The American Journal of Pathology, Vol. 174, No. 3, March 2009

DOI: 10.2353/ajpath.2009.080860

Molecular Pathogenesis of Genetic and Inherited Diseases

Premature Terminal Differentiation and a Reduction in Specific Proteases Associated with Loss of ABCA12 in Harlequin Ichthyosis

Anna C. Thomas, Daniel Tattersall, Elizabeth E. Norgett, Edel A. O'Toole, and David P. Kelsell

From the Centre for Cutaneous Research, Institute of Cell and Molecular Science, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, United Kingdom

One of the primary functions of skin is to form a defensive barrier against external infections and water loss. Disrupted barrier function underlies the most severe and often lethal form of recessive congenital ichthyosis, harlequin ichthyosis (HI). HI is associated with mutations in the gene that encodes the ABC transporter protein, ABCA12. We have investigated the morphological and biochemical alterations associated with abnormal epidermal differentiation and barrier formation in HI epidermis. An *in vitro* model of HI skin using human keratinocytes retrovirally transduced with shRNA targeting ABCA12 in a three-dimensional, organotypic co-culture (OTCC) system has also been developed. A robust reduction in ABCA12 expression had a dramatic effect on keratinocyte differentiation and morphology comparable with that observed in HI skin, including a thicker epidermis and abnormal lipid content with a reduction in nonpolar lipids. As seen in HI epidermis, proteins that are normally expressed in late differentiation were highly dysregulated in the ABCA12-ablated OTCC system. These proteins were expressed in the stratum basale and also in the stratum spinosum, indicative of a premature terminal differentiation phenotype. Expression of the proteases kallikrein 5 and cathepsin D was dramatically reduced in both HI epidermis and the OTCC model. These data suggest that ABCA12 is a key molecule in regulating keratinocyte differentiation and transporting specific proteases associated with desquamation. ***(Am J Pathol 2009, 174:970–978; DOI: 10.2353/ajpath.2009.080860)***

Harlequin ichthyosis (HI; MIM 242500) is the most severe and often lethal form of recessive congenital ichthyosis.[1–3] Infants born with HI have hard, thick skin covering most of their body. The skin forms large diamond-shaped plates resembling armor plating separated by deep red fissures, which restrict movement.[4] These skin abnormalities also affect the shape of the eyelids and lips, causing ectropion and eclabion, respectively. HI sufferers can also have malformations or autoamputation of the fingers and toes because of constricting bands of skin *in utero*. Because of the impaired barrier function of the skin, neonates struggle to control water loss, regulate temperature, and are more susceptible to infection and have feeding difficulties.[5] In addition, the tightened skin produced can cause breathing difficulties leading to respiratory failure. From our cohort data of more than 70 unrelated families, 45% of HI-affected neonates die soon after birth (if not still-born). Disease from ~6 months onwards resembles a nonbullous congenital ichthyosiform erythroderma.[6] However, the skin barrier still remains severely compromised in HI patients, and they will always have problems with thermal regulation, have increased *trans*-epidermal water loss, and be at a greater risk of microbial infection.

The gene defective in HI is *ABCA12*, a member of the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily of active transporters. *ABCA12* mutations are responsible for all HI cases analyzed to date with the majority of mutations being either nonsense substitution or frameshift.[7–10] ABCA12 localizes to lamellar granules (LG) in normal epidermal keratinocytes.[8] The exact role of the ABCA12 protein in LG lipid transport is unknown, but it is hypothesized that ABCA12 transports glucosyl-

Supported by BDF Newlife, the British Skin Foundation, the Wellcome Trust (VIP award), and the Ichthyosis Support Group (to E.O.T. and D.P.K.).

Accepted for publication December 4, 2008.

Address reprint requests to David P. Kelsell, Queen Mary University of London, Barts and the London School of Medicine and Dentistry, Centre for Cutaneous Research, Institute of Cell and Molecular Science, 4 Newark St., Whitechapel, London, E1 2AT, UK. E-mail: d.p.kelsell@qmul.ac.uk.

ceramide into the LGs and then the ABCA12-positive LGs secrete the lipid into the extracellular space to form the intercellular lipid layer.[8] Recent evidence has found that peroxisome proliferators-activated receptor (PPAR)-γ, PPAR-β, and PPAR-δ activators stimulate the expression of *ABCA12* mRNA in cultured human keratinocytes.[11] It was also found that liver X receptor (LXR) activators also increased *ABCA12* mRNA expression but to a lesser extent than the PPAR activators. The PPAR and LXR activators have been shown to stimulate downstream effects in keratinocytes related to differentiation including increased LG secretion and lipid synthesis.[12]

The histology of HI skin is striking. The most obvious abnormality is the sheer thickness of the stratum corneum (SC) and indeed the entire epidermis.[8,13] With closer histological inspection, the nuclei become flattened early on in differentiation but are retained in the cornified layer (parakeratosis) and hyperkeratosis and hypergranulosis have also been noted as hallmarks of HI skin.[4] In addition to the increased epidermal thickness, an abnormal localization of epidermal lipids has been described.[14,15]

A mouse knockout for ABCA12 has been described as having a postnatal lethal phenotype (line NIH-01279; neonatal lethal Mouse Genome Informatics). Recently, the Akiyama group[16] has generated and characterized an ABCA12-null mouse. The phenotype resembles that of newborn HI skin plus reveals alveolar collapse as an additional phenotype linked to the nonviability in these mice. The expression of ABCA12 in normal fetal skin development and the grafting of HI keratinocytes on immunodeficient mice have also been described.[17] To complement these studies and because of the lethality of the ABCA12-null mice, we have generated and characterized an *in vitro* human model of HI skin using shRNA targeting ABCA12 in a normal keratinocyte cell line to study the role of ABCA12 in human epidermis.

Materials and Methods

Cell Lines

The immortalized keratinocyte cell line NEB1[18] was cultured in 3:1 Dulbecco's modified Eagle's medium-F12 medium, supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 10 ng/ml epidermal growth factor, 10×10^{-10} mol/L cholera toxin, 5 μg/ml transferrin, 2×10^{-11} mol/L liothyronine, and 50 U/ml penicillin-G and 50 μg/ml streptomycin sulfate. Primary keratinocytes extracted from face lift skin were grown in co-culture with γ-irradiated mouse 3T3 fibroblast cells in the same media.

Retroviral Transduction

The pSUPERIOR-retro-puro shRNA system (Oligoengine, Seattle, WA) was used to suppress the expression of ABCA12. Four pairs of oligos were designed as follows (sense sequence only shown): shRNA1: 5′-GATCCCGGAACTCCCAGGAAATAGCTTCAAGAGAGCTATTTCCTGGGAGTTCCTTTTTA-3′, shRNA2: 5′-GATCCCGGAGCACCTTCTCCTATATTTCAAGAGAATATAGGAGAAGGTGCTCCTTTTTA-3′, shRNA3: 5′-GATCCCGGACAGAGCTACCTCTATGTTCAAGAGACATAGAGGTAGCTCTGTCCTTTTTA-3′, shRNA4: 5′-GATCCCGCAAATGCATCTGCCCAGATTCAAGAGATCTGGGCAGATGCATTTGCTTTTTA-3′, scrambled control: 5′-GATCCCAAGCGCGGCAATAATCCTTTTCAAGAGAAAAGCGTAGGGTACTCTGTTTTTTA-3′. Subcloning of the oligos, transfection of the packaging cell line, production of retrovirus, and transduction of target cells were performed as per the manufacturer's instructions. shRNA1 was selected for subsequent experiments described in this article.

Skin Biopsies and Organotypic Co-Culture (OTCC)

Normal skin was obtained from redundant skin and a skin biopsy from a Bangladeshi male HI patient, age 14, homozygous for the *ABCA12* mutation 6378delGC,[7] was cryo-mounted before frozen sectioning. Consent and ethical approval was obtained for these studies. OTCC was performed as described previously.[19,20]

Tissue Staining

Each tissue was cut in half such that one half was snap-frozen while embedded in Cryo-M-Bed (Bright) and was stored at −80°C. The other half was fixed in 4% paraformaldehyde and embedded in paraffin. Sections were cut at 5 to 6 μm in thickness. Hematoxylin and eosin (H&E) staining was performed on frozen sections following standard protocol.

Epidermal Thickness Measurements

Nine frozen sections were cut from different positions of each OTCC and three measurements of each section were taken at random using the measuring tool with the MetaMorph software (Molecular Devices, Sunnyvale, CA) on an Eclipse microscope (Nikon, Tokyo, Japan). Statistical analysis was performed using an unpaired two-tailed *t*-test as described previously.[20]

Nile Red Lipid Analysis

A stock solution containing 0.05% Nile Red in acetone was diluted to 2.5 μg/ml with 75:25 glycerol:water, followed by brisk vortexing. A drop of the glycerol-dye solution was applied to each tissue section and immediately covered with a coverslip. Slides were then viewed using a Nikon Eclipse TE2000-S fluorescence microscope. Pictures were taken separately of the green and red fluorescence and then subsequently merged.

Antibodies

The ABCA12 antibody was generated by Harlen (Oxford, UK), using the 15-amino acid ABCA12 (NM_173076) motif present at residues 2504 to 2519 (QLHFPKTYLK-

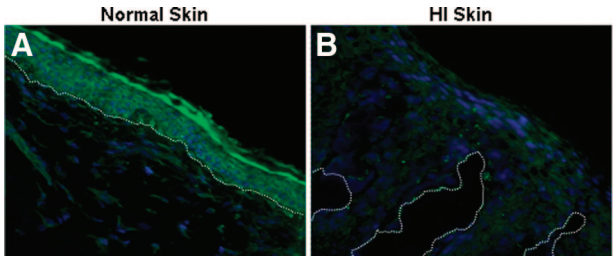

Figure 1. A and **B:** IHC with the ABCA12 antibody (green). **A:** In normal skin the strongest staining appears in the stratum granulosum. **B:** ABCA12 expression was significantly reduced/absent in the skin of this HI patient. DAPI-stained nuclei are shown in blue and the dermal-epidermal junctions are marked by a **dotted white line**.

DQHLS) plus a cysteine for conjugation. The rabbit polyclonal antibody obtained was then G-protein-purified before use, and used at a dilution of 1:500 for immunofluorescence. All other antibodies were used at 1:200 dilution. The LEKT1 antibody was a gift from Dr. W.L.Di (Institute of Child Health, UCL, London, UK). The involucrin (clone SY5) and K2e (clone LHK2E) antibodies were obtained from CRUK (Cancer Research UK, London, UK). The mouse anti-transglutaminase 1 (Biogenesis, Poole, UK), mouse anti-filaggrin (Biomeda, Foster City, CA), rabbit anti-KLK5 and goat anti-KLK7 (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-cathepsin D (Abcam, Cambridge, UK) are available commercially.

Immunohistochemical Staining

Frozen sections were air-dried for 30 minutes and permeabilized in 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 15 minutes. Sections were washed in PBS, blocked in 0.2% gelatin from cold water fish skin for 15 minutes, and incubated with primary antibody for 1 hour. After three PBS washes, fluorescent secondary antibody (donkey anti-rabbit or donkey anti-mouse Alexa Fluor 488; Molecular Probes, Eugene, OR) was added at a 1/1000 dilution and incubated for 1 hour in the dark. Sections were washed three times in PBS, incubated with 4,6-diamidino-2-phenylindole (DAPI) (0.125 μg/ml) for 5 minutes, and washed three more times. Sections were mounted with immunomount reagent (Thermo Shandon, Waltham, MA) and viewed under a Nikon Eclipse TE2000-S microscope.

Confocal Immunofluorescence

Immunocytochemistry was performed using a standard protocol. Briefly, NEB1 cells that had been seeded onto glass coverslips were fixed in 4% paraformaldehyde for 30 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. Nonspecific binding was blocked with 3% bovine serum albumin for 15 minutes before the cells were incubated in primary antibody solution for 1 hour. After washing, the cells were incubated with the relevant Alexa Fluor secondary antibodies (Molecular Probes) before being washed and mounted in Shandon Immumount (Thermo Electron Corporation, Waltham, MA) containing 10 μg/ml of DAPI. Cells were imaged using a Zeiss 510 confocal microscope (Zeiss, Thornwood, NY).

Results

ABCA12 Expression Is Absent in HI Skin

Immunofluorescence demonstrated ABCA12 was expressed throughout the normal interfollicular epidermis with prominent expression in the stratum granulosum (Figure 1A). Immunohistochemistry (IHC) with the ABCA12 antibody showed ABCA12 expression was significantly reduced/absent in the skin of this HI patient (Figure 1B).

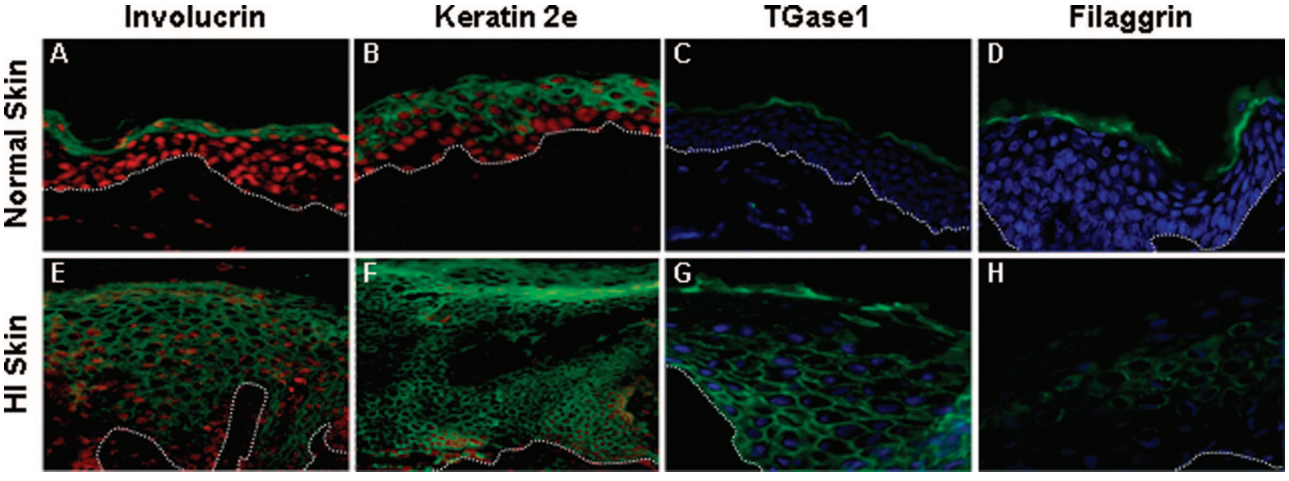

Figure 2. IHC of normal and HI patient skin biopsies with markers of late epidermal differentiation revealed an abnormal expression pattern in HI epidermis. In normal skin, the expression of involucrin (**A**) was observed primarily in the granular layer with keratin 2e (K2e, **B**) expressed variably throughout the suprabasal layers. In contrast, in HI skin both involucrin (**E**) and K2e (**F**) were expressed throughout the entire epidermis. The TGase 1 (**C**) and filaggrin (**D**) were expressed primarily in the SC in normal skin, however, in HI skin, TGase 1 (**G**) was expressed throughout the entire epidermis including the basal layer. **H:** Filaggrin was expressed throughout the upper spinous layer but was significantly reduced/absent from the SC. DAPI- or propidium iodide-stained nuclei are shown in blue and red, respectively, and the dermal-epidermal junctions are marked by a **dotted white line**.

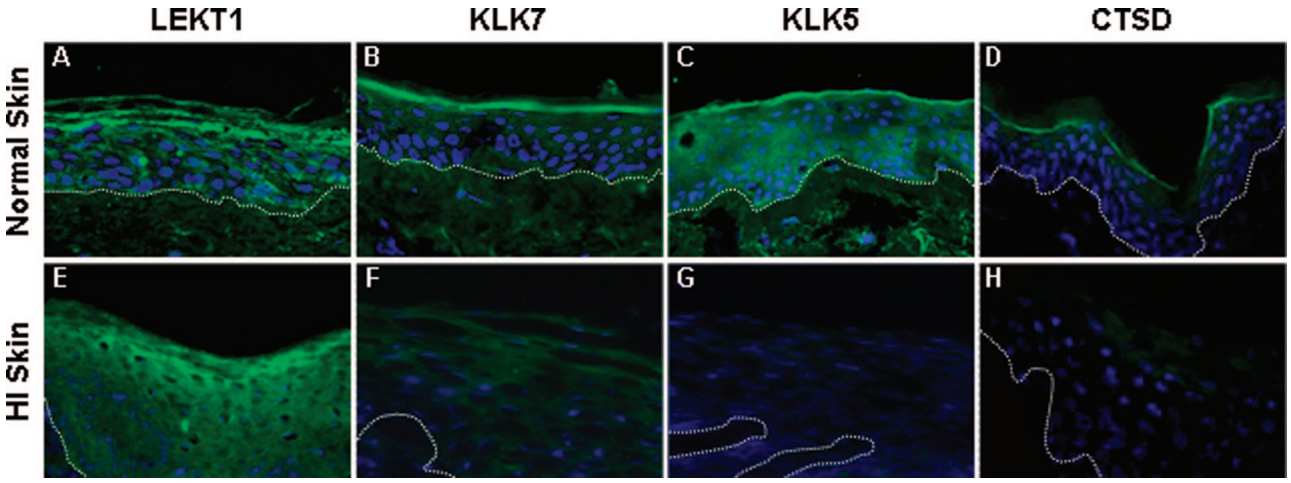

Figure 3. IHC of normal and HI patient skin biopsies with LG markers revealed KLK5 and CTSD (but not LEKT1 and KLK7) are significantly reduced/absent in HI skin. There was no obvious difference between the expression pattern of LEKT1 in normal (**A**) compared with HI skin (**E**) skin, being expressed in the granular and upper spinous layers. Kallikrein (KLK) 7 was localized to the stratum granulosum, in both normal skin (**B**) and HI skin (**F**), with some fainter expression in the lower keratinocyte layers in HI skin. In contrast, KLK5 (**C**) and cathepsin D (CTSD, **D**) were present in normal skin in the stratum granulosum and the SC, and the stratum granulosum and upper spinous layers, respectively, but both were significantly reduced/absent from HI skin (**G** and **H**). DAPI-stained nuclei are shown in blue, and the dermal-epidermal junctions are marked by a **dotted white line**.

Abnormal Epidermal Differentiation and Protease Transport in HI Skin

IHC analysis with antibodies raised against proteins expressed in late epidermal differentiation including involucrin, keratin 2e (K2e), transglutaminase 1 (TGase1), and filaggrin revealed an abnormal expression pattern in HI epidermis. Involucrin and K2e are primarily expressed in the granular suprabasal layers of normal control skin (Figure 2, A and B). In contrast, both K2e and involucrin were expressed throughout the entire suprabasal epidermis of the HI skin biopsy, with K2e expression also in the basal layer (Figure 2, E and F). The localization of both TGase1 and filaggrin was primarily in the SC in normal skin (Figure 2, C and D). This localization was consistent with the respective roles of these proteins in the epidermis. TGase1 is involved in the interprotein cross-linking of the cornified envelope.[21] Filaggrin is a product of proteolytic cleavage of profilaggrin and is thought to aggregate intermediate filaments during terminal differentiation.[22] However, in HI skin TGase1 localization was membranous and was expressed throughout the entire epidermis including the basal layer (Figure 2G). The pattern of filaggrin expression was also abnormal in HI skin; it was absent from the SC but present throughout the upper spinous layer supporting a previously reported error in filaggrin processing in HI skin (Figure 2H).[13,14] The filaggrin antibody also detects profilaggrin.

The expression of a number of LG-associated components was analyzed in HI and normal skin by immunofluorescence including lympho-epithelial Kazal-type-related inhibitor 1 (LEKT1), kallikrein (KLK) 7, KLK5, and cathepsin D (CTSD). LEKT1 was expressed throughout the epidermis but most strongly in the granular and upper spinous layers (Figure 3A) where it is associated with the LG network.[23] There was no obvious difference in the expression of LEKT1 between HI and control skin (Figure 3, A and E). In normal skin, KLK7 was identified in the stratum granulosum (Figure 3, B and F). However, some fainter expression of KLK7 was additionally identified in lower keratinocyte layers in HI skin. KLK5 expression in normal skin was observed in the upper epidermal layers but was significantly reduced/absent from the HI skin biopsy (Figure 3, C and G). CTSD was present in the stratum granulosum and upper spinous layers of normal skin but was also significantly reduced/absent from HI skin (Figure 3, D and H).

Nonpolar Lipids Reduced in HI Skin

Nile Red analysis of lipids was performed to compare the lipid profile between the HI skin biopsy and normal control skin (Figure 4, A and B). Nile Red fluoresces green in the presence of nonpolar lipids (such as ceramides) and red in the presence of polar lipids (such as phospholipids and sphingomyelin). Pictures were taken to detect both red and green fluorescence separately and subsequently merged. In the SC of normal skin, a bright yellow-gold color is observed indicating the presence of both polar and nonpolar lipids. However, in HI skin the vast majority of lipid was polar because only red fluorescence was detected with no obvi-

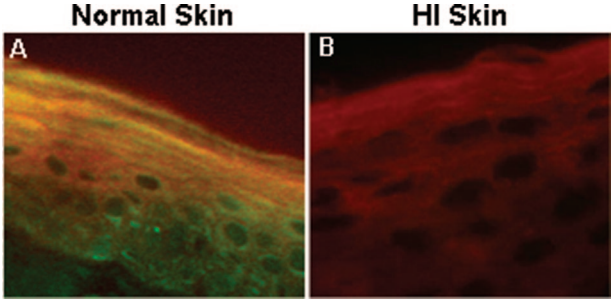

Figure 4. Nile Red analysis reveals a disruption in the lipid profile in HI skin. **A:** The SC of normal skin contains both polar lipids (red) and nonpolar lipids (green). Expression of both lipid species is shown as yellow-gold in the merged image. **B:** In HI skin the vast majority of lipid is polar, with little green nonpolar lipid staining present.

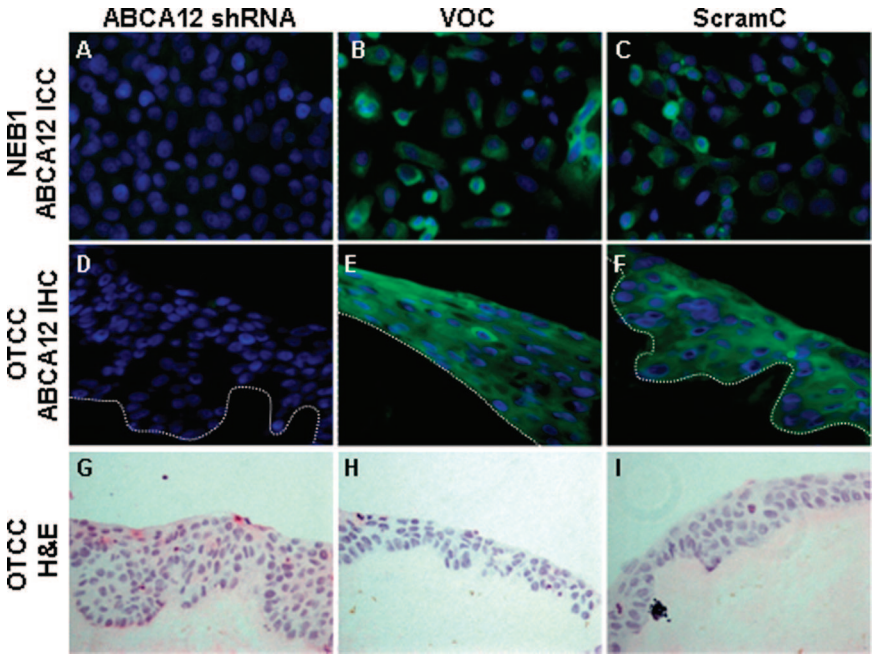

Figure 5. H&E reveals a similar morphology in ABCA12 knockdown OTCCs than that seen in HI patients. Immunocytochemistry (**A–C**) demonstrating successful knockdown in NEB1 keratinocytes (**A**) compared with the VOC (**B**) and scrambled control (ScramC, **C**). IHC of OTCCs demonstrates that ABCA12 was strongly expressed throughout the epidermis in VOC (**E**) and ScramC (**F**) but was significantly reduced in the ABCA12 shRNA knockdown OTCC (**D**). H&E staining for the knockdown OTCC (**G**) showed a thicker and more disorganized epidermis compared with the control OTCCs (**H** and **I**). Dermal-epidermal junctions are marked by a **dotted white line**.

ous yellow-gold color seen after the pictures were merged. The nonpolar ceramides comprise the bulk of the lipid contained within the lipid bilayers of the SC and are formed from the hydrolysis of glucosylceramide (GlcCer) transported via the LG system.[24] There is evidence to suggest ABCA12 is involved in the transport of GlcCer into the LG system. Human keratinocytes lacking functional ABCA12 show an abnormal intracellular localization of GlcCer that is resolved after functional gene transfer.[8]

Development of an in Vitro *Skin Model for HI*

To ascertain if the abnormal epidermal differentiation and LG transport identified in HI skin was attributable specifically to lack of functional ABCA12, three-dimensional OTCCs using NEB1 cell line keratinocytes retrovirally transduced with a pSUPERIOR.retro.puro vector containing an ABCA12 shRNA construct. After shRNA retroviral transduction of NEB1 keratinocytes, cells were fixed onto coverslips for immunocytochemical analysis of ABCA12 expression. Robust ABCA12 knockdown was achieved (Figure 5A). OTCC using this shRNA construct was created on three separate occasions along with vector-only control (VOC) and a scrambled control (ScramC) (Figure 5, B and C). These cultures were allowed to grow at the air-liquid interface for 10 days.

IHC with the ABCA12 antibody showed the protein was strongly expressed throughout the VOC (Figure 5E) and ScramC (Figure 5F) epidermis but was significantly reduced in the ABCA12 shRNA culture (Figure 5D). ABCA12 shRNA OTCCs (Figure 5G) formed a thicker and more disorganized epidermis compared with the VOC and ScramC sample (Figure 5, H and I). Retention of the nuclei was seen in the SC of all samples and is a characteristic of HPV16 immortalized keratinocytes in OTCC. Because the thickness of OTCCs varies in a single tissue block, the average thickness of the epidermis generated from each cell type was calculated. Using an unpaired two-tailed *t*-test, the average epidermal thickness of the ABCA12 shRNA co-cultures was significantly greater than that of the VOC and ScramC co-cultures ($^{***}P < 0.001$) (Figure 6). The result was repeated in all three sets of OTCCs (data not shown). Therefore, the increase in epidermal thickness and reduction in ABCA12 expression identified in ABCA12 shRNA co-cultures shows that this *in vitro* skin model reproduces key aspects of HI diseased skin.

Abnormal Epidermal Differentiation and Protease Transport Replicated in ABCA12 Knockdown OTCCs

Frozen sections from the OTCCs were stained with antibodies against involucrin, K2e, and TGase1. As was

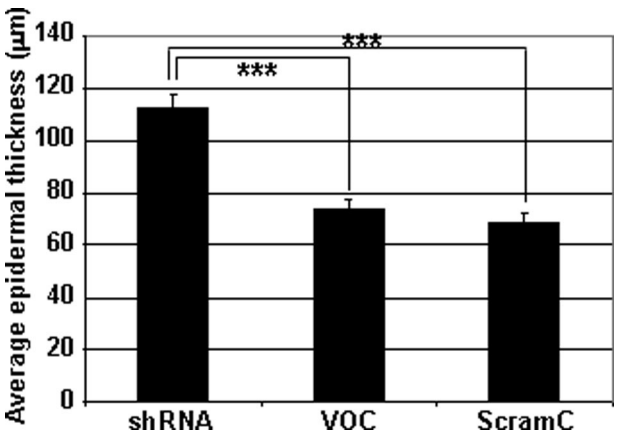

Figure 6. The epidermis is significantly thicker in ABCA12 knockdown OTCCs. Error bars represent SEM, $n = 27$. An unpaired, two-tailed *t*-test showed that the average epidermal thickness of the ABCA12 shRNA OTCC was significantly greater than the VOC ($^{***}P < 0.001$) and scrambled control (ScramC) ($^{***}P < 0.001$). The result was repeated with two different ABCA12 shRNA constructs in three separate sets of OTCCs (data not shown).

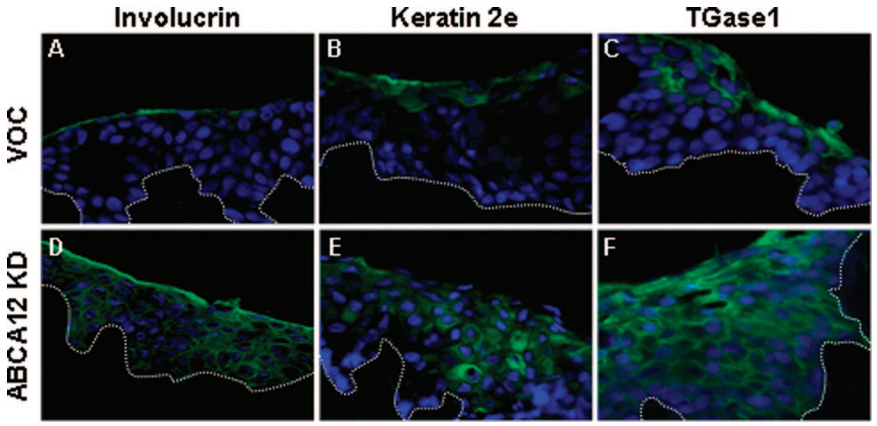

Figure 7. IHC of normal and ABCA12 knockdown OTCCs with markers of late epidermal differentiation reveal an abnormal expression pattern in the knockdown OTCC. In VOC OTCCs, involucrin (**A**), keratin 2e (K2e, **B**), and transglutaminase 1 (TGase1, **C**) were expressed in the upper layers of the epidermis, as expected. In contrast, in the ABCA12 knockdown OTCCs, the involucrin (**D**), K2e (**E**), and TGase1 (**F**) staining is present throughout the entire epidermis. DAPI-stained nuclei are shown in blue, and the dermal-epidermal junctions are marked by a **dotted white line**.

observed using the HI skin biopsy (Figure 2E), in the ABCA12 knockdown OTCC involucrin was expressed throughout the entire epidermis even in the basal layer (Figure 7D). This was in contrast to the VOC (Figure 7A), where involucrin was expressed only in the uppermost keratinocytes. K2e is expressed mainly in the stratum granulosum in the VOC OTCC (Figure 7B) but was expressed primarily throughout the epidermis of ABCA12 knockdown samples (Figure 7E). TGase1 is normally expressed in the upper layers of the epidermis as observed in control OTCCs (Figure 7C). In ABCA12 knockdown samples, TGase1 expression was observed throughout the epidermis (Figure 7F).

LG components were also analyzed by immunofluorescence in the frozen OTCC sections and mirrored the observations seen in HI skin. LEKT1 showed a similar pattern of expression between ABCA12 knockdown samples and controls (Figure 8, A and E). The protein was expressed throughout the epidermis but most strongly in the upper epidermis. Like LEKT1, the expression of KLK7 was similar between ABCA12 knockdown and control OTCCs (Figure 8, B and F). Expression of KLK7 is mostly confined to the spinous and granular layers of co-cultures. KLK5 was located throughout the epidermis of control samples (Figure 8C) but was significantly reduced/absent in all ABCA12 knockdown co-cultures analyzed (Figure 8G). Similarly, ABCA12 knockdown OTCCs all showed an almost total absence of CTSD staining (Figure 8H), whereas in control co-cultures CTSD expression was observed only in the uppermost layer of the OTCC (Figure 8D). To follow-up this observation, ABCA12 co-localization studies were performed in keratinocytes and demonstrated evidence of ABCA12 co-localizing with KLK5 and CTSD but not with KLK7 or LEKT1 (Figure 9).

Discussion

Analysis of HI skin and the *in vitro* HI skin model has provided some insight into how loss of ABCA12 affects the biology of the epidermis. The most striking abnormalities associated with HI were the thickness of the epidermis, premature terminal differentiation, an abnormal lipid profile, and an absence of specific LG proteins. The ABCA12 knockdown OTCC replicated many of the ob-

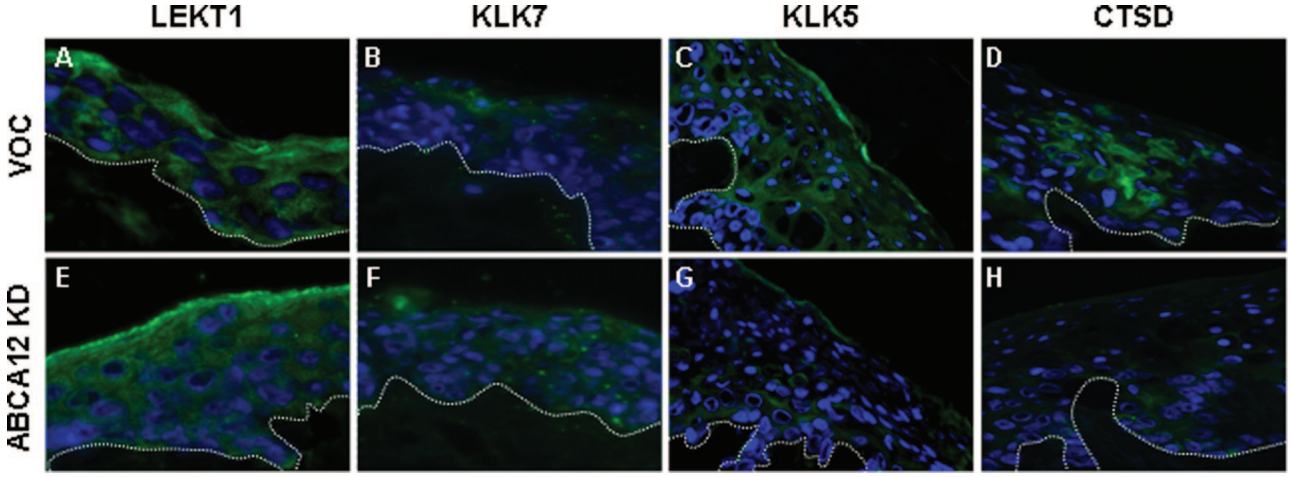

Figure 8. IHC of normal and ABCA12 knockdown OTCCs with LG markers reveals the levels of KLK5 and CTSD (but not LEKT1 and KLK7) are significantly reduced in knockdown OTCCs. In normal, VOC OTCCs, expression of LEKT1 (**A**) and kallikrein (KLK) 7 (**B**) was observed throughout the epidermis but most strongly in the upper layers. The localization was identical in the ABCA12 knockdown OTCCs (**E** and **F**). KLK5 (**C**) and cathepsin D (CTSD, **D**) were similarly expressed in the upper and middle layers of the VOC OTCCs, respectively, but the expression levels were significantly reduced in the ABCA12 knockdown OTCCs (**G** and **H**). DAPI-stained nuclei are shown in blue, and the dermal-epidermal junctions are marked by a **dotted white line**.

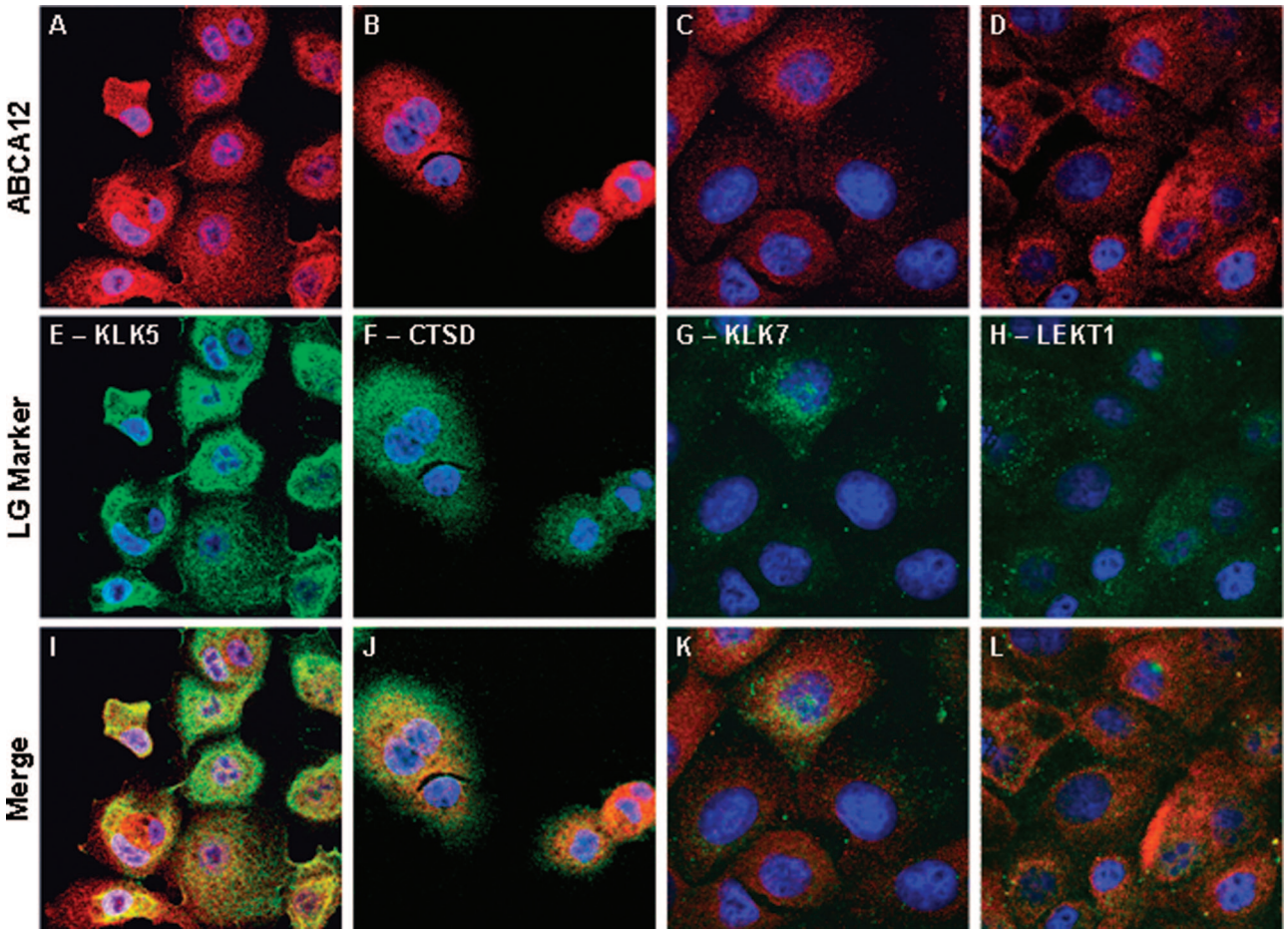

Figure 9. Co-localization between ABCA12 (red) and specific LG markers (green) by confocal microscopy. ABCA12 (**A**–**D**) partially co-localizes with kallikrein (KLK) 5 (**E**) and cathepsin D (CTSD, **F**), but not KLK7 (**G**) and LEKT1 (**H**) in NEB1 keratinocytes. **I**–**L** represent the merged panels.

servations seen in HI patient material, indicating that nonfunctional ABCA12 is the cause of the HI-associated epidermal abnormalities identified.

It has previously been documented that one of the clinical features of HI is an increased thickness of the SC and the epidermis as a whole.[13] H&E of the HI skin biopsy and ABCA12 knockdown OTCCs confirmed this increased epidermal thickness. The thickness of the SC associated with HI is most likely caused by aberrant desquamation resulting in the retention of the thick scale. This may be in part because certain proteases that are expressed or activated as products of late differentiation are not transported to the SC in HI skin because of an ineffective LG system.

We have identified that the LG components KLK5 and CTSD are significantly reduced/absent from HI skin and the OTCC HI model. Both of these proteins are important for desquamation; KLK-5 is expressed in the stratum granulosum and transported to the SC, via the LG network, where it is thought to form a proteolytic cascade where KLK5 activates itself as well as KLK7.[25] Once active it is thought that both these enzymes perform desquamation by digesting desmoglein 1, desmocollin-1, and corneodesmosin.[26–28] CTSD also has a role in epidermal desquamation through corneodesmosome degradation.[29] CTSD is activated by ceramides derived from acid sphingomyelinase.[30] The fact that ceramides may be significantly reduced/absent from the SC of HI skin could additionally hinder the role of CTSD during desquamation. Therefore, without the transport of these proteins to the SC in HI, it is likely that normal desquamation cannot occur and the SC remains abnormally thick.

Abnormal synthesis or metabolism of LG-related lipids has previously been suspected as the main pathological cause of HI.[13,14] The structure of ABCA12 and the fact that many of the proteins in the ABC superfamily have a role in energy-dependent active transport of substances across membranes led to the hypothesis that HI may in part be attributable to defective lipid transport. The Akiyama group[8] demonstrated defective lipid transport by a congested pattern of glucosylceramide (GlcCer) staining in keratinocytes with defective ABCA12. The authors then show recovery after corrective gene transfer.[8] Nile Red analysis supports the finding that ABCA12 is likely to transport GlcCer into the LG system in preparation for transport to the intercellular space. GlcCer is enzymatically hydrolyzed into different ceramides in the transition from the SG to the SC.[24] It is likely that ceramides are absent from the HI skin biopsy (and the OTCCs to some degree) because no nonpolar lipids were detected. The nonpolar ceramides are a key part of

the skin barrier so their loss would have a profound effect on barrier function.

However, a key question raised is why are there diminished or absent LGs in HI skin as other ABC transporters are also expressed in the skin and have been shown to be involved in the transportation of lipids in the epidermis. Overexpression of ABCA7 in HeLa cells results in an increased expression of intracellular and cell-surface ceramide as well as intracellular phosphatidylserine. It is suggested by the authors that ABCA7 may play a functional role in LG lipid homeostasis in the epidermis.[31] In addition the transporters ABCC1, ABCC3, and ABCC4 translocate amphiphilic anions, such as conjugates of lipophilic compounds with glutathione, glucuronate, and sulfate.[32] It has also been suggested that ABCB1 and ABCG1 are involved in phospholipid transport, whereas ABCB1 and ABCG1 may be involved in the translocation of cholesterol.[31] Therefore if these, and other, ABC transporters are expressed in the skin and are involved in lipid transport why is it not possible for them to compensate for the lack of ABCA12 found in HI? In this study, we provide evidence that ABCA12 may have other functions in the skin in addition to lipid loading.

IHC with the markers of late epidermal differentiation (involucrin, K2e, TGase1, and filaggrin) identified that the expression pattern of these proteins is highly abnormal in HI compared with normal control skin. Using both HI skin and ABCA12 knockdown OTCCs, the localization of TGase 1, involucrin, and K2e was irregular with expression seen throughout the epidermis compared with their localization in the top layers of controls. Similarly, filaggrin expression was observed throughout the upper spinous layer in HI skin, but was absent from the SC suggesting filaggrin is not being processed effectively. It has previously been reported that there is a defect in the conversion of profilaggrin to filaggrin in patient skin.[14,33] The highly abnormal expression pattern of late epidermal differentiation markers suggests that nonfunctional ABCA12 affects the tightly controlled program of early epidermal differentiation and terminal differentiation is initiated prematurely.

The proteins LEKT1, KLK7, KLK5, and CTSD are all markers of the LG system.[23,25,34] Expression of these LG proteins was analyzed in the HI skin biopsy and ABCA12 knockdown OTCCs. CTSD and KLK5 expression was significantly reduced/absent in both HI skin and ABCA12 knockdown OTCCs but LEKTI and KLK7 were expressed. These observations are in accordance with the immunocytochemical co-localization experiments. There was a degree of co-localization between ABCA12 and both KLK5 and CTSD but no evidence of co-localization between ABCA12 and either KLK7 or LEKT1. These results add to the hypothesis that CTSD and KLK5 are localized together with ABCA12 in the LG system. It suggests that LEKT1 and KLK7 are separately localized to ABCA12 and are still translocated within the LG system indicating that some areas of the LG system are still formed in HI. Some LGs are present in HI patients even though they are diminished and some appear abnormal.[13,14] It is possible that ABCA12 is important for the formation process of some but not all parts of the LG system. The lack of KLK5 and CTSD transport to the SC via ABCA12-mediated LG may in part be responsible for the defect in desquamation detected in HI. In conclusion we provide evidence that the function of ABCA12 is not limited to just lipid loading of LG but also in the formation of at least some of the LG network and in the complex regulation of early epidermal differentiation.

Acknowledgment

We thank Dr. Andrew Ilchyshyn for clinical contributions.

References

1. Hsu WY, Chen JY, Lin WL, Tsay CH: [Harlequin fetus—a case report]. Zhonghua Yi Xue Za Zhi (Taipei) 1989, 43:63–66
2. Moreau S, Salame E, Goullet de Rugy M, Delmas P: Harlequin fetus: a case report. Surg Radiol Anat 1999, 21:215–216
3. Sarkar R, Sharma RC, Sethi S, Basu S, Das R, Mendiratta V, Sardana K, Kakar N: Three unusual siblings with harlequin ichthyosis in an Indian family. J Dermatol 2000, 27:609–611
4. Buxman MM, Goodkin PE, Fahrenbach WH, Dimond RL: Harlequin ichthyosis with epidermal lipid abnormality. Arch Dermatol 1979, 115:189–193
5. Moskowitz DG, Fowler AJ, Heyman MB, Cohen SP, Crumrine D, Elias PM, Williams ML: Pathophysiologic basis for growth failure in children with ichthyosis: an evaluation of cutaneous ultrastructure, epidermal permeability barrier function, and energy expenditure. J Pediatr 2004, 145:82–92
6. Haftek M, Cambazard F, Dhouailly D, Reano A, Simon M, Lachaux A, Serre G, Claudy A, Schmitt D: A longitudinal study of a harlequin infant presenting clinically as non-bullous congenital ichthyosiform erythroderma. Br J Dermatol 1996, 135:448–453
7. Kelsell DP, Norgett EE, Unsworth H, Teh MT, Cullup T, Mein CA, Dopping-Hepenstal PJ, Dale BA, Tadini G, Fleckman P, Stephens KG, Sybert VP, Mallory SB, North BV, Witt DR, Sprecher E, Taylor AE, Ilchyshyn A, Kennedy CT, Goodyear H, Moss C, Paige D, Harper JI, Young BD, Leigh IM, Eady RA, O'Toole EA: Mutations in ABCA12 underlie the severe congenital skin disease harlequin ichthyosis. Am J Hum Genet 2005, 76:794–803
8. Akiyama M, Sugiyama-Nakagiri Y, Sakai K, McMillan JR, Goto M, Arita K, Tsuji-Abe Y, Tabata N, Matsuoka K, Sasaki R, Sawamura D, Shimizu H: Mutations in lipid transporter ABCA12 in harlequin ichthyosis and functional recovery by corrective gene transfer. J Clin Invest 2005, 115:1777–1784
9. Thomas AC, Cullup T, Norgett EE, Hill T, Barton S, Dale BA, Sprecher E, Sheridan E, Taylor AE, Wilroy RS, Delozier C, Burrows N, Goodyear H, Fleckman P, Stephens KG, Mehta L, Watson RM, Graham R, Wolf R, Slavotinek A, Martin M, Bourn D, Mein CA, O'Toole EA, Kelsell DP: ABCA12 is the major harlequin ichthyosis gene. J Invest Dermatol 2006, 126:2408–2413
10. Thomas AC, Sinclair C, Mahmud N, Cullup T, Mellerio JE, Harper J, Dale BA, Turc-Carel C, Hohl D, McGrath JA, Vahlquist A, Hellstrom-Pigg M, Ganemo A, Metcalfe K, Mein CA, O'Toole EA, Kelsell DP: Novel and recurring ABCA12 mutations associated with harlequin ichthyosis: implications for prenatal diagnosis. Br J Dermatol 2008, 158:611–613
11. Jiang YJ, Lu B, Kim P, Paragh G, Schmitz G, Elias PM, Feingold KR: PPAR and LXR activators regulate ABCA12 expression in human keratinocytes. J Invest Dermatol 2008, 128:104–109
12. Schmuth M, Jiang YJ, Dubrac S, Elias PM, Feingold KR: Thematic review series: skin lipids. Peroxisome proliferator-activated receptors and liver X receptors in epidermal biology. J Lipid Res 2008, 49:499–509
13. Milner ME, O'Guin WM, Holbrook KA, Dale BA: Abnormal lamellar granules in harlequin ichthyosis. J Invest Dermatol 1992, 99:824–829
14. Dale BA, Holbrook KA, Fleckman P, Kimball JR, Brumbaugh S, Sybert VP: Heterogeneity in harlequin ichthyosis, an inborn error of epidermal keratinization: variable morphology and structural protein expres-

sion and a defect in lamellar granules. J Invest Dermatol 1990, 94:6–18
15. Akiyama M, Shimizu H, Yoneda K, Nishikawa T: Collodion baby: ultrastructure and distribution of cornified cell envelope proteins and keratins. Dermatology 1997, 195:164–168
16. Yanagi T, Akiyama M, Nishihara H, Sakai K, Nishie W, Tanaka S, Shimizu H: Harlequin ichthyosis model mouse reveals alveolar collapse and severe fetal skin barrier defects. Hum Mol Genet 2008, 17:3075–3083
17. Yamanaka Y, Akiyama M, Sugiyama-Nakagiri Y, Sakai K, Goto M, McMillan JR, Ota M, Sawamura D, Shimizu H: Expression of the keratinocyte lipid transporter ABCA12 in developing and reconstituted human epidermis. Am J Pathol 2007, 171:43–52
18. Morley SM, Dundas S, James J, Brown RA, Sexton C, Navsaria HA, Leigh IM, Lane EB: Temperature sensitivity of the keratin cytoskeleton and delayed spreading of keratinocyte lines derived from EBS patients. J Cell Sci 1995, 108:3463–3471
19. Ojeh NO, Frame JD, Navsaria HA: In vitro characterization of an artificial dermal scaffold. Tissue Eng 2001, 7:457–472
20. Man YK, Trolove C, Tattersall D, Thomas AC, Papakonstantinopoulou A, Patel D, Scott C, Chong J, Jagger DJ, O'Toole EA, Navsaria H, Curtis MA, Kelsell DP: A deafness-associated mutant human connexin 26 improves the epithelial barrier in vitro. J Membr Biol 2007, 218:29–37
21. Hennings H, Steinert P, Buxman MM: Calcium induction of transglutaminase and the formation of epsilon(gamma-glutamyl) lysine crosslinks in cultured mouse epidermal cells. Biochem Biophys Res Commun 1981, 102:739–745
22. Presland RB, Kimball JR, Kautsky MB, Lewis SP, Lo CY, Dale BA: Evidence for specific proteolytic cleavage of the N-terminal domain of human profilaggrin during epidermal differentiation. J Invest Dermatol 1997, 108:170–178
23. Ishida-Yamamoto A, Deraison C, Bonnart C, Bitoun E, Robinson R, O'Brien TJ, Wakamatsu K, Ohtsubo S, Takahashi H, Hashimoto Y, Dopping-Hepenstal PJ, McGrath JA, Iizuka H, Richard G, Hovnanian A: LEKTI is localized in lamellar granules, separated from KLK5 and KLK7, and is secreted in the extracellular spaces of the superficial stratum granulosum. J Invest Dermatol 2005, 124:360–366
24. Hamanaka S, Hara M, Nishio H, Otsuka F, Suzuki A, Uchida Y: Human epidermal glucosylceramides are major precursors of stratum corneum ceramides. J Invest Dermatol 2002, 119:416–423
25. Sondell B, Thornell LE, Egelrud T: Evidence that stratum corneum chymotryptic enzyme is transported to the stratum corneum extracellular space via lamellar bodies. J Invest Dermatol 1995, 104:819–823
26. Simon M, Jonca N, Guerrin M, Haftek M, Bernard D, Caubet C, Egelrud T, Schmidt R, Serre G: Refined characterization of corneodesmosin proteolysis during terminal differentiation of human epidermis and its relationship to desquamation. J Biol Chem 2001, 276:20292–20299
27. Caubet C, Jonca N, Brattsand M, Guerrin M, Bernard D, Schmidt R, Egelrud T, Simon M, Serre G: Degradation of corneodesmosome proteins by two serine proteases of the kallikrein family, SCTE/KLK5/hK5 and SCCE/KLK7/hK7. J Invest Dermatol 2004, 122:1235–1244
28. Descargues P, Deraison C, Prost C, Fraitag S, Mazereeuw-Hautier J, D'Alessio M, Ishida-Yamamoto A, Bodemer C, Zambruno G, Hovnanian A: Corneodesmosomal cadherins are preferential targets of stratum corneum trypsin- and chymotrypsin-like hyperactivity in Netherton syndrome. J Invest Dermatol 2006, 126:1622–1632
29. Igarashi S, Takizawa T, Yasuda Y, Uchiwa H, Hayashi S, Brysk H, Robinson JM, Yamamoto K, Brysk MM, Horikoshi T: Cathepsin D, but not cathepsin E, degrades desmosomes during epidermal desquamation. Br J Dermatol 2004, 151:355–361
30. Egberts F, Heinrich M, Jensen JM, Winoto-Morbach S, Pfeiffer S, Wickel M, Schunck M, Steude J, Saftig P, Proksch E, Schutze S: Cathepsin D is involved in the regulation of transglutaminase 1 and epidermal differentiation. J Cell Sci 2004, 117:2295–2307
31. Kielar D, Kaminski WE, Liebisch G, Piehler A, Wenzel JJ, Mohle C, Heimerl S, Langmann T, Friedrich SO, Bottcher A, Barlage S, Drobnik W, Schmitz G: Adenosine triphosphate binding cassette (ABC) transporters are expressed and regulated during terminal keratinocyte differentiation: a potential role for ABCA7 in epidermal lipid reorganization. J Invest Dermatol 2003, 121:465–474
32. König J, Nies AT, Cui Y, Leier I, Keppler D: Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. Biochim Biophys Acta 1999, 1461:377–394
33. Akiyama M, Dale BA, Smith LT, Shimizu H, Holbrook KA: Regional difference in expression of characteristic abnormality of harlequin ichthyosis in affected fetuses. Prenat Diagn 1998, 18:425–436
34. Ishida-Yamamoto A, Simon M, Kishibe M, Miyauchi Y, Takahashi H, Yoshida S, O'Brien TJ, Serre G, Iizuka H: Epidermal lamellar granules transport different cargoes as distinct aggregates. J Invest Dermatol 2004, 122:1137–1144