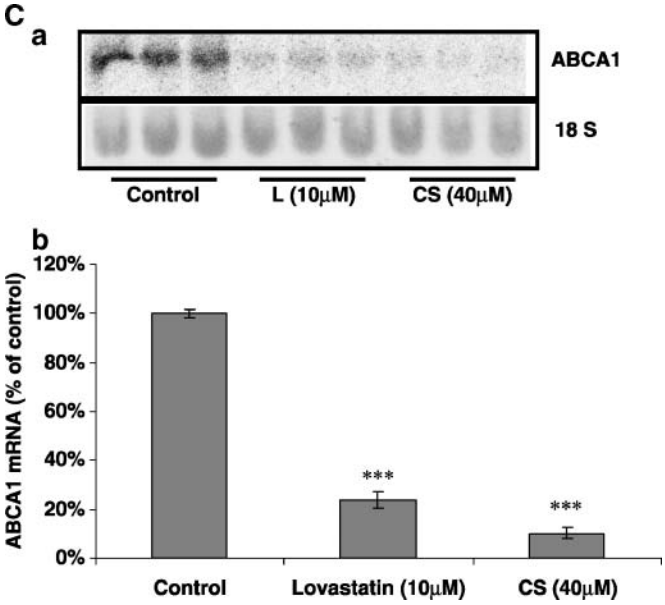

Fig. 6. Continued.

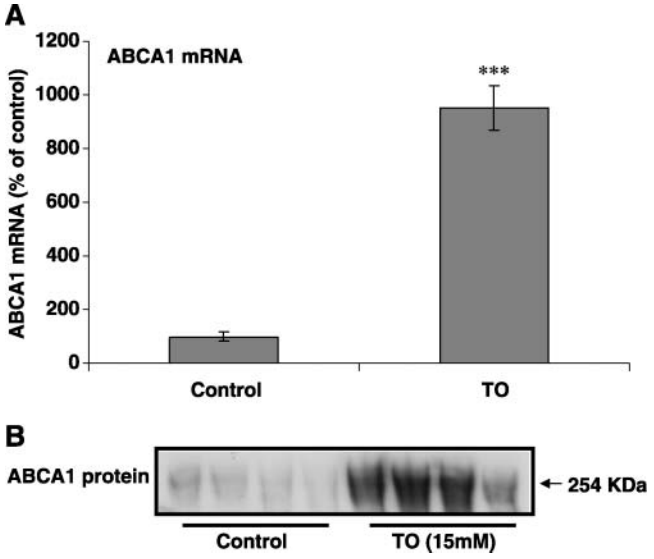

Fig. 8. Topical application of LXR activator increases ABCA1 mRNA and protein expression in vivo. A: Hairless mice were topically treated with the LXR activator TO (15 mM) twice per day for 3.5 consecutive days, and epidermis was collected. ABCA1 mRNA levels were determined by real-time PCR, and protein levels were determined by Western blot analysis as described. ABCA1 mRNA data obtained from real-time PCR are expressed as percentages of control, plotted, and presented as means ± SEM. Each group contained six mice for real-time PCR analysis and four mice for Western blot analysis, and epidermis was isolated from each individual mouse, processed separately, and analyzed as an individual sample. Similar results were obtained when the experiment was repeated with epidermal samples prepared from a different batch of mice. B: A representative Western blot is shown. *** $P < 0.001$.

in both the upper and lower layers of the epidermis. Because the outer epidermis (stratum corneum, stratum granulosum) contains more differentiated keratinocytes than the lower layer (stratum spinosum, stratum basale), these data are consistent with the data in undifferentiated versus differentiated keratinocytes (Fig. 1), further demonstrating that ABCA1 mRNA levels are not regulated by diferentiation. Yet, after topical application of the LXR activator TO to mouse skin for 3.5 days, an ~10-fold increase in both ABCA1 mRNA (**Fig. 8A**) and protein (Fig. 8B) levels was observed. Thus, LXR activation stimulates ABCA1 expression both in vitro and in vivo.

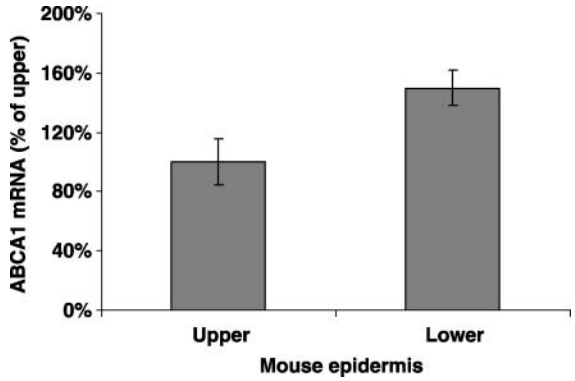

Fig. 7. ABCA1 mRNA is expressed in both upper and lower layers of mouse epidermis. Total RNA was isolated from both the upper and lower layers of mouse epidermis and subjected to real-time PCR analyses. ABCA1 mRNA was determined as described. Data are expressed as percentages of control (upper layer), plotted, and presented as means ± SEM. Each group contained six mice, and epidermis was isolated from each individual mouse, processed separately, and analyzed as an individual sample. Similar results were obtained when the experiment was repeated with epidermal samples prepared from a different batch of mice.

ABCA1 expression is regulated inversely by permeability barrier requirements

Cholesterol is required for the formation of the permeability barrier (5); therefore, we next determined ABCA1 expression after acute barrier disruption. Three hours after barrier disruption induced by tape stripping, ABCA1 mRNA levels decreased by 64% (**Fig. 9A**). This decrease in ABCA1 occurred in both the upper and lower layers of the epidermis (Fig. 9B). Similarly, disruption of the barrier by a second method, acetone treatment, also resulted in a reduction in ABCA1 mRNA level (Fig. 9C).

Furthermore, ABCA1 mRNA was present in fetal rat epidermis from estimated gestational age days 17–22, and a significant decrease in ABCA1 mRNA levels occurred at days 18–22 (**Fig. 10**), which coincides with the peak time of lamellar body generation and the formation of a competent permeability barrier (40). Together, these results suggest that in response to permeability barrier disruption, ABCA1 expression is decreased, which would increase the cholesterol available for lamellar body synthesis and barrier formation.

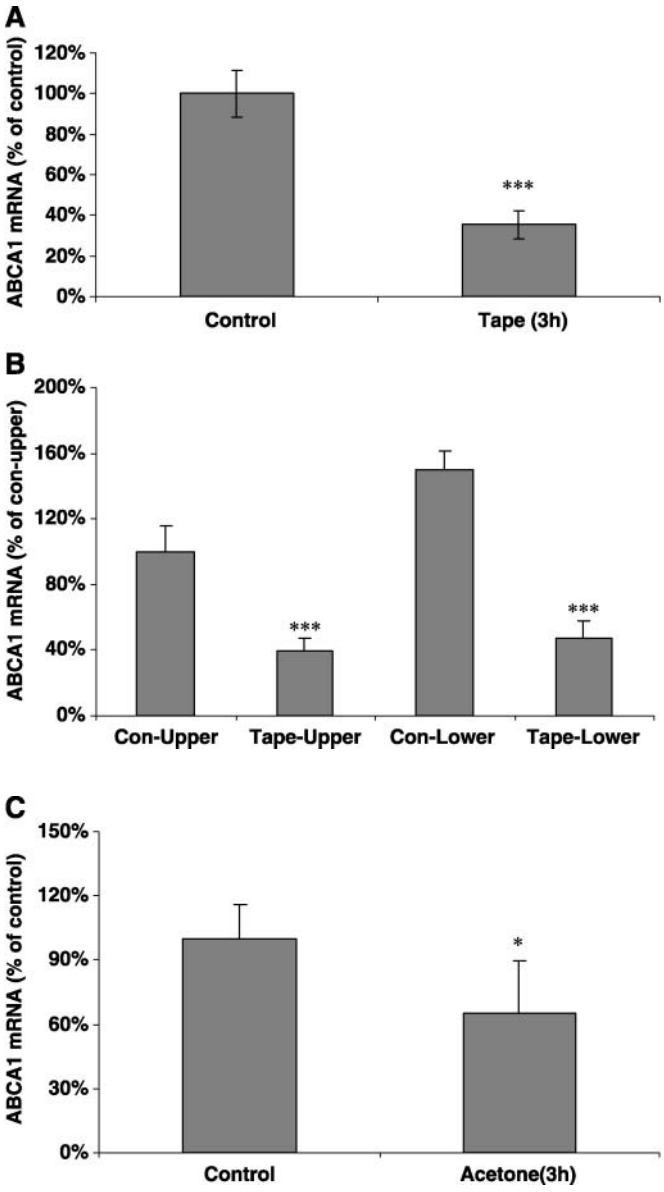

Fig. 9. Acute barrier disruption decreases ABCA1 mRNA levels. Three hours after disrupting the barrier by either tape stripping (A, B) or acetone treatment (C), the whole epidermis (A, C) or the epidermal fraction (B; upper or lower epidermis) of mouse was isolated and real-time PCR was performed to determine ABCA1 mRNA levels. Data are expressed as percentages of control, plotted, and presented as means ± SEM. Each group contained six mice for tape stripping and control (Con) or five mice for acetone treatment and control; epidermis was isolated from each individual mouse, processed separately, and analyzed as an individual sample. Similar results were obtained when the experiment was repeated with epidermal samples prepared from a different batch of mice. * $P < 0.05$, *** $P < 0.001$.

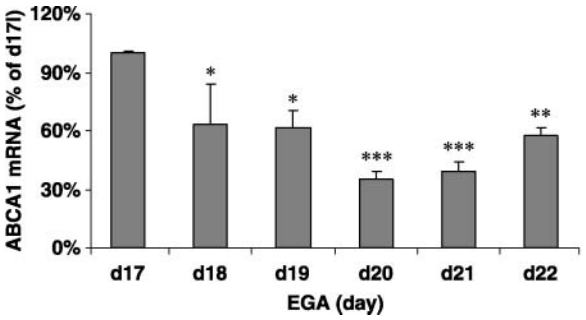

Fig. 10. ABCA1 mRNA levels decrease during fetal barrier ontogenesis. The whole epidermis was isolated from fetal rats of gestational age days 17–22, and total RNA was isolated and subjected to real-time PCR analysis as described. Data are expressed as percentages of day 17 values (as basal level), plotted, and presented as means ± SEM. Four samples were analyzed for each time point. For days 19, 20, 21, and 22, epidermis was isolated from a single fetus, processed separately, and analyzed as an individual sample. For days 17 and 18, epidermis was pooled from two to three fetuses for each sample. Similar results were obtained when the experiment was repeated with epidermal samples prepared from different batches of fetal rats. EGA, estimated gestational age. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

DISCUSSION

Cutaneous permeability is dependent on the formation of extracellular, lipid-enriched membranes located in the outermost layers of the epidermis, the stratum corneum. Approximately 25% of the lipid in these membranes is cholesterol, with the remainder consisting of free fatty acids and ceramides (1). Both cholesterol and the other lipids are delivered to the extracellular space when stratum granulosum cells secrete lamellar body contents (1). After acute disruption of the permeability barrier, a rapid secretion of preformed lamellar bodies occurs, followed by a marked increase in cholesterol synthesis, as required for the formation of nascent lamellar bodies and the regeneration of the barrier (41). Topical applications of statins inhibit epidermal cholesterol synthesis, blocking the formation of lamellar bodies, provoking a delay in barrier recovery (5, 42). In addition to stimulating lamellar body secretion and formation, disruption of the permeability barrier also leads to an increase in DNA synthesis and cell proliferation (predominantly in the basal layer of the epidermis), which also requires the production of abundant cholesterol (43).

After cutaneous permeability barrier disruption, the expression of a variety of genes is regulated to ensure a net increase in cellular cholesterol levels. Specifically, the mRNA levels of a number of key enzymes required for cholesterol synthesis, including HMG-CoA synthase, HMG-CoA reductase, farnesyl diphosphate synthase, and squalene synthase, increase rapidly (4, 44). Similarly, the mRNA levels of a number of lipoprotein receptors that increase the uptake of cholesterol into the cell, including the LDL receptor and scavenger receptor class B type I, increase (3, 4). Here, we demonstrate that the mRNA levels of ABCA1, a transporter that plays a key role in the efflux of cholesterol from cells, instead decreases after acute barrier disruption by either acetone treatment or repeated tape stripping. Moreover, during fetal epidermal development, the mRNA levels of ABCA1 decrease at the time when lamellar body formation and secretion is form-

ing the permeability barrier. A decrease in cholesterol efflux from keratinocytes would facilitate the formation of lamellar bodies by ensuring an available supply of cholesterol. Thus, a decrease in ABCA1 expression in stratum granulosum cells (upper epidermis) when accelerated lamellar body formation is required after permeability barrier disruption is not unexpected. However, we demonstrated a decrease in ABCA1 mRNA levels not only in the stratum granulosum but also in the lower epidermis. We speculate that because of the marked increase in DNA synthesis and cell proliferation in the lower epidermis that occurs after permeability barrier disruption (43), there is an increased cellular requirement for cholesterol in the keratinocytes of the lower epidermis. In support of this hypothesis, we have demonstrated in previous studies an increase in cholesterol synthesis in both the upper and lower epidermis after barrier disruption (38).

Studies from other laboratories have shown that cholesterol levels in cells regulate ABCA1 (45). Here, we demonstrate that cellular cholesterol levels also regulate ABCA1 expression in keratinocytes. When the cellular cholesterol supply increased by either providing exogenous cholesterol (LDL) or increasing synthesis by providing mevalonate, ABCA1 expression was upregulated. Conversely, when the cellular cholesterol content decreased by incubating cells with either statins or CS, inhibitors of HMG-CoA reductase that inhibit cholesterol synthesis, ABCA1 expression was repressed. Yet, it is well recognized that cholesterol does not directly regulate ABCA1 expression. Rather, cholesterol is converted to oxysterols (not yet identified) that activate LXR. Within the promoter of ABCA1, there is a response element that binds LXR (RXR), and in the presence of either an LXR or RXR ligand, ABCA1 transcription is stimulated (45). PPAR activators have also been shown to stimulate ABCA1 expression, and they do this indirectly by increasing LXR-α levels (23, 46). Here, we demonstrate that in keratinocytes, PPARα or PPARβ/δ activators also increase ABCA1 mRNA levels, likely secondary to an increase in LXR-β levels. In support of this hypothesis, treatment of keratinocytes with PPARα or PPARβ/δ activators significantly increased mRNA levels of LXR-β (2.6- and 2.8-fold respectively; unpublished observation), which is the predominant receptor in keratinocytes/epidermis (28). In contrast, PPARγ activator decreased LXR-β mRNA expression [by 58% ($P = 0.019$); unpublished observation], which may explain the lack of ABCA1 stimulation by PPARγ activator in this study (Fig. 2B, D).

We speculate that after acute permeability barrier disruption or during fetal development when the barrier is first being formed, the increased requirements of cholesterol for lamellar body formation decrease cellular cholesterol levels, leading to a decrease in the formation of the oxysterols that activate LXR. When this occurs, the expression of ABCA1 declines, which decreases cholesterol efflux, thereby helping to maintain cellular cholesterol levels. On the other hand, the increases in the enzymes necessary for cholesterol synthesis and the receptors involved in the uptake of cholesterol in lipoproteins would also help to maintain cellular cholesterol levels (47). Yet, the regulation of the expression of these genes is not via the oxysterol-LXR pathway but rather by the proteolytic activation of sterol-regulatory element binding proteins, a process that is regulated by the sterol content of the endoplasmic reticulum (48).

In both macrophages and liver, there are three alternative ABCA1 transcripts (exons 1b, 1c, and 1d); each arises from different exon 1 sequences that are spliced into exon 2, and all produce full-length proteins (25). Yet, in response to a high-fat diet, exon 1d is preferentially increased in the liver and exon 1b is increased in macrophages (26). Interestingly, in this study, we found that all three alternative transcripts are present in CHKs; upon LXR activation, the expression of these three transcripts is upregulated to a similar extent (Fig. 4). Thus, in response to environmental stimuli, the expression pattern of ABCA1 transcripts in different tissues varies. Finally, although in macrophages ABCA1 expression is regulated by other factors, including ATRA (49, 50), 8-Br-cAMP (51), tumor necrosis factor-α, interleukin-6, and LPS (52), in CHKs, there was no effect of any of these compounds (data not shown). The mechanism of the tissue-specific regulation of these various stimulators remains unclear.

In summary, cholesterol homeostasis is very important in keratinocytes because of the unique needs of keratinocytes for cholesterol. In this study, we demonstrate that a key transporter in cholesterol efflux, ABCA1, is present in keratinocytes, is regulated by cellular cholesterol levels and LXR and PPAR activation, and decreases when cholesterol is required for permeability barrier formation.

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