Regulation of ABCG1 expression in human keratinocytes and murine epidermis[S]

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Abstract **ABCG1, a member of the ATP binding cassette superfamily, facilitates the efflux of cholesterol from cells to HDL. In this study, we demonstrate that ABCG1 is expressed in cultured human keratinocytes and murine epidermis, and induced during keratinocyte differentiation, with increased levels in the outer epidermis. ABCG1 is regulated by liver X receptor (LXR) and peroxisome proliferator-activated receptor-δ (PPAR-δ) activators, cellular sterol levels, and acute barrier disruption. Both LXR and PPAR-δ activators markedly stimulate ABCG1 expression in a dose- and time-dependent fashion. PPAR-γ activators also increase ABCG1 expression, but to a lesser degree. In contrast, activators of PPAR-α, retinoic acid receptor, retinoid X receptor, and vitamin D receptor do not alter ABCG1 expression. In response to increased intracellular sterol levels, ABCG1 expression increases, whereas inhibition of cholesterol biosynthesis decreases ABCG1 expression. In vivo, ABCG1 is stimulated 3–6 h after acute barrier disruption by either tape stripping or acetone treatment, an increase that can be inhibited by occlusion, suggesting a potential role of ABCG1 in permeability barrier homeostasis. Although *Abcg1*-null mice display normal epidermal permeability barrier function and gross morphology, abnormal lamellar body (LB) contents and secretion leading to impaired lamellar bilayer formation could be demonstrated by electron microscopy, indicating a potential role of ABCG1 in normal LB formation and secretion.**—Jiang, Y. J., B. Lu, E. J. Tarling, P. Kim, M-Q. Man, D. Crumrine, P. A. Edwards, P. M. Elias, and K. R. Feingold. **Regulation of ABCG1 expression in human keratinocytes and murine epidermis. *J. Lipid Res.* 2010.** 51: **3185–3195.**

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Cholesterol levels are tightly regulated in cells, and maintenance of cholesterol homeostasis is of particular importance in keratinocytes/epidermis. In addition to being an essential component of all cell membranes, cholesterol is required in differentiated keratinocytes to form lamellar bodies (LBs) (1, 2). Secretion of these unique organelles delivers lipids, including cholesterol, which mediate permeability barrier function, to the extracellular spaces of the stratum corneum (SC) (2). 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, following permeability barrier disruption, epidermal cholesterol synthesis increases (3), as do the levels of receptors such as the LDL receptor and scavenger receptor class B, member 1 that enhance the uptake of cholesterol into keratinocytes (4, 5). Inhibition of cholesterol synthesis perturbs permeability barrier function (6), and a selective deficiency in cholesterologenesis largely accounts for the barrier abnormality in chronologically aged epidermis (7, 8). Cholesterol is also the precursor of an important bioregulatory molecule in keratinocytes, cholesterol sulfate (CS), which regulates epidermal keratinocyte differentiation (9–11) and corneocyte desquamation by diverse mechanisms (12, 13).

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Abbreviations: apoA-I, apolipoprotein A-I; CHK, cultured human keratinocyte; Cig, ciglitazone; CS, cholesterol sulfate; ER, endoplasmic reticulum; GI, GI251929; GW, GW 610742X; H and E, hematoxylin and eosin; KO, knockout; LB, lamellar body; LXR, liver X receptor; PPAR, peroxisome proliferator-activated receptor; 22R, 22(R)-OH-cholesterol; SC, stratum corneum; SG, stratum granulosum; TEWL, transepidermal water loss; TO, TO901317; TS, tape stripping; WT, wild type.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of six figures.

Very recently, gene mutations of either sterol-Δ^{24}-reductase (14, 15), which converts desmosterol to cholesterol, or 3β-hydroxysterol-Δ^{7}-reductase (unpublished observations), which converts 7-hydrocholesterol to cholesterol, led to impaired epidermal development and the death of animals a few hours after birth, further emphasizing the key role of cholesterol for normal permeability barrier homeostasis.

Cholesterol homeostasis is regulated by a balance between de novo synthesis of cholesterol, the uptake of cholesterol from lipoproteins, and cholesterol efflux. The efflux of cholesterol from cells is mediated by ABCA1 (16–18). Previously, we reported that ABCA1 is expressed in cultured human keratinocytes (CHKs) and murine epidermis, and that liver X receptor (LXR) activation markedly stimulates ABCA1 mRNA and protein levels in CHKs and mouse epidermis (19). Similar to LXR, activation of other nuclear receptors, including peroxisome proliferator-activated receptor-α (PPAR-α), PPAR-β/δ, and RXR, also increase ABCA1 expression in CHKs (19). Additionally, increases in cholesterol levels in keratinocytes induced by LDL or mevalonate stimulate ABCA1 expression, while inhibiting cholesterol synthesis with statins, or CS decreases ABCA1 expression in CHKs (19). Accordingly, following acute barrier disruption by either tape stripping (TS) or acetone treatment, ABCA1 expression declines (19), a change that should attenuate cholesterol efflux, thereby making more cholesterol available for regeneration of the barrier. In support of this notion, during fetal rat epidermal development, ABCA1 expression decreases at days 18–22 of gestation, leaving more cholesterol available for the critical period of barrier formation (19).

Recently, another membrane protein, ABCG1, was identified and was shown to be strongly responsive to cellular cholesterol status in macrophages, suggesting a role in cholesterol efflux (18, 20). ABCG1 is a member of a subfamily of half-transporters possessing one transmembrane domain and a single large intracellular region containing one ATP binding/hydrolysis cassette (20, 21). ABCG1 is abundantly expressed in multiple tissues (lung, brain, kidney, and spleen), and in many cells (macrophages, lymphocytes, epithelial cells, and endothelial cells) that require cholesterol for synthetic processes. In macrophages, ABCG1 is highly induced by modified LDL or specific oxysterols, leading to an increase in cellular cholesterol efflux to HDL (22–25). Specifically, the transfer of lipids mediated by ABCG1 requires phospholipid-containing acceptors such as HDL, phospholipid/apolipoprotein A-I (apoA-I)-containing discs, or small, phospholipid-only unilamellar vesicles (20, 26). In animal models, targeted disruption of ABCG1 causes massive deposition of neutral lipids and phospholipids in lungs, particularly in alveolar macrophages (27–29). Thus, ABCG1 is a key transporter for cholesterol homeostasis in certain tissues.

Little is known about the role of ABCG1 in keratinocytes/epidermis. Because epidermis is a rapidly turning over tissue that also requires a large amount of cholesterol for the formation of the cutaneous permeability barrier, we hypothesized that keratinocytes would express ABCG1. In the present study, we determined the expression and regulation of ABCG1 using CHKs, mouse epidermis, and *Abcg1*-null mice as model systems, and compared the pattern of ABCG1 regulation with those of ABCA1 and ABCA12.

MATERIALS AND METHODS

Materials

Hairless female mice (hr/hr), 6–8 weeks old, were purchased from Charles River Laboratories (Wilminton, MA). ABCG1 knockout (KO) mice, 8 weeks old, were generated as previously described in C57BL/6 strain (28). 22(R)-OH-cholesterol (22R), clofibrate, WY14643, CS, and lovastatin were purchased from Sigma (St. Louis, MO). PPAR-γ activators ciglitazone (Cig), and LXR synthetic ligand TO901317 (TO) were purchased from Cayman Chemical Co. (Ann Arbor, MI). The PPAR-δ activator GW 610742X (GW) and PPAR-γ activator GI251929 (GI) were a generous gift from Dr. Tim Willson (GlaxoSmithKline). Human LDL was from Intracel Resources, LLC (Frederick, MD). All reagents and supplies for real-time quantitative PCR (QPCR) were obtained as described previously (30). Primary polyclonal antibodies against ABCG1 and GAPDH were purchased from Novus Biologicals (Littleton, CO) and Santa Cruz Biologicals, Inc. (Santa Cruz, CA), respectively. All other reagents for Western blot: NuPAGE® Novex Precast gels (4–12% Bis-Tris), buffers, protein standards, and detection kits were purchased from Invitrogen (Carlsbad, CA). Secondary antibody (biotinylated goat anti-rabbit antibody) was from Vector Laboratories (Burlingame, CA).

Epidermal function studies

Transepidermal water loss (TEWL) (mg/cm^2/h) measurements were performed using an electrolytic water analyzer (Meeco; Warrington, PA) 2 and 4 h after tape stripping (3-fold increase in TEWL), and percent barrier recovery was determined as described previously (31).

Keratinocyte culture

Human foreskin keratinocytes were isolated by a modification of the method of Pittelkow and Scott (32) under an Institutional Review Board approval protocol (University of California, San Francisco). For a typical experiment, the second passage of keratinocytes was seeded and maintained in 0.07 mM Ca^{2+} 154CF medium (Cascade Biologics, Inc.; Portland, OR). 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 or absence of various reagents or vehicle control, with three to five dishes of cells for each treatment group, as described previously (30). Each experiment was repeated at least once using a separate batch of cells.

Microscopy and immunohistochemistry

Following the removal of fur, tissue samples were fixed as previously reported (33), and sections (5 μm) were made to detect ABCG1 levels using primary polyclonal antibodies against ABCG1 (1:200) (33). It should be noted that using this antibody, we could detect ABCG1 (stain in dark blue) in epidermis from wild-type (WT) but not from *Abcg1*-null mice (see supplementary Fig. IV). However, when we obtained additional batches of anti-ABCG1 antibodies from the same company, the specificity of the antibodies was not adequate, inasmuch as we observed staining in the epidermis of both WT and *Abcg1*-null mice. Hence, all immunohistochemistry ABCG1 data presented in this paper are

generated from the first batch of primary antibodies we obtained from Novus Biologicals on August 4, 2006. Nonspecific binding of secondary IgG was not seen when the primary antibody was omitted (negative control). For Nile Red staining, frozen sections (5 μm) were prepared and processed as previously described in detail (34).

Animal treatment

Adult mouse dorsal skin was treated by topically applying either vehicle (100% acetone) or the LXR activator TO (15 mM) twice a day for 3 days. At the end of treatment, the dorsal skin was collected and the epidermis was isolated as described previously (5). The small amount of acetone applied (~50 μl) does not alter TEWL.

Acute barrier disruption model

Adult mouse skin was treated either by gently applying acetone-soaked cotton balls for 5–10 min (~500 μl) or by sequential applications of cellophane tape as described previously (3, 19, 35). In another set of mice, occlusion was carried out. Briefly, following acute barrier disruption either by acetone treatment or by TS, skin was covered with a tightly fitted, water-impermeable membrane (one finger of a latex glove) immediately after treatment, for various periods of time. For the acetone model, mice treated with cotton balls soaked in 0.9% (w/v) sodium chloride served as controls. For the TS model, untreated animals served as controls.

Institutional approval

The use of animals and all experimental procedures were approved by the appropriate committees, including the Institutional Animal Care and Use Committee of the San Francisco Veterans Affairs Medical Center.

Epidermis preparation

For epidermis isolation, mouse skin was placed dermis-side down in 10 mM EDTA in calcium-magnesium-free PBS, pH 7.4, for 35–50 min at 37°C (5).

Total RNA isolation and real-time PCR

Total RNA isolation, first-strand cDNA synthesis, and real-time PCR were carried out as described previously (19). The primer sequences for PCR are listed in **Table 1**. The PCR reaction was performed in duplicate; gel electrophoresis and melting curve analyses were performed to confirm accurate PCR product sizes and absence of nonspecific bands. The expression levels of each gene were normalized against cyclophilin (human keratinocytes) or 36B4/cyclophilin (mouse epidermis) using the comparative C_T method.

Western blot analysis

Western blots were carried out according to the manufacturer's protocol. Briefly, the membrane fraction was prepared from either CHKs or mouse epidermis, and proteins were fractionated on precast gradient gels (4–12%) and transferred to polyvinylidene difluoride (0.2 μM pore size) membranes overnight (4°C). The proteins on the membrane were subsequently probed with polyclonal primary antibodies against ABCG1 (1:1000). An identical blot was probed with anti-GAPDH antibody to verify equal loading of protein.

Electron microscopy

For each animal, two sets of samples (*Abcg1* KO or WT mouse skin; n = 2) were prepared using two different methods: reduced osmium-tetroxide fixation to visualize LB secretion and overall SC formation, and ruthenium-tetroxide fixation to visualize the SC bilayers. For each method and sample, approximately 25–40 images were examined (36). Ultrathin sections (60 nm) were contrasted with uranyl acetate and lead citrate, and examined with a Zeiss 10Å electron microscope (Carl Zeiss, Inc.; Thornwood, NY) operated at 60 kV.

Assessment of LB density and secretion

The procedures used for quantifying LB density were previously described in detail (37). Briefly, for LB quantification, ⩾10 micrographs were taken randomly from different, nonoverlapping regions at the stratum granulosum (SG)-SC interface at 16,000× magnification of each sample. Empty LB was defined as lacking 2/3 of LB content, and quantified on ⩾10 random micrographs at 63,000× magnification from each sample. Micrographs were then coded, randomized, and evaluated by separate observers. The same set of micrographs (low-magnification) were also used for quantifying LB secretion, which is defined as the ratio of each area filled by secreted LB content (point) over the length (in cm) of the bottom surface of the first SC cell layer, determined with a digital planimeter.

Statistical analysis

All data are expressed as mean ± SEM, using Student's *t*-test for comparison between two groups. Comparisons within multiple groups were subjected to a one-way ANOVA test, followed by Dunnett's posthoc test to analyze the variance between two

TABLE 1. The primer sequence for real-time PCR

Gene	Primer sequence	Amplicon (bp)
ABCG1 (human)	(F) 5′-CTGGTGAACAACCCTCCAGT-3′ (R) 5′-ATCTGCTGGGTTGTGGTAGG-3′	160
Involucrin (human)	(F) 5′-CTGCCTGAGCAAGAATGTGA-3′ (R) 5′-TGCTCTGGGTTTTCTGCTTT-3′	110
Cyclophilin (human)	(F) 5′-TCTCCTTTGAGCTGTTTGCAG-3′ (R) 5′-CACCACATGCTTGCCATC-3′	326
ABCA1 (mouse)	(F) 5′-GCAGATCAAGCATCCCAACT-3′ (R) 5′-CCAGAGAATGTTTCATTGTCCA-3′	68
ABCA12 (mouse)	(F) 5′-ACAGGAATGGCCTTCATCAC-3′ (R) 5′-AACATGGTGCCCTGAGAAAC-3′	317
ABCG1 (mouse)	(F) 5′-GAAGTGGCATCAGGGGAGTA-3′ (R) 5′-AAAGAAACGGGTTCACATCG-3′	124
36B4 (mouse)	(F) 5′-GCGACCTGGAAGTCCAACTAC-3′ (R) 5′-ATCTGCTGCATCTGCTTGG-3′	110
Cyclophilin (mouse)	(F) 5′-GGCCGATGACGAGCCC-3′ (R) 5′-TGTCTTTGGAACTTTGTCTGCAA-3′	63

groups. Differences in values are considered significant at $P < 0.05$.

RESULTS

ABCG1 mRNA expression is upregulated during keratinocyte differentiation

In response to exogenous calcium, keratinocytes undergo differentiation. We initially determined whether ABCG1 mRNA levels were altered during calcium-induced keratinocyte differentiation. As shown in **Fig. 1**, ABCG1 mRNA is readily induced (3.7-fold) after incubation of keratinocytes in low calcium (0.03 mM) for 2 days, and further increases after 4 days (6.3-fold) and 7 days (12-fold). In parallel, high calcium (1.2 mM) induces an even greater increase in ABCG1 mRNA levels after 2 days (5-fold), 4 days (8.9-fold), and 7 days (22-fold) (Fig. 1). A concurrent increase in mRNA levels of involucrin, an early differentiation marker for keratinocytes, is also evident (Fig. 1; insert). Together, these data show that ABCG1 mRNA expression is stimulated during keratinocyte differentiation. Because ABGG1 mRNA is induced in CHKs in both low- and high-calcium medium, in the subsequent experiments, we focused on changes in ABCG1 expression using keratinocytes grown in low-calcium medium.

ABCG1 mRNA expression is regulated by sterol levels in CHKs

Incubation of CHKs with LDL, which increases cellular cholesterol levels, stimulates ABCG1 mRNA expression (~2.7-fold; $P < 0.01$) (**Fig. 2A**). Moreover, treatment of cells with either CS or lovastatin, which both inhibit cholesterol synthesis, markedly decreases ABCG1 mRNA expression (57% and 92%, respectively, $P < 0.01$) (Fig. 2B). These results indicate that ABCG1 is regulated by altered cellular sterol levels in keratinocytes.

Activation of LXR and PPAR-δ increases ABCG1 mRNA expression in CHKs

We next examined the effects of LXR, PPAR (α, δ, γ), RAR, RXR, and VDR activation on ABCG1 expression. In our previous studies, we demonstrated that whereas ligand activation of LXR, PPAR-α, PPAR-δ, or RXR stimulates ABCA1 expression (38), activation of PPAR-γ, PPAR-δ, or LXR increases ABCA12 expression in CHKs (30). In this study, we further demonstrated that ABCG1 mRNA levels markedly increase following treatment with either LXR activators (22R: 24-fold; TO: 36-fold), or PPAR-β/δ activator (GW: 10-fold). However, activation of PPAR-γ only slightly increases ABCG1 mRNA levels (Cig: 1.8-fold; GI: 2.6-fold; $P < 0.05$) (**Fig. 3**). LXR activator 22R also stimulates ABCG1 expression dose-dependently (**Fig. 4A**). The increase in ABCG1 mRNA occurs rapidly (~3 h), and is sustained over an extended period of time (24 h) (Fig. 4B). Similarly, a PPAR-β/δ activator (GW) also induces ABCG1 mRNA expression in a time- and dose-dependent fashion (data not shown). In contrast, activation of other nuclear hormone receptors, including PPAR-α, RAR, RXR, or VDR, had no effect on ABCG1 mRNA expression (Fig. 3). The absence of an effect of vitamin D3 is probably due to the short duration of our experiments (24 h), inasmuch as previous studies reported that vitamin D3 requires ≥30 h to induce the expression of involucrin and transglutaminase I, classic differentiation markers (39).

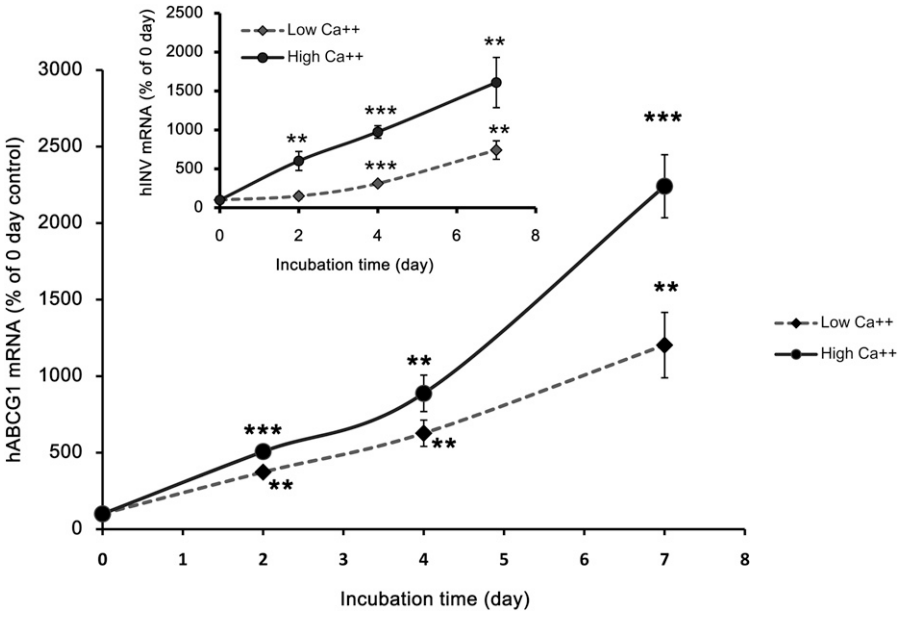

Fig. 1. ABCG1 mRNA expression increases during keratinocyte differentiation. Cultured human keratinocytes (CHKs) were cultured in medium containing either 0.03 or 1.2 mM Ca^{2+} for various periods of time (0, 2, 4, or 7 days). Real-time PCR was performed to measure mRNA levels of ABCG1, involucrin (INV), and cyclophilin (housekeeping gene). Data are expressed as percentage of 0 day control (100%) and presented as mean ± SEM (n = 4). ** $P < 0.01$; *** $P < 0.001$ compared with 0 time point.

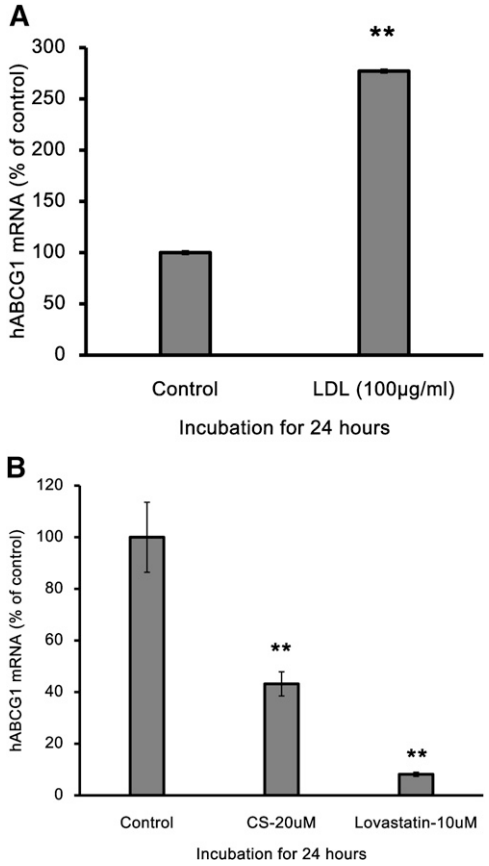

Fig. 2. ABCG1 mRNA expression is regulated by cellular sterol levels. CHKs were incubated with or without 100 μg/ml LDL in 0.03 mM Ca^{2+} medium for 24 h (A). Alternatively, cells were incubated with either cholesterol sulfate (CS, 20 μM), or lovastatin (10 μM), or vehicle (DMSO) for 24 h (B). ABCG1 and cyclophilin mRNA levels were determined as described. Data are expressed as percentage of control (100%) and presented as mean ± SEM (n = 4). ** $P < 0.01$ compared with control.

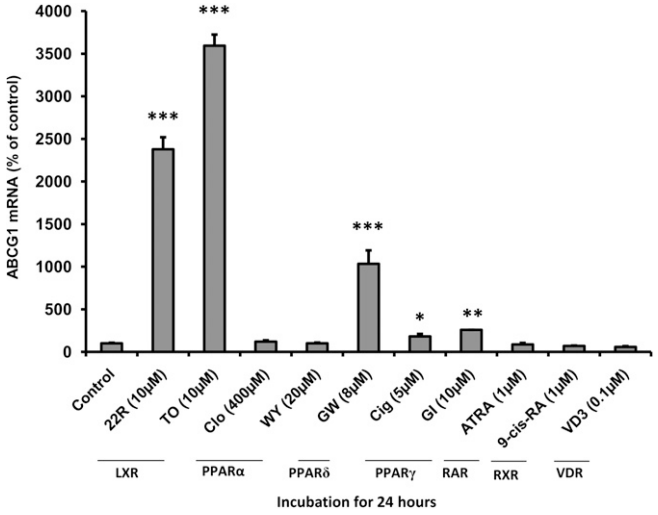

Fig. 3. Activation of liver X receptor (LXR) and peroxisome proliferator-activated receptor-δ (PPAR-δ) increases ABCG1 mRNA expression. CHKs were incubated in 0.03 mM Ca^{2+} medium in the presence or absence of various activators of PPAR-α, PPAR-δ, PPAR-γ, RAR, RXR, VDR, or vehicle (ethanol) for 24 h. Note that 9-*cis*-RA can activate both RXR and RAR. Real-time PCR was performed to measure mRNA levels of ABCG1 and cyclophilin as described. Data are expressed as percentage of control (100%) and presented as mean ± SEM (n = 5). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with control. ATRA, all-trans retinoic acid; TO, TO901317; Clo, clofibrate; WY, WY14643; GW, GW 610742X; Cig, ciglitazone; GI, GI251929. VD3, 1α,25-dihydroxyvitamin D3.

LXR and PPAR-δ activators increase ABCG1 protein levels in CHKs

We next assessed whether LXR or PPAR-δ activation also increases ABCG1 at the protein level. As expected, LXR activation by 22R dramatically increases ABCG1 protein mass in a time-dependent manner (2–6-fold) (**Fig. 5A**). Another nonsterol synthetic LXR activator, TO, also significantly increases ABCG1 protein levels (data not shown). Finally, the PPAR-δ activator GW also significantly increases ABCG1 protein levels (1.7-fold) following 24 h incubation (Fig. 5B).

Topical LXR activator increases ABCG1 expression in vivo

We next examined whether induction of ABCG1 expression by LXR activation occurs in vivo. When topically applied on mouse skin for 3 days, the LXR activator TO significantly increases ABCG1 mRNA levels (~3.8-fold) in the epidermis (**Fig. 6A**). In parallel, ABCG1 protein levels also increase (~2.7-fold) in TO-treated epidermis, determined by either Western blot (Fig. 6B) or immunohistochemistry (see supplementary Fig. I). Immunohistochemical staining also showed that ABCG1 localizes to suprabasal layers (see supplementary Fig. I; insert for enlargement), where epidermal LBs are produced and secreted (40). A similar increase in ABCG1 immunostaining is also observed in the epidermis following topical treatment with the PPAR-δ ligand GW (data not shown). Together, these results indicate that LXR and PPAR-δ activation stimulates ABCG1 expression both in CHKs in vitro and in mouse epidermis in vivo.

Acute barrier disruption stimulates ABCG1 expression

Much of epidermal cholesterol synthesis is directed toward the maintenance of a competent permeability barrier. To assess whether ABCG1 could contribute to epidermal barrier function, we next determined ABCG1 expression following acute barrier disruption by TS. Three hours after TS, ABCG1 mRNA levels increase (TS: 195% ± 39% vs. control; 100% ± 2.6%; $P = 0.072$), with a further increase at 6 h (TS: 350% ± 89% vs. control; $P < 0.05$), which returns to near baseline at 24 h (**Fig. 7**). In parallel, the intensity of ABCG1 immunostaining also increases at 3 h and 6 h (see supplementary Fig. IIA: III vs. IV). To assess whether the increase in ABCG1 expression can be linked to barrier function, we next examined whether the TS-induced increase in ABCG1 can be blocked by artificial restoration of the barrier by occlusion. The increase in ABCG1 protein following TS can be inhibited by occlusion, which artificially restores barrier function to normal, at both 3 h (see supplementary Fig. IIB: II vs. III) and 6 h (data not shown). Moreover, the increase in ABCG1

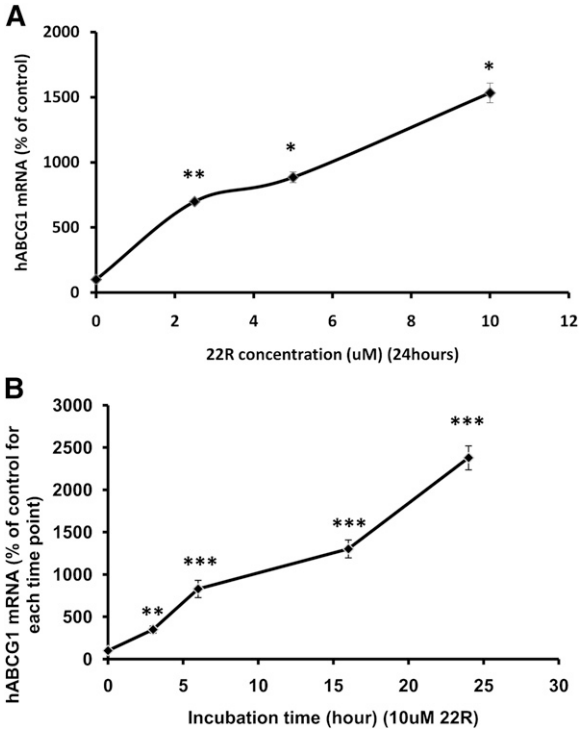

Fig. 4. The LXR activator 22R increases ABCG1 mRNA expression in a dose- and time-dependent manner. CHKs were incubated with 22R at various doses (0, 2.5, 5.0, and 10 μM) or vehicle (ethanol) in 0.03 mM Ca^{2+} medium for 24 h (A). Alternatively, cells were cultured in the presence of 10 μM 22R or vehicle (ethanol) in the same medium for various periods of time (0, 3, 6, 16, and 24 h) (B). Real-time PCR was performed to measure mRNA levels of ABCG1 and cyclophilin. Data are expressed as percentage of control (100%) and presented as mean ± SEM (n = 4). For the time course study, data are presented as percentage of control (in the absence of 22R) for each matched time point. Similar results were obtained when the experiment was repeated with a different batch of cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with control.

mRNA after TS can also be partially inhibited by occlusion (see supplementary Fig. IIC). Similarly, disruption of the permeability barrier by a second, unrelated method, organic solvent (acetone) treatment, also significantly increases ABCG1 mRNA levels at 3 h (acetone: 202% ± 22% vs. control: 100% ± 12%; $P < 0.05$), with a further increase at 6 h (acetone: 320% ± 90% vs. control; $P < 0.05$) in the epidermis (**Fig. 8**), and a parallel increase in the intensity of ABCG1 immunostaining is also observed 3 h after acetone treatment (see supplementary Fig. III: III vs. II). Again, the acetone-induced increase in ABCG1 protein can be inhibited by occlusion (see supplementary Fig. III: IV vs. III). Thus, acute permeability barrier disruption stimulates ABCG1 expression in mouse epidermis, suggesting an important role for ABCG1 in barrier recovery.

Abcg1 KO mice demonstrate abnormal LB contents and secretion, but normal permeability barrier function

To further investigate whether ABCG1 is an important physiological mediator for permeability barrier formation, we next examined epidermal morphology and permeability barrier function in *Abcg1* KO and WT mice. As shown in supplementary Fig. IV, ABCG1 is expressed in both the epidermis and hair follicles in WT mice, but is absent in *Abcg1* KO mice. Overall epidermal morphology of *Abcg1* KO mice, examined by hematoxylin and eosin staining, does not appear to differ from that of WT mice (data not shown). In contrast, electron microscope studies reveal differences in LB density, content, and secretion at the interface between the SC and SG layers, whereas LBs in WT mouse epidermis show normal density, content, and secretion (**Fig. 9,** inserts A+B). There are a large number of empty LBs in *Abcg1* KO epidermis (Fig. 9, insert C), along with a decrease in the quantities of LB density and secreted lamellar material (Fig. 9, insert D; **Table 2** for quantitation data). Although the disrupted LB secretion and lamellar bilayer formation indicate a potential role of ABCG1 in normal LB formation and secretion, we could not identify significant differences in either basal TEWL (data not shown), a measure of permeability barrier function, or in lipid content, measured by Nile Red fluorescence staining (see supplementary Fig. V). Additionally, the kinetics of permeability barrier recovery following acute barrier disruption by TS reveals no differences between *Abcg1* KO and WT mice (data not shown). Interestingly, in *Abcg1*-null mice, there is a compensatory increase in mRNA levels of ABCA1 (see supplementary Fig. VI), a lipid transporter that is functionally related to ABCG1. In contrast, mRNA levels for ABCA12, which is not involved in cholesterol efflux, are significantly decreased in the skin of *Abcg1*-null mice (see supplementary Fig. VI).

DISCUSSION

The present study demonstrates that ABCG1 is expressed in both human keratinocytes and murine epidermis. Similar to observations in other cell types (20), cellular cholesterol levels regulate ABCG1 expression in human keratinocytes. Whereas incubation of human keratinocytes with LDL, which increases cellular cholesterol levels, stimulates ABCG1 expression, inhibition of cholesterol synthesis with either statins or CS, which decreases cellular cholesterol levels, reduces ABCG1 expression. Moreover, activation of LXR with either sterol or nonsterol LXR activators markedly stimulates ABCG1 expression in human keratinocytes, as has been reported for other cells (20, 21). Topical treatment of murine skin with LXR activators also increases ABCG1 expression in murine epidermis. Functional LXR response elements have been identified in both murine and human ABCG1 promoters (41–43). It is likely that in keratinocytes and other cells, cholesterol is metabolized to oxysterols following uptake and activates LXR, thereby stimulating ABCG1 expression. In addition, activation of PPAR-δ also markedly stimulates ABCG1 expression in human keratinocytes and murine epidermis, whereas activation of PPAR-γ only modestly stimulates ABCG1, as shown for PPAR-γ activators in macrophages (44–46). Thus, the regulation of ABCG1 in keratinocytes is very similar to that previously reported in other cell types.

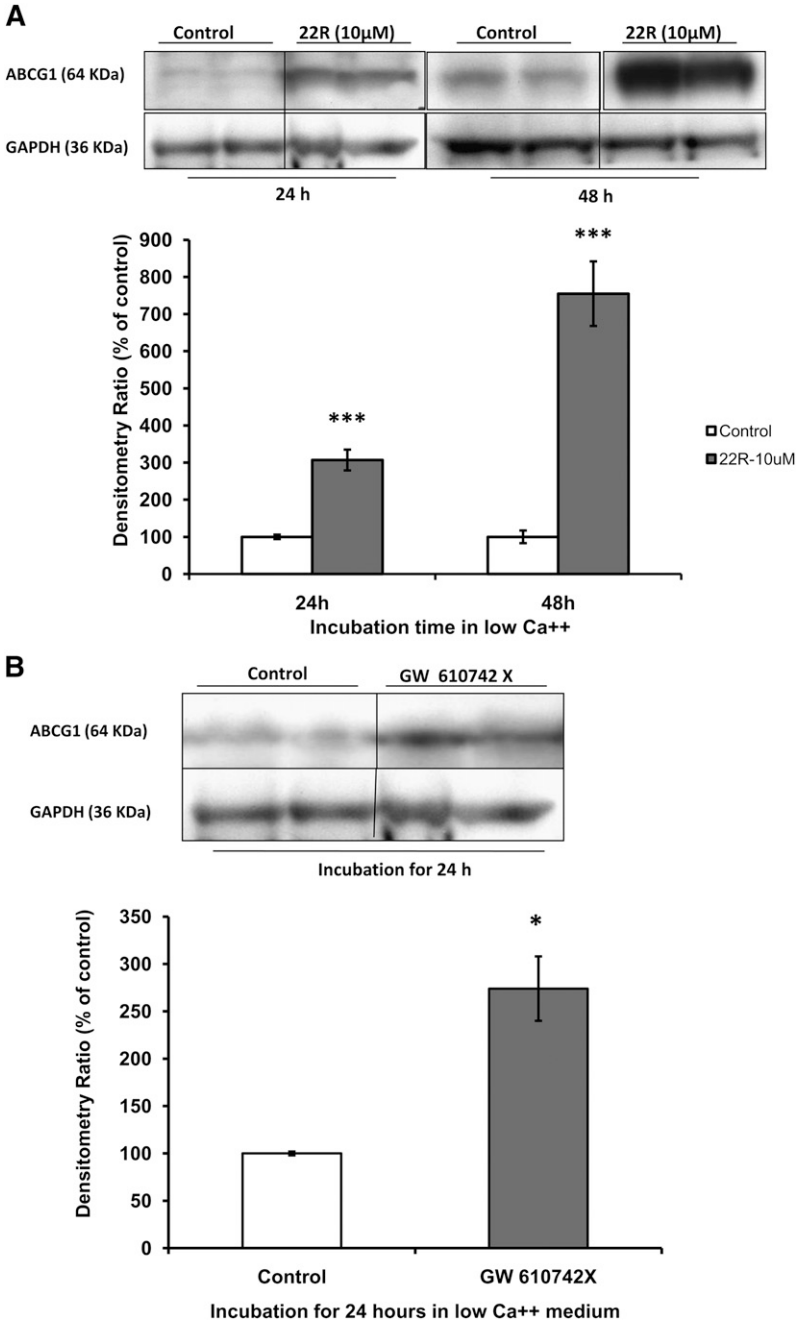

Fig. 5. LXR and PPAR-δ activators increase ABCG1 protein expression. CHKs were incubated with either vehicle (ethanol), 22R (10 μM) (A), or GW 610742X (8 μM) (B) in 0.03 mM Ca^{2+} medium for the indicated periods of time. The whole-cell extract was prepared and subjected to Western blot analysis to determine ABCG1 or GAPDH protein levels as described. Data are expressed as percentage of vehicle control densitometry (100%) and presented as mean ± SEM (n = 4–5). Representative blots are shown. * $P < 0.05$; *** $P < 0.001$ compared with control.

ABCG1 facilitates the efflux of cholesterol from cells to HDL and other lipoproteins (47). Accordingly, overexpression of ABCG1 increases the efflux of cholesterol, whereas cholesterol efflux decreases in cells deficient in ABCG1 (28, 47, 48). The ability to enhance cholesterol efflux is also facilitated by another ABC transporter, ABCA1 (47). Notably, ABCA1 is also expressed in human keratinocytes and murine epidermis, and the regulation of ABCA1 and ABCG1 is similar (19), i.e., the expression of both of these transporters is stimulated by increased cellular cholesterol levels and by LXR activation (19). One can therefore postulate that in keratinocytes, these transporters play similar roles in cellular cholesterol homeostasis by facilitating cholesterol efflux. However, there are some notable differences in the regulation of ABCA1 and ABCG1 expression in human keratinocytes and murine epidermis. First, ABCG1 expression increases with keratinocyte differentiation, whereas ABCA1 expression is not affected (19). As shown here, both time in culture and exogenous calcium stimulate both keratinocyte differentiation and the expression of ABCG1. Moreover, immunohistochemical staining of murine epidermis demonstrates increased expression of ABCG1 in the outer, more-differentiated epidermis (staining was particularly notable in the SG). Second, disruption of the permeability barrier by either TS or acetone treatment rapidly increases ABCG1 expression in murine epidermis. Furthermore, providing an artificial permeability barrier immediately after barrier disruption inhibits this increase, indicating that the increase in ABCG1 expression is specifically related to permeability

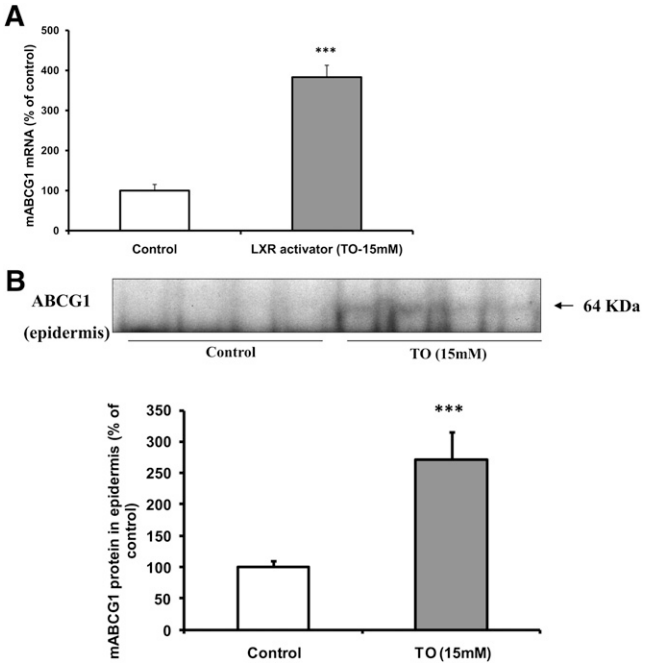

Fig. 6. LXR activation increases ABCG1 mRNA and protein levels in mouse epidermis. Hairless mice were topically treated with either vehicle (ethanol) or the LXR activator TO901317 (15 mM) for 3 days, and the epidermis was isolated for measuring ABCG1 and 36B4 (housekeeping gene) mRNAs by real-time PCR (A), or ABCG1 protein levels by Western blot (B). Data are expressed as percentage of vehicle control densitometry (100%) and presented as mean ± SEM (n = 6). A representative blot is shown (B). *** $P < 0.001$ compared with control.

barrier dysfunction, and is not a nonspecific injury response. In contrast, permeability barrier disruption induces an opposite effect on ABCA1 expression in murine epidermis, resulting in decreased expression (19). These differences in the regulation of ABCA1 and ABCG1 expression in keratinocytes suggest that they may have divergent functions in the epidermis.

A major function of the skin is to form a permeability barrier between the hostile environment and the organism (49). This permeability barrier is essential for survival and localizes in the outer layer of the epidermis, the SC, where it is mediated by extracellular lipid lamellar membranes composed primarily of three lipids: ceramides, fatty acids, and cholesterol (49). These lipids are delivered to the SC extracellular spaces by the secretion of LBs, specialized organelles synthesized by differentiated keratinocytes (49). The formation of LBs requires cholesterol, phospholipids, and glucosylceramides. Inhibition of the synthesis of any of these lipids in SG cells results in abnormal LBs and abnormal permeability barrier function (49). One can postulate that the decrease in ABCA1 following permeability barrier disruption will decrease cholesterol efflux, thereby allowing additional cholesterol to be available for LB formation. The increase in ABCG1 following permeability barrier disruption and its localization in the outer epidermis suggest that it has a role other than cholesterol efflux.

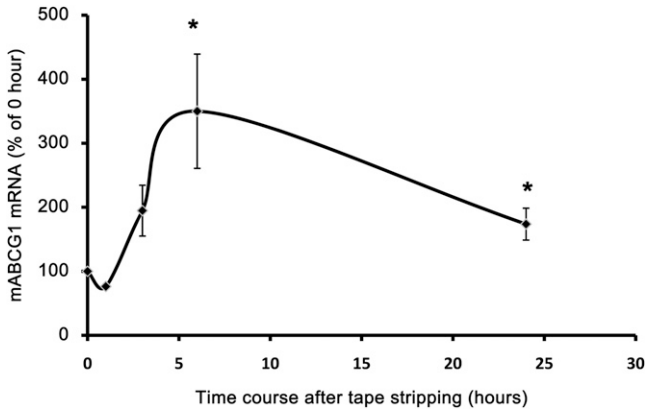

Fig. 7. Permeability barrier disruption by tape stripping (TS) increases ABCG1 mRNA expression in mouse epidermis. At 0, 1, 3, 6, or 24 h following acute barrier disruption by TS, hairless mouse epidermis was collected and subjected to analysis for ABCG1 and 36B4 mRNAs by real-time PCR. Data are expressed as percentage of nontreatment control (100%) and are presented as mean ± SEM (n = 6–7). The representative graph is shown from two different sets of animals with similar results. * $P < 0.05$ compared with nontreatment control.

How lipids are incorporated into LBs is still largely unknown, but studies have shown that the absence of ABCA12 results in abnormal LBs and a defective permeability barrier (50, 51). By yet-to-be-defined mechanisms, ABCA12 facilitates the transport of glucosylceramides into LBs (50–52). Of note is that similar to our observations with ABCG1, keratinocyte differentiation and PPAR and LXR activators stimulate ABCA12 expression in keratinocytes (30). When topically applied to hairless mice, PPAR and LXR activators also stimulate epidermal lipid synthesis, increase LB secretion, and accelerate extracellular lipid processing in vivo (53). However, in contrast to ABCG1, ABCA12 expression is not stimulated by permeability bar-

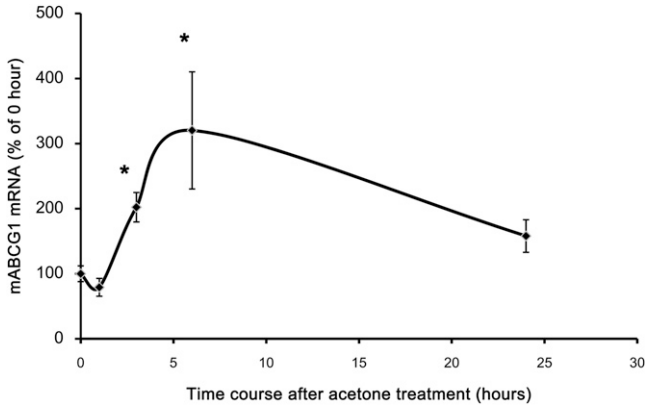

Fig. 8. Permeability barrier disruption by acetone treatment increases ABCG1 mRNA expression in mouse. Following acute barrier disruption by acetone treatment, epidermis was collected at 0, 1, 3, 6, and 24 h and subjected to analysis for ABCG1 and 36B4 mRNAs by real-time PCR. Data are expressed as percentage of control (100%) and presented as mean ± SEM (n = 5). The representative graph is shown from two different sets of animals with similar results. * $P < 0.05$ compared with control.

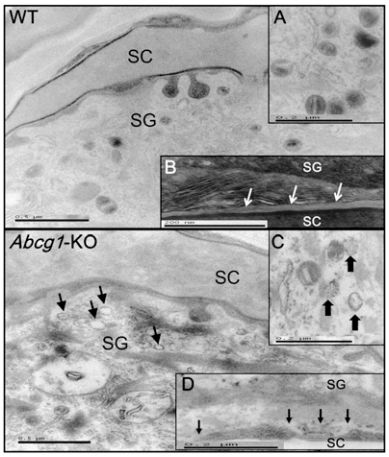

Fig. 9. *Abcg1* knockout (KO) mouse epidermis displays abnormalities in the lamellar body (LB) secretory system with impaired lamellar bilayer formation. Skin samples were collected from both *Abcg1* KO and wild-type (WT) mice, postfixed with either reduced osmium-tetroxide or ruthenium-tetroxide, and processed for electron microscopy as described in MATERIALS AND METHODS. At the interface of the stratum granulosum (SG) and stratum corneum (SC), abundant, normal-looking LBs are present in WT mouse epidermis (A, insert), and secreted LB contents are processed into lamellar bilayers (B, arrows). In *Abcg1* KO mice, however, a large number of LBs are empty (C, arrows), with disrupted lamellar bilayer formation (D, arrows).

rier disruption (unpublished observations). ABCG1 expression is increased in the outer epidermis similar to that of ABCA12. These observations suggest that ABCG1 could perhaps play a role in the intracellular movement of lipids and the formation of LBs. Although the cellular localization of ABCG1 remains controversial (22, 26, 54–57), other studies have also suggested a role for ABCG1 in intracellular lipid movement. Specifically, ABCG1 is found to localize in the endoplasmic reticulum (ER) and membrane vesicles of brain cells (58). Additionally, ABCG1 is present in intracellular vesicles of neurons and astrocytes that function to facilitate cholesterol transport from the ER to the plasma membrane, where it is available for efflux to exogenous sterol acceptors such as HDL or lipidated apoE (58). In the present study, we observed that the LBs in SG cells of *Abcg1* KO mice had a decrease in lamellar membranes and that there was an increased number of empty LBs. Additionally, the quantity of secreted lamellar material at the interface of the SG and SC was decreased in ABCG1-deficient mice. These observations suggest a role for ABCG1 in the formation of LBs analogous to what has been observed for ABCA12.

Despite the abnormalities mentioned above, however, epidermal morphology, basal permeability barrier function, and permeability barrier repair following barrier disruption were not significantly altered in ABCG1-deficient mice. This indicates that whereas ABCG1 may play a role in the movement of lipid into LBs, it is not essential. In contrast, ABCA12 deficiency in both humans and mice results in severe cutaneous abnormalities and defective permeability barrier function (50, 51, 59). This indicates that other pathways cannot compensate for the absence of ABCA12 but do compensate for the absence of ABCG1. In macrophages, studies have shown that there are several pathways that mediate the efflux of cholesterol and that the absence of any one pathway does not result in the absence of cholesterol efflux. For example, it has been estimated that ABCG1 accounts for only approximately 20% of the cholesterol efflux, with other pathways accounting for the other 80% in macrophages (60, 61). In support of this notion, there is a compensatory increase in ABCA1 mRNA levels in the skin of *Abcg1*-null mice compared with the WT. We would speculate that multiple pathways mediate the incorporation of cholesterol and phospholipids into LBs in keratinocytes, so the absence of any single pathway may result in relatively subtle morphological abnormalities but no functional defects. Additionally, there is an excess of lipid lamellar membranes in the SC and only a modest number of lipid membranes is required to provide normal permeability barrier function. When we tape-strip skin, we remove lipid with each stripping, but it takes at least three to four tape strips before we begin to see increases in TEWL, a sensitive marker of cutaneous permeability. Similarly, when we treat with acetone, we remove lipid, but initially, there is no change in TEWL. It takes quite a bit of acetone treatment before we observe increases in TEWL. These observations indicate that there is

TABLE 2. Measurement of LB density, LB secretion, and empty LB percentage in WT versus Abcg1 KO mouse epidermis

Animals	LB density	LB secretion	Empty LB
	(LB/unit area ± SEM)[a]	(point/cm ± SEM)[b]	(%) (Mean ± SEM)[d]
WT ($Abcg1^{+/+}$)	8.92 ± 1.33	0.661 ± 0.085	10.82 ± 2.45
KO ($Abcg1^{-/-}$)	6.85 ± 0.58	0.457 ± 0.035[c]	31.06 ± 5.04[e]

LB, lamellar body; WT, wild-type; KO, knockout.
[a] Data represent mean ± SEM in 10 random electron micrographs from each animal, evaluated by two blinded observers. Each group (WT and KO) contains two animals; experiment repeated once with similar results.
[b] LB secretion was determined by the number of LB contact points per length (cm) at the stratum granulosum/stratum corneum (SC) junction of the first SC cell layer. Data represent the mean ± SEM (n = 10 randomly selected, coded micrographs from four WT mice and four Abcg1 KO mice).
[c] Significantly different ($P < 0.05$) as compared with WT samples.
[d] Data represent mean ± SEM in 10 random electron micrographs at high magnification from each animal, determined by the ratio of empty LB versus the total LB count. Each group (WT and KO) contains two animals; experiment repeated once with similar results.
[e] Significant difference ($P < 0.01$) as compared with WT samples.

a substantial redundancy and that one needs a considerable decrease in the usual number of lipid lamellar membranes before one observes functional abnormalities in permeability barrier function. One can postulate that ABCG1 KO mice are able to form a sufficient number of lamellar membranes to provide normal barrier function, but on the EM, we can see abnormalities in the LB secretory system.

In conclusion, the present study demonstrates that ABCG1 is expressed in human keratinocytes and murine epidermis. The function of ABCG1 in the epidermis is not clear, but it may play a role in the formation of LBs.

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