Oxysterol Stimulation of Epidermal Differentiation is Mediated by Liver X Receptor-β in Murine Epidermis

László G. Kömüves,*§[1] Matthias Schmuth,†‡[1] Ashley J. Fowler,*§ Peter M. Elias,*§ Karen Hanley,*§ Mao-Qiang Man,*§ Arthur H. Moser,‡ Jean-Marc A. Lobaccaro,¶ Mary L. Williams,* David J. Mangelsdorf,¶ and Kenneth R. Feingold*†‡§

Departments of *Dermatology and †Medicine, University of California, San Francisco, California, U.S.A.; ‡Medical and §Dermatology Services, Department of Veterans Affairs Medical Center, San Francisco, California, U.S.A.; ¶Howard Hughes Medical Institute and Department of Pharmacology, University of Texas South-western Medical Center, Dallas, Texas, U.S.A.

Liver X receptor-α and -β are members of the nuclear hormone receptor superfamily that heterodimerize with retinoid X receptor and are activated by oxysterols. In recent studies we found that treatment of cultured human keratinocytes with oxysterol-stimulated differentiation, as demonstrated by increased expression of involucrin and transglutaminase, and inhibited proliferation. The aims of this study were to determine: (i) whether oxysterols applied topically to the skin of mice induce differentiation in normal epidermis; (ii) whether this effect is mediated via liver X receptor-α and/or liver X receptor-β; and (iii) whether oxysterols normalize epidermal morphology in an animal model of epidermal hyperplasia. Topical treatment of normal hairless mice with 22(R)-hydroxycholesterol or 24(S),25-epoxycholesterol resulted in a decrease in epidermal thickness and a decrease in keratinocyte proliferation assayed by proliferating cell nuclear antigen staining. Moreover, oxysterol treatment increased the levels of involucrin, loricrin, and profilaggrin protein and mRNA in the epidermis, indicating that oxysterols stimulate epidermal differentiation. Additionally, topical oxysterol pretreatment improved permeability barrier homeostasis. Whereas liver X receptor-$\alpha^{-/-}$ mice revealed no alterations in epidermal differentiation, the epidermis was thinner in liver X receptor-$\beta^{-/-}$ mice than in wild-type mice, with a reduced number of proliferating cell nuclear antigen positive cells and a modest reduction in the expression of differentiation markers. Topical oxysterol treatment induced differentiation in liver X receptor-$\alpha^{-/-}$ mice whereas in liver X receptor-$\beta^{-/-}$ mice there was no increase in the expression of differentiation markers. Whereas both liver X receptor-α and liver X receptor-β are expressed in cultured human keratinocytes and in fetal rat skin, only liver X receptor-β was observed on northern blotting in adult mouse epidermis. Finally, treatment of hyperproliferative epidermis with oxysterols restored epidermal homeostasis. These studies demonstrate that epidermal differentiation is regulated by liver X receptor-β and that oxysterols, acting via liver X receptor-β, can induce differentiation and inhibit proliferation *in vivo*. The ability of oxysterols to reverse epidermal hyperplasia suggests that these agents could be beneficial for the treatment of skin disorders associated with hyperproliferation and/or altered differentiation. ***Key words: keratinocytes/proliferation/permeability barrier/loricrin/involucrin. J Invest Dermatol 118:25–34, 2002***

Epidermal differentiation is required for the formation of the stratum corneum that provides both the mechanical and permeability barrier between the external environment and the internal milieu (Fuchs, 1990; Jackson *et al*, 1993; Eckert *et al*, 1997). The permeability barrier, which is essential for terrestrial life, is sustained by extracellular, lipid-enriched lamellar membranes in the stratum corneum. These extracellular lipid-enriched lamellar membranes inhibit water and aqueous solute movement (Elias and Menon, 1991; Jackson *et al*, 1993). Corneocytes in the stratum corneum are postmitotic, terminally differentiated, anuclear keratinocytes with a rigid cornified envelope that provides the extraordinary mechanical strength to the stratum corneum (Reichert *et al*, 1993; Steinert, 1995). The cornified envelope is formed by extensive, covalent cross-linking of involucrin, loricrin, and other proteins (Steinert and Marenkov, 1995, 1997). These proteins are expressed in a spatially and temporally regulated pattern as suprabasal keratinocytes undergo terminal differentiation during their upward migration towards the surface of the epidermis, and therefore, they serve as markers of the differentiation process (Fuchs, 1990; Eckert *et al*, 1997). The cross-linking of these proteins is catalyzed by the

Manuscript received May 7, 2001; revised September 13, 2001; accepted for publication September 29, 2001.

Reprint requests to: Dr. Kenneth R. Feingold, Metabolism Section (111F), Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94121. Email: kfngld@itsa.ucsf.edu

Abbreviations: 24(S)25EPOCHOL, 24(S),25-epoxycholesterol; 22(R)-OHCHOL, 22(R)-hydroxycholesterol; DIC, differential interference–contrast; PCNA, proliferating cell nuclear antigen; LXR, liver X receptor; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; TEWL, transepidermal water loss.

[1]These authors contributed equally to this work.

keratinocyte-specific enzyme, transglutaminase I, which is also expressed late in keratinocyte differentiation (Fuchs, 1990; Steinert and Marenkov, 1995, 1997; Eckert *et al*, 1997). The viable precursors of corneocytes are the terminally differentiated keratinocytes of the stratum granulosum, which are characterized by the presence of keratohyalin granules, containing profilaggrin and loricrin as well as numerous lipid-containing lamellar bodies (Fuchs, 1990; Elias and Menon, 1991; Jackson *et al*, 1993; Eckert *et al*, 1997). The proliferation rate of cells in the basal layer is balanced by the rates of apoptosis and corneocyte formation in the outer epidermis (Fuchs, 1990; Eckert *et al*, 1997). Despite the importance of keratinocyte differentiation for epidermal function, the factors that regulate keratinocyte differentiation are not well understood (Fuchs, 1990; Eckert *et al*, 1997).

Nuclear hormone receptors are transcription factors that regulate many cellular functions, including cellular differentiation (Mangelsdorf *et al*, 1995; Kliewer *et al*, 1999). The nuclear receptor superfamily has been divided into four major subgroups according to their dimerization and DNA binding properties (Mangelsdorf *et al*, 1995). The class II subfamily consists of nuclear receptors that heterodimerize with retinoid X receptor (RXR). Members of this subfamily usually bind to direct repeats separated by a variable number of spacer nucleotides (Mangelsdorf *et al*, 1995). Stimulation of several of the receptors in this subgroup, including the retinoic acid activated receptor (RAR), vitamin D receptor, and peroxisome proliferator activated receptor (PPAR)-α, regulates keratinocyte proliferation and differentiation (Bikle, 1996; Eichner *et al*, 1996; Fisher and Voorhees, 1996; Kang *et al*, 1996; Hanley *et al*, 1998; Kömüves *et al*, 2000a,b). Moreover, keratinocyte differentiation is abnormal in transgenic mice that overexpress RAR or RXR dominant negative mutations in the epidermis, further showing the importance of nuclear hormone receptors that heterodimerize with RXR (Imakado *et al*, 1995; Saitou *et al*, 1995; Feng *et al*, 1997). Additionally, in mice with a deficiency of RXR-α localized to the epidermis, epidermal hyperplasia occurs suggesting keratinocyte hyperproliferation, which is consistent with the inhibition of proliferation observed with vitamin D and PPAR-α ligands (Li *et al*, 2000).

Recently, we have shown that liver X receptor (LXR) -α and LXR-β are expressed in cultured human keratinocytes and in fetal rat epidermis (Hanley *et al*, 1999, 2000). Moreover, we have demonstrated that certain oxysterol ligands for LXR increased cornified envelope formation, as well as the levels of involucrin and transglutaminase protein and mRNA in normal human keratinocytes, indicating that oxysterols stimulate keratinocyte differentiation *in vitro* (Hanley *et al*, 2000). In contrast, oxysterols inhibited DNA synthesis *in vitro* by approximately 50% (Hanley *et al*, 2000). Lastly, we have shown that oxysterols accelerate the development of the epidermis in fetal rats, accelerating the formation of the cutaneous permeability barrier (Hanley *et al*, 1999).

The purpose of this study was to determine: (i) whether oxysterols, known to be ligands for LXR – 22(R)-hydroxycholesterol (22(R)OHCHOL) and 24(S),25-epoxycholesterol (24(S)25EPOCHOL) – when applied topically to the skin of mice induce differentiation in normal epidermis; (ii) whether this effect is mediated via LXR-α and/or LXR-β; and (iii) whether oxysterols normalize epidermal morphology in a hyperplastic animal disease model.

MATERIALS AND METHODS

Animals and tissue preparation Adult hairless mice 6–10 wk of age (Simonsen, Gilroy, CA or Charles River, Wilmington, MA) were treated topically twice a day for 4 d with 22(R)OHCHOL (Sigma, St Louis, MO) (500 nM, 200 μM, 500 μM dissolved in polypropylene glycol–ethanol 3:1 or in ethanol; 0.1 ml applied to 2 cm^2 area on one flank). Moreover, 24(S)25EPOCHOL (a gift from Timothy Willson, Nuclear Receptor Discovery Research, GlaxoSmithKline, Research Triangle Park, NC) was applied to the flank in a similar fashion as 22(R)OHChol (500 nM in polypropylene glycol–ethanol 3 : 1). Control hairless mice were treated with vehicle alone in an identical fashion. The experiments were done in triplicate.

LXR-$\alpha^{-/-}$ (four adults), LXR-$\beta^{-/-}$ (four adults), and LXR-$\alpha\beta^{-/-}$ (three adults) deficient mice were produced as described previously (Peet *et al*, 1998). These ''knockout'' animals and age- and sex-matched controls (LXR-$\alpha\beta^{+/+}$ adults) of the same genetic background were gently shaved and then topically treated twice a day for 4 d with either 22(R)OHCHOL in ethanol, or with ethanol alone.

Epidermal hyperproliferation was induced in male hairless mice by repeated barrier abrogation with acetone, twice a day, for 5 d, as previously described (Denda *et al*, 1996). For an additional 4 d these animals were treated topically with either 22(R)OHCHOL or vehicle as described above.

Cutaneous permeability barrier function was determined by measuring transepidermal water loss (TEWL) with a Meeco electrolytic water analyzer, as described previously (Kömüves *et al*, 2000a,b). Barrier repair was measured by first disrupting the barrier by repetitive, gentle wipes with acetone or by repetitive tape stripping with cellophane tape until the TEWL levels were 3–7 mg per cm^2 per h, and then determining TEWL at 3, 6, 16, and 24 h as an index of barrier homeostasis (Kömüves *et al*, 2000a,b).

Skin samples were fixed overnight in 4% formaldehyde (freshly prepared from paraformaldehyde (Sigma) and embedded in paraffin (ParaplastPlus, Fisher Scientific, Pittsburgh, PA). Sections (5 μm for immunohistochemistry, 15 μm for *in situ* hybridization) were collected on SuperfrostPlus slides (Fisher Scientific), and processed as described below.

Immunohistochemistry Affinity-purified rabbit antibodies, specific for involucrin, profilaggrin/filaggrin, and loricrin (Covance/BabCo, Berkeley, CA), and a biotinylated monoclonal anti-proliferating cell nuclear antigen (anti-PCNA) antibody (CalTag Laboratories, Burlingame, CA) were used. Affinity-purified biotinylated goat anti-rabbit IgG, affinity-purified biotinylated goat anti-mouse IgG, and ABC-peroxidase (ABC-PO) were purchased from Vector (Burlingame, CA). Immunohistochemical localization of these proteins was performed as described previously (Kömüves *et al*, 1998, 2000a,b), and peroxidase activity was revealed with diaminobenzidine (Vector). For the detection of PCNA proteins, antigen retrieval was performed with microwave treatment in 10 mM citrate buffer, pH = 6.0. Omission of the first antibodies, or incubation with the substrate solution resulted in no signal, showing that nonspecific binding of the second antibody and/or ABC-PO, or endogenous peroxidase activity were not contributing to the signals obtained.

***In situ* hybridization** Biotin-labeled RNA probes to detect loricrin and profilaggrin mRNA were synthesized from linearized cDNA sequences (a gift from S. Yuspa, NIH), using reagents supplied by Roche Molecular Biochemicals (Indianapolis, IN). *In situ* hybridization was performed as described previously (Kömüves *et al*, 2000a), with the following modifications. The sections were hybridized at 45°C and the hybridization of the biotin-labeled probes to endogenous mRNA was detected with ABC-PO (Vector). Biotinylated tyramide (CSA kit, DAKO, Carpinteria, CA) was used for signal amplification, followed by incubation with ABC-alkaline phosphatase (Vector). Alkaline phosphatase activity was developed with VectorRed substrate (Vector). Hybridization with biotin-labeled, sense control probes resulted in no signal, indicating the specificity of hybridization with the anti-sense probes. Omitting the biotin-labeled anti-sense probes from the hybridization cocktail resulted in no signal, which demonstrated that only biotin-containing RNA hybrids were detected. Moreover, incubation with the BCIP/NTBT substrate reagents alone resulted in no staining, showing that endogenous alkaline phosphatase activity within the tissues did not contribute to the signal obtained.

Microscopy and imaging The tissue samples were analyzed with an Olympus BX50 microscope using 20 × UplanApo and 40 × UplanFl objectives with bright-field or differential interference–contrast (DIC) illumination. Images were acquired with a DEI 750 CCD camera (Optronics, Goleta, CA), controlled by BioQuant Nova imaging software (R&M Biometrics, Nashville, TN). The digitized images were resized to 300 dpi resolution and assembled using Photoshop 5.5 (Adobe Systems, Mountain View, CA). Apart from adjusting the background intensities (using the Level tool in Photoshop), and cropping to size, no other digital tools were applied to the images. The figures were printed with Epson Stylus Photo 870 inkjet printer. Epidermal thickness was determined using a computer-generated micrometer (Carl Zeiss Vision, Munich, Germany). Epidermal thickness was defined as the distance between the basement lamina and the stratum granulosum–stratum

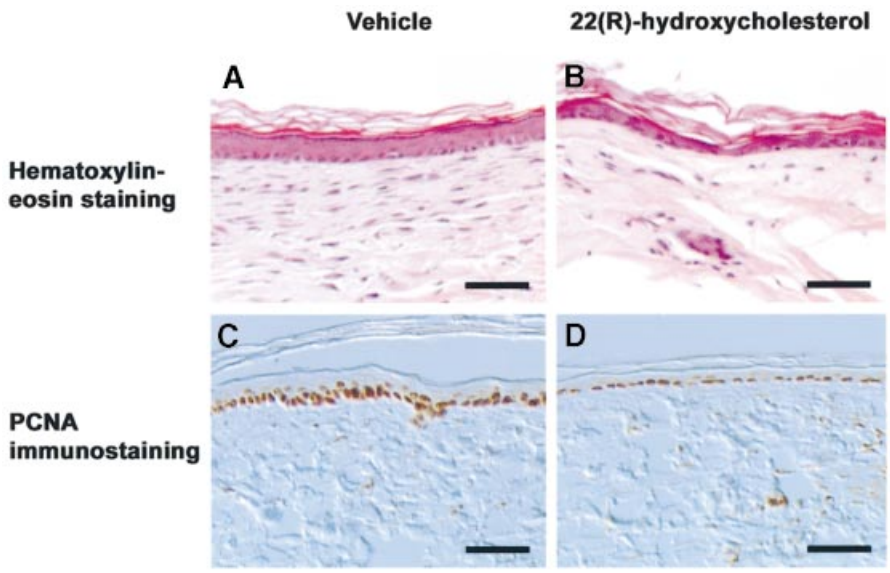

Figure 1. Effects of topical oxysterol treatment on epidermal morphology. Hairless mouse skin was treated for 4 d, twice a day with 22(R)OHCHOL (*B*, *D*). Control animals were treated with vehicle (*A*, *C*). Hematoxylin–eosin-stained sections showed a decrease in the thickness of the epidermis (*A vs B*). Immunohistochemical detection of PCNA (a protein expressed in cycling cells) revealed a decrease in proliferating cell number in the epidermis of 22(R)OHCHOL-treated animals (*D*) as compared with the control (*C*). *A*, *B*: bright-field illumination, *C*, *D*: DIC illumination. *Scale bar*: 50 μm.

corneum interface. Epidermal proliferation was determined by quantitating PCNA positive nuclei in the basal layer of the epidermis per unit length of basement membrane.

Northern blotting To separate whole epidermis from full thickness skin, skin was placed in 10 mM ethylenediamine tetraacetic acid in calcium-free and magnesium-free phosphate-buffered saline, pH 7.4, for 35 min at 37°C. The epidermis was removed by scraping with a scalpel blade. RNA from epidermis and liver was prepared by a variation of the guanidinium thiocyanate method, as described previously (Jackson *et al*, 1992). Total RNA was purified and added to oligo(dT)-cellulose to obtain poly(A)$^+$ RNA. Northern blots were prepared as described previously (Jackson *et al*, 1992).

RESULTS

Oxysterols induce epidermal differentiation We first asked whether oxysterols induce keratinocyte differentiation in intact mice, as previously shown in cultured keratinocytes (Hanley *et al*, 2000). Topical applications of several concentrations (500 nm, 200 μM, 500 μM) of 22(R)OHCHOL (**Fig 1**), or 500 nm 24(S)25EPOCHOL (not shown) for 4 d resulted in a decrease in epidermal thickness as compared with vehicle-treated controls (**Fig 1**, oxysterol 10.97 ± 0.69 μm, one to two epidermal cell layers *vs* vehicle 20.19 ± 0.49 μm, $p < 0.001$, three to four epidermal cell layers). Moreover, these treatments also resulted in a decrease in the proliferative pool of epidermal keratinocytes, as assayed by PCNA immunostaining (oxysterol 13.2% PCNA positive nuclei per unit length of basement membrane *vs* vehicle 32.8%, $p < 0.001$). The occasional suprabasal, PCNA-positive cells that occur in vehicle-treated epidermis (**Fig 1*C***) were completely eliminated by oxysterol treatments (**Fig 1*D***).

On the other hand, involucrin, filaggrin, and loricrin protein levels all increased in oxysterol-treated epidermis (**Fig 2**). Whereas we observed increased labeling with the respective antibodies, staining remained restricted to the granular and upper spinous epidermal cell layers. The changes were especially pronounced for involucrin (**Fig 2*A vs* 2*B,C***) and profilaggrin/filaggrin (**Fig 2*D vs* 2*E*, *F***), but were less evident with loricin, perhaps because of its restricted localization to the stratum granulosum (**Fig 2*G vs* 2*H*, *I***). Moreover, profilaggrin (**Fig 3*A vs* 3*B*, *C***) and loricrin (**Fig 3*D vs* 3*E*, *F***) mRNA levels increased following oxysterol treatment, suggesting that the increase in the protein markers of differentiation was due to increased mRNA levels. Thus, topical 22(R)OHCHOL or 24(S)25EPOCHOL treatments induce the differentiation of epidermal keratinocytes. None of these epidermal changes were observed on the contralateral side or outside of the treated area (not shown). Therefore, the observed changes reflect a direct, local action of the oxysterols tested, rather than a secondary, systemic effect of these compounds. Taken together, these results clearly establish that, as in cultured human keratinocytes, oxysterols can modulate and upregulate epidermal differentiation *in vivo* in murine skin.

Pretreatment with oxysterols accelerates barrier repair The end product of epidermal differentiation is the provision of the cutaneous permeability barrier (Elias and Menon, 1991; Jackson *et al*, 1993). We previously demonstrated that oxysterols stimulate *in utero* development of the permeability barrier in fetal skin (Hanley *et al*, 1999). Therefore, we next determined the effects of topical oxysterols on permeability barrier homeostasis in adult mice. We observed no differences in basal TEWL following repeated oxysterol treatment; however, sites pretreated with 22(R)OHCHOL for 3 d demonstrated accelerated barrier repair kinetics compared with vehicle-treated sites after barrier disruption with either acetone or tape stripping (**Fig 4**). Thus, topical oxysterol treatment improves barrier homeostasis in adult mice.

Keratinocyte differentiation is impaired in LXR-β deficient mice Whereas these experiments indicate that oxysterols are capable of regulating epidermal keratinocyte differentiation and barrier homeostasis following topical treatment, they do not distinguish between receptor-specific *vs* unrelated mechanisms. Therefore, we next determined whether the absence of either of the two LXR receptors would impair epidermal keratinocyte differentiation. The morphologic appearance of the epidermis (**Fig 5*A vs* 5*C***); localization of involucrin (**Fig 6*A vs* 6*C***), filaggrin (**Fig 7*A vs* 7*C***), and loricrin (**Fig 7*G vs* 7*I***) proteins; and finally, the expression of profilaggrin (**Fig 8*A vs* 8*C***) and loricrin (**Fig 8*G vs* 8*I***) mRNA were indistinguishable in LXR-$\alpha^{-/-}$ mice *vs* wild-type (LXR-$\alpha\beta^{+/+}$) mice. These findings indicate that the absence of LXR-α does not affect keratinocyte terminal differentiation *in vivo*. In contrast, in either LXR-$\beta^{-/-}$ (**Fig 5*B***, 21.42 ± 1.17 μm, one to two epidermal cell layers) or LXR-$\alpha\beta^{-/-}$ mice (not shown) the epidermis was thinner than in wild-type skin (**Fig 5*A***, 35.81 ± 2.13 μm, $p < 0.001$, two to three epidermal cell layers) with a reduced number of PCNA-positive cells in the basal layer (**Fig 5*E***, 7.6% per unit length of basement membrane in $\beta^{-/-}$ *vs* 16% in $\alpha^{-/-}$ *vs* 23.6% in wild type, $p < 0.001$). Moreover, the expression of differentiation markers at both the protein and mRNA levels was slightly reduced in LXR-$\beta^{-/-}$ mice in comparison with wild-type mice (**Figs 6** and **7**). These observations suggest that signaling via LXR-β regulates the differentiation of normal epidermis; however, the absence of major cutaneous abnormalities in LXR-$\beta^{-/-}$ or LXR-$\alpha\beta^{-/-}$

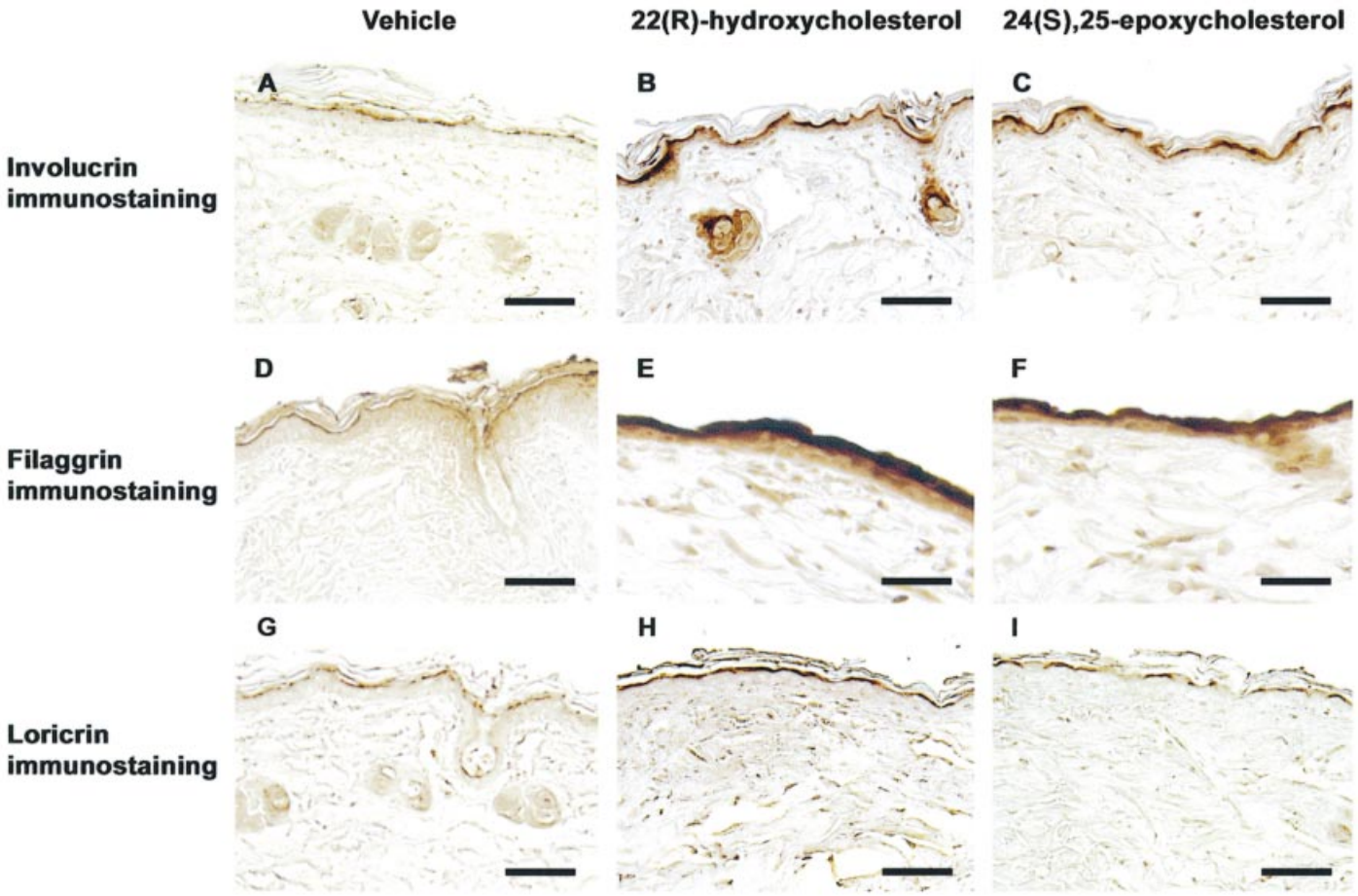

Figure 2. Effects of topical oxysterol treatment on the localization of involucrin, filaggrin, and loricrin proteins. Hairless mouse skin was treated for 4 d, twice a day with 22(R)OHCHOL (*B*, *E*, *H*) or 24(S)25EPOCHOL (*C*, *F*, *I*). Control animals were treated with vehicle (*A*, *D*, *G*). Involucrin, filaggrin, and loricrin proteins were detected with specific affinity-purified rabbit antibodies followed by goat biotinylated anti-rabbit IgG and ABC-PO. Peroxidase activity was revealed with diaminobenzidine substrate. Notice the prominent increase in involucrin (*B*, *C*) and filaggrin (*E*, *F*) staining in 22(R)OHCHOL (*B*, *E*) or 24(S)25EPOCHOL (*C*, *F*) treated animals as compared with the control (*A*, *D*). *Scale bar*: 50 μm.

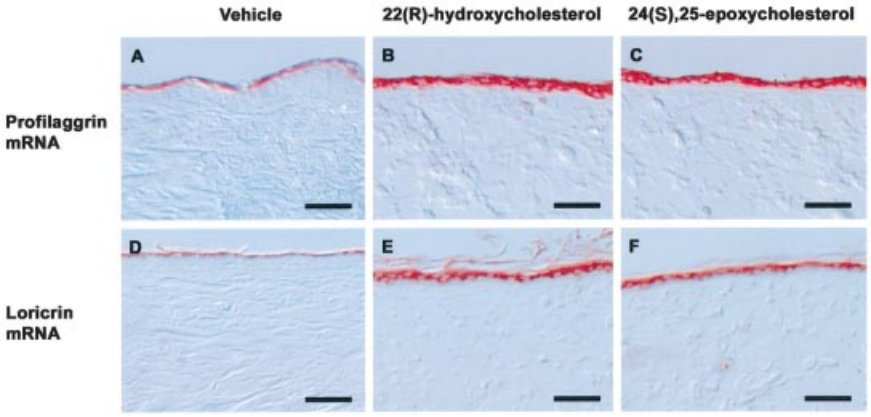

Figure 3. Effects of topical oxysterol treatment on the expression of profilaggrin and loricrin mRNA. Hairless mouse skin was treated for 4 d, twice a day with 22(R)OHCHOL (*B*, *E*) or 24(S)25EPOCHOL (*C*, *F*). Control animals were treated with vehicle (*A*, *D*). Profilaggrin and loricrin mRNA were localized with specific, biotin-labeled anti-sense riboprobes (red signal). Notice the prominent increase in profilaggrin (*B*, *C*) and loricrin (*E*, *F*) staining following treatment with 22(R)OHCHOL (*B*, *E*) or 24(S)25EPOCHOL (*C*, *F*) as compared with the control (*A*, *D*). *A–F*: DIC illumination. *Scale bar*: 50 μm.

knockout mice indicates that LXR-β may be only one of several factors that regulate epidermal differentiation, and that in its absence other pathways can compensate, maintaining the basic organization of the epidermis. Likewise, barrier recovery studies did not show any difference between wild-type and knockout mice (data not shown), indicating that the morphologic abnormalities do not result in functional impairment.

The effects of oxysterols on epidermal differentiation are mediated by LXR-β To determine whether oxysterols induce differentiation by specifically activating LXR, we initially determined the effects of topical treatment with 22(R)OHCHOL for 4 d in mice deficient both in LXR-α and LXR-β (LXR-$\alpha\beta^{-/-}$ double knockout mice). As seen above in hairless mice, in the wild-type (LXR-$\alpha\beta^{+/+}$) mice oxysterol treatment increased the

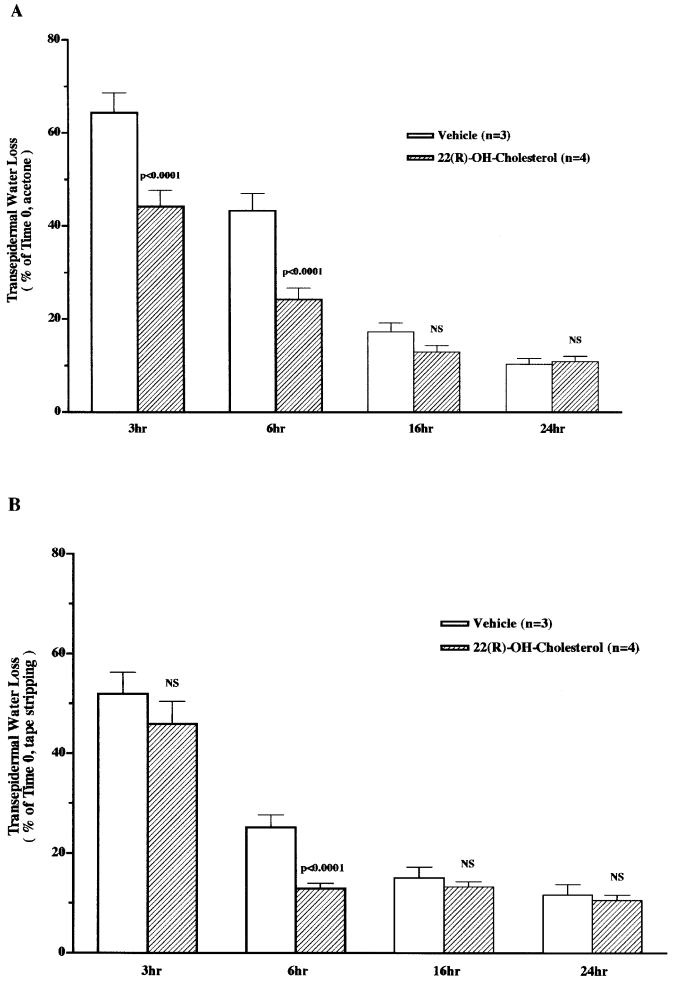

Figure 4. Effects of topical oxysterol treatment on epidermal permeability barrier homeostasis. Male hairless mice were treated with vehicle or 22(R)OHCHOL twice a day for 4 d. Next the animals were treated with acetone (*A*) or repeated tape stripping (*B*) until the TEWL reached 6–8 mg per cm^2 per h. TEWL was measured at 3, 6, 16, and 24 h and recovery of barrier function is expressed as percentage of time zero. Data are presented as mean ± SEM.

expression of differentiation markers, including involucrin, profilaggrin/filaggrin, and loricrin (**Figs 6–8**); however, in genetically matched LXR-$\alpha\beta^{-/-}$ double knockout mice, deficient in both LXR-α and -β, there were no changes in the levels of these differentiation markers (data not shown). These results indicate that the oxysterol induction of differentiation is mediated by LXR-α and/or LXR-β.

To determine whether LXR-α and/or LXR-β is important in mediating these effects, we next examined the effects of topical oxysterols in LXR-$\alpha^{-/-}$ or LXR-$\beta^{-/-}$ deficient mice (single knockout mice). In LXR-$\alpha^{-/-}$ mice topical 22(R)OHCHOL for 4 d induced an increase in involucrin (**Fig 6*C*** *vs* **6*F***), filaggrin (**Fig 7*C*** *vs* **7*F***), and loricrin (**Fig 7*I*** *vs* **7*L***) immunoreactivity, similar to that observed in wild-type (LXR-$\alpha\beta^{+/+}$) mice. Again immunolabeling was restricted to the granular and upper spinous cell layers. The changes induced by topical oxysterol application in LXR-$\alpha^{-/-}$ as well as in wild-type (LXR-$\alpha\beta^{+/+}$) epidermis, were even more pronounced for profilaggrin (**Fig 8*A*** *vs* **8*D***, and **8*C*** *vs* **8*F***) and loricrin (**Fig 8*G*** *vs* **8*J***, and **8*I*** *vs* **8*L***) mRNA expression. These results indicate that LXR-α is not required for the oxysterol induction of epidermal differentiation.

In contrast, in LXR-$\beta^{-/-}$ mice topical 22(R)OHCHOL application for 4 d failed to increase expression of epidermal differentiation markers, including involucrin (**Fig 6*B*** *vs* **6*E***), filaggrin (**Fig 7*B*** *vs* **7*E***), and loricrin (**Fig 7*H*** *vs* **7*K***). The lack of response to topical oxysterol treatment in the epidermis of LXR-$\beta^{-/-}$ mice was even more apparent when profilaggrin (**Fig 8*B*** *vs* **8*E***) and loricrin mRNA levels (**Fig 8*H*** *vs* **8K**) were compared by *in situ* hybridization. These observations indicate that LXR-β is essential for the oxysterol stimulation of epidermal keratinocyte differentiation, and that LXR-α cannot compensate for the absence of LXR-β.

LXR-β is the predominant LXR isoform in adult murine epidermis Previous studies have demonstrated that both LXR-α and LXR-β are expressed in human keratinocytes grown in culture and in fetal rat epidermis (Hanley *et al*, 1999, 2000). As the observations described above indicate that LXR-β is essential for the oxysterol-induced epidermal differentiation, we next examined if both LXR-α and LXR-β are expressed in adult mouse epidermis. In contrast to human keratinocytes and fetal rat epidermis, northern blotting demonstrated that LXR-β is the predominant LXR isoform expressed in adult mouse epidermis (**Fig 9**). The predominance of LXR-β in adult mouse epidermis is consistent with our observations that the expression of differentiation markers is modestly reduced in LXR-$\beta^{-/-}$ mice and oxysterol-induced differentiation does not occur in mice deficient in LXR-β.

Oxysterols normalize epidermal homeostasis in hyperproliferative epidermis Finally, we tested the ability of topical oxysterols to normalize epidermal morphology in a murine model of repeated barrier abrogation, which is characterized by epidermal hyperplasia and aberrant keratinocyte differentiation (Denda *et al*, 1996; Kömüves *et al*, 2000b). When 22(R)OHCHOL was applied to hyperplastic epidermis, there was a decrease in epidermal thickness in 22(R)OHCHOL-treated animals, as compared with the vehicle-treated mice (**Fig 10**, oxysterol 22.74 ± 1.41 μm, two epidermal cell layers *vs* vehicle 38.64 ± 1.87 μm, $p < 0.0001$, three to four epidermal cell layers). Moreover, a prominent transitional cell layer appeared between the stratum granulosum and stratum corneum (**Fig 10*B***), indicative of accelerated terminal differentiation, i.e., more rapid conversion of nucleated keratinocytes into corneocytes. These observations demonstrate that topical oxysterols can restore normal morphology in hyperproliferative epidermis.

DISCUSSION

In this study, we demonstrate that the topical application of 22ROHChol and 24(S)25EPOCHOL, two oxysterols that activate LXR, stimulate epidermal differentiation *in vivo*. Topical application of oxysterols increased the expression of loricrin, filaggrin, and involucrin, proteins that are expressed in the outer epidermis and are required for the formation of the cornified envelope and keratohyalin granules. Thus, oxysterols stimulate the latter stages of differentiation of keratinocytes in intact murine epidermis. Moreover, treatment with oxysterols accelerates the ability of the epidermis to restore cutaneous barrier function following barrier disruption by tape stripping or acetone treatment. The cutaneous permeability barrier is the end product of epidermal differentiation, and thus it is a functional marker of epidermal differentiation. Additionally, our studies further demonstrate that topical application of oxysterols inhibits epidermal proliferation. These observations extend previous studies, where we demonstrated that oxysterols stimulate differentiation and inhibit cell proliferation when added to human keratinocytes in culture (Hanley *et al*, 2000). With PPAR-α ligands, we and others have shown a similar induction of differentiation and inhibition of proliferation in keratinocytes both *in vivo* and *in vitro* (Hanley *et al*, 1998; Rivier *et al*, 1998; Kömüves *et al*, 2000a). Pertinently, most studies with retinoids or vitamin D have shown that these nuclear hormone ligands display markedly different effects when applied topically to animals as compared with their *in vitro* effects. Retinoids inhibit

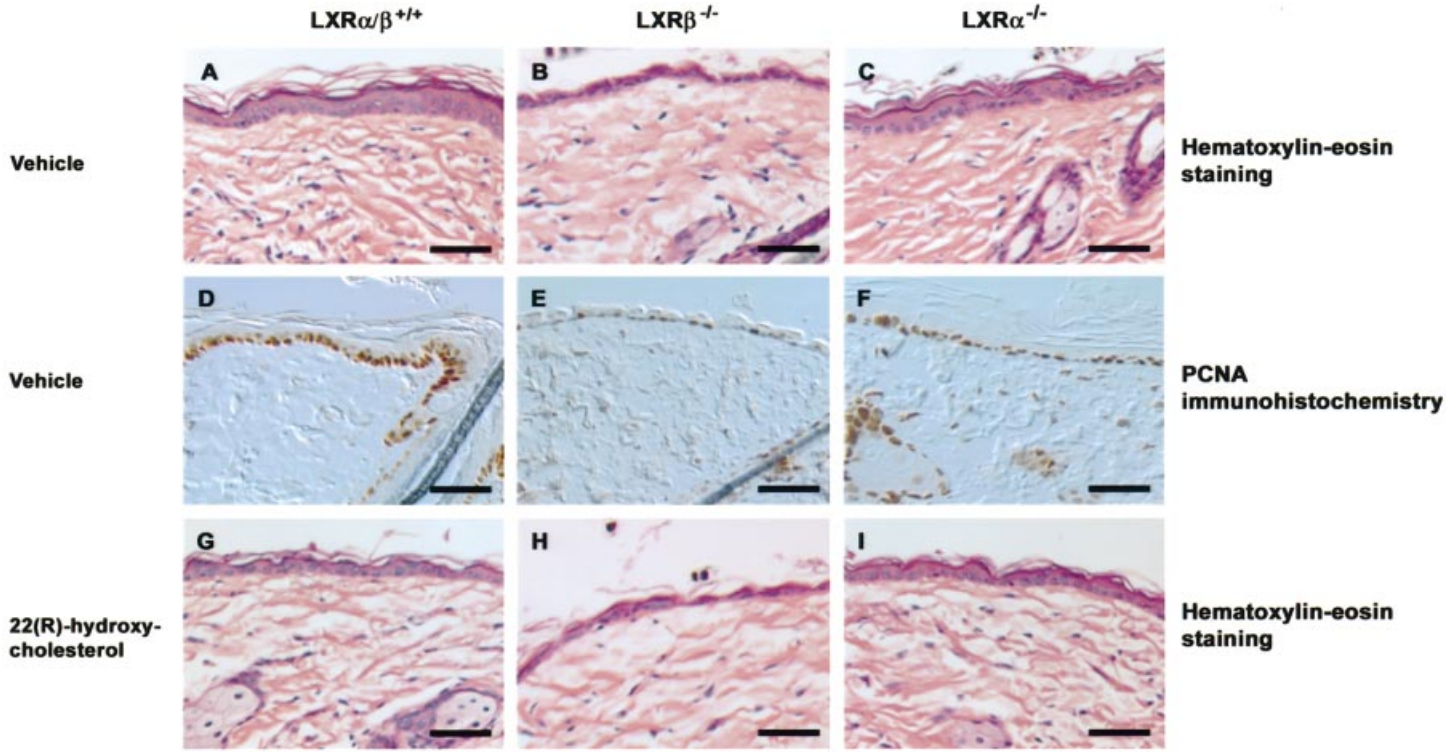

Figure 5. Effects of topical oxysterol treatment on epidermal morphology in LXR-$\beta^{-/-}$ and LXR-$\alpha^{-/-}$ mice. Shaved skin of LXR-$\beta^{-/-}$ (*B*, *E*, *H*), LXR-$\alpha^{-/-}$ (*C*, *F*, *I*), and wild-type, control (LXR-$\alpha\beta^{+/+}$, *A*, *D*, *G*) mice were treated for 4 d, twice a day with 22(R)OHCHOL (*G*, *H*, *I*). Hematoxylin–eosin-stained sections showed the epidermis of LXR-$\beta^{-/-}$ (*B*) animals was thinner as compared with wild-type control (LXR-$\alpha\beta^{+/+}$, *A*) or LXR-$\alpha^{-/-}$ (*C*) mice. Moreover, immunohistochemical detection of PCNA (a protein expressed in cycling cells) revealed a decrease in proliferating cells in the epidermis of LXR-$\beta^{-/-}$ mice (*E*) compared with wild-type control (LXR-$\alpha\beta^{+/+}$) or LXR-$\alpha^{-/-}$ mice (*D*, *F*). Topical 22(R)OHCHOL treatment resulted in a decrease in epidermal thickness in wild-type (LXR-$\alpha\beta^{+/+}$) control (*A vs G*), and LXR-$\alpha^{-/-}$ (*C vs I*) mice; however, the epidermal thickness in LXR-$\beta^{-/-}$ mice remained unaffected (*B vs H*) following topical 22(R)OHCHOL treatment. *A–C*, *G–I*: bright-field illumination, *D–F*: DIC illumination. *Scale bar*: 50 μm.

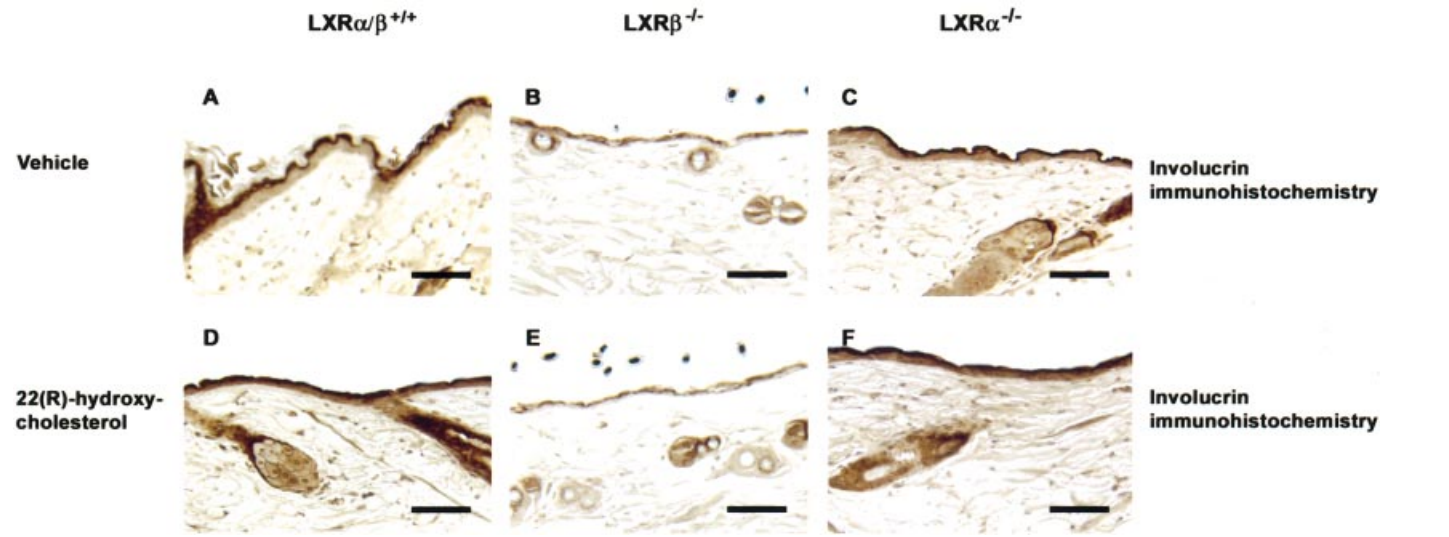

Figure 6. Effects of topical oxysterol treatment on involucrin localization in LXR-$\beta^{-/-}$ and LXR-$\alpha^{-/-}$ mice. Shaved skin of LXR-$\beta^{-/-}$ (*B*, *E*), LXR-$\alpha^{-/-}$ (*C*, *F*), and wild-type control (LXR-$\alpha\beta^{+/+}$, *A*, *D*) mice were treated for 4 d, twice a day with 22(R)OHCHOL (*D–F*) or with vehicle (*A–C*). Immunohistochemical detection of involucrin showed an increased staining in wild-type (LXR-$\alpha\beta^{+/+}$) control (*A vs D*) and LXR-$\alpha^{-/-}$ (*C vs F*) mice following 22(R)OHCHOL treatment; however, no such changes were seen in the epidermis of LXR-$\beta^{-/-}$ mice (*B vs E*). *Scale bar*: 50 μm.

keratinocyte differentiation *in vitro*, while stimulating differentiation when applied to intact animals (Brown *et al*, 1994; Eichner *et al*, 1996; Fisher and Voorhees, 1996; Kang *et al*, 1996; Hanley *et al*, 1998). Conversely, 1,25$(OH)_2$-vitamin D_3 inhibits proliferation and stimulates differentiation *in vitro*, while stimulating proliferation when applied *in vivo* (Pillai and Bikle, 1991; Bikle, 1996; Kang *et al*, 1996). In contrast, with both oxysterols and PPAR-α ligands, the *in vitro* and *in vivo* effects on keratinocyte proliferation and differentiation are identical (Hanley *et al*, 1998, 2000; Kömüves *et al*, 2000a; this paper).

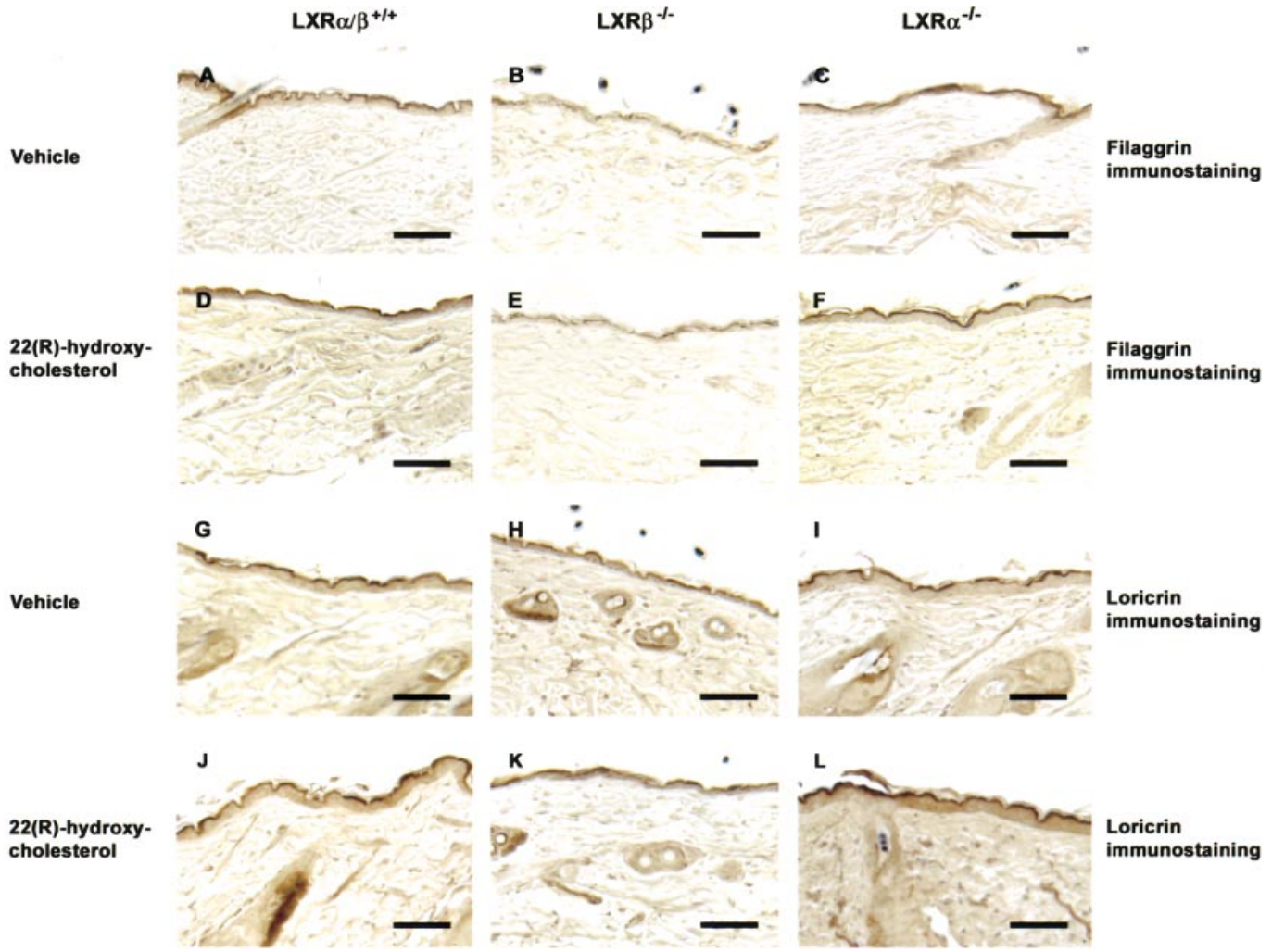

Figure 7. Effects of topical oxysterol treatment on filaggrin and loricrin localization LXR-$\beta^{-/-}$ and LXR-$\alpha^{-/-}$ mice. Shaved skin of LXR-$\beta^{-/-}$ (*B*, *E*, *H*, *K*), LXR-$\alpha^{-/-}$ (*C*, *F*, *I*, *L*), and wild-type control (LXR-$\alpha\beta^{+/+}$, *A*, *D*, *G*, *J*) mice were treated for 4 d, twice a day with 22(R)OHCHOL (*D–F*, *J–L*) or with vehicle (*A–C*, *G–I*). Immunohistochemical detection of filaggrin (*A–F*) and loricrin (*G–L*) showed an increased staining in the epidermis of wild-type (LXR-$\alpha\beta^{+/+}$) control (*A vs D*, *G vs J*), and LXR-$\alpha^{-/-}$ (*C vs F*, *I vs L*) mice following 22(R)OHCHOL treatment. On the contrary, both filaggrin (*B vs E*) and loricrin (*H vs K*) localization remained unchanged in the epidermis of LXR-$\beta^{-/-}$ mice. *Scale bar*: 50 μm.

This study further demonstrates that the oxysterol induction of differentiation in adult mouse epidermis is mediated via LXR-β, the predominant LXR isoform expressed in adult murine epidermis. The expression of differentiation markers is reduced in mice deficient in LXR-β. Moreover, topical oxysterols do not induce differentiation in LXR-β-deficient mice, whereas oxysterols induce differentiation in wild-type or LXR-α-deficient mice. Furthermore, LXR-α is either not expressed or expressed at low levels in murine epidermis. Thus, LXR-β is essential for the oxysterol stimulation of differentiation, and LXR-α cannot compensate for the absence of LXR-β. These studies do not rule out a role for LXR-α in the epidermis of species where it may be expressed in greater abundance.

To our knowledge this is one of the first examples of a biologic response that is mediated exclusively by LXR-β. In the liver, LXR-α is essential for the increase in cholesterol 7-α-hydroxylase that occurs with cholesterol feeding, and the presence of LXR-β in the liver of LXR-α-deficient mice cannot compensate for the absence of LXR-α (Peet *et al*, 1998). Recently, other genes important for cholesterol metabolism have been shown to be regulated by LXR. Specifically, the adenosine triphosphate-binding cassette transporters, ABC1 and ABC8, which mediate the efflux of cholesterol out of cells, and cholesterol ester transfer protein, a protein important in reverse cholesterol transport are stimulated by the RXR/LXR heterodimer (Costat *et al*, 2000; Luo and Tall, 2000; Repa *et al*, 2000b; Venkateswaran *et al*, 2000). Furthermore, very recent studies have shown that in addition to regulating cholesterol metabolism, stimulation of LXR increases lipogenesis by increasing the expression of the transcription factor SREBP-1c (Repa *et al*, 2000a; Schultz *et al*, 2000). The present study demonstrates that LXR stimulation plays a part in regulating keratinocyte differentiation, a process that is not known to be linked directly to lipid metabolism.

The mechanism by which activation of LXR-β stimulates the expression of the proteins involved in keratinocyte differentiation has been explored in our *in vitro* studies (Hanley *et al*, 2000). We have demonstrated that oxysterols increase the levels of involucrin mRNA when added to human keratinocytes, and that this increase is due to an increase in transcription. Using a series of truncations and deletions of the involucrin promoter upstream of a luciferase reporter, we identified an oxysterol-responsive region spanning nucleotides –2452 bp to –1872 bp. A single base pair mutation of the AP-1 site contained in this region (AP-1–5, –2117 to –2111 bp) abolished oxysterol responsiveness. Moreover, oxysterols increase the expression of an AP-1 reporter construct transfected into keratinocytes and gel shift experiments demonstrated increased

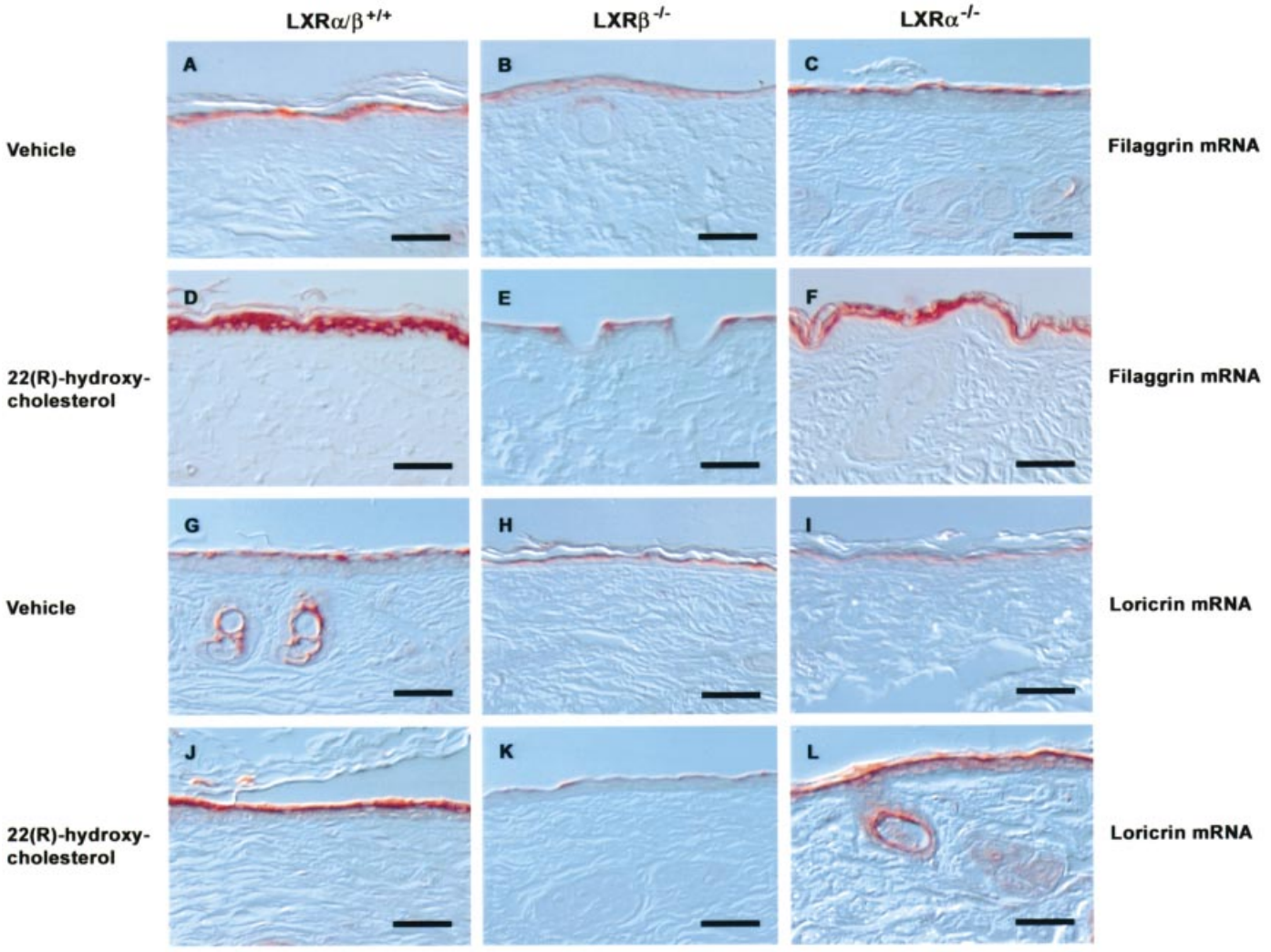

Figure 8. Effects of topical oxysterol treatment on profilaggrin and loricrin mRNA expression in LXR-$\beta^{-/-}$ and LXR-$\alpha^{-/-}$ mice. Shaved skin of LXR-$\beta^{-/-}$ (*B*, *E*, *H*, *K*), LXR-$\alpha^{-/-}$ (*C*, *F*, *I*, *L*) and wild-type control (LXR-$\alpha\beta^{+/+}$, *A*, *D*, *G*, *J*) mice were treated for 4 d, twice a day with 22(R)OHCHOL (*D–F*, *J–L*) or with vehicle (*A–C*, *G–I*). *In situ* hybridization with anti-sense biotin-labeled riboprobes were used to localize profilaggrin (*A–F*) and loricrin (*G–L*) mRNA. Sense control riboprobes did not yield a signal under identical conditions. Both profilaggrin and loricrin anti-sense probes indicated an increased expression in the epidermis of wild-type (LXR-$\alpha\beta^{+/+}$) control (*A vs D*, *G vs J*), and LXR-$\alpha^{-/-}$ (*C vs F*, *I vs L*) mice following 22(R)OHCHOL treatment; however, neither profilaggrin (*B vs E*) nor loricrin (*H vs K*) mRNA expression changed in the epidermis of LXR-$\beta^{-/-}$ mice. *A–L*: DIC illumination. *Scale bar*: 50 μm.

binding of nuclear extracts from oxysterol-treated keratinocytes to AP-1–5. Pertinently, this same AP-1–5 site mediates the increase in involucrin expression stimulated by 12-*O*-tetradecanoyl-phorbol-13-acetate, calcium, and PPAR-α ligands (Welter *et al*, 1995; Banks *et al*, 1998; Kömüves *et al*, 2000a; Ng *et al*, 2000). As a number of the other proteins that increase during keratinocyte differentiation also are known to be regulated by AP-1 transcription factors (Eckert *et al*, 1997), one can postulate that oxysterols act through LXR-β to increase AP-1 activity thereby coordinately stimulating the expression of a variety of proteins required for keratinocyte differentiation.

As noted above, the expression of differentiation markers was slightly reduced in untreated LXR-β- or LXR-αβ-deficient mice compared with wild-type mice, suggesting that LXR-β plays a part in regulating normal epidermal keratinocyte differentiation. Paradoxically, given the decrease in epidermal thickness and PCNA staining with oxysterol treatment of normal animals, in LXR-β- and LXR-αβ-deficient mice the thickness of the epidermis and PCNA staining was slightly reduced. The explanation for this decrease is unclear but it is worth noting that in LXR-α-deficient mice basal levels of cholesterol 7-α hydroxylase in the liver are increased despite an inability of oxysterols to further increase expression (Peet *et al*, 1998). It is postulated that LXR-α plus ligand stimulates the expression of cholesterol 7-α hydroxylase but that LXR-α alone inhibits expression. In animals deficient in LXR-α basal expression of cholesterol 7-α hydroxylase is therefore increased. Whether a similar phenomenon is occurring with regards to keratinocyte proliferation is unknown. Notably, the gross cutaneous phenotypes of LXR-β- and LXR-αβ-deficient mice are normal indicating that multiple factors regulate epidermal development and differentiation, and that the absence of LXR-β alone is not sufficient to result in substantial abnormalities in the epidermis. It is likely that the activation of other nuclear hormone receptors, such as PPAR-α and the vitamin D receptor, can compensate for the absence of LXR-β.

Cholesterol synthesis is very active in the epidermis and large quantities of cholesterol are required for the formation of lamellar bodies (Feingold, 1991). Stress to the epidermis such as ultraviolet light, air pollution, and aging increases the oxidative state of keratinocytes thereby potentially increasing the formation of oxysterols (Ozawa *et al*, 1991; Darr and Fridovich, 1994; Fuchs *et al*, 1995; Yamazaki *et al*, 1999). Moreover, cholesterol is

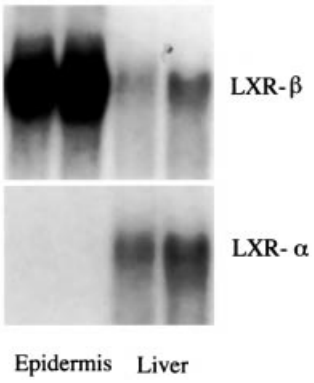

Figure 9. Expression of LXR in mouse epidermis. Poly(A)(+) mRNA was isolated from the epidermis of hairless mice or liver as described in the *Materials and Methods*. Ten micrograms were loaded per lane. In the case of epidermis, each lane represents mouse epidermis pooled from two animals. Northern analysis was performed, and blots hybridized with either LXR-α or LXR-β cDNA as described in *Materials and Methods*.

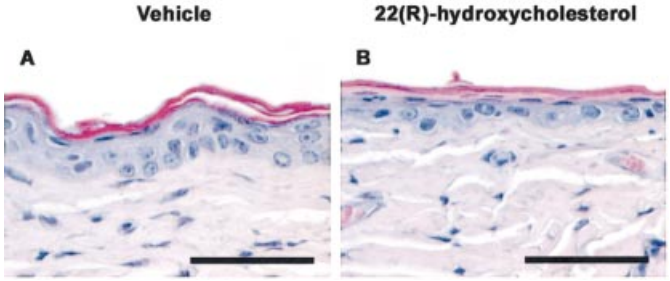

Figure 10. Effects of topical oxysterol treatment on hyperproliferative epidermis. Epidermal hyperproliferation was induced by repeated barrier abrogation (twice a day treatment with acetone, for 5 d). For an additional 4 d the epidermis was treated, twice a day with vehicle (*A*) or with 22(R)OHCHOL (*B*). Hematoxylin–eosin-stained sections. *Scale bar*: 50 μm.

converted to oxysterols, some of which are LXR ligands, by P450 enzymes that are expressed in many tissues, including the epidermis (Jugert *et al*, 1994; Russell, 2000). Although the levels of specific oxysterols in the epidermis and keratinocytes are not yet known, it is possible that oxysterols may serve as endogenous regulators of keratinocyte differentiation and development. Finally, our studies have also demonstrated that topical treatment with oxysterols can normalize the epidermal hyperplasia seen after repeated barrier disruption, suggesting that LXR ligands could be used therapeutically to treat a variety of skin disorders.

In conclusion, this study demonstrates that naturally occurring oxysterols stimulate epidermal differentiation and that this effect is mediated via LXR-β. It is possible that oxysterols may be clinically useful for the treatment of cutaneous disorders due to impaired differentiation and/or increased proliferation.

We thank Timothy Willson (Nuclear Receptor Discovery Research, GlaxoSmithKline, Research Triangle Park, NC) for his generous gift of 24(S),25-epoxycholesterol. This work was supported by NIH grants HD 29706, AR 29706, and PO 039448, and VA Research Funding. M.S. was supported by a grant from the Austrian Science Fund (Project J2112).

REFERENCES

Banks EB, Crish JF, Welter JF, Eckert RL: Characterization of human involucrin promoter distal regulatory region transcriptional activator elements—A role for Sp1 and AP1 binding sites. *Biochem J* 331:61–68, 1998

Bikle DD: 1,25 OH_2D_3-modulated calcium induced keratinocyte differentiation. *J Invest Dermatol Symp Proc* 1:22–27, 1996

Brown LJ, Geesin JC, Rothnagel JA, Roop DR, Gordon JS: Retinoic acid suppression of loricrin expression in reconstituted human skin cultured at the air–liquid interface. *J Invest Dermatol* 102:886–890, 1994

Costat P, Luo Y, Wang N, Tall AR: Sterol-dependent transactivation of the ABC1 promoter by LXR/RXR. *J Biol Chem* 275:28240–28245, 2000

Darr D, Fridovich I: Free radicals in cutaneous biology. *J Invest Dermatol* 102:671–675, 1994

Denda M, Wood LC, Emami S, Calhoun C, Brown BE, Elias PM, Feingold KR: The epidermal hyperplasia associated with repeated barrier disruption by acetone treatment or tape stripping cannot be attributed to increased water loss. *Arch Dermatol Res* 288:230–238, 1996

Eckert RL, Crish JF, Robinson NA: The epidermal keratinocyte as a model for the study of gene regulation and function. *Physiol Rev* 77:397–424, 1997

Eichner R, Gendimenico GJ, Kahn M, Mallon JP, Capetola RJ, Mezick JA: Effects of long-term retinoic acid treatment on epidermal differentiation *in vivo*, specific modification in the programme of terminal differentiation. *Br J Dermatol* 135:687–695, 1996

Elias PM, Menon GM: Structural and biochemical correlates of the epidermal permeability barrier. *Adv Lipid Res* 24:1–26, 1991

Feingold KR: The regulation and role of epidermal lipid synthesis. *Adv Lipid Res* 24:57–82, 1991

Feng X, Peng ZH, Di W: Suprabasal expression of a dominant-negative RXR-alpha mutant in transgenic mouse epidermis impairs regulation of gene transcription and basal keratinocyte proliferation by RAR-selective retinoids. *Genes Dev* 11:59–71, 1997

Fisher GJ, Voorhees JJ: Molecular mechanisms of retinoid actions in skin. *FASEB J* 10:1002–1013, 1996

Fuchs E: Epidermal differentiation. The bare essentials. *J Cell Biol* 111:2807–2814, 1990

Fuchs J, Schurer N, Mibrad R: Environmental stress and reactive oxidants in skin. In: Fuchs, F, Packer, R (eds). *Oxidative Stress in Dermatology*. New York: Marcel Dekker, 1995: pp 413–456

Hanley K, Jiang Y, He SS: Keratinocyte differentiation is stimulated by activators of the nuclear hormone receptor PPAR-alpha. *J Invest Dermatol* 110:368–375, 1998

Hanley K, Kömüves LG, Bass NM: Fetal epidermal differentiation and barrier development *in vivo* is accelerated by nuclear hormone receptor activators. *J Invest Dermatol* 113:788–795, 1999

Hanley K, Ng DC, He SS: Oxysterols induce differentiation in human keratinocytes and increase AP-1 dependent involucrin transcription. *J Invest Dermatol* 114:545–553, 2000

Imakado S, Bickenbach JR, Bundman DS: Targeting expression of a dominant-negative retinoic acid receptor mutant in the epidermis of transgenic mice results in loss of barrier function. *Genes Dev* 9:317–329, 1995

Jackson SM, Wood LC, Lauer S, Taylor JM, Cooper AD, Elias PM, Feingold KR: Effect of cutaneous permeability barrier disruption on HMG CoA reductase, LDL receptor and apoprotein E mRNA levels in the epidermis of hairless mice. *J Lipid Res* 33:1307–1314, 1992

Jackson SM, Williams ML, Feingold KR, Elias PM: Pathobiology of the stratum corneum. *West J Med* 158:279–285, 1993

Jugert FK, Agarwal R, Kuhn A, Bickers DR, Merk HF, Mukhtar H: Multiple cytochrome P450 isozymes in murine skin. induction of P450, 1A, 2B, 2E, and 3A by dexamethasone. *J Invest Dermatol* 102:970–975, 1994

Kang S, Li X-Y, Voorhees JJ: Pharmacology and molecular action of retinoids and vitamin D in skin. *J Invest Dermatol Symp Proc* 1:15–21, 1996

Kliewer SA, Lehmann JM, Willson TM: Orphan nuclear receptors: Shifting endocrinology into reverse. *Science* 284:757–760, 1999

Kömüves LG, Hanley K, Jiang Y, Elias PM, Wiliams ML, Feingold KR: Ligands and activators of nuclear hormone receptors regulate epidermal differentiation during fetal rat skin development. *J Invest Dermatol* 111:429–433, 1998

Kömüves LG, Hanley K, Lefebvre AM: Stimulation of peroxisome proliferator-activated receptor-α promotes epidermal keratinocyte differentiation *in vivo*. *J Invest Dermatol* 115:353–360, 2000a

Kömüves LG, Hanley K, Man MQ, Elias PM, Williams ML, Feingold KR: Keratinocyte differentiation in hyperproliferative epidermis. Topical application of peroxisome proliferator-activated receptor-α activators restores tissue homeostasis. *J Invest Dermatol* 115:361–367, 2000b

Li M, Indra AK, Warot X, *et al*: Skin abnormalities generated by temporally controlled RXRalpha mutations in mouse epidermis. *Nature* 407:633–636, 2000

Luo Y, Tall AR: Sterol upregulation of human CETP expression in vitro and in transgenic mice by an LXR element. *J Clin Invest* 105:513–520, 2000

Mangelsdorf DJ, Thummel C, Beato M: The nuclear receptor superfamily: The second decade. *Cell* 83:835–839, 1995

Ng DC, Shakaee S, Lee D, Bikle DD: Requirement of an AP-1 site in the calcium response region of the involucrin promoter. *J Biol Chem* 275:24080–24088, 2000

Ozawa N, Yamazaki S, Chiba K, Aoyama H, Tomisawa H, Tateishi M, Watanabe T: Occurrence of cholesterol 7-alpha- and 7-beta-hydroperoxides in rat skin as aging markers. *Biophys Biochem Res Commun* 178:242–247, 1991

Peet DJ, Turley SD, Ma W, Janowski BA, Lobaccaro J-MA, Hammer RE, Mangelsdorf DJ: Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR-alpha. *Cell* 93:693–704, 1998

Pillai S, Bikle DD: Role of intracellular free calcium in the cornified envelope

formation of keratinocytes, differences in the mode of action of extracellular calcium and 1,25 dihydroxyvitamin D_3. *J Cell Physiol* 146:94–100, 1991

Reichert U, Michel S, Schmidt R: The cornified cell envelope. A key structure of the terminally differentiated keratinocytes. In: Darmon, M, Blumenberg, M (eds). *Molecular Biology of the Skin*. San Diego: Academic Press, 1993: pp 107–297

Repa JJ, Liang G, Ou J: Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXR-alpha and LXR-beta. *Genes Dev* 14:2819–2830, 2000a

Repa JJ, Turley SD, Lobaccaro J-MA: Regulation of absorption and ABC1 mediated efflux of cholesterol by RXR heterodimers. *Science* 289:1524–1529, 2000b

Rivier M, Safonova I, Lebrun P, Griffiths CE, Ailhaud G, Michel S: Differential expression of peroxisome proliferator-activated receptor subtypes during the differentiation of human keratinocytes. *J Invest Dermatol* 111:1116–1121, 1998

Russell DW: Oxysterol biosynthetic enzymes. *Biochem Biophys Acta* 1529:126–135, 2000

Saitou M, Sugai S, Tanaka T, Shimouchi K, Fuchs E, Narumiya S, Kakizuka A: Inhibition of skin development by targeted expression of a dominant-negative retinoic acid receptor. *Nature* 374:159–162, 1995

Schultz JR, Tu H, Luk A: Role of LXR in control of lipogenesis. *Genes Dev* 14:2831–2838, 2000

Steinert PM: A model for the hierarchical structure of the human epidermal cornified cell envelope. *Cell Death Differ* 2:23–35, 1995

Steinert PM, Marenkov LN: The proteins elafin, filaggrin, keratin intermediate filaments, loricrin and SPRs are isopeptide crosslinked components of the human epidermal cornified cell envelope. *J Biol Chem* 270:17702–17711, 1995

Steinert PM, Marenkov LN: Involucrin is an important early component in the assembly of the epidermal cornified cell envelope. *J Biol Chem* 272:2021–2030, 1997

Venkateswaran A, Repa JJ, Lobaccaro J-MA, Bronson A, Mangelsdorf DJ, Edwards PA: Human white/murine ABC8 mRNA levels are highly induced in lipid loaded macrophages: a transcriptional role for specific oxysterols. *J Biol Chem* 275:14700–14707, 2000

Welter JF, Crish JF, Agarwal C, Eckert RL: Fos-related antigen Fra-1, junB, and junD activate human involucrin promoter transcription by binding to proximal and distal AP1 sites to mediate phorbol ester effects on promoter activity. *J Biol Chem* 270:12614–12622, 1995

Yamazaki S, Ozawa N, Hiratsuka A, Watanabe T: Quantitative determination of cholesterol 5-alpha-, 7-alpha-, and 7-beta-hydoperoxides in rat skin. *Free Rad Biol Med* 27:110–118, 1999