Novel insights into transcriptional changes during keratinocyte differentiation and ultraviolet radiation response

Doctoral thesis

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Budapest
2009

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List of abbreviations

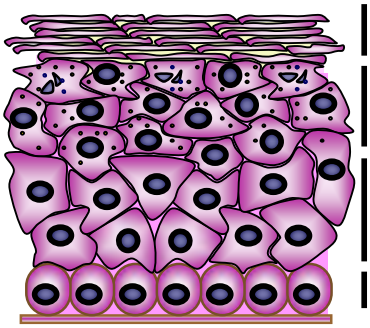
22(R)	22(R)-OH-cholesterol
25-OH	25-OH-cholesterol
9-cis-RA	9-cis-retinoic acid
ABC transporter	ATP binding cassette (ABC) transporter
AK	adenylate kinase
ALOX12B	12R-lipoxygenase
ALOXE3	lipoxygenase-3
ATP	adenosine triphosphate
ATRA	all-trans retinoic acid
C12	C12-Alkylamine
C12-SLC	C12-Alkylamine-Salicylate
Cig	Ciglitazone
COUPTFA	chicken ovalbumin upstream promoter transcription factor alpha
COUPTFB	chicken ovalbumin upstream promoter transcription factor beta
D3	Calciferol
DAX1	DSS-AHC critical region on the X chromosome, gene 1
ER beta	estrogen receptor beta
FFA	free fatty acids
FXR	farnesoid X receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI	GI 251929X
GlcCer	Glucosylceramide
GluCer	Glucosylceramide
GO	gene ontology
GR	glucocorticoid receptor
GW	GW610742
HIF	hypoxia inducible factor
HMOX1	heme oxygenase 1
iNOS	inducible nitric oxide synthase
IκBα	NFκB inhibitor, alpha
KBM	keratinocyte basal media
LB	lamellar body
LDH	lactate-dehydrogenase
LOX	Lipoxygenase
LXR	liver X receptor
MAPK	mitogen activated protein kinase
NFκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NHEK	normal human epidermal keratinocyte
PI3K	phosphatidil inositol 3 kinase
PPAR	peroxisome proliferators-activated receptor

PPARA	peroxisome proliferator activated receptors alpha
PPARD	peroxisome proliferator activated receptors beta/delta
PPARG	peroxisome proliferator activated receptors gamma
PS	Phytosphingosine
PS-C18	Stearoyl-Phytosphingosine
PS-C6	Hexanoyl-Phytosphingosine
PS-SLC	Phytosphingosine-Salicylate
PVDF	Polyvinylidene fluoride
RARA	retinoic acid receptors alpha
RARG	retinoic acid receptors gamma
Ret	Retinol
RORA	retinoid-related orphan receptor alpha
ROS	reactive oxygen species
RXRA	retinoid X receptor alpha
RXRB	retinoid X receptors beta
SA	Sphinganine
SA-C18	Stearoyl-Sphinganine
SA-C6	Hexanoyl-Sphinganine
SA-SLC	Sphinganine-Salicylate
SC	stratum corneum
SF-1	steroidogenic factor 1
SG	stratum granulosum
SLC	Salicylat
SM	Sphingomyelin
SO	Sphingosine
SO-C18	Stearoyl-Sphingosine
SO-C6	Hexanoyl-Sphingosine
SO-SLC	Sphingosine-Salicylate
SPR	small proline rich protein
SREBP1C	sterol regulatory element-binding protein-1C
SULT2B1	sulfotransferase family, cytosolic, 2B, member 1
TGM1	transglutaminase 1
TO	TO901317
TR	thyroid receptor
Tro	Troglitazone
UV	Ultraviolet
UVB	ultraviolet B radiation
VD3	1α, 25-dihydroxyvitamin D_3
VDR	vitamin D receptor
VEGF	vascular endothelial growth factor
WY	WY14643

Introduction

Preface

The skin is the largest human organ and it provides the first line of defense protecting the body from dehydration, injury, infection, physical and environmental effects, yet its importance is often overlooked. Amongst the numerous cells present in the skin epidermal keratinocytes are the predominant cell type and also the major contributors to these protective functions. They undergo an elaborate terminal differentiation process to form a constantly renewing mechanical and chemical barrier between the body and the environment. Transient proliferating cells of the epidermal basal layer leave this compartment and through morphologically evident changes create the spinous and granular layers and provide building blocks for the formation of the corneal layer (1). During this process intermediate filaments and various anchoring proteins that connect the cells and provide their attachment to neighboring structures are systematically replaced and the lipid composition is also altered (**Figure 1**).



Layer	Basal Layer	Spinous Layer	Granular Layer	Corneal Layer
proteins and structural changes	keratin5/14	keratin1/10	keratin2e, involucrin, profilaggrin, loricrin SPR1&2 transgutamainase 1	cornified envelope nucleus disappears
lipids	phospholipids triglicerides glicerophospholipids	production and storage of sphingolipids FFA, cholesterol	extracellularization of FFA, cholesterol, cholesterol-sulfate, sphingolipids	lipid lamellae (mixture of mainly FFA, cholesterol and sphingolipids)

Differentiation

Figure 1 Keratinocyte differentiation involves changes in the proteome and lipid composition of keratinocytes. (SPR: small proline rich protein; FFA: free fatty acids)

These changes take place to create a mechanically interconnected shield around the body and to enable the creation of the lamellar layer, an indispensable component of the

water permeability and chemical barrier in the epidermis. Even though there seem to be distinct mechanisms for the creation of the mechanical and chemical barriers these are not at all independent processes. They are interconnected by the homeostatic regulatory networks of the skin. A good example for the mutual reliance of the mechanical (proteins) and the water permeability barrier (lipids) on each other is the grossly similar clinical presentation found in lamellar ichtyosis type 1 and type 2 caused by mutations of transglutaminase 1 or ABCA12 respectively (2;3). Transglutaminase 1 is an enzyme responsible for covalently interconnecting structural proteins, while ABCA12 is understood to be a transporter of sphingolipid species in the epidermis. Dermatological diseases (atopic dermatitis, psoriasis and various genodermatoses ect.) showing keratinocyte differentiation disturbance, acute UV damage and drug treatment have all been found to disrupt the barrier functions of the epidermis, while barrier disruption by organic solvents is known to alter keratinocyte differentiation. The formation of the epidermal barrier relies on the controlled transcriptional changes governing the cells through their gradual terminal differentiation process, and barrier lipids (sphingolipids) are also known to regulate the epidermal differentiation process (4).

Even though keratinocyte differentiation regulation has been widely studied and much has been uncovered on the role of p63, Notch, NFκB, homeobox transcription factors and nuclear hormone receptors in this process the full scope of regulatory changes taking place in the epidermis are still not well understood (5). There is limited knowledge on the molecules regulating keratinocyte differentiation, and a complete list of the genes showing altered expression during keratinocytes differentiation is also lacking. In the present work we aimed to assess transcriptional changes taking place in keratinocytes after treatment with potential novel sphingolipids, and aimed to also find previously undescribed genes regulated during keratinocyte differentiation (6). To gain further insight into the background of transcriptional changes taking place in the skin we focused on the nuclear receptor regulation of ABCA12 the recently identified sphingolipid transport protein of the epidermis. Moreover as epidermal lipid changes are an important hallmark of ultraviolet radiation effect on the epidermis, we studied the regulatory effects of UVB on hypoxia signaling to identify the potential new regulatory mechanism responsible for UV epidermal effects.

The following pages will provide a focused insight into the current knowledge on the molecular biology of epidermal keratinocyte differentiation, with emphasis on keratinocyte sphingolipid metabolism, the regulatory role of nuclear receptors in keratinocytes and the effects of UVB radiation on the epidermis also identifying potential links between UVB and hypoxia signaling.

Changes in the cellular proteome during keratinocyte differentiation

The importance of keratinocyte differentiation lies in the essential role this process plays in the production of the epidermal barrier. During this process cells of the basal layer undergo a closely regulated sequential transformation. The most important known highlights of this cellular differentiation process are summarized below, whith an emphasis on cellular networks used as controls or studied during our work.

The **basal layer** of the epidermis, the layer of cells in direct contact with the basement membrane contains a pool of proliferating cells. Basal keratinocytes can be divided into two functional cell groups based on their commitment to differentiation: epidermal stem cells and transit amplifying cells. True stem cells have low abundance in the skin and are understood to only seldom give rise to epithelial progenitor cells (7). These epidermal stem cells of the interfollicular epidermis are characterized by a high proliferative capacity but low proliferative activity in intact skin. They do not have well established cellular markers however, they are known to express high levels of integrin β1 while showing low expression of transferrin receptor (8). Like all keratinocytes, cells of the basal layer possess a network of 10-nm keratin intermediate filaments (IFs). Keratin 15 was reported to be a keratin-species specific for epidermal progenitor cells of the basal layer hair bulge (9). The cells immediately derived from stem cells are the so called **transit amplifying cells**. They expand the population of basal cells by limited active divisions. Epidermal fatty acid-binding protein (E-FABP / FABP5) was identified in transit amplifying cells signaling the, highlighting the importance of lipid metabolism in keratinocytes (10;11). All basal keratinocytes express high levels of integrin α6 and adult basal cells contain keratin 5 and 14 while fetal basal cells keratin 19 (12;13).

The transit amplifying cells detach from the basement membrane and after a few cycles of cell division and migrate upward towards the surface of the skin (12) giving rise to **cells of the stratum spinosum**. After losing direct contact with the basement

membrane the first change to take place is the rearrangement of the IF networks to increase the tensile strength of keratinocytes. Cells achieve this by synthesizing large amounts of new keratins, which assemble into bundles of more resilient IFs. Induction of the expression of keratins 1 and 10 is one of the earliest changes, paralleled by the downregulation of keratins 5, 14 as keratinocytes leave the basal compartment. In order for IFs to properly function they need to be anchored to desmosomes (cell-cell junctions) (12;14). The cell spanning desmosome linked IF networks enable distribution of tensile strain over the entire tissue. Although desmosomes are present in cells from the basal layer to the granular layer specific desmosomal proteins are expressed in a differentiation-dependent manner similar to IFs. For example, in case of desmosomal cadherins, desmoglein (Dsg) 1 and desmocollin (Dsc) 1 are expressed in a gradient, with the highest levels found in the superficial layers, while Dsg 3 and Dsc 3 show an opposite pattern with highest expression in the basal layer which fades away gradually as differentiation progresses (15). Similarly, the armadillo family member plakophilin (PKP) 1 and 2 proteins show differentiation specific epidermal distribution. PKP1 is concentrated in junctions of the superficial layers, whereas PKP2 was identified predominantly in desmosomes deeper in the epidermis(16).

Filaggrin is a well known differentiation marker, which is normally expressed in advanced epidermal differentiation. Filaggrin along with trichohyalin are the two major intermediate filament associated proteins, which interact with keratin filaments in the skin. These two proteins initially accumulate in cytoplasmic granules called keratohyalin or trichohyalin granules, which are prominent morphological hallmarks of differentiation in the epidermis and the inner root sheath of hair follicles (17). These cellular organells are most prominent in **cells of the stratum granulosum**. After undergoing modification the contents of these granules are released into the cytoplasm of the fully mature cells where they play a role in the aggregation of keratin filament bundles during cornification (17). The importance of these structural components of the epidermis are easily demonstrated by the numerous dermatological diseases that arise from the structural alteration of modified expression of these proteins, and by the 80% contribution of these molecules to the total mammalian epidermal protein mass.

During the differentiation process the keratinocytes also undergo metabolical alteration to become ready for the relatively fast and marked morphological

transformation needed to create the stratum corneum. Thus in the upper spinosus and granular layers the cells start accumulating large amounts of lipids, and precursor forms of enzymes needed in the final phases of keratinocyte differentiation (18;19). These lipids are responsible for providing a water-impermeable barrier (for details please see page 12).

To produce a functional human **stratum corneum** one additional major transformation needs to take place. Living keratinocytes of the SG need to transform into flattened corneocytes. Keratinocyte prepare for this final transformation during their outward journey through the epidermis by accumulating structural proteins, enzymes and lipids in their storage organelles (keratohyalin granules and lamellar bodies). The accumulation and immediate availability of these components enables the cellular transformation to take place fast when keratinocytes reach the upper granular layer (19). The cells here cease transcriptional and soon also metabolic activity and undergo a special form of programmed cell death. Their nucleus is fragmented and mitochondria disintegrate while transglutaminases (TGM1, TGM3, TGM5) connect the preformed large arrays of proteins creating a compact proteinaceus scaffold in place of the once living cell. While their fromer lipid content forms lipid lamellae. In the generation of the protein scaffold the most abundant protein species used as building blocks are keratin filaments, filaggrin, involucrin, loricrin and members of the small proline-rich protein family (20;21) (**Figure 2**). The formed outer stratum corneum is constantly regenerated from the pool of differentiating keratinocytes, replacing the loss due mechanical and chemical insults. Normally the full turnover of cells takes 28 days (22;23).

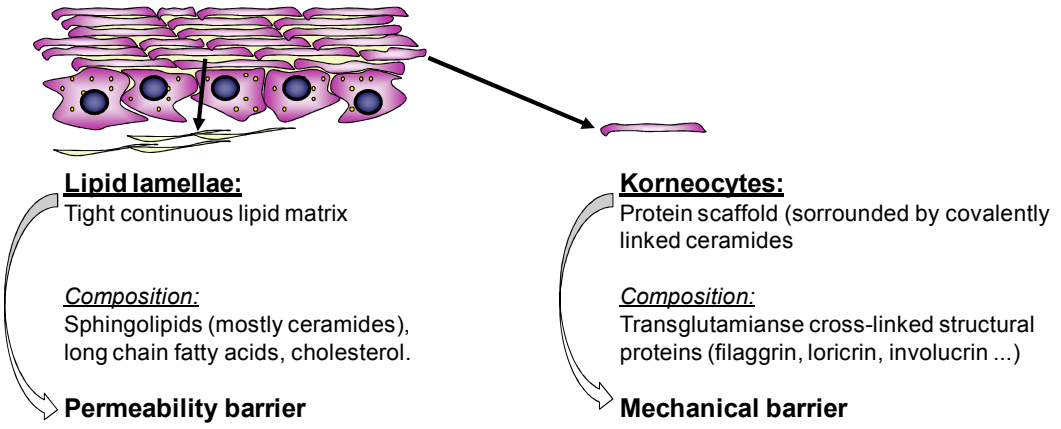


Figure 2 Structure and main function of the stratum corneum.

Although many aspects of the structural organization of the skin and keratinocyte differentiation have been studied, our understanding of these processes are to date incomplete. Due to the very different functional and morphological characteristics of basal and suprabasal layer keratinocytes, as examples of this text block have shown there are numerous genes that are differentially expressed. Some of these genes have long been used as differentiation markers and have been valuable in assessing the characteristics of epithelial tumors (24). Moreover some later discussed keratinocyte differentiation genes have also emerged as primary targets for drug therapy (RAR, PPARs, VDR) in dermatological diseases. Although our knowledge of the keratinocyte differentiation process has gradually expanded in recent decades many of the genes suspected to play a role it are yet to be identified.

Lipids in the skin

The main structural lipids in epidermis

As discussed earlier the conversion of living keratinocytes into dead corneocytes, the building blocks of the corneal layer, not only involves changes in the cellular proteome. Barrier formation also inevitably requires lipids to enable compartmentalization of stable aqueous environments. Thus it is not surprising that for the creation of a well functioning epidermal barrier, specific changes in the lipid composition of differentiating keratinocytes are also necessary (25;26).

During keratinocyte differentiation an increase in the cellular content of very long chain highly saturated fatty acids, cholesterol sulfate, unesterified cholesterol and sphingolipids takes place paralleled by a decrease of glycerophospholipids, triglycerides, and very long chain polyunsaturated fatty acids (27). Sphingolipids contribute to approx. 50% of the stratum corneum (SC) lipids, with a predominance of ceramides (28-30). Some of the lipids are either stored in storage forms in epidermal keratinocytes or converted to intermediaries to enable accumulation of these molecules without negatively affecting cellular functions. Large amount of cholesterol is converted to cholesterol-sulfate, which is only hydrolyzed back to cholesterol in the stratum corneum (31). While ceramides are converted to glucosylceramide (GlcCer) by

ceramide glucosyltransferase most likely to enable their accumulation in lamellar granules (19), and rapid production of ceramides only takes place at the straum granulosum (SG) and SC interface by hydrolysis of this GlcCer pool and cellular sphingomyelin (SM) (30;32;33). The epidermal lipids along with numerous proteins involved in their maturation and organization of the epidermal barrier are mostly accumulated in **lamellar body granules** (Odland bodies) in cells of the SG. The exact method of lipid deposition on the SG/SC boundary and the formation of the lipid lamellae is still dabated. It most likely involves an active transport mechanism during which the content of lamellar bodies empties into the interstitial space similar to how the content of secretory lysosomes are extruded from cells, with highly energy demanding mechanisms also involved in the formation of the lamellar structure of the inter-corneocyte lipid matrix. Although alternatively less energy demanding mechanisms may also be responsible for formation of this unique lamellar lipid structure (18;34;35).

At least 9 major classes of non-corneocyte-bound **ceramide** species have been reported in the epidermis along with four classes of covalently bound ceramides (36;37). These molecules are unique due to differences mostly in their fatty acid side chains. The role of ceramides in the skin is understood to be divers. In part ceramides are building blocks of the epidermal barrier. ω-OH Cer species are covalently bound to surface proteins of the corneal envelope (CE) in such a high amount that they effectively replace the normal cell membrane and form a lipid layer around the corneocytes. This lipid mantel promotes mechanical attachment of coenocytes into the lipid matrix by creating a lipophilic outer layer (29;38-40) (**Figure 2**). In addition Cer species are also present in the lipid lamellae of the stratum corneum. Their importance is highlighted by the observation that depletion of SC Cer content by blocking the cleavage of GlcCer and SM into Cer results in increased transepidermal water loss and impaired barrier function. Moreover, ceramides need to be present along with cholesterol and fatty acids in the topically applied mixture to allow for normal barrier repair after disruption with acetone (41). However, beyond the structural role of Cer species they are also important regulators of cellular function through their influence on the apoptotic pathway, which is a part of the terminal differentiation of keratinocytes (42-45).

To get a better insight into the role of ceramides in the epidermis first their structure and pathways of generation and their known regulatory function needs to be discussed. **Ceramides are the most basic sphingolipid structures.** Sphingolipids are lipids derived from an aliphatic amino alcohols, mostly sphingosine (SO) and in some cases sphinganine (SA) or phytosphingosine (PS) (**Figure 3**). They are constituents of lipid membranes and as it has been lately identified also possess regulatory roles (46).

Several subtypes of sphingolipids can be distinguished based on the molecular group attached to the 1-OH group of the aliphatic amino alcohol. The most basic structures are ceramides, while modification of this structure yields sphingomyelins, cerebrosides, lactosylceramides and gangliosides (**Figure 4**). The character of sphingolipids is markedly modified by the length and structure of the fatty acid chain attached to the aliphatic amino alcohol via an amide bond (typically acyl chains are 16–24 carbon atoms long)(46;47).

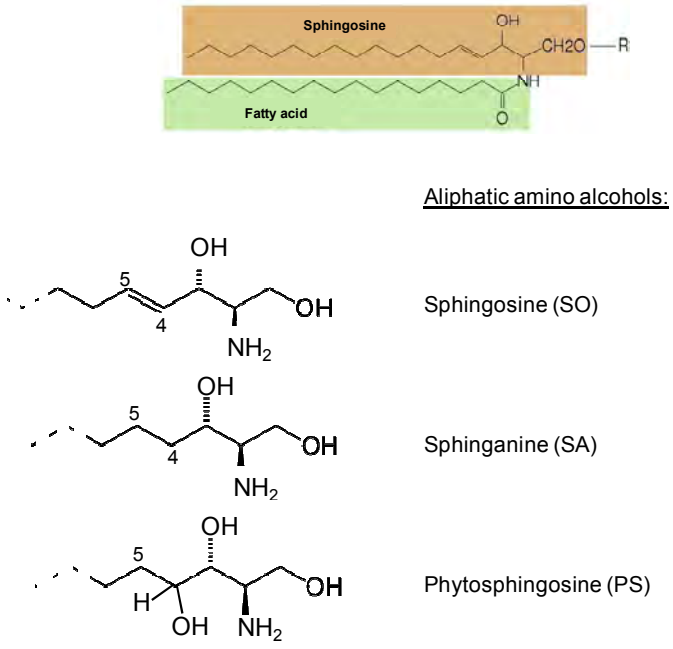
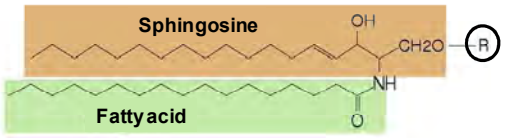


Figure 3 The aliphatic amino alcohol component of sphingolipids



Group linked to 1-hydroxy group	Name of sphingolipid
———	Ceramide
Phosphorylcholine / Phosphoroethanolamine	Sphingomyelin
Glucose / Galactose	Cerebroside
2-4 Galactose molecules	Lactosylceramide
Complex sugar structures with sialic acid	Gangliosides

Figure 4 Sphingolipid subtypes based on 1-hydroxy group modification.

The *de novo* synthesis of sphingolipids starts in the endoplasmic reticulum (ER) from serine and palmitoyl-CoA (**Figure 5**). The rate limiting step of ceramide generation is the first step catalyzed by the serine palmitoyl transferase. Initially the ceramide synthesis pathway leads to the creation of sphingaine based ceramide (N-acil-sphinganine), which still in the ER is converted into sphingosine ceramide (N-acil-sphingosine). These can later be modified in the Golgi to produce a full range of sphingolipids the cell requires for its normal function (48).

Although *de novo* synthesis is important, majority (50-90%) of cellular sphingolipids are known to be synthesized in the so called salvage pathway from partially degraded components of cellular sphingolipids (**Figure 5**) (49). Degradation of sphingolipids takes place in the late endosomes and lysosomes. Glycosphingolipids are first sequentially degraded by an array of exohydrolases, resulting in the release of monosaccharide units and eventually ceramide (50), while sphingomyelin is converted to ceramide by acid sphingomyelinase. Ceramides are hydrolyzed by acid ceramidase to sphingoid backbones and free fatty acids, these then can easily leave the lysosomes as the lysosomes are permeable for these degradation products in contrast to ceramides (49). Sphingoid bases and fatty acids can be reassembled into ceramide by entering synthesis pathways or may generate sphingosine-1-phosphate(49;51).

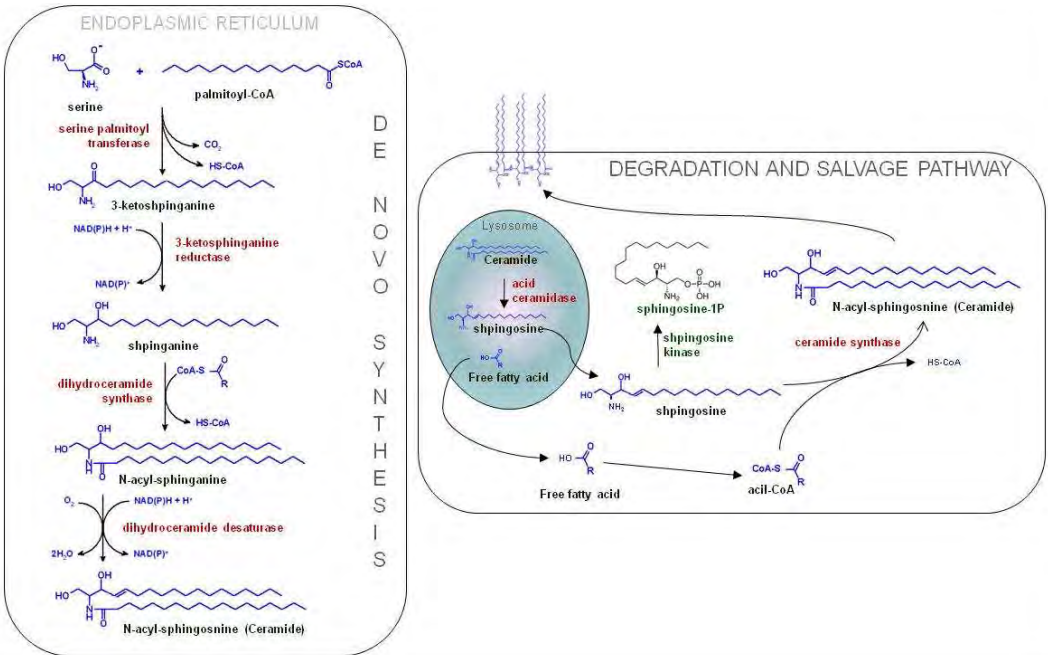


Figure 5 Major pathways of sphingolipid synthesis.

The cellular roles of sphingolipids are wide reaching. They are important structural components of cellular membranes, and especially complex sphingolipids are noted for creating the chemically and mechanically stable outer leaflet of the plasma membrane. Moreover they are known to be involved in the formation of plasma membrane lipid rafts(52). Their cell surface presence may constitute a species or cell type specific signal. The ABO blood group antigens, for example, are glycolipid carbohydrate moieties on the surface of cells or on serum glycoproteins (53). Their cell surface presence and structure renders these molecules important contributors to the regulation of cell adhesion, targets of pathogen cell surface binding and mediator of pathogen internalization (viruses: HIV, Ebola; bacteria: A .naeslundii, H. pylori; toxins: botulinium, tetanus) (54). Furthermore their interaction with integral membrane proteins have been shown to take part in the regulation of among other proteins fibroblast growth factor receptor, platelet derived growth factor receptor and epidermal growth factor receptor, by influencing receptor dimerization and even ligand binding (55).

The regulatory role of sphingolipids in keratinocytes

Close transcriptional regulation is needed to harmonize the differentiation process and the metabolic shift. SP1, AP1, AP2 and GATA family members have all been proven to be involved in keratinocyte differentiation but the identification of nuclear hormone receptors brought a new era of understanding keratinocyte lipid metabolism(56;57). RARs, RXRs, VDR and PPARs and LXRs are all members of this protein family influencing epidermal homeostasis and as regulators of lipid metabolism in other tissues, they are understood to be responsible for orchestrating keratinocyte water permeability barrier formation(58).

Lipids are not only targets of these regulatory processes, but lipid metabolites also regulate some of the homeostatic circuits. This is also the case in sphingolipid metabolism. The most widely used keratinocyte modulators of proliferation and differentiation, retinoids, VD3, and their analogues influence sphingolipid metabolism (59-62), while sphingolipids themselves were identified as regulators of keratinocyte function (42-45).

Numerous sphingolipids are known to be regulatory molecules influencing cellular homeostasis via their various receptors (63). **Ceramides** directly modify cellular signaling by binding to kinse supressor of Ras, ceramide activated protein kinase, protein kinase C-zeta, phosphoinositide-3 kinase and cathepsin-D(63). Depending on the stimuli converging on other pathways the status of the cells and the given cell type ceramides may induce apoptosis, stimulate or inhibit proliferation and can induce inflammation (63;64). In the epidermal keratinocytes ceramides are not believed to be only involved in cell death signaling, as discussed earlier they are essential components of the permeability barrier and either directly or via their degradation products thought to be involved in the regulation of cell differentiation(65;66).

Ceramides via the above mentioned mechanisms act mostly as intracellular messengers. **Sphingosine-1-phoshate**, however, also has a long ranging hormone like effect(63;67). It is generated by sphingosine kinase from sphingosine and is mainly associated with HDL in the circulation(67). It is involved in the regulation of the egress of immune cells from lymphoid organs, T- and B-cell trafficking(68). It has also been shown to play a role in the regulation of angiogenesis, vascular stability and

permeability(69). Many of its cellular effects are mediated by sphingosine-1-phosphate receptors present on the surface of numerous cell types, and effect of this molecule are greatly dependent on receptor expression and downstream signaling. Keratinocytes also express sphingosine-1-phosphate receptors the activation of which confers anti-proliferative, anti-apoptotic activity on these cells(62;70). Moreover sphingosine-1-phosphate has also been shown to possess antimicrobial activity(71).

The balance between ceramide levels and sphingosine-1-phosphate, seems to be a general rheostat of cellular homoeostasis, targeting the cells towards either differentiation or apoptosis or proliferation and cell survival (62;72). Sphingosine-1-phosphate and long chain ceramide-1-phosphate were found to stimulate cell proliferation in this rheostat (73;74), while cell permeable short chain ceramides as well as long chain ceramides cause growth inhibition and induce differentiation in normal keratinocytes (75;76) or even apoptosis in the immortalized HaCaT keratinocytes cell line (77;78). When both compounds are added, ceramides have been shown to specifically antagonize the mitogenic effects of sphingosine-1-phosphate and ceramide-1-phosphate (73), but were unable to antagonize the effects of insulin, epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) (79). The downstream effects of the sphingolipid rheostat involve modulation of protein kinase C (PKC) isoforms (26), mitogen-activated protein kinase (MAPK), c-jun N-terminal kinase and peroxisome proliferator activated receptors (PPARs) (80), however much of its regulation is still unknown.

Impaired stratum corneum lipid barrier is repaired **in healthy skin** by increased lipid biosynthesis, lipid processing and accelerated exocytosis of lamellar bodies. Therefore topically applied ceramides are thought to enhance the recovery of damaged skin, either by directly contributing to the mature ceramide species found in the stratum corneum, or by providing precursors for the ceramide biosynthesis pathway to enhance endogenous ceramide production (81). Even though a large amount of knowledge has accumulated concerning the effect of single sphingolipid species in keratinocytes, and they are used in cosmetic compounds, to our knowledge no genome wide analysis of their effects on gene expression during the early stages of keratinocyte differentiation and no comprehensive comparison of different sphingolipid derivatives has been carried out.

Adenosine triphosphate binding cassette (ABC) transporters in the skin; the genetic alterations behind lamellar ichthyosis

Generation of the lipid lamellae of the epidermis requires the metabolic processing and ordered extracellular deposition of lipids. Proteins responsible for lipid translocation have recently come in the crosshairs of scientific interest (82). **Adenosine triphosphate binding cassette (ABC) transporters** are an evolutionary conserved group of seven families containing 48 proteins responsible for ATP dependent translocation of substrates through cellular membrane surfaces (83;84). They are characterized by different substrate specificity and tissue expression and many of them are involved in transmembrane lipid transport (85;86). ABCA1 has been shown to facilitate cholesterol and choline backbone phospholipid export (87;88). While ABCB1 (MDR1) and ABCB4 (MDR3) were shown to translocate phospholipids between the inner and outer plasma membrane leafet (89) and ABCD1 (adrenoleukodystrophy transporter) functions as a peroxisomal transporter of very long chain highly saturated fatty acids (90). Given the major role of ABC transporter species in cellular lipid organization and the importance of lipids in the epithelial barrier it is not surprising that ABC transporters are transcriptionally regulated during keratinocyte differentiation (82).

Several ABC cassette transporters have been found to be associated with human skin diseases. ABCB2 and ABCB3 polymorphisms were found to be associated with the appearance of psoriasis and ABCC6 has been identified as the gene mutated in pseudoxantoma elasticum and ABCA12 mutations have been found to result in the clinical presentation of lamellar or harlequin ichthyosis (2;91;92). However, only ABCA12 mutations are thought to effect keratinocytes directly. The mutations of the mentioned other three genes (ABCB2, ABCB3, ABCC6) are thought to abrogate skin morphology and function indirectly by altering functions of other cell types (fibroblasts and cells of the dermal immune response) or via indirect metabolic effects.

Lamellar ichthyosis (LI) is a rare (1 / 300,000-500,000) autosomal recessive inherited skin disease characterized by the presence of large adherent pigmented scales. To date 4 loci (LI1, LI2, LI3, LI5) have been identified in the background of the disease, with the underlying mutated genes found in 3 of these 4 locations. At the LI1

locus TGM1 (transglutaminase 1), at LI2 ABCA12 and at LI5 the mutation of two lipoxygenases (ALOXE3 and ALOX12B) were proven to be responsible for the disease phenotype **(Table** 1**)** (2;93;94). Harlequin ichthyosis is the most severe often fatal congenital ichthyosis, in which harge diamond-shaped scales are apperent and may lead to abnormal contractions and tush malformation of the nose mouth and eyes. Currently it is becoming evident severe forms of genetic mutation of genes also implicated in lamellar ichthyosis may be responsible for the appearance of this disorder (2;95)

Locus	Gene
Lamellar Ichthyosis 1	transglutaminase 1
Lamellar Ichthyosis 2	ABCA12
Lamellar Ichthyosis 3	still unknown
Lamellar Ichthyosis 5	Lipoxygenase-3 (ALOXE3) 12R-lipoxygenase (ALOX12B)

Table 1 Lamellar ichthyosis loci and the mutated genes

Lipoxygenases (LOX) are iron (non-heme) containing dioxygenases that can insert molecular oxygen into cis,cis-1,4-pentadiene containing polyunsaturated fatty acids. The two recently discovered LOX genes, which are associated with lamellar ichthyosis form subsequent elements of a metabolic pathway, which clearly affects epidermal differentiation. Although ALOXE3 is a member of the LOX family, it does not possess the usual enzymatic activity typical of this protein family. Rather, ALOXE3 converts the product (12*R*-HPETE) of ALOX12B a typical epidermal LOX into an epoxyalcohol isomer. Moreover it can also convert other LOX products such as 12*S*-HPETE and 15*S*-HPETE to related epoxyalcohol products, although with lower catalytic efficiency. The role of these molecules in keratinocyte differentiation are to date unknown, even though the importance of these genes in the process is well established (96;97). Transglutaminase 1 has long been known as a crucial enzyme in keratinocyte differentiation (please see details in the keratinocyte proteome section on page 11) and the fact that its mutations may cause a disease with similar presentation to

proteins involved in keratinocyte lipid metabolism shows the striking inter-reliance of keratinocyte lipid metabolism and the structural changes of cornification (21).

ABCA12 is a recently characterized member of the ATP-binding cassette transporter family. It is highly expressed in the skin and testis. With its predicted 293kDa molecular weight it is one of the largest members of the ABC transporters. Its sequence is highly conserved in mammals. It was postulated form the proven function of other members of its family and the profound morphological and microscopical effects of its mutation that it is involved in the transportation of lipid derivatives within the epidermis. Thus it was not surprising that ABCA12 has recently been found to be involved in the trafficking of glucosylceramide to lamellar bodies in keratinocytes (98;99). ABCA12 mutations not only cause lamellar ichthyosis. Severe mutations in this gene were identified to result in the appearance of Harlequin ichthyosis, an often fatal keratinizing disorder (2;95). The mechanism responsible for these changes is thought to be the abrogated transportation of sphingolipids, and deficient formation and secretion of lamellar bodies in ABCA12 mutation (2;95). These findings indicate the critical role of sphingolipid transport in epidermal physiology, and make ABCA12 regulation an important aspect of epidermal sphingolipid homeostasis. Although the regulation of several members of the ABC transporter protein family have been thoroughly studied only the keratinocyte differentiation dependent upregulation of ABCA12 expression in cultured human keratinocyte (CHK) has been described and the regulatory molecules mediating this process are unknown (99;100). As members of the nuclear receptor protein family have often been implicated in ABC transporter regulation in various cellular model systems and human tissues, the question whether nuclear receptor species known to be responsible for the regulation of epidermal barrier functions of the human skin are involved in the expression of ABCA12 arose.

Nuclear receptors in keratinocyte differentiation and their potential for regulating lipid transport within the epidermis

General considerations: subtypes and structure of nuclear receptors

The ultrastructure of the epidermis and the well orchestrated expression of its mechanical structural elements are of paramount importance in distributing the mechanical forces throughout large areas of tissue, greatly contributing to the tensile strength of the skin. A gradual gene expression change that allows the compositional modification of this mechanical scaffold while maintaining its integrity is required for the continuous renewal of the barrier. To fully understand how the barrier works, we also need to uncover the regulatory machinery responsible for the gradual conduction of the differentiation process. One should not forget that transcriptional regulation is a result of the interplay of a large array of proteins the functional interactions of which are still only vaguely understood. Due to their direct effect on transcription, their hormone responsiveness and the availability of synthetic ligands nuclear hormone receptors are undoubtedly one of the most exiting family of regulatory proteins from the clinical perspective. Therefore in this section the most important information on our current knowledge of the differentiation dependent regulation of gene expression in keratinocytes by nuclear hormone receptors will be discussed along with biologically relevant other functions of these receptors. We will discuss in detail: peroxisome proliferator-activated receptors (PPARs), retinoic acid receptors (RAR), retinoid x receptors (RXR), vitamin D receptor (VDR) and liver X receptor (LXR).

Nuclear receptors (NRs) are ligand sensitive transcriptional regulators localized, as suggested by their names in the cell nucleus. The members of this group of evolutionally highly conserved proteins have 3 structural regions important for understanding their function (**Figure 6**): 1. The transactivation region is responsible for part of the protein interactions that enable the modulation of transcription by nuclear receptors. 2. The DNA binding domain (DBD) attaches the receptor to its response element (RE), and it also serves as a week dimerization interface for other receptors. 3. The last noteworthy structural component of the receptor is the ligand binding (LBD)

domain, which is responsible for the ligand selectivity and serves as the major dimerization interface and an equally important transactivation site (101;102).

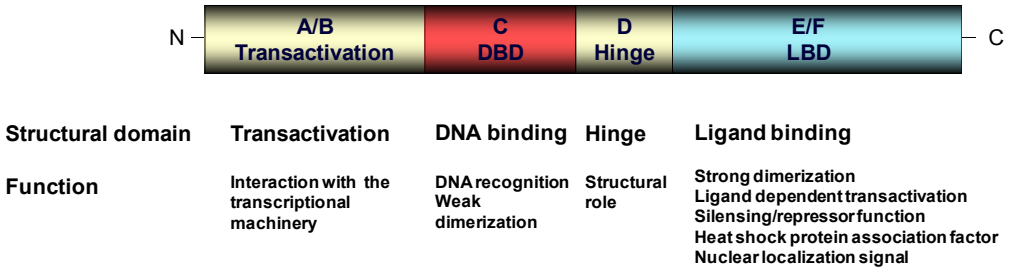


Figure 6 The structure of nuclear receptors and functional role of their domains.

Nuclear hormone receptors form one of the largest family of transcriptional regulator proteins(103). They are expressed with specific tissue selectivity and they regulate metabolism, proliferation and differentiation, cell death and even immunologic responses(101). NRs are capable of modulating transcription in the "old-fashioned" way of binding their response elements, or by non DNA dependent protein-protein interactions(104). Most nuclear receptors bind DNA in dimer form(102). The dimerization partner is mostly retinoid X receptor (RXR) in heterodimers, and another protein of the same receptor in homodimers(102). These DNA bound receptors depending on the presence of ligands acquire different protein complexes while attached to the DNA. Usually ligand binding changes receptor bound repressor protein complexes to activators, therefore increasing the efficiency of transcription(105;106).

Mode of action and functional role of nuclear receptors

Nuclear receptor ligands have been used in human therapy long before the identification of the receptors themselves. One of the earliest target organs was the skin, where retinoic acid and its derivatives revolutionized the treatment of acne, and have been in use very effectively to ameliorate a wide range of other diseases(107). Later with the discovery of the actual receptors their functional importance in the skin started to be appreciated (the NRs described in the skin are listed in **Table 2**). Now it is clear

that they similarly to their effects in other cells are capable of modulating the most important biologic networks of keratinocytes, and thus are capable of altering barrier formation(58).

Before detailing our current knowledge on the role of specific nuclear receptors in the regulation of epidermal functions one has to highlight the fact that nuclear receptors are not lonely regulators of transcription acting by themselves. They lack a direct effector mechanism being capable of altering gene expression and therefore they rely on the presence and action of co-regulator proteins to fulfill their physiological roles(105;106). The direct interacting proteins can be divided into two main groups according to their effects on transcription. Coactivators that are termed after their ability to positively regulate the transcriptional machinery form the bigger group, whereas corepressors that play an opposite role are less numerous. The binding of cofactors is regulated by the structural changes in the nuclear receptors. Depending on the receptor the cofactor and the ligands, interaction can be mediated in many different ways. Most often agonist binding induces a change in the protein structure of the receptor, which results in changing the corepressor to a coactivator. Although these direct binding cofactors form the heart of the scaffold of proteins that assemble around active regulatory sites they are still not the final effectors (108;109). The direct modulation of transcription is thought to be mainly achieved by the anchoring and activation of histone acetyltransferases (HAT) and histone deacetylases (HDACs) (110) (**Figure 7)**.

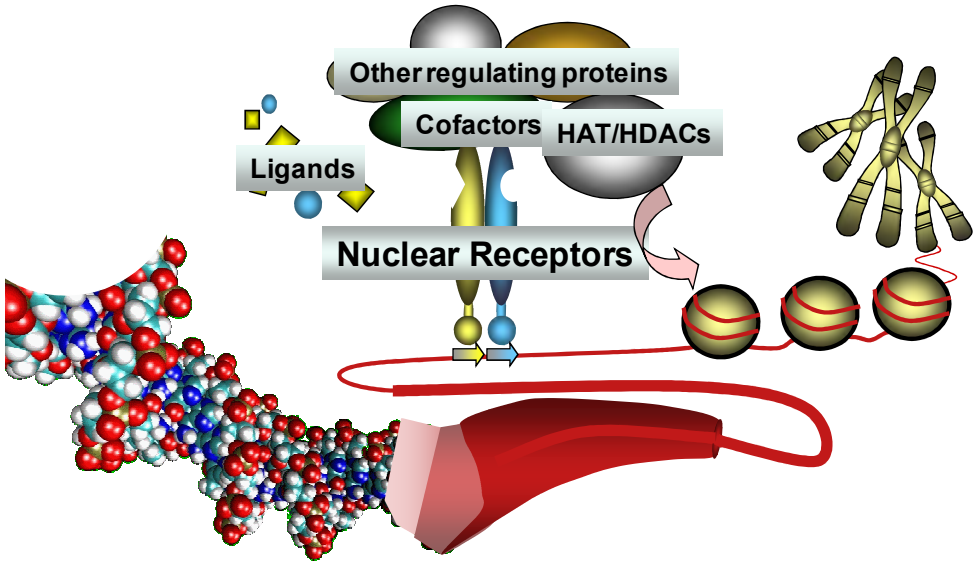


Figure 7 Schematic picture of transcriptional regulation by nuclear receptors, through histone acetylation (histone acetyltransferases (HAT) and histone deacetylases (HDAC)

Best known epidermal nuclear receptors

Although the expression of several nuclear receptors have been described in the epidermis (**Table 2**). So far only a small number of these receptors have been recognized as important regulators of epidermal homeostasis. In the following pages the our knowledge on these already recognized important nuclear receptors will be summarized.

The dermatological use of retinoids (vitamin A derivatives) preceded description of their mechanism of action and discovery of their receptors (107). There are two major groups of retinoid responsive hormone sensitive nuclear receptors one termed **retinoic acid receptors** (RAR) and the so called **retinoid X receptors** (RXR). Both of these groups of retinoid receptors have three subtypes (alpha, beta and gamma), which show different tissue specificity. RARs and RXRs are activated by 9-cis-retinoid acid, while all-trans-retinoic acid only activates RARs (111). The functional retinoid receptor is a heterodimer of a RAR and an RXR. It influences transcriptional activity similarly to other nuclear receptors via a cofactor complex anchored to the response element of the promoter by the receptor heterodimer. RXR is the obligate dimerization partner of numerous other nuclear receptors such as PPARs, VDR, TR; thus, the effects

of retinoids are wide-ranging and basically involve all tissues and stages of ontogenesis, from embryogenesis to adult life (111).

UniGene	Full name of nuclear receptor	Abbreviation	Systemic name	Supporting literature
Hs.126608	glucocorticoid receptor	GR	NR3C1	(112)
Hs.443150	estrogen receptor beta	ER beta	NR3A2	(113)
Hs.361071 Hs.1497	**retinoic acid receptors (alpha, gamma)**	**RARA RARG**	**NR1B1 NR1B3**	(114)
Hs.20084 Hs.388034	**retinoid X receptors (alpha, beta)**	**RXRA RXRB**	**NR2B1 NR2B2**	(115)
Hs.2062	**vitamin D receptor**	**VDR**	**NR1I1**	(116)
Hs.534037 Hs.387667 Hs.519689	**peroxisome proliferator activated receptors (alpha, gamma, delta)**	**PPARA PPARG PPARD**	**NR1C1 NR1C3 NR1C2**	(117)
Hs.724 Hs.187861	thyroid receptor	TR		(118)
Hs.438863	**liver X receptor**	**LXRA LXRB**	**NR1H3 NR1H2**	(119)
Hs.133209	farnesoid X receptor	FXR	NR1H4	(120)
Hs.361748 Hs.347991	chicken ovalbumin upstream promoter transcription factor (alpha, beta)	COUPTFA COUPTFB	NR2F1 NR2F2	(121;122)
Hs.60770	retinoid-related orphan receptor alpha	RORA	NR1F1	(123)
Hs.268490	DSS-AHC critical region on the X chromosome, gene 1	DAX1	NR0B1	(124)
Hs.157037	steroidogenic factor 1	SF-1	NR5A1	(124)

Table 2 Nuclear receptors expressed in the epidermis (in bold receptors assessed for their ABCA12 expression regulating effect)

Within the epidermis RAR-alpha and gamma and RXR-alpha and beta are the major isoforms of these receptors, and heterodimeric complexes of these receptors as well as the above mentioned RXR heterodimers are responsible for retinoid effect on the keratinocytes (114;115;125). The sum of the wide reaching effects of retinoid treatment in keratinocytes is the inhibition of cell differentiation, with differing effects on keratinocyte proliferation depending on the pre-treatment state of the cells (126;127). These effects render retinoids effective in the treatment of psoriasis, ichthyosis, acne

and rosacea, moreover also making retinol an ideal compound as a control for antidifferentiation effects in drug studies(128).

Peroxisome proliferator activated receptors (PPAR) are relatively newly identified orphan nuclear receptors which have attracted significant attention due to their crucial role in lipid homeostasis and owing to their potential to regulate cell differentiation. PPARs form permissive heterodimers with RXR, thus allowing both PPAR and RXR ligands to regulate transcription of target genes(101;102). The effects of PPAR regulation were mostly studied in the liver (PPAR alpha), adipocytes (PPAR gamma), mononuclear cells (PPAR gamma) and in skeletal muscle (PPAR delta)(129). Their importance in regulating keratinocyte function has only lately been recognized(58;117). PPAR delta is the most abundant PPAR subtype in the skin, followed by PPAR alpha and PPAR gamma(117). PPAR alpha knock out mice models show in utero delay in the formation of the corneal layer, a transient and initial delay in wound healing and a decrease in beta glucocerebrosidase production (130). PPAR gamma null mice after placental rescue exhibit no obvious skin defects. In PPAR delta heterozygous (+/-) mice TPA induced hyperplasia is enhanced while keratinocyte migration and wound healing is reduced(131).

The marked up-regulation of PPAR-gamma and PPAR-alpha during keratinocyte differentiation provides evidence that PPAR subtypes orchestrate metabolic changes taking place during terminal differentiation of these cells. PPAR delta was shown to remain high during keratinocyte differentiation (132) and was significantly up-regulated by conditions inducing keratinocyte proliferation (133). Furthermore ligands of PPARs were effective inhibitors of keratinocyte proliferation and specific ligands for each receptor were shown to up-regulate differentiation markers (134;135).

Vitamin D receptor is a unique member of the group of nuclear receptors due to the fact that it is not only present in the skin and plays a regulatory role in keratinocytes but endogenous production of its ligand involves the UVB mediated photolysis of 7-dehidrocholesterol taking place in the skin. Although much of the produced pro-hormone is activated during subsequent hydroxylation steps within the liver and kidney it has recently been shown that the epidermis is also capable of producing active vitamin D3, which might play a role locally (136). Moreover, non

conventional endongenous vitamin D derivatives might also influence VDR activation and effect cellular functions in the epidermis (137). Vitamin D receptor activation affects skin homeostasis and keratinocytes in several aspects. It has an antiproliferative effect by increasing CDK inhibitor (p15, p21, p27) activity and also confirms pro differentiation effects on osteoclasts myeloid progenitors and keratinocytes (138;139). The keratinocyte differentiation enhancing effect of vitamin D receptor is partly thought to be due to the regulation of intracellular Ca2+ concentration (140) and the direct effect of vitamin D on the promoters of numerous keratinocyte differentiation genes (141). However, keratinocyte differentiation is not only dependent on the effects of VDR and calcium. It is becoming clear that VDR function besides the presence of its ligand is profoundly affected by the cofactor complexes available for the receptor binding. This phenomenon is also thought to be responsible for the apparent inability of vitamin D to induce the differentiation of the VDR expressing squamous cell carcinoma cells (139;142). Active VD3 mediated sphingomyelin hydrolysis via an autocrine loop involving TNF-alpha signaling has also been documented in keratinocytes (61;143). The produced ceramides were thought to be responsible for induction of apoptosis. However, active VD3 treatment in physiological doses does not lead to keratinocyte cell death, on the contrary it enhances keratinocyte survival (62). The cell survival promoting and differentiation enhancing effects are thought to both contribute to the generation of a functional epidermal barrier. Due to its clear pro-differentiation effect active VD3 is used in the treatment of psoriasis (144), and is often utilized as a control keratinocyte differentiation inducing compound also in experimental settings.

Liver X receptors (LXRs) also use RXR as an obligatory heterodimerization partner similar to PPARs, VDR and RARs. They were originally described as orphan receptors and oxysterols (22(R)-hydroxycholesterol (22(R)-Chol), 24(S)-hydroxycholesterol, 24(S),25-epoxycholesterol) were only later identified as their ligands(102). Two members of this receptor family have been identified. LXR-alpha is expressed predominately in the liver with also notable expression found in the spleen, adrenal gland, kidney and the small intestine (145), whereas LXR-beta is ubiquitously expressed (146). Both LXRs have been found in fetal rat epidermis (119). Oxysterols are formed in amounts proportional to cellular cholesterol load(147). Therefore LXRs are thought to operate essentially as cholesterol sensors protecting the cells from

cholesterol overload(147). In order to fulfill this role their activation results in the decrease of intestinal cholesterol absorption, stimulates cholesterol efflux from cells to high density lipoproteins via ABCA1 and ABCG1(148), in the liver activates the conversion of cholesterol to bile acids, and bile acid excretion. Moreover, LXR also activates de novo fatty acid synthesis by stimulating the expression of sterol regulatory element-binding protein-1C (SREBP1C)(147;149;150). Recently, topical application of oxysterols to murine epidermis and oxysterol administration to human keratinocytes was found to stimulate keratinocyte differentiation and epidermal permeability barrier formation (151;152). However, the targets of LXR in the epidermis responsible for these effects are to date obscure.

Models for studying epidermal homeostasis and gene expression

Model systems for studying keratinocyte homeostasis

Primary cultures of normal human keratinocytes (NHEK) have been a helpful tool for dermatological research. Keratinocytes for primary culture are isolated most often from neonatal foreskin after circumcision, however similar results were also obtained by keratinocyte isolated from abdominal or mammary reduction plastic surgery specimens. Generally methods of keratinocyte isolation require mechanical separation of subcutaneous tissue and a lengthy dispase digestion for dermo-epidermal separation, followed by trypsin digestion. Nowdays the isolated cells are expanded in special FBS free growth media supplemented among other things most importantly with insulin, epidermal growth factor and bovine pituitary extract, making elaborate use of feeder cell layers unnecessary (153). Although these isolated cells have been shown to mimic the homeostatic function of proliferating keratinocytes of the basal layer and are also capable of undergoing differentiation similarly to keratinocytes in the skin, thus providing an excellent model system, they can only be cultured for a few passages as they fast become senescent and lose their primary characteristics (154;155). The *in vitro* differentiation process of primary human keratinocytes can be triggered by raising the Ca^{2+} content of the media and by vitamin D treatment, moreover cell-cell contact and thereby confluence of the cells is also capable of initiating the differentiation of keratinocytes in primary cultures (156-162).

In many experimental systems primary human keratinocytes have also successfully be substituted by the use of **HaCaT** (**H**uman **a**dult low **Ca**lcium and high **T**emperature) cells, a non carcinogenic immortal human epidermal cell line that received its name after the process that was used to isolate it from a pool of primary human keratinocytes (163;164). These cells are also capable of undergoing differentiation similar to primary keratinocytes and have been widely used in UV experiments (163;165). Their use is popular due to their much cheaper culture costs and great similarity to primary human keratinocytes.

The human epidermis is not composed of a single cell layer. Therefore to better mimic it, models, which provide an *in vitro* three dimensional epidermis like structure of differentiating human keratinocytes have also been developed, termed **skin-equivalent models**. These models are created by raising keratinocytes to air liquid barrier and are promising alternatives to *in vivo* studies in some experimental setups (153;166). Although they are the closest alternative to human skin these models are often unsuitable for detailed studies on specific aspects of keratinocyte homeostasis.

Genome wide gene expression analysis in keratinocyte differentiation research

Although genome wide gene expression analysis has been widely available for determining the transcriptional profile of cells for close to a decade surprisingly little work has been conducted in an attempt to identify the whole array of keratinocyte differentiation genes responsible for the diverse changes that take place in the skin. Although an internet search using PubMed for publications discussing “keratinocyte” and “differentiation” in their abstracts along with the terms “microarray” or “GeneChip” yields 65 results these publications are of little value to someone wishing to understand normal homeostatic changes taking place in keratinocytes. Most of these papers focus on disease conditions or treatment modalities and many discuss non-epithelial keratinocytes. Moreover a high portion of these projects were designed to understand the effects of the used compounds and are unsuitable for expending our general understanding on keratinocyte differentiation (167;168). Therefore it is not surprising that most of our current data on keratinocyte differentiation genes have been the side product of research aiming to understand the role of vitamin D, Ca^{2+}, N-acetyl-L-cystein, retinoids and inflammatory mediators on keratinocytes, or were identified when

the ultrastructural elements of the epidermis were studied (116;167-169). In this hypothesis driven approach only expression of genes with suspected roles in keratinocytes or established mutations in dermatological conditions were analyzed. Even the few non-hypothesis driven studies focusing on keratinocyte differentiation only provided a few interesting candidate genes and fell short of naming other targets for dermatological research. While some other studies were conceptually unable to provide a large number of meaningful keratinocyte differentiation gene candidates due to study design (170;171). Due to the fact that to date the search for keratinocyte differentiation related genes has mostly been hypothesis driven only barely more than 150 genes have so far been shown to undergo differentiation state depend regulation with validated evidence in the epidermis or in keratinocyte cultures (172). These are mostly genes coding structural proteins and enzymes of metabolic processes responsible for the creation of intact stratum corneum, like transglutaminase 1 (TGM1), proteinase inhibitor 3 (PI3), corneodesmosin, involucrin, filaggrin and regulators of keratinocyte homeostasis (retinoic acid receptors, peroxisome proliferator activated receptors, vitamin D receptor ect.) (173-178). This relatively small number of genes cannot possibly explain the wide array of changes taking place during the differentiation related complete restructuring of this cell type, therefore many aspects of this complex process are still suspected to be unknown.

Genome wide gene expression analysis in assessing keratinocyte differentiation

Even though microarray analysis obtains reliable and reproducible information on the mRNA levels of large number of genes form samples its use has long been limited mostly to the identification of candidate genes or gene groups in biological processes. Lately the need of individualized medicine lead to the wider use of pathway based analysis methods, however, so far it has only seldom been used to establish the effects of drug candidates in keratinocyte differentiation(179). Moreover even when it has been used: for example in the study conducted by Gazel et al. although JNK inhibitors were found to increase the expression of numerous keratinocyte differentiation genes and promote keratinocyte differentiation, yet the method of analysis failed to provide a plausible comparison of the achieved differentiation induction with that of know keratinocyte differentiation compounds (170). Yet given

the ability of mRNAs of keratinocyte differentiation genes to signal pro- or anti-differentiation effects better use of microarray technology in establishing keratinocyte differentiation effects of drug candidates can be envisioned (180).

Ultraviolet radiation and the potential role for the involvement of HIF1-alpha in its epidermal effects

Ultraviolet radiation: General considerations

Ultraviolet (UV) radiation is the portion of the electromagnetic spectrum, which is shorter then visible light but longer than X-ray radiation (**Figure 8).** From the dermatologic and biologic perspective UVB (290-320nm) and UVA (320-400nm) are of greatest importance as these are the wavelength ranges that pass through the atmosphere and reach the surface of the Earth. Although UVC is also emitted by the sun it is readily absorbed by the oxygen and ozone content of the atmosphere, which latter is also responsible for the elimination of much of the UVB that reaches the top of the atmosphere (**Figure 9).** Radiation under the wavelength of 290 does not reach the sea level due these natural filtering effects of the atmosphere. The UV dose reaching the Earth's surface shows great seasonal changes (181).

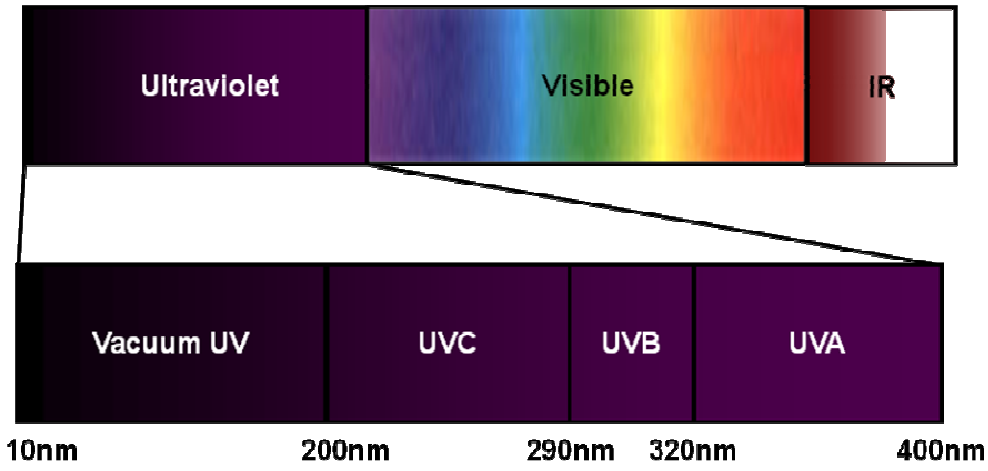


Figure 8 UV radiation in the electromagnetic spectrum. UV radiation spans the 10nm to 400nm region of the electromagnetic spectrum and is classified based on its physical and biological properties in the four distinct categories depicted in the figure. As the lower threshold of UVB 280nm is often also discussed and occasionally some authors use 315nm as the higher threshold when discussing this radiation.

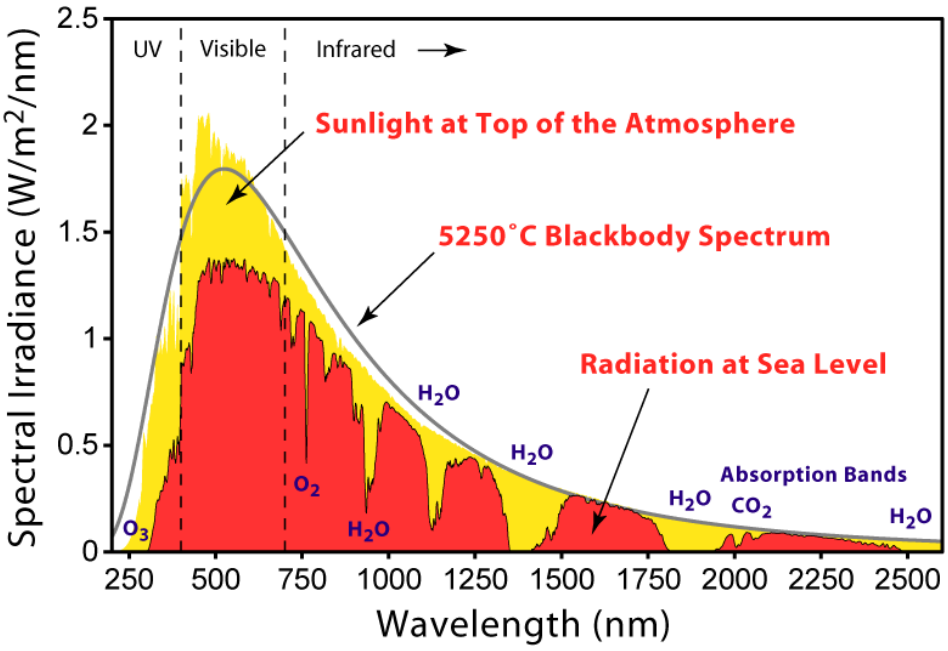


Figure 9 Solar radiation spectrum. The yellow shaded graph represents the electromagnetic radiation reaching the top of the Earth's atmosphere, while the red graph depicts the radiation reaching the surface of the planet at see level. The light emission by the sun shows similar distribution similar to a blackbody at 5250°C (the sun's surface temperature). Some of the light is absorbed by gases present in the atmosphere with specific absorption bands. The marked absorption of especially lower wavelength UV radiation by the atmosphere can be easily appreciated in the figure. (Creative Commons; based on the American Society for Testing and Materials Terrestrial Reference Spectra).

UV radiation is relatively low energy ionizing radiation, which is nevertheless capable of altering chemical structures absorbing it and can generate free radicals. UVB and UVC can directly excite DNA most often leading to the generation of cyclobutane pyrimidine dimers and (6-4) photoproducts. Normally these modified nucleotides are eliminated by nuclear excision repair, however, when excess amount of damage takes place in the cells and repair mechanism are saturated these alterations can lead to either apoptotic cell death or the generation of so called UV signature mutations passed along to progeny (182). UVA on the other hand is not capable of directly damaging DNA, however, as it generates reactive oxygen species (ROS) it also contributes to the mutagenic effects of UV radiation (136;183).

The epidermal effect of ultraviolet radiation

UVB irradiation is a predominant factor in acute photodamage of the skin. Furthermore, chronic sun exposure leads to various inflammatory and malignant skin alterations. Although repair mechanisms at lower UVB doses are able to reverse the majority of the damage, higher doses induce substantial genetic abnormalities (28). According to the World Cancer Report, skin cancer constitutes nearly 30% of all newly diagnosed malignancies in the world (29), solar UV radiation (particularly its UVB component) is an established cause of about 90% of skin cancers. Large amount of data has been published on the ability of UV light to modulate signal transduction pathways related to growth, differentiation, diseases, carcinogenesis and angiogenesis of skin. Important mediators of UV-induced alterations have already been identified, yet a clear, comprehensive view on the underlying regulatory mechanisms is still not available.

Solar ultraviolet (UV) radiation is a well known and extensively studied environmental factor effecting epidermal homeostasis. It is needed for the endogenous production of vitamin D and its therapeutic effects are harnessed in the treatment of dermatological and immunological conditions (136;184;185). However via direct DNA damage and indirect reactive oxygen species mediated mutagenesis (136;183) exposure to the UV light it is also the sole most important risk factor responsible for both initiation and progression of non-melanoma skin tumors, the highest incidence human malignancies (186;187). Moreover UV exposure induced sunburn has also been shown to be a risk factor for the development of melanoma, the human tumor showing the highest incidence increase in the past decades(188;189). Owing to its longer wavelength UVA penetrates deeper in the skin and is thought to be the most important factor in the photoaging of the dermis. The effects of UV radiation are not confined to the cell nucleus and to its mutagenic potential. The major acute effects of UV irradiation on normal human skin include sunburn, inflammation, erythema, tanning, and local or systemic immunosuppression(190). There is overwhelming evidence that UVB radiation also alters cellular metabolic function, leading to functional changes in the skin, which might also modify the carcinogenic properties of UV light and could also play a role in its therapeutic value. Detrimental effect of UV light on the barrier function of the skin barrier function was found (191;192). Moreover alteration in the lipid profile of the epidermis, decrease in the level of covalently bound ceramide and dysfunctional

lamellar body genesis and secretion were observed in keratinocytes after UVB irradiation (192-196). Furthermore as a characteristic feature of the various pathologic alterations caused by UVB the stimulation of angiogenesis also takes place, which precedes the development of the malignancies(197;198). Vascular endothelial growth factor (VEGF) is a key mediator of the angiogenic response (199). Upregulation of VEGF is an important factor in tumor vascularization and VEGF is also a regulator of angiogenesis in skin inflammation (200;201). The role of VEGF as a cutaneous damage promoting agent has also been shown after UVB exposure (202). Furthermore, it has been demonstrated that UVB induces VEGF expression in vivo and in various in vitro systems including human keratinocytes (203). VEGF is not only regulated by UV radiation but it is also one of the best characterized targets of hypoxia signaling.

Hypoxia inducible factor 1-alpha and its potential role in the epidermis

Hypoxia inducible factor (HIF) is a heterodimer transcription factor, which functions as the master regulator of oxygen homeostasis. It is comprised of two subunits. HIF-1 beta is constitutively expressed and serves as the dimerazition partner of other transcription factors (it is also called aryl hydrocarbon receptor), while HIF-1 alpha's expression is under the control of the cellular O_2 concentration, and major signal transduction pathways (204). Therefore the availability of the created HIF-1 transcription factor complex is thought to solely depend on the presence of HIF-1alpha making this latter the master regulator of oxygen homeostasis (205). Hypoxia mediates HIF levels by inhibiting proteosomal degradation of HIF 1-alpha. This takes place due to the decreased hydroxylation capability of HIF-1alpha prolyl hydroxilases in hypoxic environment. The hydroxilized proline residues of HIF-1alpha are needed to enable binding of the von Hippel-Lindau tumor suppressor, the recognition component of an E3 ubiquitin-protein ligase, which by constant ubiquitination in normoxic environment targets HIF-1alpha for proteosomal degradation. In hypoxic environment the activity of the above described process is abrogated and the resulting fast increase in the levels of HIF-1alpha mediate a large number of adaptive processes by modulating the concomitant upregulation of HIF1 targets (206). Target genes of HIF1-alpha include numerous genes responsible for adjusting cellular metabolism to the low oxygen microenvironment by enhancing anaerobic energy production (glucose transporter 1 and

3, hexokinase 1 and 2, phosphofructokinase L, glyceraldehyde-3-phosphate dehydrogenease ect.) and by increasing the availability of oxygen (erythropoietin, vascular endothelial growth factor (VEGF), VEGF receptor, nitric oxide synthase 2 ect.) and numerous other genes the precise role of which in hypoxia have not yet been determined (transferrin, transglutaminase 2, heme oxygenase-1 (HMOX1))(204). The basic oxygen dependent regulation of hypoxia gene expression is complicated by the ability of other cellular signal transduction pathways such as the phosphatidil inositol 3 kinase (PI3K) – Akt, the mitogen activated protein kinase (MAPK) and the Ca2+/calmodulin kinase-dependent pathways to activate HIF1-alpha signaling by inducing protein expression and increasing DNA binding activity of the protein under non-hypoxic conditions (207). The role of ractive oxygen species (ROS) in HIF1-alpha stabilization has also been suggested (208). The implications of these signaling mechanisms have also been suggested in various skin tumors and after UV exposure (204;209). Taken together these data suggest that UVB response could involve HIF1-alpha activation and hypoxia signaling in keratinocytes. This assumption was further supported by the findings showing, that besides VEGF, other HIF-1 target genes such as heme oxygenase-1 (HMOX1) and inducible nitric oxide synthase (iNOS) are also regulated by UV light in the skin (210-213). Interestingly recent observation suggesting that hypoxia sensing via HIF-1alpha in mouse keratinocytes is a potent contributor to systemic hypoxic adaptation directly highlight the importance of hypoxic signaling in keratinocytes, and unequivocally point to the systemic role of epithelial hypoxic responses (214).

Aims

The epidermis protects the body from dehydration, chemical agents, infection and physical environmental effects. Conditions affecting keratinocyte differentiation such as dermatological diseases (atopic dermatitis, psoriasis and various genodermatoses ect.), acute UV damage and drug treatment have all been found to alter the barrier functions of the epidermis, while barrier disruption by organic solvents is known to alter keratinocyte differentiation. Although the inter-reliance of these processes and the importance of undisturbed keratinocyte differentiation are well understood and have been extensively studied, many aspects of keratinocyte differentiation are still unknown, along with regulatory changes governing this process. Therefore during our studies we initially aimed to set up, and validate a fast and easy to use system, enabling quantitative comparison of compound effect, for testing suspected keratinocyte differentiation modifying sphingolipid compounds. Moreover we also aimed to identify previously undescribed novel keratinocyte differentiation genes, to better understand the homeostatic system guiding keratinocytes through the differentiation process, and to provide a more complete list of transcriptional changes during the process. Focusing on the effects of two specific regulatory networks of keratinocyte homeostasis, we studied the potential for the role of the hypoxia system in mediating some of the effects of UVB radiation in keratinocytes, and studied the nuclear receptor regulation of the recently identified keratinocyte ceramide transporter, ABCA12.

Specific aims

1. To set up a fast, efficient system for testing compounds with suspected keratinocyte differentiation modifying potential.
    a. To verify the ability of the keratinocyte differentiation system to effectively signal pro- or anti-differentiation effects of known keratinocyte differentiation modulator compounds.
        i. To validate the ability of the model to sense the effects of keratinocyte differentiation modulators by the analysis of the expression of known keratinocyte differentiation genes.

ii. To validate the ability of the mean gene expression of a selected list of keratinocyte differentiation genes to signal keratinocyte differentiation modifying effects.
iii. To find new potential keratinocyte differentiation genes in the model by studying the gene expression profile.
iv. To validate interesting keratinocyte differentiation induced genes within a longer term calcium induced *in vivo* keratinocyte differentiation model.

b. To test the keratinocyte differentiation modifying potential of various sphingolipid compounds by studying the gene expression changes of a high number of keratinocyte differentiation genes, in order to select the most promising compounds for further use.
i. Cytotoxicity measurement of the test compounds to determine dosage during the study.
ii. To determine the effects of compounds on keratinocyte differentiation.

2. To study the gene expression regulation of ABCA12, the epidermal sphingolipid transporter.
a. To understand ABCA12 gene expression regulation during differentiation in keratinocyte cell culture.
b. To identify the effects of nuclear receptor ligands on ABCA12 gene expression in keratinocytes.
3. To analyze the effect of UVB radiation on hypoxia signaling in keratinocytes.
a. To establish of the optimal UVB dose for the study.
b. To determine the timeline of HIF-1alpha protein expression regulation *in vitro* after low dose broadband UVB treatment.
c. To determine the mRNA expression of HIF-1alpha and HIF-1alpha target genes after UVB.
d. To study the potential regulatory mechanism responsible for the UVB regulation of HIF-1alpha.

Methods

Reagents, chemicals and technical equipment

The most commonly used chemicals and inhibitors were purchased from either Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Fairlawn, NJ) unless otherwise stated. Troglitazone (Tro), ciglitazone (Cig) and TO901317 (TO) were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). PPAR-gamma activator GI 251929X (GI) and PPAR-delta activator GW 610742X (GW) were obtained from GlaxoSmithKline (Brentford, UK), while 1α,25-Dihydroxyvitamin D_3 (VD3) was purchased from BIOMOL International (Plymouth Meeting, PA). 22(R)-OH-cholesterol (22R), clofibric acid (CLO), WY14643 (WY), all-*trans*-retinoic acid (ATRA), and 9-*cis*-retinoic acid (9-*cis*-RA) were purchased from Sigma (St Louis, MO). Molecular grade chemicals such as TRI Reagent were obtained from either Sigma or Fisher Scientific (Fairlawn, NJ). All reagents and supplies for Real-time PCR were purchased from Applied Biosystems (Foster City, CA).

Abbreviation	Full name
Ret	Retinol
D3	Calciferol
PS	Phytosphingosine
SO	Sphingosine
SA	Sphinganine
SLC	Salicylat
PS-C6	Hexanoyl-Phytosphingosine
SO-C6	Hexanoyl-Sphingosine
SA-C6	Hexanoyl-Sphinganine
PS-C18	Stearoyl-Phytosphingosine
SO-C18	Stearoyl-Sphingosine
SA-C18	Stearoyl-Sphinganine
SO-SLC	Sphingosine-Salicylate
SA-SLC	Sphinganine-Salicylate
PS-SLC	Phytosphingosine-Salicylate
C12	C12-Alkylamine
C12-SLC	C12-Alkylamine-Salicylate

Table 3 List and abbreviation of compounds tested for their ability to influence keratinocyte differentiation.

For keratinocyte differentiation studies retinol and calciferol, as control compounds were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Sphingolipid compounds were made available by Degussa GmbH (currently known as Evonic GmbH, Düsseldorf, Germany) (**Table 3**). Compounds were diluted in the 50% mixture of ethanol and water and were administered at non-toxic concentrations as determined by the cytotxocitity assays.

Cell culture

HaCaT cells

HaCaT immortalized human keratinocyte cell line was obtained from Norbert E. Fusenig. Cells were grown at 37°C under normoxic conditions (5% CO_2, 95% air) in D-MEM (GIBCO, Carlsbad, CA, USA) medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (0.1mg/ml) in 10 cm diameter Petri dishes (Sarstedt, Nümbrecht, Germany).

Normal human epidermal keratinocyte (NHEK) isolation and culture

Skin samples were obtained from female donors undergoing plastic surgery. Keratinocytes were isolated after overnight by dermo-epidermal separation in Dispase II (2.4 U/ml, Roche Diagnostics, Germany) and by subsequent trypsin digestion (2hrs in 0.005% trypsin, 0.1 % glucose, 0.8 % NaCl, and 0.4 % KCl). Cells were seeded and propagated in KBM media (Cambrex Bioscience, Walkersville, Maryland) supplemented with 1 μM insulin, 1 μM cortisol, 50 μg/ml Gentamycin, 50 ng/ml Amphotericin, 0.4% bovine pituitary extract and 0.06 mM $CaCl_2$. Cultures of NHEK cells were split at 60-70% confluence. Medium was replaced every 2-3 days and cell morphology evaluated by phase contrast microscopy.

In nuclear receptor ligand ABCA12 mRNA expression studies second passage human keratinocytes from foreskin were seeded and maintained in 0.07mM Ca++ serum-free keratinocyte medium (154CF) with growth supplement (Cascade Biologies, Inc., Portland, OR). After cells were attached to the culture dish, the medium was switched to either low (0.03 mM) or high calcium (1.2 mM) medium. In a typical experiment, cells were treated with reagent at optimized concentration at pre-confluence (60%-70%) in either low or high calcium conditions, and were harvested at 80%-100% confluence.

Cells were either treated with nuclear receptor ligands or control keratinocytes were treated with vehicle (0.05% ethanol or DMSO).

Hypoxia treatment

HaCaT cells were grown until 70-90% confluence, to maintain even cell density throughout various experiments. Fresh medium was added 1 day before starting the experimental procedure. As a positive control, HIF-1α stabilization was induced in experiments where the Petri dishes were incubated on 37°C for 4 hours in a hypoxia chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) in a gas mixture containing 1% O_2, 5% CO_2 and 94% N_2 (data not shown).

NHEK differentiation model

Although three dimensional epidermal models were found to be useful in drug testing prior to human trials (215;216) they are inherently incapable of distinguishing between drug effects on keratinocytes at specific stages of differentiation. Furthermore they also require the addressing of drug delivery problems at an early phase in the study, which unnecessarily limits initial experiments. Therefore we deemed three dimensional skin equivalent models to be unsuitable for use in the preliminary testing of potential keratinocyte differentiation modifying compounds and opted for a model of early confluence induced differentiation or a 10 day calcium induced differentiation.

Confluence induced differentiation was carried out by growing second passage cells to 80-90% confluence. The experiment was initiated before the appearance of any visual signs of keratinocyte differentiation (day 0). After media change NHEK cells were either administered control or test compounds or were only administered empty vehicle (ethanol) and were allowed to grow to confluence. Final ethanol concentration was 0.05%. Cells were harvested on day 0 (controls), day 1 and day 4 (controls and compound treatments). At each time point and treatment modality three 60cm^2 culture dishes were harvested from keratinocytes of each of the skin donors to enable isolation of enough RNA for microarray studies, and real time quantitative PCR.

Calcium induced differentiation experiments were carried out by switching growth media (0.06mM Ca^{2+}) to 1.2mM Ca^{2+} containing fully supplemented Keratinocyte Basal Medium (KBM; Cambrex Bioscience, Walkersville, Maryland).

Cells were cultured in 60cm^2 culture dishes and were harvested every second day until 10 days after differentiation induction.

Cytotoxicity study

Keratinocytes were cultured in 24-well plates until confluence, and test substances were administered subsequently along with controls (solvent control, SDS and H_2O_2). Cells were harvested at the time of cell treatment (day 0), 24 hours later (day 1) and 96 hours later (day 4). Three different tests were carried out on two keratinocyte samples derived from different isolations to assess cytotoxicity of compounds. **Lactate-dehydrogenase (LDH)** activity was measured in the culture supernatant (S) and the remaining cell monolayers (M) by spectrometry of NADH conversion. The percentage of LDH release was calculated from the ratio of S/(S+M)(40). **Adenylate kinase release** and cellular ATP content were used to determined ToxLight and ViaLight plus assays (Cambrex, Rockland, ME, USA) respectively. Assays were carried out according to the manufacturer's suggestions. Whole cell protein content was measured according to the method of Bradford (41). Enzyme activity and **ATP content** were calculated relative to controls with the help of Excel (Microsoft, Redmond, WA, USA) and a scoring system was applied to enable combined evaluation of the results. Mild (1.5-2x higher), intermediate (2-5x higher) and high (over 5x higher) specific cytotoxicity values were determined depending on the LDH release and adenylate-kinase release in samples compared to controls. Results were also scaled according to the cellular ATP content of the cells compared to controls (mild (70-50%), intermediate (50-20%) and high cytotoxicity (under 20%)). Compounds showing at least intermediate cytotoxicity with more than 2 assays or high cytotoxicity in one assay and low in at least one were considered cytotoxic at the given time point.

Cell harvesting

For protein studies cells were harvested in 200µl lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, 1% NP40) supplemented with protease and phosphatase inhibitors (1 mM Na_3VO_4, 1 mM PMSF, 10 mM benzamidine, 20 mM NaF, 1 mM para-nitrophenyl-phosphate, 10 µM lactacystin and 1:100 diluted SIGMA Inhibitor Cocktail). Samples were sonicated for 20 seconds (Sonic Dismembrator 300, Artek

Systems Corporation, Framingdale, NY, USA) and centrifuged (10000rcf, 10 minutes, 4°C) and aliquots of the supernatant were stored at -20°C until protein-measurement (DC Protein Assay reagents, BioRad, Hercules, CA, USA) and for gel-electrophoresis. Experiments were repeated at least twice to minimize inter-experimental errors.

UV irradiation

For broad-band UVB irradiation an FS-20 fluorescent lamp (Westinghouse Electric, Pittsburgh, PA, USA) was used. Its emission spectrum is depicted in (**Figure 10**). Prior to experiments, regular UVB dosimetry was performed by a VLX-3W radiometer equipped with a CX-312 sensor (Vilber Lourmat, France) to allow constant dose delivery.

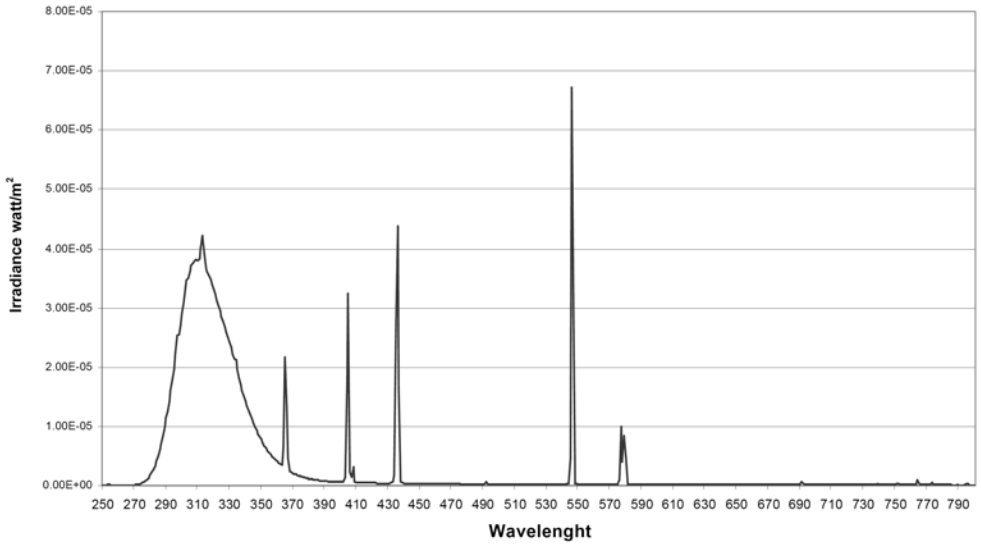


Figure 10 Emission spectrum of the UVB light source

To minimize unwanted environmental influence cells were removed from the CO_2-incubator for less than 1 minute during UVB-treatment. The culture medium was thoroughly removed and cells were irradiated under a thin liquid-film. Petri dishes were kept at 37°C to minimize temperature-fluctuations. After irradiation the removed media was reintroduced to the cells. In experiments where wortmannin was used after the UV irradiation, the cell culture media were immediately reintroduced, and supplemented

with wortmannin at a final concentration of 10nM. Mock-treatments were carried out the same way with the exception of UV irradiation.

In order to determine biological efficacy of UV irradiation the percentage of apoptotic cells was determined by flow-cytometry. Briefly: media of HaCaT cultures (10ml Petri dishes) were removed, followed by 2x10ml 37 °C PBS washing. The washing PBS and the media were collected in 50 ml Falcon tubes placed on ice. The adherent cells were mobilized using 0.05% trypsin EDTA solution (2ml, 10 minutes, 37 °C). Detached cells were collected by 2x5ml 37°C PBS washing and were combined with previously removed media and PBS. 1ml FCS was applied to samples in Falcon tubes to fully inactivate trypsin. Tubes were centrifuged (270g, 8 minutes, 8 °C) and pellet was resuspended in 5ml 1%FCS containing PBS. 500µl aliquots of this suspension were placed in polystyrene flow-cytometry tubes and were washed twice in 1%FCS PBS (200g, 5 minutes, 8 °C). Flow-cytometric analysis of apoptosis was carried out using the Annexin V, propidium iodide (PI) method. Staining kit was purchased from Roche (Indianapolis, IN, USA) and staining was carried out according to the manufacturers' protocol. Flow-cytometry was carried out on a Becton-Dickinson FACScan (Franklin Lakes, NJ, USA) flow-cytometer.

Western bloting

Polyacrylamide gel-electrophoresis and transfer reagents, equipments and membranes for protein-blotting were obtained from BioRad (Hercules, CA, USA). For immunoblotting, an anti-HIF-1α antibody was purchased from Becton Dickinson Transduction Laboratories (Lexington, KY, USA), whereas the anti-phospho-AKT (Ser 473), and anti-AKT antibodies were from Cell Signaling Technology (Beverly, MA, USA). Horseradish-peroxidase conjugated secondary anti-mouse antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Western Lightning™ Chemiluminescence Reagent Plus was purchased from PerkinElmer Life Sciences Boston, MA, USA. Samples were boiled in Laemmli loading buffer for 5 min and equal amounts of proteins were separated on a 7.5% sodium dodecylsulphate (SDS)-polyacrilamide gels. The proteins were transferred to nitrocellulose or PVDF membrane, and immuno-detected with specific primary and horseradish-peroxidase labeled secondary antibodies. The antibodies were detected using chemiluminescence

reagent blue-sensitive radiography film. Equal protein loading was verified by Ponceau staining of the membrane or an identical blot was probed with anti-GAPDH antibody to verify the equal loading of protein.

For ABCA12 protein expression analysis whole cell extract was prepared from keratinocytes. 80-100µg proteins were fractionated on pre-cast gels (3-8%) and transferred to PVDF membranes, overnight at 4°C using standard western blotting protocols. The proteins on the membrane were subsequently probed with polyclonal primary antibodies against human ABCA12, and then visualized by horseradish peroxidase-conjugated anti-rabbit secondary antibody using the Enhanced Chemiluminescence (ECL) Western Blotting Detection System Kit. Membranes were then exposed to CL-XPosure film. An identical blot was probed with anti-GAPDH antibody to verify the equal loading of protein (data not shown).

RNA isolation

For microarray experiments and most real-time PCR assays total RNA was extracted from cultured cells using the RNeasy Protect midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Purity and integrity of the RNA was determined by Agilent 2100 bioanalyzer with the 6000 Nano LabChip reagent set (Agilent Technologies, USA). RNA concentration was measured spectrophotometrically and samples were stored at -80 °C. During HIF-1alpha and hypoxia experiments RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, traces of the DNA were removed by DNA-*free* DNase Treatment and Removal kit by Ambion (Austin, TX, USA) and integrity of RNA was verified by denaturing agarose-gel (1.5%) electrophoresis.

Real-time RT-PCR (TaqMan™) analysis

First-strand cDNA synthesis was performed with the Reverse Transcription System from Promega (Madison, WI, USA) according to the manufacturer in 40 ml reaction cocktails containing 2 µg of RNA each. The reaction mixture was incubated at 42 ºC for 60 min, followed by heat inactivation of the enzyme at 95 ºC for 5 min. After cooling samples on ice for 5 min, the cDNA was stored at -20 ºC. Real-time RT-PCR analysis was performed with an ABI7900HT machine (Applied Biosystems, Chicago, IL, USA).

All TaqMan RT-PCR reagents, including primers and probes, were purchased from Applied Biosystems. TaqMan analysis was performed with predesigned and optimized Assay on Demand mixtures (Applied Biosystems). Reaction parameters were as follows: 2-min 50 °C hold, 30-min 60 °C hold, and 5-min 95 °C hold, followed by 45 cycles of 20-s 94 °C melting and 1-min 60 °C annealing/extension. Measurements were performed in triplicates. Results were analyzed with an ABI sequence detector software version 2.0 or 2.1 (Applied Biosystems). Relative quantitation was performed according to the delta-delta-Ct method with 18S ribosomal RNA (rRNA) used as normalizer gene. Samples were run in triplicates and at least 3 independent experiments were preformed to enable thorough verification of results.

Alternatively in HaCaT UVB studies reverse transcription of 1 μg RNA from each sample was carried out using Ambion's "RETRO*script"* kit following the manufacturer's protocol. Real time quantitative PCR was carried out in optical-capped PCR plates (Bio-Rad, Hercules, CA, USA) on a iCycler Real Time PCR machine (Bio-Rad) using TaqMan universal PCR master mix or SYBR green PCR Master Mix (Applied Biosystems (ABI), Foster City, CA, USA). cDNA derived from 1.25 ng RNA was used in each reaction. Gene-specific TaqMan probe and primer mixtures were purchased from ABI (VEGF:Hs00173626_m1; HMOX1: Hs00157965_m1; ACTB: Hs99999903_m1), whereas HIF-1α primers (5'CCAGCAGACTCAAATACAAGAACC ; 5'TGTATGTGGGTAGGAGATGGAGAT) were purchased from Invitrogen (Carlsbad, CA, USA). All measurements on the iCycler were carried out in 25 μl total volumes and triplicate measurements. In these studies human beta-actin mRNA levels served as normalizers. Data analysis was carried out using the delta-delta-Ct method according the recommendations of ABI. Samples were run in triplicates and at least 3 independent experiments were preformed to enable thorough verification of results.

In studies aiming to identify the effect of nuclear receptor ligand stimulation on ABCA12 expression total RNA was isolated using TRI reagent and first strand cDNA for PCR was synthesized using an iScript^{TM}cDNA Synthesis Kit (BioRad, Hercules, CA, USA) following manufacturer's protocol. The relative mRNA levels of total ABCA12 gene expression and individual alternatively spliced transcripts (ABCA12-L and -S) were determined using a Mx3000P™ Real-Time PCR System (Stratagene, La

Jolla, CA, USA). The primer sequences for determination of ABCA12 expression are listed in **Table 4**. Quantitative PCR was performed using 20µl total volume with the SYBR Green Q-PCR Master Mix (BIO-RAD, Hercules, CA) according to the manufacturer's protocol. The PCR reaction was performed in duplicate, with 3~4 samples in each group (n=3~4). Experiments were repeated at least once using a different batch of cells to ensure reproducibility. 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 (an invariant transcript) using the comparative C_T method, and expressed as percentage of control, with the control as 100%. Data were expressed as mean ± SEM. Comparison between two groups was carried out using two-tail unpaired *t* test.

Gene	Primer sequence	Amplicon (bp)	
ABCA12	(F) 5'-ACAGGAATGGCCTTCATCAC-3' (R) 5'-AACATGGTGCCCTGAGAAAC-3'	317	AY033486
ABCA12-L	(F) 5'-ACCTGGGATGGTTTTCACTG-3' (R) 5'-TGCTTGGAGATAAGGTCTGTCC-3'	116	NM_173076
ABCA12-S	(F) 5'-TGCAACTAACGAAGGTTTCAGA-3' (R) 5'-CATCCTCATTCCACACATGC-3'	144	NM_015657
Cyclophilin	(F) 5'-TCTCCTTTGAGCTGTTTGCAG-3' (R) 5'- CACCACATGCTTGCCATC-3'	326	Y00052

Table 4 Sequences of primers used for ABCA12 real-time quantitative PCR (217)

DNA microarray and microarray data analysis

Gene expression profiles were determined using Affymetrix HGU133 plus 2.0 GeneChips (Affymetrix, Santa Clara, CA), which represent over 39000 human gene transcripts or transcript variants. To minimize interindividual differences in gene expression and to enrich transcriptional effects of spontaneous or compound driven keratinocyte differentiation 2 µg of total RNA from 3 different normal human kerationcyte cell lines were pooled at each timepoint from every treatment modality and control group to generate complementary RNA (cRNA). To further increase precision of the experiments 2 independent control pools were used for 1 and 4 day incubations.

The remaining amount of RNA was used for real-time RT-PCR validation in single samples. Six micrograms of pooled total RNA was converted to complementary DNA (cDNA) using a T7-oligo-d(T)24 primer and SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA). Second-strand cDNA synthesis and blunt ending was performed using T4 DNA polymerase, Escherichia coli DNA ligase, and T4 polynucleotide kinase. Following phenol-chloroform extraction, cDNA was used for in vitro transcription reaction using the T7 BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY) to produce biotinylated cRNA. Thereafter, the labeled cRNA was purified using RNeasy Mini Kit columns (Qiagen) and fragmented by incubation at 94°C for 30 minutes. The fragmentation was checked by microcapillary electrophoresis on an Agilent 2100 bioanalyzer, and 30 μg of biotinylated cRNA was split into 2 parts and hybridized to U133A and U133B GeneChips, respectively, for 16 hours at 45°C with constant rotation. All 16 microarrays were processed in an Affymetrix GeneChip Fluidics Station 400. Following staining with streptavidin-conjugated phycoerythrin and washing cycles, the microarrays were scanned using the GeneArray Scanner (Agilent Technologies). Expression signals for each transcript and comparisons between different samples were calculated with the Affymetrix Gene- Chip software MAS5.0 and Microsoft Excel (Microsoft Corp., Redmond, WA). In addition, a special biomedical-oriented pathway analysis of significantly regulated genes was performed. All data are expressed as fold changes in transcript levels relative to the control group.

To understand whether keratinocyte underwent differentiation in the short term confluence model, results of microarray analysis were sorted in Microsoft Excel, and lists of genes that underwent greater than 2.5 lineal up- or downregulation from day 0 through day 1 to day 4 were created. The contents of these lists were subjected to a search using Cytoscape 2.5 and the Bingo 2.0 plugin and significantly overrepresented Go annotations were listed.

Gene expression data are usually easy to compare when there are directional differences in genes regulated and when the experiments result in profound changes in at least clusters of the transcriptional network. The task however becomes much harder when in comes to mostly quantitatively different changes. In order to tackle this problem we had to come up with easily comprehendible and meaningful gene

expression data capable of quantifying the differentiation modifying potential of several compounds in a confluence induced differentiation system of NHEK cells. Most studies use the expression of at most a couple of a hand full of differentiation markers, and describe the differentiation state of the cells by describing the expression level of these few genes. However, as the differentiation process involves many genes analyzation of the expression of only a few of these can be misleading. The alternative of using numerous individual expression values to signal changes in gene sets is although probably more suitable, nevertheless it would still not have enabled quantitative assessment of changes and allowed comparison of effects to known keratinocyte differentiation modifying compounds(170). Therefore we decided to look at the average gene expression changes of compounds compared to controls in a large set of known keratinocyte differentiation genes powerfully regulated by differentiation in the studied system. In order to do this, first extensive literature search was carried out and a list of 150 known keratinocyte differentiation related genes was created. However, as the experimental models used in keratinocyte differentiation studies are very prolific, and varied in timescale, model cell type and intercellular architecture the subset of these genes, which showed regulation in our confluence induced differentiation model had to be determined. Therefore in the second step microarray data from 3 NHEK cell batches were compared to 1 and 4 days differentiated samples of the same batches, and gene expression changes in the 150 proposed keratinocyte differentiation genes were analyzed. 53 of these 150 genes were found to be linearly regulated by at least 1.9 fold in the 4 day confluence induced differentiation model. The expression levels of these genes were therefore used to analyze alterations in the differentiation state of the keratinocytes. From this set of 53 genes only 2 were downregulated and 51 were upregulated by confluence induced keratinocyte differentiation. Gene expression average was calculated from the signal change levels of individual genes. In case of the 2 downregulated genes the reciprocal value of the signal change was used therefore their decrease (the expected pro-differentiation change) increased the gene expression average and their increase (anti-differentiation change) decreased the gene expression average. Thus upon treatment with a compound pro-differentiation changes were all represented by a fold change value greater than 1 with greater magnitude differentiation induction represented by the greater numbers. Anti-differentiation potential was

represented by fold-change values lower than 1 with lower average numbers representing greater anti-differentiation potential. The geometric mean was calculated from the gene expression values of individual genes and was used to assess the effects of compounds on keratinocyte differentiation. Normality of samples were analyzed by the Shapiro-Wilks W-test using the StatsDirect software (StatsDirect Ltd., Cheshire, UK). The Wilcoxon matched-pair signed-ranks test was used to assess the differences between control and compound treated samples.

To compare the proposed keratinocyte differentiation gene lists with genes regulated in psoriasis, we reanalyzed the gene expression data of uninvolved and lesional skin of 13 patients with plaque-type psoriasis (GDS2518) obtained from the Gene Expression Omnibus (GEO)(12). The original analysis of this dataset was too rigorous, taking only genes that showed 2 fold regulation with p-values below 0.00001 in the paired t-test and below 0.001 in the paired Wilcoxon test yielding only 179 differentially expressed genes between lesional and non lesional skin (12). To allow better statistical assessment of similarities between genes differentially regulated in psoriasis and our list of keratinocyte differentiation genes first the psoriasis dataset was reanalyzed by Significance Analysis of Microarrays (SAM, http://www-stat.stanford.edu/~tibs/SAM/). The two class paired analysis of the dataset yielded 477 significantly upregulated and 732 significantly downregulated probe sets in psoriasis vs. non lesional skin of matching patients (SAM threshold 2 fold, 0.0% false discovery rate). The keratinocyte differentiation datasets were converted from the U133 Plus 2.0 format to U133A format by excluding all probsets that were not represented in the psoriasis study due to platform differences using Microsoft Office Access (Redmond, WA, USA). The overrepresentation of the resultant keratinocyte differentiation lists in the similarly regulated psoriasis datasets were statistically assessed by Pearson's chi-square test (http://faculty.vassar.edu/lowry/VassarStats.html).

Statistical analysis

Statistical analysis of experiments was carried out as noted in the respective sections of the materials and methods. All experiments were carried out in triplicates unless otherwise noted.

Results

Establishing the keratinocyte differentiation model and testing effects of sphingolipid compounds

Confluence induced differentiation provides a suitable model system

Messenger RNA expression of four established keratinocyte differentiation markers, transglutaminase 1, filaggrin, involucrin, and corneodesmosin was upregulated 4 days after induction of differentiation by confluence (**Figure 11**).

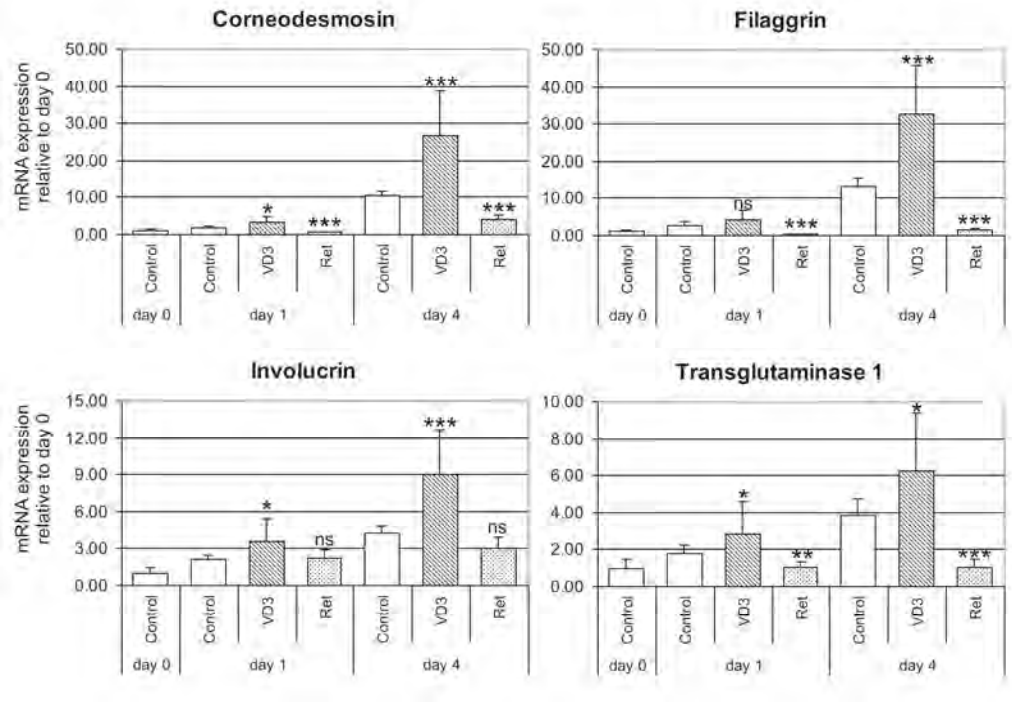


Figure 11 Regulation of the expression of individual differentiation marker genes in NHEK during 4 days of confluence stimulated differentiation by TaqMan® realtime RT-PCR. 25 µM retinol or 50 nM calciferol (VD3) were administered on day 0 for day 1 experiments and on day 0 and on day 3 for day 4 long experiments. The data are presented as mean + SD. (Statistical significance of difference vs. control of the respective day: *: $p < 0.05$, **: $p < 0.001$, ***: $p < 0.0001$). (6)

Retinol, a known keratinocyte differentiation inhibiting agent, prevented the induction of transglutaminase 1, filaggrin, keratin 10, and corneodesmosin, while VD3, a known

inducer of differentiation enhanced the expression of these differentiation markers even further (**Figure 11)**.

UniGene	**Gene name**	**Short name**	**Affymetrix**	**Fold**
Hs.250236	late cornified envelope 1E	LCE1E	1559224_at	69.5
Hs.234766	late cornified envelope 2B	LCE2B	207710_at	57.9
Hs.707	Keratin 2A	KRT2A	207908_at	36.8
Hs.80828	Keratin 1 (epidermolytic hyperkeratosis)	KRT1	205900_at	35.5
Hs.302034	phospholipase A2, group IIF	PLA2G2F	221416_at	32.3
Hs.25333	interleukin 1 receptor, type II	IL1R2	205403_at	30.6
Hs.432416	Trichohyalin	THH	213780_at	27.9
Hs.375103	late cornified envelope 1B	LCE1B	1560531_at	27.3
Hs.251680	Loricrin	LOR	207720_at	26.1
Hs.516420	Skin-specific protein	xp33	216718_at	22.8
Hs.139389	cystatin E/M	CST6	206595_at	22.2
Hs.201877	DESC1 protein	DESC1	220431_at	17.1
Hs.433629	small proline rich protein 4	SPRR4	1552620_at	16.6
Hs.520006	Corneodesmosin	CDSN	206192_at	14.0
Hs.490253	small proline-rich protein 2G	SPRR2G	236119_s_at	13.1
Hs.297413	matrix metalloproteinase 9	MMP9	203936_s_at	12.0
Hs.531478	Filaggrin	FLG	215704_at	11.7
Hs.407618	desmoglein 4	DSG4	1561330_at	11.5
Hs.112408	S100 calcium binding protein A7 (psoriasin 1)	S100A7	205916_at	11.4
Hs.105924	defensin, beta 4	DEFB4	207356_at	11.0
Hs.148641	cathepsin H	CTSH	202295_s_at	10.2
Hs.151254	kallikrein 7 (chymotryptic, stratum corneum)	KLK7	205778_at	8.6
Hs.19413	S100 calcium binding protein A12 (calgranulin C)	S100A12	205863_at	7.4
Hs.148590	Cornifelin	CNFN	224329_s_at	7.0
Hs.2633	desmoglein 1	DSG1	206642_at	6.8
Hs.2022	transglutaminase 3	TGM3	206004_at	6.3
Hs.129719	transglutaminase 5	TGM5	207911_s_at	6.3
Hs.282997	glucosidase, beta; acid (includes glucosylceramidase)	GBA	209093_s_at	5.9
Hs.406998	sulfotransferase family, cytosolic, 2B, member 1	SULT2B1	205759_s_at	5.9
Hs.550492	phospholipase A2, group IVB (cytosolic)	PLA2G4B	219095_at	5.5
Hs.376144	Repetin	RPTN	1553454_at	5.3
Hs.2421	small proline-rich protein 2C	SPRR2C	220664_at	4.8
Hs.139322	small proline-rich protein 3	SPRR3	218990_s_at	4.7
Hs.76171	CCAAT/enhancer binding protein (C/EBP), alpha	CEBPA	204039_at	4.3
Hs.57771	kallikrein 11	KLK11	205470_s_at	3.7
Hs.285671	bone morphogenetic protein 6	BMP6	206176_at	3.4
Hs.514913	serine or cysteine proteinase inhibitor, clade B, member 2	SERPINB2	204614_at	3.4
Hs.112341	protease inhibitor 3, skin-derived (SKALP)	PI3	203691_at	3.2
Hs.59403	serine palmitoyltransferase, long chain base subunit 2	SPTLC2	203127_s_at	3.1
Hs.508950	transglutaminase 1	TGM1	206008_at	3.1
Hs.519689	peroxisome proliferative activated receptor, delta	PPARD	208044_s_at	3.0
Hs.331555	serine protease inhibitor, Kazal type 5	SPINK5	205185_at	3.0
Hs.50915	kallikrein 5	KLK5	222242_s_at	2.9
Hs.32949	defensin, beta 1	DEFB1	210397_at	2.6
Hs.272373	interleukin 20	IL20	224071_at	2.6
Hs.500635	Envoplakin	EVPL	204503_at	2.5
Hs.415702	solute carrier family 27 (fatty acid transporter), member 4	SLC27A4	225779_at	2.5
Hs.516439	Involucrin	IVL	214599_at	2.4
Hs.2062	vitamin D (1,25- dihydroxyvitamin D3) receptor	VDR	204253_s_at	2.4
Hs.79876	steroid sulfatase (microsomal), arylsulfatase C, isozyme S	STS	203767_s_at	2.3
Hs.546248	cathepsin D (lysosomal aspartyl protease)	CTSD	200766_at	2.1
Hs.429052	integrin, beta 1	ITGB1	1553530_a_at	-1.9
Hs.529618	transferrin receptor (p90, CD71)	TFRC	207332_s_at	-2.5

Table 5 Regulation of the members of the 53 differentiation marker gene set during confluence induced keratinocyte differentiation. Fold changes represent gene expression changes of control cells at day 4 vs. day 0 levels (Affymetrix GeneChip data). (6)

Establishment of the list of known keratinocyte differentiation genes regulated during confluence induced differentiation

From the 150 keratinocyte differentiation related genes assembled by literature search, 53 were found to be regulated by at least 1.9 fold four days after induction of differentiation. From this set of 53 genes only 2 were down regulated and 51 were upregulated by confluence induced keratinocyte differentiation (**Table 5**).

The mean gene expression of several genes enables the comparison of differentiation modifying compound effect

The expression levels of these 53 genes were used to calculate the geometric means of pro-keratinocyte differentiation changes and thereby enable comparison of alterations in the differentiation state of the keratinocytes treated with control compounds and sphingolipids. Retinol and VD3 were found to modify the expression of the geometric mean of these gene expression changes as expected from their known respective inhibitory and stimulatory effects on keratinocyte differentiation (**Figure 12**). The geometric mean of the gene expression changes for retinol treatment showed a 29.38 % ($p = 0.0995$) and a 59.04 % ($p < 0.0001$) decrease at day 1 and day 4, respectively, whereas VD3 treatment resulted in increased pro-differentiation change in gene expression of these marker genes (day 1: 13.76 % ($p = 0.015$); day 4: 19.77 % ($p < 0.001$)).

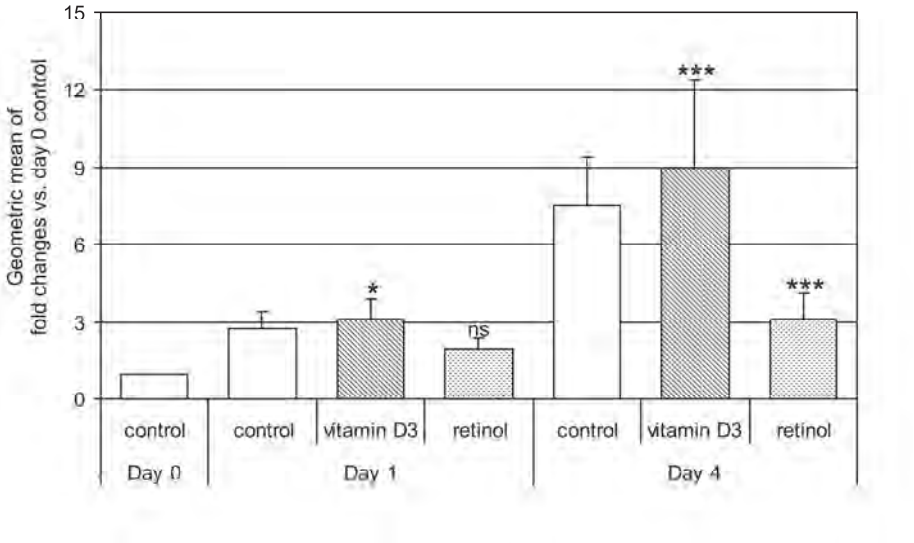


Figure 12 Combined regulation of the expression of 53 differentiation marker genes in NHEKs during 4 days of confluence stimulated differentiation by Affymetrix gene array of three pooled keratinocyte preparations. 25 µM retinol or 50 nM VD3 were added on day 0 for day 1 experiments and on day 0 and on day 3 for day 4 experiments. Data are shown as geometric mean +SEM. (Significantly different to control of the respective day with *: $p < 0.05$, **: $p < 0.001$, ***: $p < 0.0001$). All day 1 and day 4 results were significantly ($p < 0.001$) different from the control at day 0. (6)

Bioinformatic analyses finds model suitable for identifying novel keratinocyte differentiation genes

In order to further test the ability of the model to identify keratinocyte differentiation genes and therefore its potentially enable the identification of genes not yet described in keratinocyte differentiation we subjected the more than 2.5 fold up and down regulated keratinocyte confluence induced differentiation gene lists established by microarray to analysis by the Bingo tool in the Cytoscape software package (Cytoscape Consortium, http://www.cytoscape.org/). This method identified the marked overrepresentation of epidermal differentiation related gene ontology terms **(Table 6)** indicating the ability of the confluence induced *in vitro* keratinocyte differentiation system to model *in vivo* keratinocyte differentiation. Roughly a third of the genes represented in the various keratinocyte differentiation related gene ontology terms were positively identified in the upregulated gene list, even at the used relatively high fold change threshold (2.5 fold regulation).

The most significantly overrepresented annotations in upregulated genes

GO ID	Term	Total	Observed	Expected	ZScore
0008544	epidermis development	111	34	4.58	14.09
0007398	ectoderm development	122	34	5.03	13.24
0048730	epidermis morphogenesis	36	13	1.49	9.66
0009888	tissue development	268	41	11.06	9.27
0031424	Keratinization	21	9	0.87	8.93
0048513	organ development	1028	96	42.42	8.69
0009913	epidermal cell differentiation	32	11	1.32	8.61
0048856	Anatomical structure development	1747	138	72.09	8.41
0048729	tissue morphogenesis	58	15	2.39	8.34
0048731	System development	1439	117	59.38	8.01
0007275	multicellular organismal development	1927	142	79.52	7.64
0008150	biological_process	1132	552	467.38	7.63
0030216	keratinocyte differentiation	23	8	0.95	7.4

The most significantly overrepresented annotations in downregulated genes

GO ID	Term	Total	Observed	Expected	ZScore
0044237	cellular metabolic process	5969	215	138.08	8.43
0044238	Primary metabolic process	5987	214	138.49	8.27
0008152	metabolic process	6552	228	151.56	8.25
0043283	Biopolymer metabolic process	3860	155	89.29	8.11
0043170	macromolecule metabolic process	5162	188	119.41	7.76
0009987	cellular process	9530	289	220.45	7.49
0006139	nucleobase, nucleoside, nucleotide and	2865	119	66.27	7.25

Table 6 Lists of significantly overrepresented GO annotations within lists of up- and downregulated genes during keratinocyte confluence induced differentiation.

Due to a bias towards the publication of positive findings ontology terms were not capable of directly providing sufficient evidence for the validity of the list of downregulated genes, nor were the lists of known keratinocyte housekeeping genes capable of doing so. Knowing that psoriasis overwhelmingly alters keratinocyte differentiation previously published results on the differential expression of genes in psoriasis were downloaded from the Gene Expression Omnibus (GEO) from the: GDS2518 accession number experiment and datasets were reanalyzed using the significance analysis of microarray (SAM) software tool (218). At 2 fold threshold and 0% false discovery rate 477 significantly up- and 732 downregulated probe sets were

identified between samples of lesional and non affected skin from psoriatic individuals. These gene lists were compared to the lists of keratinocyte differentiation genes identified in the confluence induced short term differentiation model. Significant overrepresentation of keratinocyte differentiation gene ontology terms was seen in the upregulated psoriasis gene list similar to the overrepresentation of these terms in the genes upregulated in confluence induced keratinocyte differentiation (data not shown). Not surprisingly the probe sets from the list of keratinocyte confluence induced differentiation upregulated genes were also found to be significantly overrepresented in the list of psoriasis regulated probe sets (<0.0001). Moreover also significant overrepresentation ($p<0.001$) of probe sets from the downregulated keratinocyte differentiation list were found in the psoriasis regulated list providing proof for the validity of the list of keratinocyte downregulated genes.

Identification of potential novel keratinocyte differentiation genes

Based on the previous findings, showing the significant overrepresentation of genes related to keratinocyte differentiation in the dataset we assumed that the lists created during the microarray analysis of the confluence induced keratinocyte differentiation model could also contain novel keratinocyte differentiation genes. Therefore from the list of upregulated genes eight (RNF39, PKP3, HMOX1, ICEBERG, FOXQ1, ELMOD, FAM43A, PLA2G4B) identified as potential keratinocyte differentiation genes by microarray were selected after literature review of potential candidates. These 8 genes showed 3.7 – 24.3 fold expression upregulation during the 4 day confluence induced differentiation experiment (**Table 7**).

Gene Symbol	Gene Name	Fold Change
ELMOD	ELMO/CED-12 domain containing 1	24.25
HMOX1	heme oxygenase (decycling) 1	17.75
FAM43A	family with sequence similarity 43, member A	10.93
RNF39	ring finger protein 39	9.51
PLA2G4B	phospholipase A2, group IVB (cytosolic)	7.73
ICEBERG	ICEBERG caspase-1 inhibitor	4.76
PKP3	plakophilin 3	4.29
FOXQ1	forkhead box Q1	3.73
PI3	*peptidase inhibitor 3, skin-derived (SKALP)*	*3.03*
TGM1	*transglutaminase 1*	*3.03*

Table 7 List and fold change regulation of selected potentially interesting genes (regular font) and known keratinocyte differentiation genes (italic font) upregulated during 4 day confluence induced keratinocyte differentiation. Fold changes represent changes in gene expression signal levels from day 0 to day 4. Microarray results.

Real time quantitative PCR validation of potential novel keratinocyte differentiation genes in a longer term keratinocyte differentiation model

In order to validate the differentiation related upregulation of the selected 8 potential keratinocyte differentiation genes (RNF39, PKP3, HMOX1, ICEBERG, FOXQ1, ELMOD, FAM43A, PLA2G4B) their regulation along with the gene expression of the known keratinocyte differentiation genes (TGM1, PI3) were analyzed during calcium induced 10 day NHEK differentiation. TGM1 and PI3 were both found to be markedly upregulated (at least 2.5 fold by day 10) along with six of the tested eight potential differentiation genes (75%) in the longer term Ca^{2+} induced differentiation model (**Figure 13**). These findings not only validate the role of the selected proteins in the keratinocyte differentiation process but also verify the ability of the initial short term confluence induced keratinocyte differentiation study to identify genes that were previously not described to play a role in keratinocyte differentiation.

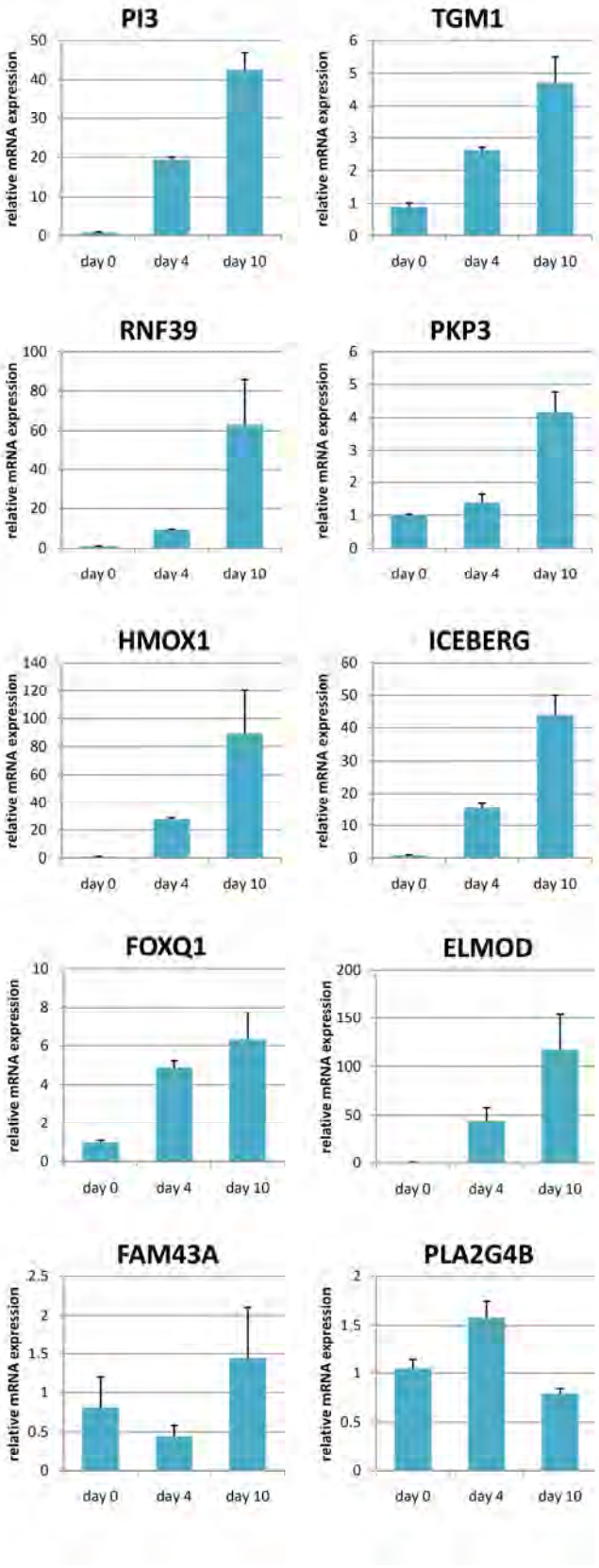


Figure 13 Gene expression after Ca^{2+} induced keratinocyte differentiation. Keratinocytes were subjected to 1.2mM Ca^{+2} induced differentiation. Cells were harvested just prior to differentiation induction (day 0) or 4 and 10 days after initiation of differentiation. Results of TaqMan quantitative PCR measurements were normalized to 18S rRNA and expressed relative to day 0 values. TGM1 and PI3 are known keratinocyte differentiation genes, while all other genes were candidates selected for verification in this longer term differentiation model after showing regulation in confluence induced keratinocyte differentiation. Error bars represent SEM (n=3).

Determining cytotoxicity of sphingolipid compounds

Molecules capable of influencing keratinocyte differentiation are of potential dermatological value. Therefore using the established confluence induced keratinocyte differentiation model we wished to test the ability of various sphingolipid compounds to modify keratinocyte differentiation.

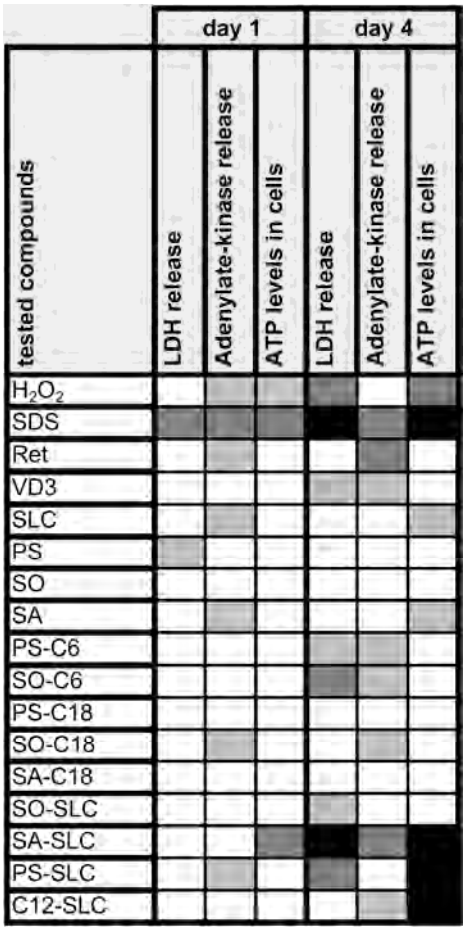


Table 8 Cytotoxicity of test substances. Specific cytotoxicity after 25 μM keratinocyte differentiation modulating compound and positive control (SDS [25μM], H2O2 [1mM]) treatment is represented by increasing darkness of the field (no effect [white], low [light gray], intermediate [dark gray] or high [black] level of cytotoxicity as described for various assays in the methods). (6)

First, however, in order to gain information on the cytotoxicity of the substances to be tested, human keratinocytes were treated with 1 μM, 5 μM, and 25 μM concentrations

of the sphingoid derivatives. Hydrogen peroxide (oxidative stress) and sodium dodecylsulfate (SDS, membrane permeabilisation) were used as positive controls. The results of 25μM compound treatment are summarized in **Table 8**. After one day of treatment no unanimous cytotoxic effects were seen in the three test systems even the at 25 μM concentration of the compounds, except for the control compounds. PS, however, increased LDH-release; Ret, SA, SO-C18, PS-SLC, SLC, and C12 resulted in elevated adenylate kinase (AK) levels in the medium and SA-SLC diminished ATP-levels within the cells. When NHEKs were incubated with the highest concentration (25μM) of the sphingolipids for 4 days, marked cytotoxicity was seen for SA-SLC, PS-SLC (LDH, AK and ATP) and C12-SLC (AK and ATP). However, 1 μM and 5 μM concentrations showed no significant difference in cytotoxicity even after 4 day treatment (data not shown). Therefore, during the keratinocyte differentiation modifying compound treatment experiments salicylate (SLC) derivatives along with SLC itself were used at 25 μM for only the day 1 incubations and at 5 μM for day 4 incubations. SLC was not found to be toxic by itself, but as it served as a control for the sphingoid-SLC conjugates, it was used in the same concentration. All other substances were used at 25 μM for both time intervals.

Sphingolipid compounds modulate keratinocyte differentiation

As the geometric mean of gene expression changes of the 53 known keratinocyte differentiation genes were shown to signal differentiation related changes this was determined in case of sphingolipid compound treatment and compared to differentiated control cells of the same respective day (**Figure 14**). PS-C6, SA, PS-SLC, and SO-SLC were the most potent promoters of differentiation after one day treatment. However, the difference vs. untreated controls was no longer as marked after 4 days of treatment, especially for PS-C6 and SA, however, differentiation-associated gene expression was still stimulated significantly as majority of keratinocyte differentiation genes showed mRNA upregulation compared to same day non treated controls. A less marked stimulation with similar kinetics was seen after incubation with PS, SO, and SA-C18, with SO showing a non-significant decrease compared to controls on day 4. SO-C6, SA-SLC, and SLC treatment showed different kinetics with increasing pro-differentiation effect from day 1 to day 4. No significant effect on keratinocyte differentiation was seen

with PS-C18 and SO-C18, whereas C12-SLC was the only compound tested that negatively regulated differentiation-associated gene expression. Interestingly SO-C6, PS-SLC, and SO-SLC were capable of more powerfully promoting KC differentiation than VD3 at both time points. Furthermore, C12-SLC steadily inhibited expression of keratinocyte differentiation genes in our experimental system, with an effect slightly less potent than the anti-differentiation effect of retinol.

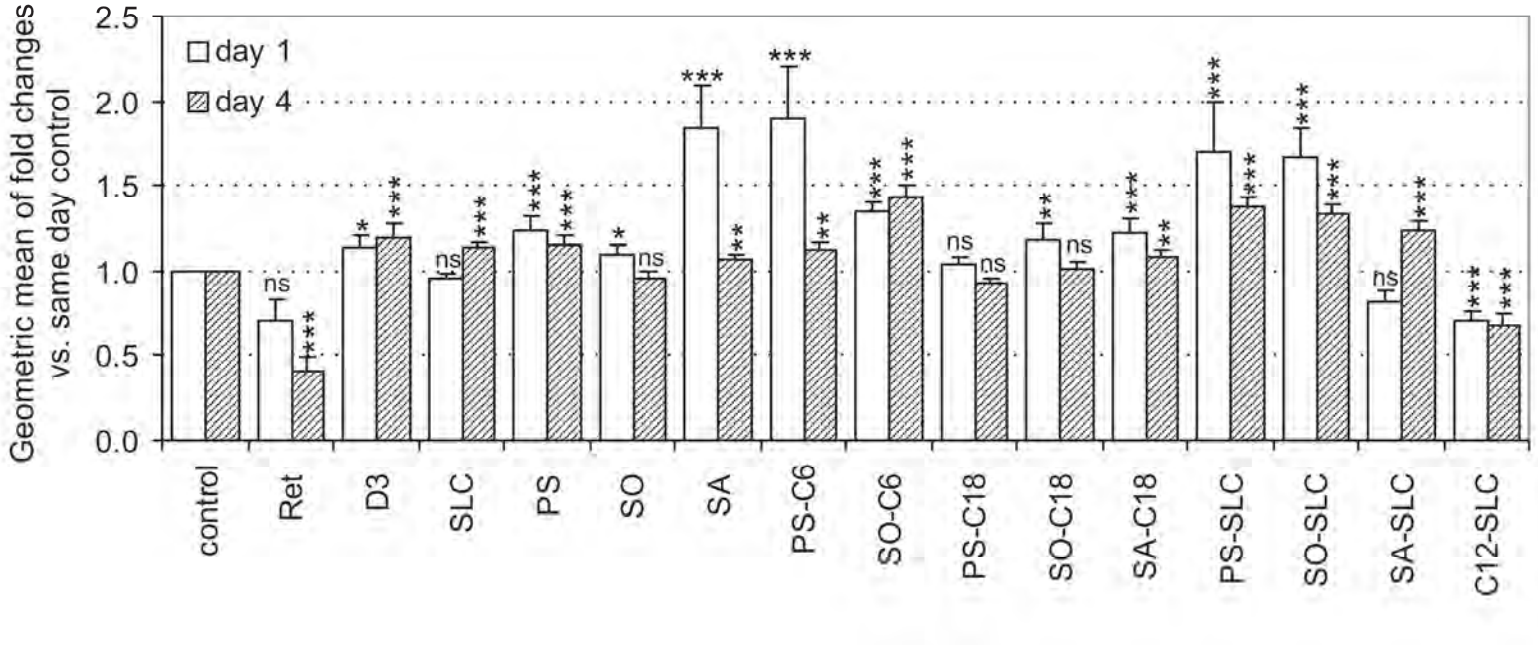


Figure 14 Sphingolipid compounds differentially regulate gene expression of the 53-keratinocyte differentiation genes. Geometric mean of gene expression changes of keratinocytes 1 and 4 days after compound treatment compared to same day controls undergoing confluence-induced differentiation are shown. Values lower than 1 represent inhibition of differentiation whereas values higher than 1 show differentiation promotion effect (significantly different to control of the respective day with *: $p < 0.05$, **: $p < 0.001$, ***: $p < 0.0001$). Error bars present SEM. (Paragh et al. Exp Derm 2008) (6)

ABCA12 regulation in human keratinocytes

ABCA12 is upregulated during keratinocyte differentiation

In normal human keratinocytes undergoing 4-day confluence induced differentiation ABCA12 mRNA underwent significant 2 fold upregulation (data not shown). In both HaCaT keratinocytes (**Figure 15**) and normal human epidermal keratinocytes (**Figure 16**) undergoing 10 day Ca^{2+} induced differentiation ABCA12 mRNA levels were markedly elevated compared to non-differentiated (0 day) controls.

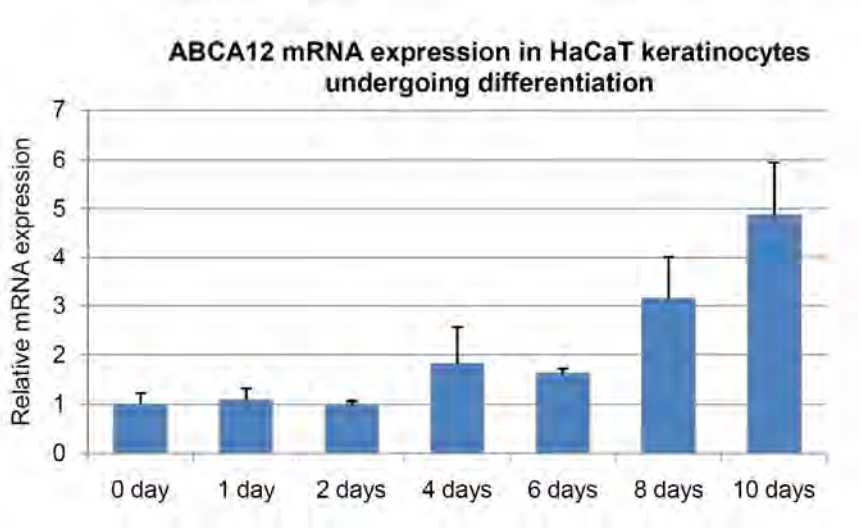


Figure 15 ABCA12 mRNA levels are upregulated during Ca^{2+} induced differentiation of HaCaT keratinocytes. Subconfluent HaCaT keratinocytes were grown in 0.06mM Ca^{2+} containing media for 3 days. At the initiation of the experiment (day 0) media was switched to 2mM Ca^{2+} containing keratinocyte growth media. Results of real time quantitative PCR experiments were normalized to 18S rRNA levels, and ABCA12 gene expression is shown relative to mRNA expression at day 0 (mean+SD).

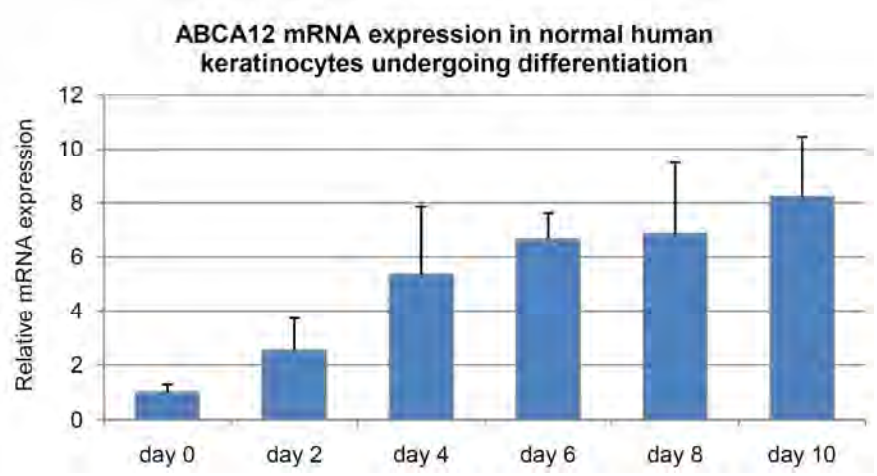


Figure 16 ABCA12 mRNA levels are upregulated during Ca^{2+} induced differentiation of normal human epidermal keratinocytes. Subconfluent NHEKs were induced to undergo differentiation by calcium switch (1.2mM Ca^{2+}) at day 0. Results of real time quantitative PCR experiments were normalized to 18S rRNA levels, and ABCA12 gene expression is shown relative to mRNA expression at day 0 (mean+SD).

The effect of the activation of LXR, PPARs, RAR, RXR and VDR on ABCA12 mRNA expression

The optimized dose for each activator was determined previously (Jiang et al., 2005). As shown in **Figure 17A** ABCA12 mRNA level increased markedly following treatment with PPAR-gamma activators (Cig: 11.8-fold; Tro: 12-fold; GI: 6.4-fold) and PPAR-delta activator (GW: 5.1-fold), after 24h incubation. In addition, while LXR activators modestly increased ABCA12 mRNA level (TO: 3.3-fold), activators of PPAR-alpha, RAR, RXR, or VDR had no effect (**Figure 17A**).

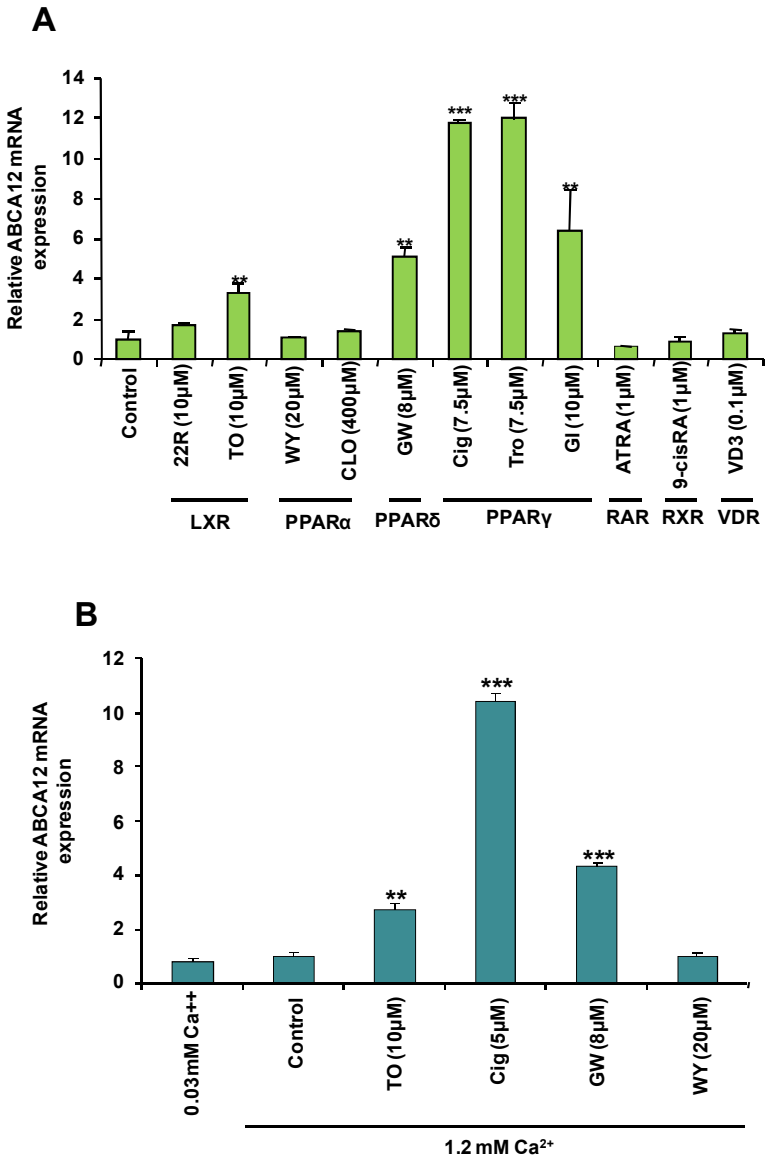


Figure 17 Activation of PPAR-gamma, PPAR-delta and LXR increase ABCA12 mRNA expression in NHEK. Cells were incubated with either vehicle control, or activators of LXR (10μM 22R or 10μM TO); PPAR-gamma (7.5μM Cig, or 7.5μM Tro, or 10μM GI); PPAR-delta GW (8μM); PPAR-alpha (20μM WY or 400μM CLO); RAR (1μM ATRA); RXR (1μM 9-cis-RA); VDR (VD3 0.1μM) in low calcium (0.03mM) medium for 24 hours (**A**). Alternatively, cells were incubated in either 0.03mM Ca^{2+} medium, or 1.2mM Ca^{2+} medium alone, or 1.2mM Ca^{2+} medium with 10μM TO, or 5μM Cig, or 8μM GW, or 20μM WY for 24 hours (**B**). Real-time quantitative PCR results of ABCA12 gene expression were to normalized to cyclophilin and are expressed relative to control. (Mean ± SEM)(**P<0.01; *** P<0.001). (217)

Moreover the up-regulation of ABCA12 by PPAR-gamma (Cig) occurred rapidly and was sustained over an extend period of time (**Figure 18A**) showing clear dose-dependence (**Figure 18B**). Similar time- and dose-dependent up-regulation of ABCA12 mRNA was induced by PPAR-delta (GW) activation (data not shown).

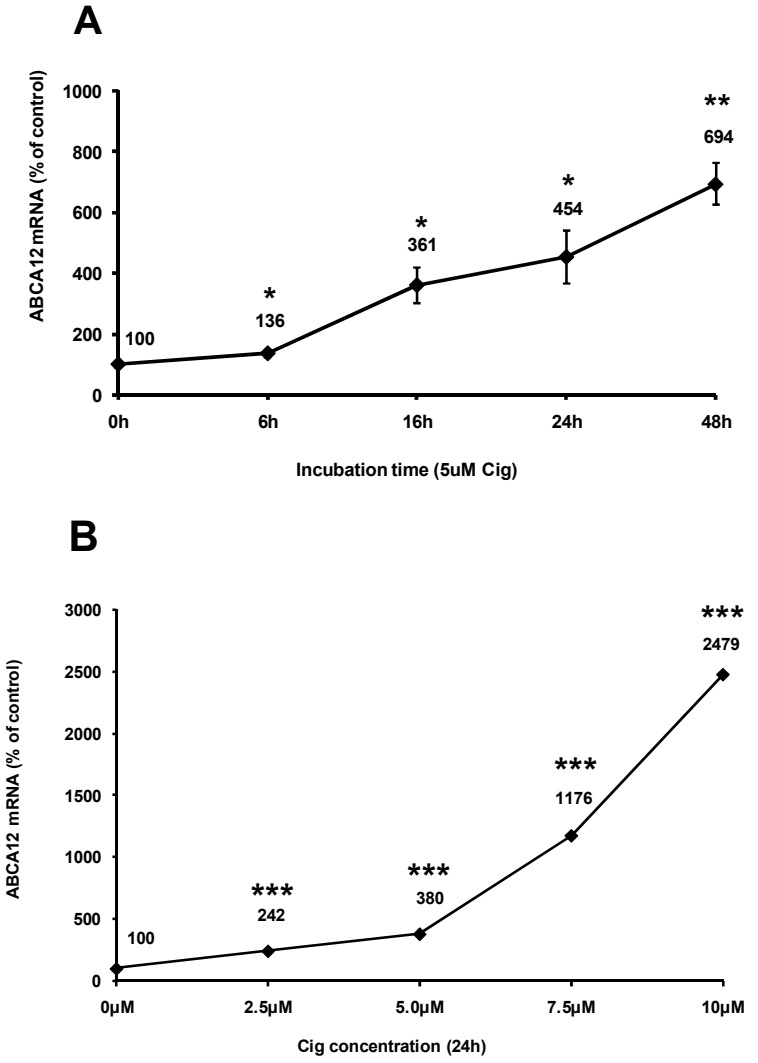


Figure 18 PPAR-gamma activation increases ABCA12 mRNA level in a time- and dose-dependent fashion. Cells were incubated with 5µM Cig for the indicated length of time in 0.03mM Ca^{2+} medium (**A**). Alternatively, cells were incubated with Cig at various concentrations in the same medium for 24 h (**B**). ABCA12 mRNA levels were normalized to cyclophilinv. Data are expressed as percentage of control (100%) and presented as mean±SEM. (*P<0.05; **P<0.01; *** P<0.001). (217)

We next assessed how PPAR and LXR activators would influence ABCA12 mRNA levels in NHEKs subjected to calcium switch (1.2mM) for 24 hours. As it can be seen in **Figure 17B** although high calcium itself did not lead to significant increase of ABCA12 mRNA due to the short incubation time ligand activation of PPAR-gamma and -delta increased ABCA12 mRNA levels by 11- and 4.5-fold, respectively also in high calcium. Similarly, LXR activation by TO increased ABCA12 mRNA level by 2.3-fold ($P<0.01$) (**Figure 17B**). Consistent with the observations in NHEK not subjected to calcium switch PPAR-alpha activation had no effect on ABCA12 mRNA levels (**Figure 17B**). The expression of ABCA12 splice variants was also studied in real-time quantitative PCR experiments. The long variant was assigned ABCA12-L and the ABCA12-S name in this study. Neither transcripts were found to be regulated by 24 hour calcium switch in NHEK (**Figure 19**).

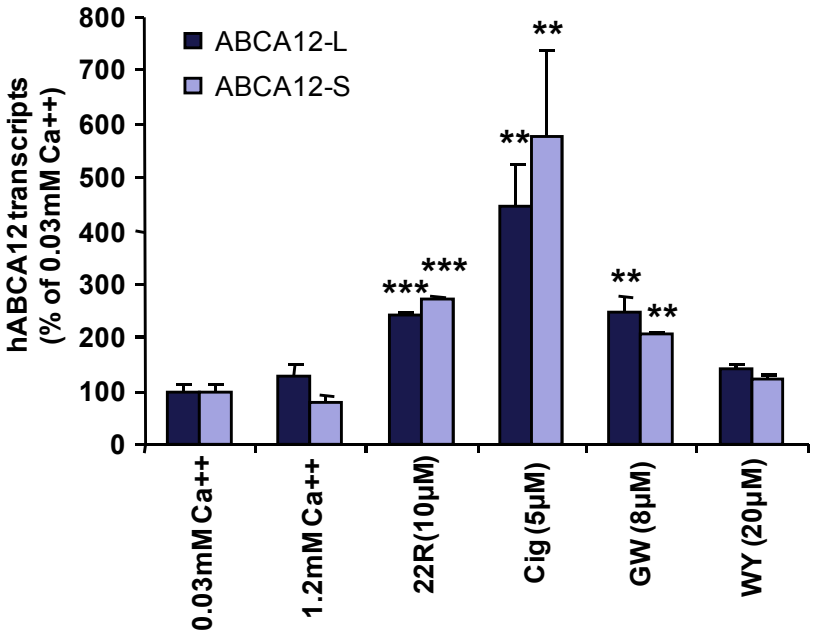


Figure 19 Both studied alternate transcripts of ABCA12 are expressed in NHEK and up-regulated by PPAR-γ, PPAR-β/δ and LXR activators. Cells were incubated in 0.03mM or 1.2mM Ca^{2+} keratinocyte growth medium alone, or in 1.2mM Ca^{2+} medium with 10µM TO, or 5µM Cig, or 8µM GW, or 20µM WY for 24 hours. Human ABCA12-L, -S and cyclophilin mRNA levels were determined by real-time PCR. ABCA12 expression was normalized to cyclophilin and data are expressed as percentage of control (100%) (mean ± SEM) (n= 6). **P<0.01; *** P<0.001.

Ct values (mean) for the basal levels of these two transcripts, hABCA12-L and -S were 25.4 and 30.9 respectively. PPAR-gamma activator Cig increased both transcripts by roughly 5 fold. Similarly, PPAR-delta (GW) or LXR activator (22R) increased both transcripts by 2- and 2.5-fold, respectively, while activation of PPAR-alpha had no effect (**Figure 19**). Similar results were obtained in absence of calcium treatment (data not shown).

Polyclonal antibodies against human ABCA12 recognized both 293.3 kDa (upper) and 257 kDa (lower bands) isotype of the protein, corresponding to gene products of ABCA12-L and –S **(Figure 20)**.

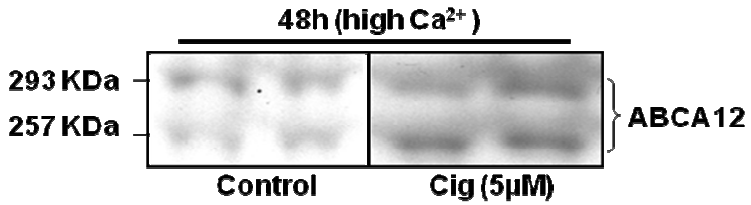


Figure 20 PPAR-gamma activation increases ABCA12 protein expression. Cells were incubated with vehicle or 5μM Cig in 1.2mM Ca^{2+} medium for 48 h. Western blot analyses to determine protein levels of ABCA12 were carried out as described. A representative blot is shown.

PPAR-gamma activation of by Cig increased ABCA12 protein expression (upper and lower band, 220% and 317% as measured by densitometry respectively) in independent batches of differentiated NHEK (Figure 23). Furthermore, activation of PPAR-delta by GW also caused a 175%~182% increase in ABCA12 protein in these cells when assessed by densitometry (**Figure 21**). Finally, both Cig and GW also stimulated ABCA12 protein in undifferentiated CHK (data not shown). Thus, PPAR-γ and –β/δ activator up-regulate both isoforms of ABCA12 at mRNA and protein levels. The function of these isofroms, however, remains unknown.

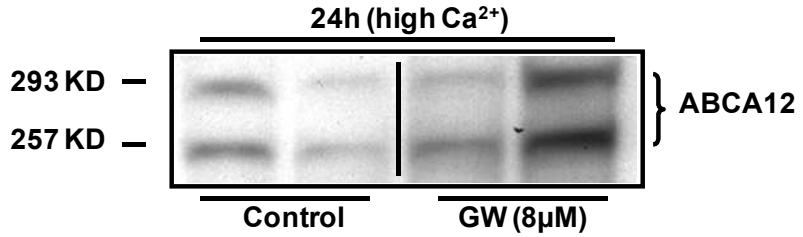


Figure 21 PPAR-delta activation increases ABCA12 protein expression. Cells were incubated with vehicle or 8μM GW in 1.2mM Ca^{2+} medium for 24 h. Western blot analyses to determine protein levels of ABCA12 were carried out as described. A representative blot is shown.

Effect of UVB irradiation on HIF-1alpha expression in keratinocytes

Cytotoxicity after UVB irradiation

To determine the lethal and sub-lethal doses of UVB in our experimental system, HaCaT cells at approximately 70-90% confluence were irradiated with broadband UVB light. The doses employed ranged from 5 to 67 mJ/cm^2. To assess the viability of the cultures, half of the Petri dishes were studied after 24 hours of treatment by light microscopy, the other half were subjected to flow cytometry after a staining by propidium iodide and annexin-V-FLUOS. Our data indicated that a one-minute UVB irradiation (approx. 67 mJ/cm^2) led to demise of all the cells, as they all detached from the surface of the Petri dish 24 hours after irradiation. On the other hand, irradiation for 30 seconds (approx. 33 mJ/cm^2) resulted in no change in confluence at 24 hours. Moreover, there was negligible loss in viability and cell-multiplication potential when cells were irradiated with 20 mJ/cm^2 UVB compared to controls. The light microscopy observations were also verified by flow cytometry (data not shown). Based on these data, we concluded that 20 mJ/cm^2 (18 sec) UVB can be safely applied in our experiments, which dose is also relevant when compared to UVB dosage *in vivo* (24,25).

Effect of UVB irradiation on HIF-1alpha level

HaCaT cells irradiated with 20 mJ/cm^2 of UVB were harvested 1 to 24 hours after treatment and HIF-1alpha protein levels were determined by western blot (**Figure 22**). We found that 3 hours after the irradiation HIF-1alpha protein levels decreased and became barely detectable. However, 12 hours after irradiation a significant increase in HIF-1alpha was observed compared to control samples. This elevated HIF-1alpha level continuously decreased during the course of the study in the next 12 hours. These alterations in HIF-1alpha levels were not present in mock treated samples.

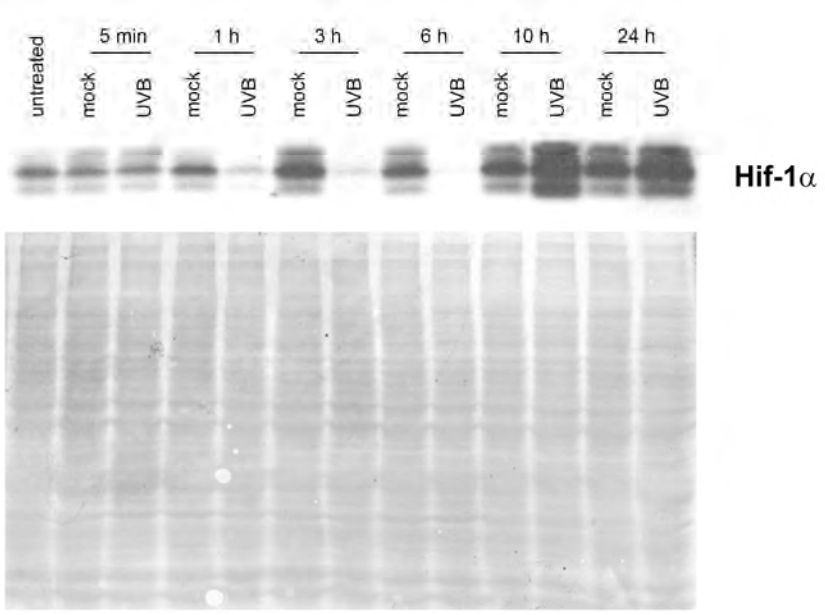


Figure 22 HIF-1α protein expression after UVB irradiation. Confluent HaCaT cells were mock- or 20 mJ/cm^2 UVB-treated and harvested after the time period indicated on the panel. HIF-1α protein levels were detected by Western-blot analysis. The lower panel depicts results of Ponceau staining showing equal protein loading. (219)

Dose-dependence was studied at doses between 6.7 and 50 mJ/cm^2 of UVB as shown in **Figure 23**. The decrease in HIF-1alpha protein was clearly visible 1 hour after UVB treatment at all doses used; the lowest value was seen at 50 mJ/cm^2. However, at the greatest dose instead of an increase seen at lower doses a decrease in the HIF-1alpha signal occurred 12 hours after UVB irradiation, which is most likely due to the overwhelming toxic effects of higher doses of UVB seen also in the cytotoxicity study. Given these observations, the best comparison appears at the 20 mJ/cm^2 dose-point, which dose is also comparable to the UVB dose basal keratinocytes are subjected to *in vivo* during sun exposure (24,25).

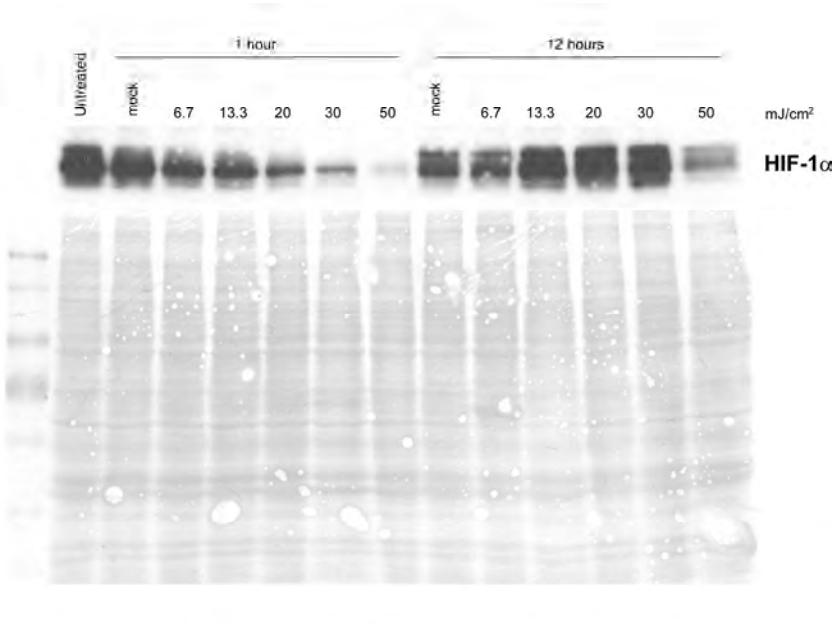


Figure 23 HIF-1α protein expression changes depend on the dosage of the UVB. Confluent HaCaT cells were mock-treated for 45 seconds or UVB-treated with the dosage indicated on the panel. Cells were harvested after 1 or 12 hours of the treatment. HIF-1α protein levels were detected by Western-blot analysis. The lower panel depicts results of Ponceau staining showing equal protein loading. (219)

Effects of repeated UVB irradiation on the expression of HIF-1alpha were also investigated. 6 hours after initial irradiation the UVB treatment was repeated and cells were harvested an additional 6 hours later, i.e. 12 hours after the initial treatment. HIF-1alpha levels in these cells were compared to the levels in those cells harvested at 6 and 12 hours after a single irradiation (**Figure 24**). As shown in figure 15 repeated irradiation six hours after the first UVB treatment causes a down-regulation of HIF-1alpha protein levels again and seems to completely abolish the upregulatory effect of UVB on HIF-1alpha observed 12 hours after a single UVB irradiation (**Figure 24 lane G**).

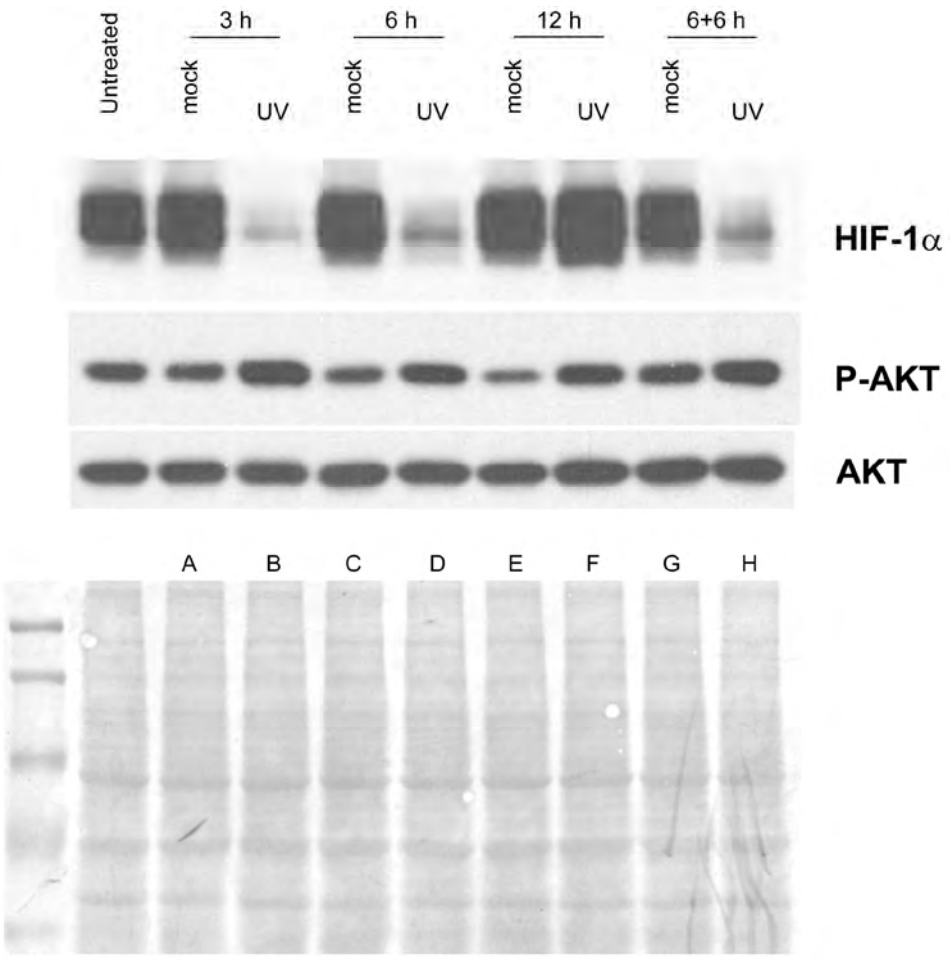


Figure 24 Repeated UVB-irradiation restarts the HIF-1α level fluctuation. Confluent HaCaT cells were mock- or 20 mJ/cm^2 UVB-treated and harvested after the time period indicated on the panel (lanes: A-F). Samples loaded in lanes G and H were re-treated after 6 hours of the first treatment, and harvested 6 hours later. HIF-1α, Ser^{473}-phosphorilated AKT, and AKT protein levels were detected by Western-blot analysis. The lowermost panel depicts results of Ponceau staining of the membrane showing equal protein loading. (219)

Effect of UVB on the expression of HIF-1α, VEGF and HMOX1 at the mRNA level

Real-time quantitative PCR was performed to study the mRNA expression of two well known HIF-1 target genes (VEGF and HMOX1) parallel to the observed HIF-1alpha changes. Moreover HIF-1α mRNA levels during the experiments were also analyzed. β-

actin expression levels were used to normalize results. We found that UVB treatment led to upregulation of both VEGF and HMOX1 mRNA levels 12 and 24 hours after UVB irradiation (**Figure 25**). HIF-1alpha mRNA showed a significant decrease 3, 12, and 24 hours after UVB treatment (**Figure 25**).

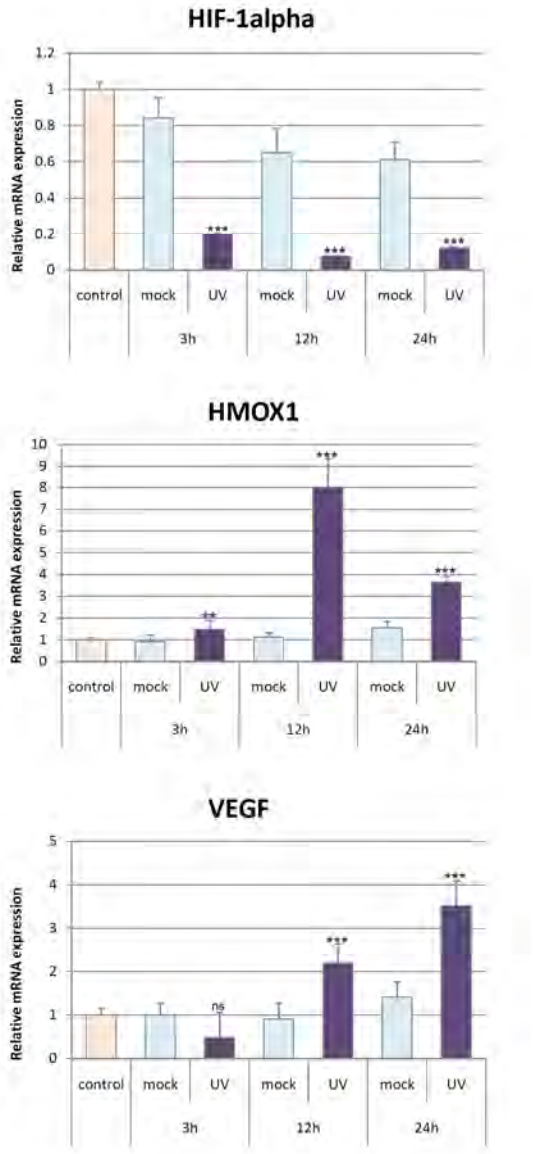


Figure 25 VEGF and HMOX1 mRNA levels upon UVB irradiation. Confluent HaCaT cells were mock- or 20 mJ/cm2 UVB-treated and harvested 3, 12 and 24 hours after treatment. Vascular Endothelial Growth Factor (VEGF), Heme Oxigenase 1 (HMOX1) and Hypoxia Inducible Factor 1α (HIF-1α) mRNA levels were analyzed by quantitative real-time PCR. Data were normalized expression of actin beta and were expressed relative to untreated controls. ns: non-significant; *: marginally significant ($p<0.05$); **: significant ($p<0.01$); ***: highly significant ($p<0.001$) differences. (219)

PI3K inhibition reduces HIF-1alpha elevation after UVB

Previous studies have shown that the PI3K regulation is an important positive regulator of HIF-1alpha in various model systems (26,27), and UV irradiation was shown to result in increased PI3K activation. Therefore, the effects of wortmannin, a specific inhibitor of the PI3K enzyme, were analyzed in our experimental setup. Wortmannin was added to the cell suspension immediately after UVB irradiation. Cells were harvested 3, 12 and 24 hours after the inhibitor treatment. Western-blot analysis showed that wortmannin effectively decreased the elevation of the HIF-1alpha protein 12 hours after UVB treatment (**Figure 26**). AKT phosphorylation was also studied by specific anti-phospho-serine AKT antibody (corresponding panels of **Figure 24**). In case of UV-treated samples increased AKT phosphorylation was found.

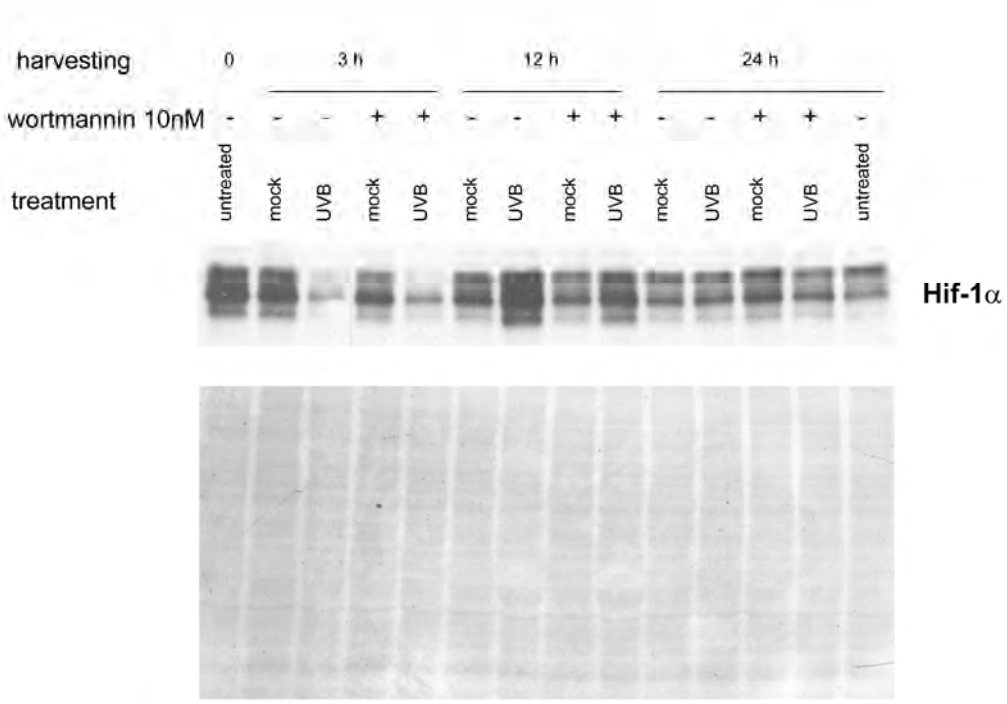


Figure 26 The PI3K/AKT pathway is involved in HIF-1alpha upregulation. Confluent HaCaT cells were mock- or 20 mJ/cm^2 UVB-treated, harvested after the time period indicated on the panel and supplemented (+) with 10 nM PI3K-inhibitor (wortmannin). HIF-1α protein levels were detected by Western-blot analysis. The lower panel depicts results of Ponceau staining showing equal protein loading. (219)

Discussion

During our studies in an attempt to widen our understanding of the processes underlying the transcriptional regulation of keratinocyte differentiation, we identified novel sphingolipid regulators of keratinocyte differentiation, and previously undescribed genes involved in the formation of the epidermis. Moreover focusing on specific transcriptional regulatory systems involved in epidermal sphingolipid homeostasis and response to environmental factors we identifying the regulatory effects of nuclear receptor ligands on ABCA12 transcription and identified the regulation of the "hypoxia machinery" by ultraviolet light.

Discussion: Establishing the confluence induced keratinocyte differentiation model and testing keratinocyte differentiation modification by sphingolipid treatment

Keratinocyte differentiation is responsible for creation of the structural building blocks of the stratum corneum and the generation of the epidermal permeability barrier (1;26). Alterations in keratinocyte differentiation and deregulation of keratinocyte differentiation gene expression are important contributors to dermatological pathogenesis (191;192;220-223). The modification of keratinocyte differentiation is of obvious dermatological and cosmetic value. In order to test the effects compounds on differentiation a proper test system had to be found. As sphingolipids can be expected to be involved in the regulation of the first phases of keratinocyte differentiation (224), the initial phase (0-4 days) of confluence induced keratinocyte differentiation was chosen to be the background for testing their ability to alter keratinocyte differentiation. Although theoretically this system was thought to provide suitable background for measurement of both pro- and anti-keratinocyte differentiation effects, without previous works using this model in the suggested context, initially the ability of confluence induced differentiation to be inhibited or accelerated by keratinocyte retinol or vitamin D3, respectively, had to be determined. Vitamin D3 is a well known to induce keratinocyte differentiation, while retinol has been described to inhibit this process. Supporting our initial assumption that short term confluence induced differentiation can both respond to anti and pro-differentiation signals messenger RNA expression of four established

keratinocyte differentiation markers, (transglutaminase 1, filaggrin, keratin 10, and corneodesmosin) was upregulated 4 days after induction of differentiation by confluence, while this induction in most cases was significantly abrogated by retinol treatment and significantly enhanced by vitamin D3 (**Figure 11**).

Many publications use a few keratinocyte differentiation genes and depending on the regulation of these genes term the studied impact pro- or anti-differentiation (75;225). Although this is sufficient to establish the direction of drug treatment effect as it could be seen from also our previous observations. However, using this method it would have been nearly impossible to quantitatively define differences between compounds. Given that selection of lead compounds for further studies requires the quantitative comparison of compound effect a different method had to be found. Microarray experiments provide an opportunity for more inclusive analysis of compound effect by measuring the expression levels of numerous genes with high precision. However, so far even when microarray has been used for assessing differentiation effects of a treatment modality the method of analysis failed to provide a plausible comparison of the achieved differentiation induction with that of know keratinocyte differentiation compounds, and dealt with genes only as a collection of individual signals (170). We however believed that microarray analysis can provide better bases of analysis when gene expression values of genes signaling similar endpoints are combined. In order to test this hypothesis, however, initially the complex regulation of the keratinocyte differentiation machinery in the confluence induced differentiation system had to be proven.

Initially therefore lists of genes regulated after confluence induction were created (data not shown). There were 932 positively and 629 negatively regulated (greater than 2.5 fold) genes identified as potential keratinocyte differentiation genes in the short term confluence induced keratinocyte differentiation system. These numbers greatly exceed the number of currently published (approx. 150) known keratinocyte differentiation genes, that could be found in PubMed (data not shown). Bingo analysis found a significant overrepresentation of the epidermal differentiation related gene ontology groups indicative of the presence of epidermal differentiation in the studied samples **(Table 6)**. Interestingly in the short term keratinocyte differentiation system with the 2.5 fold cutoff only a third of the keratinocyte differentiation related genes

forming the various epidermal differentiation related gene ontology (GO) groups were identified, indicative of either the stringency of the method or the relative difference of the subset of genes regulated in confluence induced differentiation vs. other methods that helped identify genes within the gene ontology groups, such as immunohistochemistry in human skin, Vitamin D induced differentiation and others. The fact that some of the best characterized keratinocyte differentiation genes like transglutaminase 1 and peptidase inhibitor 3 only showed a relatively modest 3 fold expression change within this model suggest that stringency could be a likely explanation for this phenomenon. Although obviously an in vitro method cannot be expected to fully mimic the complicated interactions during keratinocyte differentiation within the human body even if it provides a suitable model system for initial identification of potential keratinocyte differentiation gene candidates. Due to the overwhelming bias of the research world towards positively regulated keratinocyte differentiation genes there is only a small number of well defined characteristic negatively regulated keratinocyte differentiation markers. Nevertheless the validity of our list of downregulated genes is supported by the fact that it contains some genes, which have been shown to be downregulated during the keratinocyte differentiation, such as fibroblast growth factor receptor 2 and epidermal growth factor receptor (226;227).

Although the maked dominance of keratinocyte differentiation related GO terms indicated the presence of wide reaching keratinocyte differentiation gene expression changes in the 4 day confluence model, further verification was carried out. Hypothesizing that even genes that were previously unknown to undergo changes in keratinocyte differentiation but are truly related to this process are regulated in the confluence model, we tested the overlap of the confluence induced differentiation lists with lists of genes regulated in psoriasis. Psoriasis is characterized by an immunological disballance and marked dysregulation of keratinocyte differentiation within involved areas of the skin (218;228). Therefore, the significant overrepresentation of genes disregulated in psoriatic plaques in the confluence induced differentiation provided further evidence for the validity of the list of up-regulated genes, while unlike the gene ontology analysis, by the significant overlap of the datasets it was also able to confirm the validity of the list of downregulated keratinocyte differentiation genes.

Even though many aspects of gene expression regulation during keratinocyte differentiation have been uncovered (156;159;217;229), to date a comprehensive search for keratinocyte differentiation genes has according to our knowledge not been carried out in a controlled *in vitro* differentiation setting. Our previous observations suggested that confluence induced differentiation could identify previously unidentified keratinocyte differentiation regulated genes. Therefore the differentiation gene list could provide the first comprehensive lists of genes regulated in well characterized keratinocyte differentiation. Thus to verify that the confluence induced differentiation gene lists, enable the identification of novel keratinocyte differentiation genes 8 genes were selected from the list of 932 genes that showed lineal at least 2.5 fold upregulation during keratinocyte differentiation. These 8 genes were chosen to cover wide ranging potential processes that represent important fields of interest for dermatology from desquamation and redox-regulation to signal transduction (RNF39, PKP3, HMOX1, ICEBERG, FOXQ1, ELMOD, FAM43A, PLA2G4B) (**Table 7**). The regulation of these genes during 10 day Ca^{2+} induced differentiation was assessed in NHEK cells by quantitative RT-PCR (**Figure 13**). Six of the eight candidate genes showed marked upregulation during keratinocyte differentiation similar to positive controls. Although confluence induced short term differentiation might have some shortcomings, these results showed that in this model the regulation of keratinocyte differentiation genes takes place similarly to the more conventional long term Ca^{2+} induced keratinocyte differentiation model, which is known to mimic *in vivo* keratinocyte differentiation (156;158). Moreover the high rate of concondance (75%) between the two models provided further evidence to the validity of the gene lists as comprehensive lists of keratinocyte differentiation genes. Thus further close evaluation of the candidates within these lists will hopefully enable deeper insight into the delicate processes underlying the creation of the epidermal barrier.

After obtaining evidence that the regulation of a whole array of keratinocyte differentiation genes took place in confluence induced differentiation the new method of gene expression data analysis had to be tested. The ability of a gene expression values from large number of genes to quantify keratinocyte differentiation modifying effect of drug treatment had to be determined. Even though this might seem an obvious alternative to using single genes or an array of discreet gene expression data to identify

effects of drugs, according to our knowledge previously similar approaches have not be used in identifying the potential biologic effects of treatment modalities. Therefore to find out whether this approach could be viable first a list of well established keratinocyte differentiation genes that showed regulation during keratinocyte differentiation had to be selected.

Via literature search we assembled a list of 150 keratinocyte differentiation genes with *in vitro* or *in vivo* protein or mRNA level evidence suggesting their regulation during keratinocyte differentiation. In the confluence induced keratinocyte differentiation model during 4 days of differentiation 53 of this list of 150 keratinocyte differentiation genes showed more than 1.9 fold lineal regulation. Interestingly, even numerous late keratinocyte differentiation genes were markedly regulated and thus included in the list of 53 genes used for analysis of compound effects **(Table** 5**)**. These findings correlate with our own previous unpublished results and findings of others showing the rapid upregulation of even late keratinocyte differentiation genes takes place during *in vitro* differentiation (156). The finding that in our model only slightly more than a third of the known keratinocyte differentiation genes were found to be significantly regulated over 1.9 fold was not surprising since literature about keratinocytes is highly heterogeneous, and genes found to be expressed in keratinocyte differentiation from HaCaT, NHEK studies and also immunohistochemistry observations were included in the initial list. The overwhelming majority of up-regulated genes (51 out of 53) in the final list mirrors the bias towards publication of positive changes. Nevertheless, regulation of this list of genes can still be capable of measuring both pro- and anti-differentiation effects equally, since decreased upregulation of normally upregulated differentiation genes can signal anti-differentiation potential while increased regulation of these genes pro-differentiation effects.

The geometric mean of the gene expression changes of these 53 keratinocyte differentiation genes (**Figure 12**) showed similar pattern to the the mRNA expression levels of single marker genes (transglutaminase 1, filaggrin, keratin 10, corneodesmosin) (**Figure 11**). Moreover, changes in gene expression induced by VD3 and retinol were also similar with the 2 methods. These results validate the use of this set of 53 differentiation genes for the analysis of the keratinocyte differentiation

modifying effect of compounds. While the benefits of using more genes for analysis are quite obvious: the lower bias due to specific gene related effects of compounds, increased power during statistical assessment of the results.

Prior to testing sphingolipid compounds (**Table 3**) the adequate test dosage was determined had to be determined. Our unpublished preliminary studies indentified that μM range of these sphingolipid compounds was sufficient to study keratinocyte effects. As some sphingolipid derivatives have previously been suggested to possess pro-apoptotic effects we tested the ability of the used compounds to induce keratinocyte apoptosis (77;78). Salicylate compounds possessed a more potent pro-apoptotic effect than their non-salicylate backbones in the tested maximum concentration (**Table 8**). However, decreasing the dose of these salicylate compounds from 25μM to 5μM for the 4 days of incubation effectively minimized release of cytoplasmic materials, as observed by LDH and AK release assays and lead to normal ATP content measurements and microscopic cell evaluation. Sphingolipids have been implicated in the regulation of apoptosis in HaCaT cells and NHEKs. Excessive pro-apoptotic effects were mostly attributed to short chain ceramides, however, the literature is not consequent on the apoptotic effect of sphingolipids (77;78). Moreover it should be noted that keratinocyte differentiation and apoptosis are related entities and certain apoptotic changes such as decrease of mitochondrial membrane potential can be expected in normal keratinocytes undergoing differentiation (230). The results therefore suggests that due to the high biological activity of these compounds appropriate dosing is important to decrease the marked and rapid induction of keratinocyte terminal differentiation and cell death, and to enable utilization of their potential therapeutic value.

Salicylation of sphingolipid backbones resulted in altered differentiation influencing potential (**Figure 14**). The pro-differentiation effect of PS was seemingly potentiated by the SLC moiety, while SO, that otherwise had no notable effect on keratinocyte differentiation, was clearly transformed into a potent pro-differentiation molecule by covalent attachment of SLC, whereas the short term pro-differention potential of SA was completely abrogated and it gave place to a more prolonged differentiation enhancing effect. Thus the effect of salicylation seemed to have furnished the compounds with new character and according to our unpublished data this is not likely due to simply modifying cellular uptake of the sphingolipid backbone.

C12-SLC was utilized as a control substance for the sphingoidbase-SLC's since this lipophilic SLC derivative exhibits penetration properties similar to the other salicyl compounds (PS-SLC, SA-SLC, SO-SLC) tested in this study (data not shown). In contrast to these C12-SLC steadily inhibited expression of keratinocyte differentiation genes. After 1 day of treatment this effect was even more prominent than the anti-differentiation effect of retinol indicating the differentiation promoting effect is not based on a lipophilic SLC derivative but is dependent also on the sphingoid backbone structure.

After four days of compound treatment the effects of sphingolipid compounds were generally weaker than on day one. This might have been caused by the use of decreased concentrations of salicylic compounds used to avoid compound induced cell damage, or could also be attributed to the decreased response of the cells to exogenous sphingolipids due to the increased cellular lipid pool created by endogenous synthesis of sphingolipids after the induction of differentiation (25). The effects of the salicylate compounds were only slightly decreased and both PS-SLC and SO-SLC were able to further increase gene expression of KC differentiation genes significantly by 37.28% ($p<0.0001$) and 33.5% ($p<0.0001$) respectively after 4 days of treatment compared to controls undergoing un-modified confluence induced differentiation. Interestingly, 4 compounds were capable of more powerfully promoting KC differentiation than VD3 after 4 days of treatment in this model (SO-C6, PS-SLC, SO-SLC and SA-SLC). Of these 4 compounds 3 are novel salicylate derivatives with previously un-published keratinocyte differentiation promoting effect. However, one should note that 1 day SA-SLC treatment failed to promote keratinocyte differentiation, leading to a non-significant but apparent decrease in the expression of the differentiation genes. Thus PS-SLC and SO-SLC are the most promising candidates for application in dermatological and cosmetic applications. The in vivo activity of PS-SLC with respect to the induction of collagen and fibrillin and the repression of matrix metalloprotease 1 in photodamaged skin on protein level has been demonstrated previously (231). This taken together with our data renders it the prime candidate for potential therapeutic use.

These findings provide evidence for the usefulness of whole genome transcriptional profiling in keratinocyte differentiation modulating compound testing

and suggest that PS-SLC and SO-SLC are potent novel keratinocyte differentiation promoting agents.

Discussion: Regulatation of ABCA12 by nuclear receptor ligands

Sphingolipids are not only regulators; they are also important building blocks of the epidermal permeability barrier. Nowhere is it more evident than in the profound importance of proper ABCA12 function in the epidermis. Many members of the ATP binding cassette transporter family are expressed in the epidermis, and show differentiation related regulation (82). Due to its recent discovery little was known about ABCA12, until its mutations were identified in patients suffering from lamellar and harlequin ichthyoses (2;95). ABCA12 is highly expressed in the skin and testis. Its potential role in the skin is the trafficking of glucosylceramide to lamellar bodies in keratinocytes (98;99), the defect of which is thought to result in a grossly altered epidermis, when ABCA12 is mutated. Its upregulation by short term calcium treatment had been suggested (99;100), however, the epidermal regulation of ABCA12 expression has not be described.

ABCA12 upregulation in the epidermis parallel to keratinocyte differentiation has been shown. To test whether in vitro models could be suitable for testing the regulatory role of nuclear receptor ligands initially the effect of long term Ca^{2+} induced differentiation on the regulation of ABCA12 mRNA expression in NHEK and HaCaT keratinocytes was determined. The upregulation of ABCA12 gene expression in both cell types similar to the epidermis suggested that the in vitro models are suitable for establishing the regulatory role of nuclear receptor ligands in keratinocytes.

Numerous nuclear receptors are known to be regulators of epidermal lipid homeostasis and regulators of other members of the ABC transporter protein family (58;119;232;233), and thus could be considered potential regulators of ABCA12 expression. Specific synthetic ligands are available for these nuclear receptors. Due to their hormone responsiveness the role of these nuclear receptors in the regulation of individual genes can be determined by studying the effects of their specific agonist on the expression of potential target genes(105;106). To establish the role of the best characterized regulators of epidermal homeostasis of we examined the effect of the

activation of LXR, PPARs, RAR, RXR and VDR on ABCA12 mRNA levels (58;119;232;233). Interestingly the well known keratinocyte differentiation inductor vitamin D had no effect on ABCA12 expression after 24h treatment and neither could RAR, RXR and PPAR-alpha activators influence ABCA12 mRNA levels (141) (**Figure 17A)**. Treatment with PPAR-gamma activators (Cig, Tro, GI) and PPAR-delta (GW) and LXR (TO) activators however led to increased ABCA12 expression after 24h of ligand treatment, indicating the potential likely direct regulatory role of these receptors on the ABCA12 promoter (**Figure 17A**).

The direct regulatory role of the receptor is further supported by the significant upregulation of ABCA12 expression already by 6 hours after Cig (PPAR-gamma activator) treatment (**Figure 18A**) and the observed clear dose-dependence (**Figure 18B**). The similar time- and dose-dependence also suggest the direct regulatory role of PPAR-delta (GW) activation in ABCA12 expression.

Long term calcium induced differentiation induced keratinocyte differentiation, and previously shorter term calcium switch was also found to stimulate ABCA12 mRNA expression (99), therefore we next assessed how PPAR and LXR activators influence ABCA12 mRNA levels in NHEKs subjected to 24h calcium switch (1.2mM). High calcium itself did not lead to significant increase of ABCA12 mRNA due to most likely the short incubation in this experimental setup, but PPAR-gamma, -delta and LXR ligands regardless of the increased Ca^{2+} had similar effect as in keratinocytes not subjected to Ca^{2+} switch (**Figure 17B and Figure 17A)**. Thus, under our experimental conditions (24h calcium incubation), the used PPAR and LXR activators stimulate ABCA12 mRNA expression, independent of the induction of keratinocyte differentiation. Calcium switch neither potentiated nor abrogated the effects of nuclear receptor ligands in NHEK. Taken together these results indicate that that the mechanism underlying calcium-induced ABCA12 expression in keratinocytes seems different from that of PPAR and LXR.

ABCA1 splice variants have recently been shown to be similarly regulated by LXR lignads (232). Like in case of ABCA1 two major splice variants of the ABCA12 gene have recently been described (234;235). The long variant was assigned ABCA12-L and the ABCA12-S name in this study. The ABCA12-L (NM_173076) gene consists of 53 exons with a total length of the coding sequence of 9112 bp, which is translated

into a protein of 2595 amino acid residues (molecular weight: 293.4 kDa). ABCA12-S (NM_015657) variant lacks 8 of the 5' exons present in the long form and has a full length of 8097 bp coding for a protein of 2277 amino acid residues with a calculated molecular weight of 257 kDa. To determine which of these transcripts are expressed in NHEK specific primer pairs for both hABCA12-L and –S were designed, and used in real-time quantitative PCR experiments. Neither transcripts were found to be expressed at significantly effected by 24 hours calcium switch (**Figure 19**), and although Ct values (mean) for the basal levels of these two transcripts, hABCA12-L and -S were 25.4 and 30.9 respectively indicative of a more than 10 fold higher expression of the long isoform PPAR-gamma, PPAR-delta, LXR and PPAR-alpha activators all had essentially similar effect on had both of the isoforms (**Figure 19**) regardless of the accompanying Ca^{2+} treatment. These data suggest similar regulation of both isotypes in keratinocytes.

To test whether these mRNA isoforms also resulted in the appearance of protein products, and to verify that the mRNA level regulatory changes observed after nuclear receptor ligand treatment resulted in protein level changes in keratinocytes ABCA12 protein expression was also analyzed by western blot. Polyclonal antibodies against human ABCA12 recognized both 293.3 kDa (upper) and 257 kDa (lower bands) isotype of the protein, corresponding to gene products of ABCA12-L and –S **(Figure** 20**)** verifying the translation of both isoforms. Interestingly the observed protein level difference seemed to be less pronounced than the expected difference based on the mRNA levels of the isoforms, potentially due to the different half life of the ABCA12 isoform proteins. The western blot analysis also verified the protein level change of both isotypes of ABCA12 after PPAR-gamma and delta ligand treatment. Although the altered structure of the 2 ABCA12 proteins suggests potential distinct roles for these isoforms new studies will need to be conducted to determine their exact cellular functions.

The epidermal permeability barrier, which is a prerequisite for terrestrial life, resides in the outermost layers of the mammalian epidermis and is comprised of lamellar membranes enriched in non-polar lipids that are derived from the secretion of LB by stratum granulosum cells (236;237). Abnormal LB formation or secretion leads to the failure of the formation of a functional barrier, a severe case occurs in the disease HI. Ultra-structurally, in cultured HI keratinocytes, ABCA12 deficiency leads to an

abnormality in the formation and secretion of LBs, which is associated with alterations in the distribution pattern of glucosylceramide (99). It is hypothesized that ABCA12 mediates the transport of sphingolipids into LB, which appears to be critical for a competent barrier formation.

Previously the activation of LXR, PPAR-alpha, PPAR-delta and PPAR-gamma has been shown ameliorate effects acute barrier disruption (238-241). This could be attributed to their effects on both the protein and the lipid components of the SC. Corneocytes provide a scaffold for the organization of the lipid matrix into lammelar membranes. Activation of PPARs and LXR has been shown to stimulate keratinocyte differentiation and the formation of cornified envelope (119;134;238;239). Activation of PPARs and LXR has also been shown to stimulate LB secretion, epidermal lipid synthesis (242), as well as stimulate SULT2B1b expression, the key enzyme in the synthesis of cholesterol sulfate (243). Together these effects on the SC could account for the improvement in permeability barrier homeostasis induced by PPAR and LXR activator treatment. Our current results that demonstrate the PPAR and LXR activator mediated expression of ABCA12 provide another mechanism by which PPAR and LXR activators promote epidermal permeability barrier homeostasis, through an effect on sphingolipids translocation within the epidermis.

Discussion: Effect of UVB irradiation on HIF-1alpha expression in keratinocytes

UV radiation is a proven cytotoxic agent, a known regulator of both ABC transporters, and potent modifier of epidermal lipid profile. Furthermore UV effect leads to pronounced vascular changes (244). The pathogenic role of several hypoxia regulated genes has been proposed in UVB induced skin changes. The hypoxia pathway is regulated by hypoxia inducible factors capable of altering the efficacy of transcription by binding to the hypoxia response element in the promoters of their target genes. Several previous findings point to the involvement of hypoxia signaling in the UV mediated changes in keratinocytes. Hypoxia was shown to decrease UV irradiation induced sunburn cell formation in vivo (245). UV regulated genes include well known HIF-1 targets VEGF (202;203;246;247) iNOS (248;249) and HMOX1 (250;251).

Therefore, the possible role of HIF-1alpha in UVB radiation induced keratinocyte response has been investigated.

The regulator of hypoxia signaling is the HIF-1 transcription factor. Its regulatory effect is thought to be determined by posttranslational regulation of HIF-1alpha levels (205). Thus in order to study the potential role of hypoxia signaling in UV response first the regulation of HIF-1alpha protein level after UV radiation was analyzed. UVB radiation was repeatedly seen to cause a biphasic effect on HIF-1alpha levels in HaCaT keratinocytes. An initial decrease of the protein level, with lowest values detected at 3 hours after irradiation was visible followed by a protein level increase reaching its peak at 12 hours. 24 hours after treatment the HIF-1alpha expression of UV irradiated samples was restored to their original level (**Figure 22** and **Figure 24**).

To establish whether the changes in HIF-1alpha levels could have a downstream effect on hypoxia targets in keratinocytes the mRNA expression of well known HIF-1 targets with previously implicated UVB regulation were studied. Following UVB exposure and parallel to HIF-1alpha protein upregulation, elevated mRNA levels of HIF-1 target genes (VEGF, HMOX1) were found **(Figure 25)** in agreement with previous observations (203;210-213), suggesting the potential involvement of HIF-1alpha in the increase of VEGF and HMOX1 expression after UVB treatment. Interestingly our findings also suggest a decoupling of HIF-1alpha mRNA and protein-level expression after UVB treatment, which is not suprising in light of previous observations suggesting the paramount role of post translational regulation in determining the cellular HIF-1alpha level, and hypoxic response (228).

UV radiation can modulate various metabolic and regulatory pathways that may affect the protein level of HIF-1. These include stimulated production of ROS(252;253). UVB has been shown to induce ROS at two distinct stages: immediately following irradiation and around 3 h after irradiation(254). The initial decrease in HIF-1alpha by UVB may be attributed to ROS accumulation after UVB irradiation (203;255), although the effect of ROS on HIF-1alpha regulation is still not fully understood. UV light has also been shown to inhibit protein synthesis by the ER stress induced translational inhibition pathway (256;257). This has been demonstrated in activation of the NFκB transcription factor by UV through translational inhibition of IκBα synthesis (257).

Therefore, ER stress related inhibition of protein synthesis may also play a role alongside initial ROS accumulation and most likely these result in the initial temporary depression of HIF-1alpha by UVB. This dual effect might be a cause of the apparent "dissociation" of AKT phosphorylation and the HIF-1alpha level (**Figure 24**) after UVB treatment, which is somewhat surprising in light of recent findings indicating the crucial role of AKT/mTOR mediated HIF-1alpha upregulation(258). There is an ongoing debate about the effect of ROS on HIF-1α stabilization. Therefore our findings that UVB dramatically decreases HIF-1alpha, in the timeframe when it is known to lead to marked upregulation of ROS in keratinocytes (254) may also help better understand the complex role of ROS in hypoxic signaling.

Even though AKT mediated effects were clearly not involved in the initial down regulation of HIF-1alpha after UVB treatment based on the proposed roles of AKT mediated signaling in HIF regulation (258) and the upstream effect of PI3K in the regulation of AKT(204) the potential for PI3K inhibitors to abrogate UVB dependent HIF-1alpha upregulatation was tested. Our findings indicate that the PI3K pathway may be involved in HIF-1alpha increase after UVB irradiation. The participation of this pathway is highlighted by the inhibitory effect of wortmannin on elevated HIF-1alpha levels (**Figure 26**). Moreover our data also show phosphorylation of AKT after UVB irradiation in HaCaT cells, suggesting the induction of this pathway. Others also confirmed these findings (259). The observed increase in HIF-1alpha seen 12 hours after UVB irradiation is likely related to the HIF-1alpha protein stabilizing effect of the UV-induced PI3K enzyme. Previous studies proved the importance of the PI3K/AKT pathway on HIF-1alpha increase in several cell lines (207;260;261). However, PI3K does not exclusively exert its biologic functions through AKT phosphorylation, and AKT itself is also a target of multiple signaling pathways. Moreover the direct regulatory role of alternative pathways, such as PI3K/PKCζ (262) and p38 MAPK/AKT (263;264) might also play a role in the regulation of HIF1alpha signaling.

These findings are among the first reports showing the involvement of a HIF-1 dependent regulation in UV induced signaling, which is in agreement with the previously postulated view on HIF-1 mediated regulation as an environmental signaling pathway serving as an adaptive mechanism (265).

Conclusions

The altered differentiation of keratinocytes is an important contributor to epidermal pathogenesis (191;192;220-223). Therefore regulation of keratinocyte differentiation and transcriptional changes taking place during this process are of paramount dermatological importance. Moreover molecules capable of modifying keratinocyte differentiation are of potential dermatological and cosmetic value. Therefore we set up a system for analyzing the effects of sphingolipid compounds on keratinocyte differentiation. Assessment of the expression of more than 50 keratinocyte differentiation genes in confluence induced differentiation based on microarray measurements proved to be a valuable tool for comparing both keratinocyte differentiation promoter and inhibitor effects of control and test compounds. These findings provided evidence for the usefulness of whole genome transcriptional profiling in keratinocyte differentiation modulating compound testing, identified potent novel keratinocyte differentiation promoting agents and also gave insight into the epidermal effects of sphingolipids.

Despite the obvious importance of keratinocyte differentiation in regulating epidermal homeostasis, the transcriptional regulation and gene expression changes during this process are not well understood (116;167-169). Therefore we analyzed microarray data from keratinocytes undergoing confluence induced differentiation and identified several novel keratinocyte differentiation genes (RNF39, PKP3, HMOX1, ICEBERG, FOXQ1, ELMOD), which were also verified in a longer calcium induced differentiation model of normal human keratinocytes using TaqMan gene expression analysis. These genes suggest the involvement of previously unrecognized pathways in epidermal differentiation and may prove to be suitable targets for future drug development.

Sphingolipids are not only potential regulators of epidermal homeostasis they also serve as building blocks of the epidermal permeability barrier (236;237). ABCA12 mutations are thought to result in greatly altered epidermal structure in the form of lamellar and harlequin ichthyoses due to the abrogated transportation of sphingolipids, and deficient formation and secretion of lamellar bodies (2;95). We and others have found ABCA12 to show keratinocyte differentiation depended gene expression. Nuclear

receptors have been shown to stimulate lamellar body secretion and epidermal lipid metabolism (242), as well as cholesterol sulfate synthesis (243). LXR, PPAR-alpha, PPAR-delta and PPAR-gamma activation have been shown to improve permeability barrier homeostasis by both effecting the protein and lipid constituents of the epidermal permeability barrier (238-241). The findings presented here further demonstrate that PPAR and LXR activators increase ABCA12 expression, which may enhance sphingolipid trafficking, thereby providing another mechanism by which activators of these nuclear receptors promote epidermal permeability barrier integrity.

UVB radiation is one of the most important environmental stressors affecting the epidermis (182). Besides its direct genotoxic effect it influences most epidermal homeostatic networks (191;192). Moreover pronounced vascular changes also take place in the skin after UV irradiation (244). The pathogenic role of several hypoxia regulated genes has been proposed in these UVB induced skin changes (VEGF, HMOX1). Therefore our findings suggesting that HIF-1alpha undergoes a biphasic response to UVB in keratinocytes followed by transcriptional regulation of VEGF and HMOX1 provide evidence for the role of HIF-1alpha regulation in the UVB response. Given HIF-1alpha's potential to convey cellular metabolism from an energy rich oxidative to a low energy hypoxic state, its role in downregulating the highly energy dependent cellular metabolism of lipids after UVB can be suspected (204;265).

During our studies we analyzed the transcriptional changes taking place during keratinocyte differentiation, the transcriptional effects of sphingolipid derivatives and specific regulatory networks in the epidermis. The identified novel changes and regulatory mechanisms broaden our understanding of the processes underlying epidermal homeostasis and differentiation and may provide new potential targets for treating dermatological diseases such as atopic dermatitis and psoriasis.

Summary

The epidermis is the major interface between the human body and the environment. It protects against dehydration, chemical agents, infection and physical environmental effects. The barrier function is mutually susceptible to alterations in the normal keratinocyte differentiation process and direct barrier disruption by chemical agents or physical effects such as ultraviolet (UV) light capable of both inflicting direct and indirect barrier damage. Given the paramount importance of keratinocyte differentiation in epidermal homeostasis it might not be surprising that therapeutic agents capable of altering keratinocyte differentiation via transcriptional modification such as retinoids and active vitamin D proved to be invaluable tools in the treatment of dermatologic diseases. During our studies we aimed to uncover new aspects of the transcriptional regulation of the keratinocytes. We established a short term confluence induced normal human keratinocyte differentiation model, and validated the ability of GeneChip gene-expression analysis to test differentiation effects of sphingolipid derivatives. Analysis of expression data of more than 50 known keratinocyte differentiation genes found some of the test compounds to be potent inducers of keratinocyte differentiation. Moreover we identified previously not described keratinocyte differentiation genes involved in redox regulation and skin immune homeostasis, and verified their differentiation related expression also by long term (10 day) Ca^{+2} induced keratinocyte differentiation. When studying transcriptional regulation of the lamellar ichthyosis 2 gene, the sphingolipid transporter ABCA12, we established a potential role for nuclear receptor ligands in the therapy of this debilitating genodermatosis. Aiming to uncover novel aspects of UV mediated transcriptional changes in keratinocytes we identified the biphasic response of hypoxia factor 1-alpha to UV light, and thus a link between epidermal effects and the hypoxia system. Taken together, during our work focusing on transcriptional changes in keratinocyte differentiation and homeostasis we uncovered new genes regulated during the process, a new regulatory pathway (HIF-1alpha) involved in the maintenance of epidermal homeostasis. Furthermore we found an important new target (ABCA12) for previously indentified homeostatic regulatory circuits (nuclear receptors) in keratinocytes and identified several potential novel therapeutic agents (salicyl-sphingolipid derivatives), which modify keratinocyte differentiation.

Összefoglalás

Az epidermisz alkotja a legfontosabb, az emberi szervezet integritását a környezettől védelmező felszínt, megakadályozza a dehidrációt, védelmez kémiai behatások, fertőzések és potenciális fizikai károsító tényezőkkel szemben. Ezen védelmi, barrier funkciók károsodását egyaránt eredményezheti a keratinocyta differenciáció felborulása, és direkt hatások kémiai károsodás vagy fizikai behatás pl. ultraibolya (UV) sugárzás. A zavartalan keratinocita differenciáció epidermális barrierben betöltött szerepének ismeretében nem meglepő, hogy a keratinocita differenciációt módosítani képes ágensek (D vitamin, retinoidok) fontos eszközökké váltak a bőrgyógyászati betegségek kezelésében. Vizsgálatink során a keratinocinociták transzkripcionális regulációjának vizsgálatát tűztük ki célként. Létrehoztunk egy rövidtávú konfluencia indukálta keratinocita differenciációs modellt, hogy sphingolipid származékok keratinocyta differenciációs hatását vizsgáljuk. Több mint 50 gén GeneChip-pel történő génexpresszió analízise során találtunk néhány potens keratinocyta differenciációt indukáló molekulát. Továbbá azonosítottunk ezeddig nem leírt keratinocyta differenciációs géneket, melyek a bőr redox ill. immun szabályozásában vesznek részt, és verifikáltuk a differenciáció függő expressziójukat hosszútávú (10 nap) Ca^{2+} indukálta keratinocita differenciáció során is. A lamelláris ichthyosis 2 lokusz génje, az ABCA12 sphingolipid transzporter, transzkripcionális szabályozásának tanulmányozása során a gént kifejeződését fokozó magreceptor ligandokat azonosítottunk, melyek reményeink szerint később akár szerepet kaphatnak ezen genodermatózis terápiájában is. Az egyik legfontosabb epidermális stresszor, az UV sugárzás transzkripcionális hásait vizsgálvan a hipoxia indukálta faktor 1-alfa két-fázisú válaszát figyeltük meg, és ezáltal a hypoxia rendszer és az epidermális válasz összefüggését azonosítottuk. Mindent összevetve a keratinociták génexpresszió változásait különböző szinteken vizsgálva, azonosítottunk új keratinocita differenciációs géneket és új az epidermális homeostázis fentartásában szerepet játszó útvonalat (HIF-1alfa). Továbbá ismertettük régebbről ismert szabályozó elemek (magreceptorok) új célgénjét (ABCA12) a keratinocitákban, és leírtuk néhány molekulát (salicyl sphingolipid derivátumok), melyek az általunk azonosított jelentős keratinocita differenciációs hatásuk révén a későbbiekben új terápiás lehetőséget is jelenthetnek.

Acknowledgement

First and foremost I would like to express my gratitude to my mentors, Dr. Norbert Wikonkál and Prof. Dr. József Mandl, for the continuous help and support in the conduction of this work and in the preparation of this thesis, and all members of their laboratories at the Departments of Dermatology and Medical Chemistry (Semmelweis University, Budapest) without whom this work would not have been possible especially Dr. Livius Wunderlich and Prof. Dr. Gábor Bánhegyi. I would also like to express my deepest appreciation for the continuous support I recived from Professors Kárpáti and Horváth of the Department of Dermatology of Semmelweis University, Budapest. Moreover I wish to express my gratitude for the provided guidance, to our most important collaboration partner on these projects, Prof. Gerd Schmitz and his team at the Institute for Clinical Chemistry and Laboratory Medicine of the University of Regensburg, and Dr. Petra Schling from the University of Heidelberg. Furthermore I also wish to thank the collaboration of Dr. Yan J. Jiang and Professors Elias and Feingold from the University of California at San Francisco, and the help provided in the field of UVB expertise by Dr. Gabriella Csík and Dr. Attila Bérces of the Department of Biophysics and Radiation Biology at Semmelweis University, Budapest. Last but not least, I would like to dedicate this work to my parents who by providing a role model inadvertently started me off on the quest to becoming a researching physician, and my wife who had to put up with my long hours in the laboratory.

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List of own publications

Publications related to theses:

1. **Paragh G,** Schling P, Ugocsai P, Kel AE, Liebisch G, Heimerl S, Moehle C, Schiemann Y, Wegmann M, Farwick M, Wikonkál NM, Mandl J, Langmann T, Schmitz. Novel sphingolipid derivatives promote keratinocyte differentiation. Exp Dermatol. 2008 Dec;17(12):1004-16. (IF=3.259)

2. Wunderlich L*, **Paragh G***, Wikonkal NM, Banhegyi G, Karpati S. and Mandl J. UVB induces a biphasic response of HIF-1α in cultured human keratinocytes. Exp Dermatol. 2008 Apr;17(4):335-42. **Authors contributed equally* (IF=3.259)

3. Jiang YJ, Lu B, Kim P, **Paragh G,** Schmitz G, Elias PM and Feingold KR. PPAR and LXR Activators Regulate ABCA12 Expression in Human Keratinocytes. J Invest Dermatol. 2008 Jan;128(1):104-9. Epub 2007 Jul 5. (IF=5.251)

Publications not related to theses:

1. **Paragh G**, Kumar SM, Rakosy Z, Choi SC, Xu X, and Acs G. RNA Interference-Mediated Inhibition of Erythropoietin Receptor Expression Suppresses Tumor Growth and Invasiveness in A2780 Human Ovarian Carcinoma Cells. Am J Pathol. 2009 Apr;174(4):1504-14. Epub 2009 Mar 5. (IF=5.697 (2008))

2. Acs G, **Paragh G**, Chuang ST, Laronga C, Zhang PJ. The Presence of Micropapillary Features and Retraction Artifact in Core Needle Biopsy Material Predicts Lymph Node Metastasis in Breast Carcinoma. Am J Surg Pathol. 2009 Feb;33(2):202-10. (IF=4.020 (2008))

3. Balogh A, **Paragh G Jr,** Juhasz A, Kobling T, Torocsik D, Miko E, Varga V, Emri G, Horkay I, Scholtz B, Remenyik E. Reference genes for quantitative real-time PCR in UVB irradiated keratinocytes. J Photochem Photobiol B. 2008 Dec 11;93(3):133-9. Epub 2008 Aug 14. (IF=1.838)

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5. Kosztáczky B, Fóris G, **Paragh G Jr,** Seres I, Zsiros E, Koncsos P, Balogh Z, Paragh G. Leptin stimulates endogenous cholesterol synthesis in human

monocytes: New role of an old player in atherosclerotic plaque formation. Int J Biochem Cell Biol. 2007;39(9):1637-45. (IF=4.009)

6. Remenyik E., Torocsik D., Balogh A., **Paragh G.,** Dózsa A., Marko L., Emri G., Horkay I. Nuclear receptros (in Hungarian). Hungarian Journal of Dermatology and Venereology 2007. Jun (no IF)

7. Seres I, Foris G, Pall D, Kosztaczky B, **Paragh G Jr**, Varga Z, Paragh G. Angiotensin II-induced oxidative burst is fluvastatin sensitive in neutrophils of patients with hypercholesterolemia. Metabolism. 2005 Sep;54(9):1147-54. (IF=2.294)

8. Paragh G, Foris G, **Paragh G Jr**, Seres I, Karanyi Z, Fulop P, Balogh Z, Kosztaczky B, Teichmann F, Kertai P. Different anticancer effects of fluvastatin on primary hepatocellular tumors and metastases in rats. Cancer Lett. 2005 May 10;222(1):17-22. (IF=3.049)

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11. **Paragh G**, Torocsik D. Effect of nuclear receptor activators on the expression of ENC-1 (in Hungarian) Student thesis, University of Debrecen, 2003 (no IF)

12. Torocsik D. **Paragh G**, Role of steroid hormone receptors in myeloid differentiation in different myeloid cell lines (in Hungarian) Student thesis, University of Debrecen, 2003 (no IF)

13. **Paragh G**, Torocsik D. Expression of nuclear receptors in human placental macrophages (in Hungarian). Student thesis, University of Debrecen, 2002 (no IF)

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