# Establishment and Characterization of an *In Vitro* Skin Disease Model Based on Filaggrin Knock Down

## DISSERTATION

Zur Erlangung des akademischen Grades des

Doktors der Naturwissenschaften (Dr. rer. nat.)

eingereicht im Fachbereich Biologie, Chemie, Pharmazie

der Freien Universität Berlin

vorgelegt von

**Dominika Henkes**

aus Freiburg i. Br.

Berlin, im August 2013

**1. Gutachter:** **Frau Prof. Dr. Monika Schäfer-Korting**
Institut für Pharmazie (Pharmakologie und Toxikologie)
Freie Universität Berlin

**2. Gutachter:** **Herr Prof. Dr. Jürgen Lademann**
Klinik für Dermatologie, Venerologie und Allergologie
Charité Universitätsmedizin Berlin

**Disputation am:** **15.11.2013**

Die nachfolgende Arbeit wurde auf Anregung und unter Anleitung von

**Frau Prof. Dr. Monika Schäfer-Korting**

in der Abteilung für Pharmakologie und Toxikologie

des Instituts für Pharmazie der Freien Universität Berlin angefertigt.

Für meine Eltern

und Holger

## Danksagung

Bei Frau Prof. Dr. Schäfer-Korting möchte ich mich für die stets intensive Betreuung sowie die beständige Förderung und Unterstützung während meiner Promotionszeit bedanken.

Herrn Prof. Dr. Lademann, Charité Berlin, danke ich für die Erstellung des Zweitgutachtens.

Für die intensive Betreuung, die Überlassung ihres „Babys“ als Forschungsthema, die stetige Förderung und Motivation möchte ich Frau Dr. Sarah Küchler, Institut für Pharmazie, FU Berlin, danken. Ihre stetige Gesprächsbereitschaft und wissenschaftliche Kompetenz haben wesentlich zum Gelingen dieser Arbeit beigetragen. Darüber hinaus bedanke ich mich für alle außeruniversitären Aktivitäten und die Freundschaft die daraus entstanden ist.

Frau Dr. Eckl und Herrn Dr. Hennies, Cologne Center for Genomics, Universität Köln, danke ich für die Bereitstellung des Protokolls zur Anzucht der Hautmodelle.

Ganz besonders bedanke ich mich bei Frau Prof. Dr. Kateřina Vávrová, Frau Michaela Sochorová und Frau Barbora Školová, Faculty of Pharmacy, Charles University of Prague, für die Durchführung der Lipidanalyse des Stratum corneums meiner Hautmodelle.

Bei Frau Karin-Briest Forch, Institut für Veterinäranatomie, FU Berlin, bedanke ich mich für die Hilfe bei der Histologie. Bei Frau Ursula Puchta, Institut für Dermatologie und Allergologie, LMU München, und bei Frau Ramona Casper, Cologne Center for Genomics, Universität Köln, möchte ich mich für die Hilfe bei dem immunohistochemischen Nachweis der Differenzierungsmarker meines Hautmodells bedanken.

Frau Prof. Dr. Plendl, Institut für Veterinäranatomie, FU Berlin, und Herrn Prof. Dr. Kleuser, Institut für Ernährungswissenschaft, Uni Potsdam, danke ich für die Betreuung im Rahmen des Graduiertenstudiengangs Biomedical Sciences der Dahlem Research School, FU Berlin.

Der Dahlem Research School, FU Berlin, danke ich ganz besonders für die Vergabe eines Promotionsanschubstipendiums und eines Reisestipendiums. Ebenso danke ich

der Frauenbeauftragten des Fachbereichs Biologie, Chemie und Pharmazie, FU Berlin, für ein Reisestipendium.

Dem AK MSK danke ich für das angenehme Arbeitsklima, die gute Zusammenarbeit und den wissenschaftlichen Austausch untereinander. Ganz besonders möchte ich danken:

- Frau Dr. Sarah Heilmann und Frau Wiebke Klipper für eine sehr angenehme Büroatmosphäre, und den stetigen gemeinsamen wissenschaftlichen und privaten Austausch.
- Herrn Christian Zoschke und Frau Nhung Do für den Mediumwechsel bei meinen Modellen an so manchem Wochenende.
- Herrn Prof. Dr. Günther Weindl für seine stetige Hilfe in praktischen Fragen.
- Frau Katharina Ackermann für die Hilfe bei den Permeationsversuchen.
- Frau Hannelore Gonska für das Isolieren der Keratinozyten und Fibroblasten und jeden gut gemeinten Rat.
- Frau Gabriele Roggenbuck-Kosch und Frau Barbara Brüggener für jegliche verwaltungstechnische Unterstützung.

Dem kleinen vietnamesischen Flüchtlingskind und Prinzessin Fuchsine danke ich für die vielen gemeinsam verbrachten Stunden des Kaffee Trinkens, die Schaumparty im Labor, das Kaktuseis und eine Freundschaft, die hoffentlich noch viele weitere Jahre andauern wird.

Meiner Apfel-Milch-Gruppe danke ich, dass wir es irgendwie doch immer geschafft haben, genügend Milch für einen Kaffee vorrätig zu haben.

Bei allen meinen Freunden, ganz besonders aber bei Frau Sarina Linz und Frau Lilian Arnold, bedanke ich mich für die jahrelangen Freundschaften - auch über jegliche Distanz!

Meinen Eltern und meinen Geschwistern danke ich für die unendliche Unterstützung und Liebe, die sie mir entgegenbringen und dass sie mir die Freiheit gelassen haben meinen naturwissenschaftlichen Forschergeist zu entdecken und auszuleben.

Holger. Für einfach Alles.

## Abbreviations

| | |
|---|---|
| AD | atopic dermatitis |
| Cer | ceramides |
| Chol | cholesterol |
| DHA | docosahexaenoic acid |
| DMEM | Dulbecco's modified eagle's medium |
| DMSO | dimethyl sulfoxide |
| EBM | endothelial cell base medium |
| EGM | endothelial cell growth medium |
| ELISA | enzyme-linked immunosorbent assay |
| EU | European Union |
| FCS | fetal calves serum |
| FFA | free fatty acids |
| FGM | fibroblast growth medium |
| FLG | filaggrin |
| ft/ft | flaky tail |
| GC | glucocorticoid |
| HUVEC | human umbilical vein cord endothelial cell |
| IFNγ | interferon-γ |
| IgE | immunoglobulin E |
| IL | interleukin |
| IV | ichthyosis vulgaris |
| KBM | keratinocyte basal medium |

| | |
|---|---|
| KD | knock down |
| KGM | keratinocyte growth medium |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NHDF | normal human dermal fibroblast |
| NHE-1 | sodium/hydrogen antiporter-1 |
| NHK | normal human keratinocyte |
| NMF | natural moisturizing factor |
| OECD | Organisation for Economic Cooperation and Development |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate buffered saline |
| PCA | pyrrolidone-5-carboxylic acid |
| RT-PCR | reverse transcription polymerase chain reaction |
| SC | stratum corneum |
| SDS | sodium dodecyl sulfate |
| $sPLA_2$ | secretory phospholipase $A_2$ |
| TNFα | tumor necrosis factor α |
| UCA | urocanic acid |

## Publications and Accepted Manuscripts

S. Küchler, **D. Henkes**, K.M. Eckl, K. Ackermann, J. Plendl, H.C. Korting, H.C. Hennies, M. Schäfer-Korting: Hallmarks of Atopic Skin Mimicked In Vitro by the Means of Skin Disease Model Based on *FLG* Knock Down. ATLA 39, 471-480, 2011

K. Vávrová, **D. Henkes**, K. Strüver, M. Sochorová, B. Školová, M. Witting, W. Friess, S. Schreml, R.J. Meier, M. Schäfer-Korting, J.W. Fluhr, S. Küchler: Filaggrin Deficiency Leads to Impaired Lipid Profile and Altered Acidification Pathways in a 3 D Skin Construct. – *Accepted of Publication in the Journal of Investigative Dermatology*

## Poster Presentations

**D. Henkes**, S. Küchler, K.M. Eckl, J. Plendl, H.C. Korting, H.C. Hennies, M. Schäfer-Korting: Establishment and Characterization of In Vitro Skin Models Mimicking Hallmarks of Atopic Skin. DPhG-Doktorandentagung, Heringsdorf, Germany, 2011

S. Küchler, **D. Henkes**, K.M. Eckl, H.C. Hennies, M. Schäfer-Korting: Mimicking Hallmarks of Atopic Skin In Vitro. Gordon Research Conference, Waterville Valley, USA, 2011

**D. Henkes**, K.M. Eckl, J. Plendl, H.C. Korting, H.C. Hennies, M. Schäfer-Korting, S. Küchler: Establishment and Characterization of in Vitro Skin Models Mimicking Hallmarks of Atopic Skin. 8th World Congress on Alternatives and Animal Use in the Life Sciences, Montréal, Canada, 2011

**D. Henkes**, M. Schäfer-Korting, S. Küchler: Filaggrin Knock-Down Skin Models – A New Option In Dermatological Research. 9th International Conference and Workshop on Biological Barriers, Saarbrücken, Germany, 2012

**D. Henkes**, H.C. Korting, M. Schäfer-Korting, S. Küchler: Investigating the Impact of Filaggrin on Skin Barrier Function In Vitro – One Step in the Direction of a Skin Model Mimicking Atopic Dermatitis? 13th PPP conference, La Grande Motte, France 2012

M. Sochorova, **D. Henkes**, W. Friess, M. Schäfer-Korting, K. Vavrova, S. Küchler: Lipid Organization and Ceramide Composition of Filaggrin Knock Down Skin Models. 13th PPP conference, La Grande Motte, France 2012

**D. Henkes**, K.M. Eckl, J. Plendl, H.C. Korting, H.C. Hennies, M. Schäfer-Korting, S. Küchler: Establishment and Characterization of in Vitro Skin Models Mimicking Hallmarks of Atopic Skin. Tag der Pharmazie, Berlin, Germany, 2012

## Awards and Scholarships

Personal Scholarship (10/09 - 12/09) by the Dahlem Research School, FU Berlin

Travel Grant by the Dahlem Research School, FU Berlin, for the 8th World Congress on Alternatives and Animal Use in the Life Sciences, Montréal, Kanada, 2011

Poster-Award 9th International Conference and Workshop on Biological Barriers, Saarbrücken, Germany, 2012

Travel Grant by the Frauenbeauftragte des Fachbereichs Biologie, Chemie und Pharmazie, FU Berlin, for the 13th PPP Conference, La Grande Motte, Frankreich 2012

# Table of Contents

# 1. Introduction

## 1.1 The Human Skin

The skin forms with a surface of about two square meters and a weight of up to ten kg in adults the biggest organ of the human body and fulfills diverse responsibilities, most of them are essential for survival (Walters and Roberts, 2002). As the outermost barrier the skin protects all other organs and tissues from varying physical, chemical and pathogenic influences such as microbial invasion, radiation or the penetration of chemical substances (Elias, 2010; Proksch *et al.*, 2008; Walters and Roberts, 2002). Furthermore, it prevents excessive loss of water and electrolytes from inside the body, is part of the sensory perception system and helps to keep the body temperature constant (Elias, 2010; Walters and Roberts, 2002). The skin is thereby a rather flexible tissue (Menon *et al.*, 2012; Walters and Roberts, 2002). The skin is composed of three layers. The epidermis is the outermost and thinnest (50 - 100 µm) layer of these, while the dermis (100 - 500 µm) and the subcutis are much thicker. The overall thickness of the skin is varying between 1.5 and 4 mm depending on the site of the body. The appendages of the skin - hair follicles, nails, sebaceous and sweat glands - are contributing to the protection of the skin and support the skin in fulfilling its responsibilities, such as the thermal regulation through secretion of sweat or the protection of all other organs of the body (Walters and Roberts, 2002).

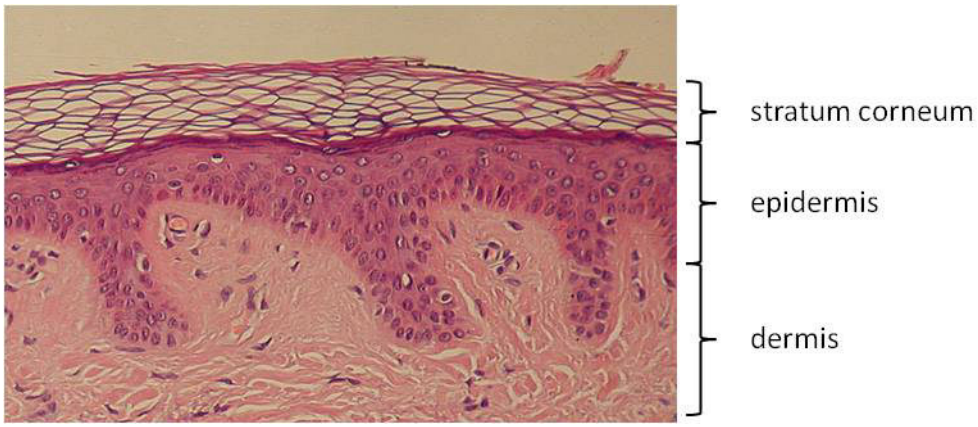


**Fig. 1 Cross-section of human skin stained with hematoxylin and eosin. Stratum corneum, epidermis and dermis can be clearly distinguished. The desquamation of corneocytes from stratum corneum when they reach the skin surface is apparent. The epidermal part is rich in cells, while the dermis is a connective tissue poor of cells (Picture: Kilbad, Fama Clamosa, License: Creative Commons by-sa 3.0.de; Source: http://commons.wikimedia.org/wiki/File:Normal_Epidermis_and_Dermis_ with_Intradermal_Nevus_10x-cropped.JPG#filelinks, accessed: 23.08.2013).**

### 1.1.1 The Epidermis

The epidermis is mainly composed of normal human keratinocytes which form about 85 % of the epidermal cells. Langerhans cells, melanocytes and Merkel cells are located in the basal or the spinous layer of the epidermis. Being free of vasculature, the epidermis is fed by the underlying dermis. The epidermis is a rather thin layer, still, it is composed of four or five layers depending on the body site, respectively. The stratum lucidum is found only in the ridged skin on the soles and palms. It's a thin, refractive layer of anucleate keratinocytes, located between the stratum granulosum and stratum corneum (SC) (Walters and Roberts, 2002). One main feature of the epidermis is the continuous self-renewing process with a total turnover once every two to three weeks through differentiation of keratinocytes which left the cell-cycle (Simpson *et al.*, 2011; Walters and Roberts, 2002). They migrate from the stratum basale through all epidermal layers up to the stratum corneum, the outermost layer of the epidermis (Candi *et al.*, 2005; Walters and Roberts, 2002). This process is accompanied by differentiation of the keratinocytes until they are fully stratified, anucleate, flattened and cornified cells (= corneocytes), which can easily be shed of off the skin surface (Walters and Roberts, 2002). Balanced proliferation of keratinocytes in the basal layer and shedding off from the stratum corneum keep the epidermal thickness constant (for review see: (Candi *et al.*, 2005). During differentiation the composition of proteins and the setup of the cytoskeleton inside the keratinocytes is completely changing (Simpson *et al.*, 2011; Walters and Roberts, 2002). Part of this process is the assembly of the cornified envelope. The cornified envelope is a scaffold of cross-linked proteins replacing the plasma membrane to which lipids from the extracellular space are covalently attached (Candi *et al.*, 2005; Kalinin *et al.*, 2002; Walters and Roberts, 2002). The cornified envelope together with the attached extracellular lipids is one major factor contributing to the barrier function of the skin (Kalinin *et al.*, 2001).

#### 1.1.1.1 The Stratum Basale

The stratum basale is the innermost layer of the epidermis making contact with the basement membrane, which is a 50 - 70 nm thick layer composed of the extracellular matrix proteins collagen IV, laminin, nidogen and fibronectin (Candi *et al.*, 2005; Simpson *et al.*, 2011; Walters and Roberts, 2002). The basement membrane divides the epidermis from the underlying dermis. The stratum basale is a single layer of highly erected keratinocytes wherein melanocytes and Merkel cells are embedded (Simpson

*et al.*, 2011; Walters and Roberts, 2002). Basal keratinocytes are attached to the basement membrane by hemidesmosomes and adherence junctions and connected among themselves by adherence junctions and desmosomes (Simpson *et al.*, 2011; Walters and Roberts, 2002). The main components of the keratin intermediate filaments of basal keratinocytes are the keratins 5 and 14 (Candi *et al.*, 2005). While basal keratinocytes are mitotic active and highly proliferative they develop upon a currently unknown stimulus to postmitotic, non-proliferative cells, detach from the basement membrane and build up the stratum spinosum (Candi *et al.*, 2005; Walters and Roberts, 2002).

#### 1.1.1.2 The Stratum Spinosum

Subsequent to the detachment from the basement membrane, keratinocytes reach the stratum spinosum, where they begin to flatten and become more and more connected among each other by desmosomes (Simpson *et al.*, 2011; Walters and Roberts, 2002). Inside of the cells the synthesis of lamellar bodies is initiated. They contain precursors of the major barrier lipids cholesterol, phospholipids, sphingomyelin and glucosylceramides. Furthermore, hydrolytic, lipid-processing enzymes, mainly β-glucocerebrosidase, acid sphingomyelinase and secretory phospholipase $A_2$ ($sPLA_2$) are part of the lamellar bodies (Feingold, 2007; Walters and Roberts, 2002). Hence, lamellar bodies are important contributors for the development of skin barrier function (Feingold, 2007). Additionally, new structural proteins are synthesized inside of the keratinocytes (for review see: (Candi *et al.*, 2005; Simpson *et al.*, 2011)). The composition of keratin intermediate filaments changes: keratin 1 and 10 are primarily expressed (Candi *et al.*, 2005). In the stratum spinosum Langerhans cells are located. Langerhans cells belong to the family of dendritic cells. Upon stimulation through antigens / haptens or other inflammatory stimuli, Langerhans cells migrate out of the epidermis to local lymph nodes and present the picked up antigens to T-lymphocytes. Thereby, the immune defense of the skin is initiated (for review see: (Kaplan, 2010)).

#### 1.1.1.3 The Stratum Granulosum

While keratinocytes differentiate further they enter the stratum granulosum. Keratohyalin granules develop inside the keratinocytes. These granules contain mainly profilaggrin, the precursor protein of filaggrin (FLG) (Walters and Roberts, 2002). FLG

itself plays an important role in skin hydration and for the development of skin barrier function, since it is responsible for aggregation and collapse of keratin intermediate filaments during keratinocyte differentiation (the role of FLG will be discussed in detail in chapter 1.2) (Brown and McLean, 2012; Sandilands *et al.*, 2009; Walters and Roberts, 2002). Coincidently, the formation of the cornified envelope is initiated by the synthesis of the major structural proteins involucrin, loricrin, trichohyalin and small proline-rich proteins (for review see: (Candi *et al.*, 2005; Kalinin *et al.*, 2002)). Involucrin is the first protein becoming attached to the inner surface of the plasma membrane, the others are cross-linked to it and among each other. Finally, the complete inner surface of the plasma membrane is covered. These attaching and cross-linking steps are catalyzed by the $Ca^{2+}$-dependent enzymes transglutaminases. They generate $N^{\varepsilon}$-(γ-glutamyl)-lysine bonds between proteins, which are mainly responsible for the insolubility of the cornified envelope (Candi *et al.*, 2005; Kalinin *et al.*, 2001; Kalinin *et al.*, 2002). Concurrent with the initiation of cornified envelope formation at the stratum granulosum - stratum corneum - interface, lamellar bodies release their content to the extracellular space by fusion of the lamellar body membrane with the plasma membrane. Thus, lipid precursors and lipid-metabolizing enzymes are released to the extracelluar space (Candi *et al.*, 2005; Kalinin *et al.*, 2002; Walters and Roberts, 2002). In the stratum granulosum, keratinocytes are connected among each other by tight junctions, which help to build up a strong barrier for big molecules. Tight junctions are composed of different proteins, including claudins, occludins and tricellulin (for review see: (Simpson *et al.*, 2011)).

#### 1.1.1.4 The Stratum Corneum

The stratum corneum is the outermost layer of the epidermis. Keratinocytes have reached their final differentiation state and developed to anucleate, dead flattened corneocytes (Elias, 2010; Kalinin *et al.*, 2002; Walters and Roberts, 2002). The plasma membrane of the cornified cells is completely replaced by the cornified envelope (Candi *et al.*, 2005). Corneocytes are connected with each other by corneodesmosomes, which derive from desmosomes through morphological changes in the transition zone between stratum granulosum and corneum (Candi *et al.*, 2005; Proksch *et al.*, 2003; Simpson *et al.*, 2011). Continuously, corneodesmosomes become degraded by proteases and corneocytes get shed of (Elias, 2010; Walters and Roberts, 2002). In the stratum corneum the precursor lipids, which were released from the lamellar bodies at the stratum granulosum - stratum corneum - interface, are

processed. While ω-hydroxyceramides attach to the cornified envelope proteins, most of the other released lipids are converted by the concurrently released enzymes to ceramides, cholesterol and free fatty acids. These lipids are aligned in intercellular lamellae, forming the lipid envelope, thus contributing to barrier function development (Candi *et al.*, 2005; Kalinin *et al.*, 2002; Walters and Roberts, 2002). Corneocytes and extracellular lipid matrix form a strong barrier. The structural behavior of the stratum corneum is often described with the term "bricks and mortar": The corneocytes are the bricks, serving mainly as physical barrier, embedded in the "mortar" lipid matrix, which covers the total space between corneocytes and represents the permeability barrier (Elias, 1983; Nemes and Steinert, 1999). Ceramides, cholesterol and free fatty acids are found in almost equimolar proportion inside the lipid lamellae. This composition is important for an intact barrier function (for review see: (Holleran *et al.*, 2006; Menon *et al.*, 2012)). One main difference of the intercellular lipid lamellae to other lipid matrices in the human body, is the absence of phospholipids in the stratum corneum (Walters and Roberts, 2002). The lamellar body-derived phospholipids are processed by secretory phospholipase $A_2$ ($sPLA_2$) to free fatty acids and glycerol. The latter has a strong water-binding capacity and, hence, contributes to stratum corneum hydration (for review see: (Feingold, 2007)). In human stratum corneum, nine major subclasses of ceramides are found. They are mainly synthesized by the degradation of glucosylceramides or sphingomyelin by β-glucocerebrosidase or acidic sphingomyelinase, respectively. Both enzymes require an slightly acidic pH for optimal activity (for review see: (Choi and Maibach, 2005; Feingold, 2007; Holleran *et al.*, 2006)). Under physiological conditions skin surface pH is between 5.0 and 5.5 (for review see: (Schmid-Wendtner and Korting, 2006), therefore being not only best for lipid processing but also limiting the colonization of pathogenic bacteria (for review see: (Chan and Mauro, 2011; Holleran *et al.*, 2006)).

### 1.1.2 The Dermis

Separated from the epidermis by the basement membrane the dermis provides plasticity and tear-resistance for the skin. Mainly fibroblasts are found there. The dermis is composed of two layers. The stratum papillare is a connective tissue where capillaries, nerve endings and immune cells are embedded. Name-giving papillae stretching out into the epidermis provide stronger adherence between epidermis and dermis. The main function of the stratum papillare is to ensure the nutrition of the epidermis and, furthermore, the transmission of mechanical stimuli to the central

nervous system. Hair, sweat and sebaceous glands have their roots and grow from here through the epidermis to the body surface. Underneath the stratum papillare, the stratum reticulare is located. It is a connective tissue with cross-linked collagen fibres, mainly contributing to elasticity of the skin (Walters and Roberts, 2002).

### 1.1.3 The Subcutis

The subcutis or hypodermis connects the skin to the underlying muscle. It is a connective tissue, mainly composed of collagen end elastin, in which lobule-shaped lipocytes are embedded. Blood vessels and nerves are located between the lipocytes. The subcutis is an energy reservoir for the skin and the rest of the body. Besides, it is able to absorb shock and hamper its implication for the underlying tissues and organs. As a fatty layer, the subcutis provides heat insulation and therefore contributes to thermal regulation (Walters and Roberts, 2002).

## 1.2 Filaggrin

### 1.2.1 Role of Filaggrin in Healthy Human Skin

Filaggrin (FLG) is an ubiquitary epithelial barrier protein and was isolated from human skin first in 1977 and initially named "stratum corneum basic protein" (Dale, 1977). Three years later the today commonly used term "filaggrin" (for: filament aggregating protein) was introduced (Steinert *et al.*, 1981). The gene is located on chromosome 1q21 inside the epidermal differentiation complex (EDC) and was successfully encoded and sequenced in 1992 (Presland *et al.*, 1992). Inside the EDC approximately sixty different genes are located, such as involucrin and loricrin. All of them are essential for epidermal maturation (for review see: (Irvine *et al.*, 2011)). In 1985 Fleckman et al. detected the protein profilaggrin in human skin and monolayer cultures of normal human keratinocytes by the use of antibodies directed against human FLG (Fleckman *et al.*, 1985). The precursor protein profilaggrin is a 400 kDa, phosphorylated, insoluble protein, which is rich in histidine. Profilaggrin is the main component of the keratohyalin granules in the stratum granulosum (for review see: (Brown and McLean, 2012; Irvine *et al.*, 2011; Sandilands *et al.*, 2009)). Structurally, the N-terminal domain of profilaggrin exhibits a $Ca^{2+}$ binding site. This suggests a possible role of $Ca^{2+}$ in profilaggrin-to-FLG processing, during which the N-terminal domain is cleaved and translocated to the nucleus. There, the N-terminal domain might play a role in the enucleation process of

keratinocytes during their differentiation (Brown and McLean, 2012; Sandilands *et al.*, 2009). The exact function of the C-terminal domain of profilaggrin is currently unknown, but it seems to be involved in the profilaggrin-to-FLG processing as well (Brown and McLean, 2012; Sandilands *et al.*, 2009). Between N- and C-terminal domain ten to twelve FLG repeats are found, the number is varying in individuals (Gan *et al.*, 1990). While keratinocytes change from granular to cornified cells, profilaggrin is processed to monomeric FLG. The degradation is initiated by an intracellular increasing $Ca^{2+}$ concentration in the outer cell layer of the stratum granulosum and is catalyzed by protein phosphatases and serine proteases (Heimall and Spergel, 2012; Sandilands *et al.*, 2009). The monomeric FLG is a 37 kDa protein, which binds to keratin intermediate filaments. Thus, FLG is supporting their aggregation into macro fibrils and leading to the collapse of the cytoskeleton. Hence, FLG is directly involved in keratinocyte flattening and squame-forming inside the stratum corneum (Brown and McLean, 2012; O'Regan and Irvine, 2008; Sandilands *et al.*, 2009). Subsequently, the collapsed and compacted keratins are cross linked by transglutaminases to form an insoluble keratin matrix, which is the scaffold for formation of the cornified envelope (Candi *et al.*, 2005; Irvine *et al.*, 2011; Sandilands *et al.*, 2009). Immediately after its release from profilaggrin, monomeric FLG is further degraded. Peptidyl arginine deiminases catalyze a citrullination step leading to a change of the net charge of FLG from basic to neutral (Sandilands *et al.*, 2009). Hence, the protein structure is unfolded making it more susceptible to proteolysis by caspase 14 to histidine (Hoste *et al.*, 2011; Irvine *et al.*, 2011; Sandilands *et al.*, 2009). Subsequently, histidine is further degraded and hydrophilic amino acids, including urocanic acid (UCA) and pyrrolidone-5-carboxylic acid (PCA), are formed (Brown and McLean, 2012; Sandilands *et al.*, 2009). As a part of the natural moisturizing factor UCA and PCA contribute to the stratum corneum hydration, since they are able to osmotically draw water into corneocytes (Elias *et al.*, 2008; Rawlings and Harding, 2004; Sandilands *et al.*, 2009). Furthermore, the role of UCA and PCA in UV protection (Mildner *et al.*, 2010; Sandilands *et al.*, 2009) and in the maintenance of skin surface pH is under discussion (Fluhr *et al.*, 2010; Krien and Kermici, 2000; Sandilands *et al.*, 2009). An acidic skin surface pH is required for the development of barrier function of the skin, since the two major ceramide generating enzymes, β-glucocerebrosidase and acidic sphingomyelinase, have their optimal activity in an acidic milieu (Choi and Maibach, 2005; Feingold, 2007; Holleran *et al.*, 2006). Furthermore, it has been demonstrated, that an elevation of skin surface pH results in impaired stratum corneum cohesion and disturbed permeability barrier homeostasis (Hachem *et al.*, 2003). The FLG degradation products UCA and PCA

might contribute to the acidification (Krien and Kermici, 2000). Still, a recent study by Fluhr et al. questions them being essential for acidification (Fluhr *et al.*, 2010). Rather, the importance of the known pathways via secretory phospholipase $A_2$ and the sodium/hydrogen antiporter-1 (NHE-1) have been strengthened (Chan and Mauro, 2011; Fluhr *et al.*, 2004; Fluhr *et al.*, 2010).

In summary, FLG is directly and indirectly important for the establishment of skin barrier function and might contribute to the hydration and acidification of the SC.

### 1.2.2 Loss-of-Function Mutations in *FLG*

Loss-of-function mutations in the gene encoding for *FLG* lead to reduced or absent FLG expression in the skin. They are well-known risk factors for the development of ichthyosis vulgaris (IV) and atopic dermatitis (AD) (Barker *et al.*, 2007; Palmer *et al.*, 2006; Sandilands *et al.*, 2006; Smith *et al.*, 2006; Weidinger *et al.*, 2006). IV and AD appear often in parallel: 37 - 50 % of all IV patients develop an associated AD, whereas only 8 % of AD patients develop IV symptoms (Smith *et al.*, 2006). Up to now, twenty-three different mutations of *FLG* are known in the European population, with the most prominent R501x and 2282del4 existing in 80 % of all mutation carriers (Brown and McLean, 2012; Heimall and Spergel, 2012; Smith *et al.*, 2006). In contrast, in the Asian population currently even thirty mutations are described with none being predominant (Heimall and Spergel, 2012). In Europe, about 10 % of the population carries at least one mutation in *FLG* (Barker et al., 2007). Loss-of-function mutations carriers produce unstable profilaggrin and therefore miss profilaggrin-to-FLG processing leading to reduced FLG content in the skin (Brown and McLean, 2012). Smith et al. demonstrated the complete absence of keratohyalin granules in skin from patients with mutations in both alleles in IV patients (Smith *et al.*, 2006). Furthermore, the FLG breakdown products, UCA and PCA, are found to be reduced in correlation to the extent of the reduced FLG content in the skin (Kezic *et al.*, 2008; Kezic *et al.*, 2011; O'Regan *et al.*, 2010).

The flaky tail (ft/ft) mouse represents the animal model of *FLG* loss-of-function mutations. Scharschmidt et al. demonstrated in the ft/ft mouse, that the mutation in *FLG* alone suffices to induce a barrier abnormality and low-grade inflammation in the skin. Permeability of water-soluble substances through the paracellular route was increased and the lamellar body secretion disturbed, while skin surface pH and transepidermal water loss were not altered (Scharschmidt *et al.*, 2009). These results

extended those from Fallon et al. which revealed an increased immunoglobulin E (IgE) serum level and epidermal hyperplasia in ft/ft mice (Fallon *et al.*, 2009).

Furthermore, in IV patients with known mutations in *FLG* a disorganization of keratin filaments concurrent with alterations in the cornified envelope structure and impaired lamellar body formation and loading was described just recently (Gruber *et al.*, 2011). Hence, loss-of-function mutations in *FLG* are major risk factors for a disturbance of normal epidermal development.

## 1.3 Common Skin Disorders Based on Mutations in *FLG*

### 1.3.1 Atopic Dermatitis (AD)

AD is a chronic skin disease with inflammatory pathogenesis, which belongs to the major public health problems in the industrialized world. Currently, the rising prevalence in children is about 20 % whereas in adults only 1 - 3 % are affected (Bieber, 2010; Brown and McLean, 2012; Leung *et al.*, 2004; Ring *et al.*, 2012a). In most cases, the first symptoms arise in the early months of life, with milk crust and eczema on the cheeks being the most prominent features (Bieber, 2010). While the patients grow older, the eczema spreads to the flexural areas, the nape and the dorsum of feet and hands (Fig. 2). Most of the children outgrow the disease, but in some cases the disease persists into adulthood, where mainly the flexural areas, the head and neck are affected (Bieber, 2010). AD is considered to be the starting point of

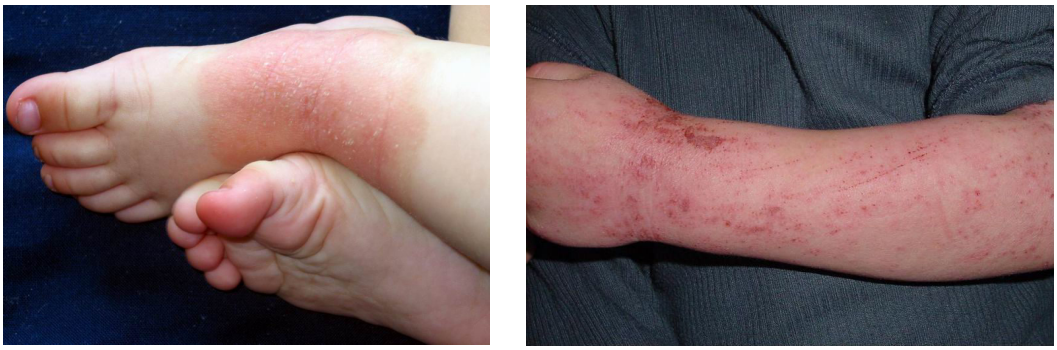

**Fig. 2 Atopic eczema on the flexural areas of a child. Right picture shows the typical inflamed and dry skin of a healing lesional area. Left picture shows the typical itch-scratch-cycle. Inflamed scratch marks are the result of unbearable pruritus (Left picture: Bernd Untiedt; License: GNU Free Documentation License, Source: http://de.wikipedia.org/wiki/Datei:Neurodermitis1.jpg; Right picture: Eisfelder, License: GNU Free Documentation License, Source: http://de.wikipedia.org/wiki /Datei: Atopic_dermatitis_child.JPG; accessed: 23.08.2013).**

the so-called atopic march, wherein patients suffer from allergic rhinitis and asthma subsequently to the development of AD (Leung *et al.*, 2004; Marenholz *et al.*, 2006). In the past, two subtypes of AD have been described in the literature: an intrinsic and an extrinsic form, with the latter having the much higher occurrence. They have been distinguished from another by the presence of elevated serum levels of immunoglobulin E (IgE) in the extrinsic subtype, which are missing in the intrinsic form (Leung *et al.*, 2004). In 2003 the World Allergy Organization redefined the term atopy: from then on only the extrinsic subtype is described by the term atopic dermatitis (Johansson *et al.*, 2004). The main features of AD are the dry and itchy skin, the inflammation caused by cutaneous hyperactivity to environmental triggers and the increased serum levels of IgE (Bieber, 2010; Leung *et al.*, 2004; Liu *et al.*, 2011). Patients are suffering from a poorer quality of life, mainly due to the consistent pruritus, which worsens during night time harmfully affecting the recreative sleep (Bieber, 2010; Spergel and Paller, 2003). Even the normal-appearing, non-lesional skin of AD patients is affected. It reveals a xerotic appearance and a marginal inflammation, as shown by a mild infiltrate with T-cells (Bieber, 2010; Leung *et al.*, 2004; Proksch *et al.*, 2003). Additionally, in acute eczema of AD patients the epidermis presents spongiosis (Bieber, 2010) and the dermis a marked infiltrate with T-cells and monocyte-derived macrophages. Furthermore, a peripheral eosinophilia is found (Bieber, 2010; Leung *et al.*, 2004; Liu *et al.*, 2011). Chronically inflamed eczematous sites show lichenification with increased epidermal thickness and hyperkeratosis (Bieber, 2010; Leung *et al.*, 2004). In the acute phase, inflammation is driven by Th2 cytokines, mainly interleukin (IL)-4 , IL-13 and IL-5. The initial Th2-based inflammatory response changes to a Th0/1-type concurrently with the switch from acute to chronic state of the eczema. In the chronic state in particular interferon-γ (IFNγ), IL-12 and granulocyte macrophage colony-stimulating factor (GM-CSF) contribute to the maintenance of the inflammation (Bieber, 2010; Leung *et al.*, 2004). The xerosis, which is found in lesional and non-lesional skin of AD patients, is accompanied by an increased transepidermal water loss (Bieber, 2010; Proksch *et al.*, 2003). Additionally, the dryness of the skin may worsen the defect in skin barrier function found in AD patients. The disturbed barrier enhances the transcutaneous uptake of potential allergens. Subsequently, these allergens stimulate inflammatory reactions, which harm the skin barrier even further (Bieber, 2010; Leung *et al.*, 2004; Proksch *et al.*, 2003). Another quite often found feature of AD is an infection of the skin with *Staphylococcus aureus*. These and other bacterial infections can be attributed to the decrease in antimicrobial peptides and the elevated skin

surface pH found in the skin of AD patients. Skin infections might contribute to a further exacerbation of the AD (Bieber, 2010; Elias, 2010; Leung, 2003).

In AD the lipid composition and content of the stratum corneum is altered. The overall lipid concentration is significantly lower compared to normal human skin. Mainly the ceramides and of these primarily ω-hydroxyceramide (= Cer 1) are affected (Choi and Maibach, 2005; Holleran *et al.*, 2006; Jakasa *et al.*, 2011; Jensen *et al.*, 2004; Jungersted *et al.*, 2010; Pilgram *et al.*, 2001; Proksch *et al.*, 2003). Pilgram et al. demonstrated a more hexagonal instead of orthorhombic lateral lipid organization in the skin from AD patients. This might cause the impaired barrier function (Pilgram *et al.*, 2001). A disordered lamellar lipid organization was also demonstrated in skin from AD patients by others and strengthened the hypothesis that the disturbed lipid organization harmfully effects skin barrier function (Choi and Maibach, 2005; Groen *et al.*, 2011). The observed changes in lipid composition and organization might be related to a defective synthesis of lamellar bodies in the stratum granulosum or a disturbed secretion of their components into the stratum granulosum - stratum corneum - interface (Cork *et al.*, 2009; Gruber *et al.*, 2011; Pilgram *et al.*, 2001; Proksch *et al.*, 2003). Moreover, changes in the sphingomyelin metabolism, such as the reduced activity of the acidic sphingomyelinase, lead to the decreased ceramide levels in AD skin (Jensen *et al.*, 2004; Proksch *et al.*, 2003). Furthermore, the ceramide-precursor processing enzymes sphingomylein deacylase and glucosylceramide deacylase are a unique feature of AD skin. These enzymes hydrolyze sphingomyelin or glucosylceramide, respectively, to their lyso-forms instead of producing ceramides, and therefore might contribute to the lower ceramide content found in AD skin (Choi and Maibach, 2005; Holleran *et al.*, 2006).

#### 1.3.1.1 AD Pathophysiology

The pathophysiology of the disease is rather complex, with an interplay between several affected genes, which can cause defects in skin barrier function and diminish the inflammatory threshold, and the environment the patient is living in, such as the exposure to potential allergens or the psychological stress (for review see: (Bieber, 2010; Elias, 2010; Leung *et al.*, 2004)). During childhood food allergies, e.g. to egg, milk or wheat, are often found. While patients get older, they often loose food allergies, but start to suffer from sensitization to inhalant allergies, like pollen or dust mite (Leung *et al.*, 2004). Psychological stress is a known exacerbating factor based on its potential

to trigger inflammatory reactions (Garg *et al.*, 2001). Scratching, caused by unbearable pruritus, leads to further mechanical impairment of the skin barrier and proceeds the inflammation, which in turn intensifies the pruritus and activates the patient to intensify scratching - a hard to break itch-scratch-cycle is initiated (Heimall and Spergel, 2012; Leung *et al.*, 2004).

In 2006 loss-of-function mutations in *FLG* were demonstrated to be one of the most important predisposing factors for AD development (Palmer *et al.*, 2006). Such mutations have been shown to lead to a more severe disease pattern (Heimall and Spergel, 2012; Sandilands *et al.*, 2009), which starts earlier in life and bears a greater risk of lasting into adulthood (Barker *et al.*, 2007). Furthermore, the patients are more susceptible to suffer from skin infections (Heimall and Spergel, 2012; Irvine *et al.*, 2011). *FLG* mutations are considered to favor all steps of the atopic march, namely the development of allergic rhinitis and asthma (Marenholz *et al.*, 2006; Weidinger *et al.*, 2008b). Still, it has to be mentioned, that only 14 - 56 % of all AD patients carry a mutation in *FLG* (O'Regan and Irvine, 2008). Furthermore, merely around 42 % of *FLG* mutation carriers develop AD, thus, other contributing factors have to be considered as well (Irvine *et al.*, 2011). In the past, AD development has been believed to be mainly caused by immunological deficiencies which in turn trigger abnormal skin barrier function development (inside-to-outside pathophysiology hypothesis) (Leung *et al.*, 2004). Just recently, with the discovery of mutations in *FLG* as predisposing factor for AD development, Elias et al. substantiated the idea of the outside-to-inside hypothesis of disease pathophysiology (Elias *et al.*, 2008). They proclaimed that patients start to suffer from a defect in skin barrier function and subsequently develop the inflammatory feature of the disease. They justified their idea with the finding that the extent of barrier dysfunction correlates with disease severity (Chamlin *et al.*, 2002; Sugarman *et al.*, 2003). Due to the non-sufficient skin barrier in AD patients, the permeation of environmental allergens into the skin is enhanced (Bieber, 2010; Jakasa *et al.*, 2011). Up taken allergens initiate the release of Th2 cytokines and therefore trigger the inflammatory process in the skin (Jensen *et al.*, 2004; Leung *et al.*, 2004; Neis *et al.*, 2006). Subsequently, the inflammation might impair the skin barrier function further (Hatano *et al.*, 2005; Howell *et al.*, 2007). The outside-to-inside hypothesis has been strengthened by studies with ft/ft mice. The animal model of *FLG* mutations exhibits a defective skin barrier and increased allergen ingress. The basal barrier abnormality is further impaired by allergens making contact with the skin (Fallon *et al.*, 2009; Scharschmidt *et al.*, 2009). Still, it is not exclusively clarified how the reduced expression of FLG leads to a defective skin barrier. Further contributing factors are

needed (Elias *et al.*, 2008; Weidinger *et al.*, 2008b). Besides, there are AD patients who do not carry a mutation in *FLG* at all (O'Regan and Irvine, 2008). Interestingly, an atopic inflammatory reaction itself can decrease the expression of FLG in the skin in AD patients, who do not carry a *FLG* mutation (Howell *et al.*, 2007).

Besides the well-known loss-of function mutations in *FLG* several other genetic loci leading to the development of AD are discussed in the literature. A polymorphism in the serine protease kallikrein-peptidase 7 gene (*KLK7*), which is encoding stratum corneum chymotryptic enzyme has been demonstrated to be associated with AD in one study (Vasilopoulos *et al.*, 2004) but could not be confirmed in another study (Weidinger *et al.*, 2008a). Since the stratum corneum chymotryptic enzyme has been discussed to be involved in profilaggrin - to - FLG processing (Descargues *et al.*, 2005; Resing *et al.*, 1995) a reduced expression of stratum corneum chymotryptic enzyme might lead to a decreased release of FLG from profilaggrin. Furthermore, the stratum corneum chymotryptic enzyme might be regulated by the serine protease inhibitor lymphoepithelial Kazal-type inhibitor (LEKTI), which is encoded by the serine protease inhibitor Kazal-type 5 gene (*SPINK5*) (Bitoun *et al.*, 2003; Komatsu *et al.*, 2002). Defects in *SPINK5* have been reported to be related to Netherton syndrome (Sprecher *et al.*, 2001). An association to AD has been suggested by some (Kato *et al.*, 2003; Weidinger *et al.*, 2008a), but could not be confirmed by others (Folster-Holst *et al.*, 2005). So far, the relevance of *KLK7* and *SPINK5* for AD development is not exclusively clarified and further studies are needed. Furthermore, the expression of thymal stromal lymphopoietin (TSLP) is upregulated in lesional skin of AD patients (Soumelis *et al.*, 2002; Ziegler and Artis, 2010). TSLP induces the Th2 inflammatory response in mouse models (Yoo *et al.*, 2005; Ziegler and Artis, 2010), thus, it might be one further predisposing factor leading to AD development. New genetic risk loci such as the *OVOL1* (ovo-like 1 involved in the regulation of the development and differentiation of epithelial tissues) or *ACTL9* (actin like protein 9) have just recently been identified, but further research needs to be done to determine the impact on AD development (Hirota *et al.*, 2012; Paternoster *et al.*, 2011).

#### 1.3.1.2 Therapeutic Options

Currently used therapeutic options are not addressing the underlying pathological mechanisms but are mainly focusing on controlling inflammation (for review see: (Elias *et al.*, 2008)). The gold standard anti-inflammatory therapy is the topical application of

glucocorticoids (Elias, 2010; Leung *et al.*, 2004). Glucocorticoids bind to their cytoplasmic receptor and translocate to the nucleus, where the transcription of pro-inflammatory cytokines (such as IL-4, IL-5 or IL-13), chemokines (such as IL-8 or eotaxin) and adhesion molecules (such as intercellular adhesion molecule (ICAM)-1 or E-selectin) is reduced through inhibition of the transcription factor NFκB (Leung *et al.*, 2004). When used under a strict therapy regime, glucocorticoids are proven to be safe and effective (Ring *et al.*, 2012a). However, side effects, such as skin atrophy, limit their long term use and the application on delicate skin areas like the face (Blume-Peytavi and Metz, 2012; Katoh, 2009; Ring *et al.*, 2012a). Therefore, topical application of glucocorticoids is limited to acute lesions in AD patients (Katoh, 2009; Ring *et al.*, 2012a). Alternatively, topical calcineurin inhibitors, such as pimecrolimus and tacrolimus, are on the market. They bind to the cytoplasmic protein macrophilin and thus inhibit the activity of the phosphatase calcineurin. Subsequently, the dephosphorylation of the nuclear factor of activated T cell protein (NF-ATp) and hence the transcription of inflammatory cytokines (such as IL-2, -4, -5) is inhibited. T cells fail to be activated (Leung *et al.*, 2004). Calcineurin inhibitors provide a good anti-inflammatory effect and have only minor acute side effects. Yet, their potential risk for triggering the development of skin cancer subsequent to their long-term use is discussed (Artik and Ruzicka, 2003; Bieber, 2010; Leung *et al.*, 2004). In contrast to topical glucocorticoids calcineurin inhibitors can be used in the face (Artik and Ruzicka, 2003). For improvement of skin hydration the use of emollients is widely-spread and has been demonstrated to reduce the use of topical glucocorticoids (Blume-Peytavi and Metz, 2012; Katoh, 2009; Ring *et al.*, 2012a). Since dry skin is one main feature of AD, emollients are recommended for daily use even in non-lesional skin (Blume-Peytavi and Metz, 2012; Ring *et al.*, 2012a). Usually, emollients contain a moisturizer such as urea or glycerol, and should be free of any possible allergens to reduce the risk of allergic sensitization (Ring *et al.*, 2012a). For severe AD in adults, which cannot be improved by topical treatment, a systemic therapy with oral glucocorticoids is indicated, but should be restricted to acute exacerbations (Ring *et al.*, 2012b). Side effects, such as ulcer or osteoporosis, limit their long-term use (Ring *et al.*, 2012b). Another systemic therapeutic option for severe, therapy-resistant cases is ciclosporin A (Artik and Ruzicka, 2003). Ciclosporin A inhibits the calcineurin phosphatase and thus leads to reduced expression of IL-2 and inhibition of the activation of T cells (Katoh, 2009). Ciclopsorin A should be used for short term only, since severe side effects, mainly nephrotoxicity, can appear (Katoh, 2009; Leung *et al.*, 2004). Since most patients show an improvement of symptoms during summer months, phototherapy with

narrow-band UV-B or UV-A light might provide another option for treatment of adults and children > 12 years (Ring *et al.*, 2012b). The inflammatory feature of AD skin is improving, most probably through alterations in cytokine production or apoptosis of immune cells (Leung *et al.*, 2004; Ring *et al.*, 2012b). However, phototherapy should not be used in the acute state of the disease and the risk for developing skin cancer needs to be considered. Currently, phototherapy is recommended as second line treatment (Ring *et al.*, 2012b). Besides pharmacological therapy, the knowledge of the patient about his disease, his personal allergens and the avoidance of other triggering factors, like psychological stress, is one major factor helping to improve the disease state and therefore the quality of life of the patient (Bieber, 2010; Elias, 2010).

### 1.3.2 Ichthyosis Vulgaris (IV)

IV belongs to the most prevalent keratinization disorders and can be attributed to a defect in one single gene: *FLG* (Sandilands et al., 2006; Sandilands et al., 2007; Smith et al., 2006). Its prevalence is about 1 in 250 British school children (Smith *et al.*, 2006; Wells and Kerr, 1966). The first symptoms usually develop early in life, are worst during childhood and improve while patients grow older but never get totally lost (Hoffjan and Stemmler, 2007; Okulicz and Schwartz, 2003; Schmuth *et al.*, 2007). Characteristic feature is the abnormal desquamation resulting in fine scaling, predominantly found on the extensor side of arms and legs. The skin looks dry and rough, hyperlinearity of the palms and soles is often found (Hoffjan and Stemmler, 2007; Okulicz and Schwartz, 2003; Schmuth *et al.*, 2007). Symptoms usually improve during summer time and in warmer climates (Okulicz and Schwartz, 2003). In histological examination, the reduced number or total absence of keratohyalin granules in the stratum granulosum is the most prominent feature (Fleckman and Brumbaugh, 2002). These findings led to the detection of *FLG* mutations as factor for IV development (Sandilands *et al.*, 2006; Sandilands *et al.*, 2007; Smith *et al.*, 2006). These mutations are inherited in an autosomal semidominant manner, resulting in a mild phenotype in heterozygous and a more severe phenotype in homozygous or compound heterozygous patients (Irvine *et al.*, 2011; Sandilands *et al.*, 2009; Schmuth *et al.*, 2007; Smith *et al.*, 2006). In contrast to AD, inflammation of the skin is no feature of IV. It is only seen in patients with associated AD (Vahlquist *et al.*, 2008). As described above, loss-of-function mutations in *FLG* lead to decreased levels of natural moisturizing factor and consequently to reduced skin hydration resulting in the dry skin phenotype found in IV patients (Irvine *et al.*, 2011).

#### 1.3.2.1 Therapeutic Options

Current therapeutic options are addressing the symptoms but do not improve the underlying pathologic mechanisms of the disease (for review see: (Krug *et al.*, 2009; Vahlquist *et al.*, 2008)). The main goals of the treatment are the hydration and the keratolysis, since dry, scaly skin is the most prominent feature of IV (Vahlquist *et al.*, 2008). For the improvement of skin hydration α-hydroxy-acids (such as lactic acid), urea or glycerol are most often used (Krug *et al.*, 2009; Okulicz and Schwartz, 2003; Vahlquist *et al.*, 2008). They provide good water-holding capacity and have only minor side effects, such as slight irritation of the skin or a burning feeling (Krug *et al.*, 2009; Vahlquist *et al.*, 2008). In addition, α-hydroxy-acids provide a keratolytic effect and therefore help to accelerate desquamation (Vahlquist *et al.*, 2008). A stronger keratolytic effect is achieved by salicylic acid or topical retinoids (Krug *et al.*, 2009; Vahlquist *et al.*, 2008). In severe cases retinoids should be administered orally (Krug *et al.*, 2009; Vahlquist *et al.*, 2008). After binding to their cytoplasmic receptor, retinoid-receptor-complexes dimerize and translocate into the nucleus where they interfere with the transcription of diverse genes, such as inflammatory cytokines. Subsequently, retinoid-receptor-complexes stimulate mitosis and the turnover of keratinocytes resulting increased desquamation of the skin (for review see: (Okulicz and Schwartz, 2003)). Since currently available therapeutic options are not addressing the underlying pathologic condition, research is strongly needed to provide better therapies for the patients.

## 1.4 *In Vitro* Skin Models

The marketing authorization of new drugs requires next to the proof of effectiveness a careful assessment of the potential risk for the user. The experiments to assess the pharmacokinetic, -dynamic and toxicological profile of the substance are usually performed in animals before clinical trials in humans are conducted (FDA, 2006; ICH, 2013). For chemical substances the requirements for the registration have just recently been changed completely with the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) legislation by the European Parliament and Council (EuropeanParliament and Council, 2006). From then on, all chemical substances - including all "old" already available chemicals - need to be registered at the European Chemicals Agency ahead of their appearance on the European market. Therefore, risk assessment of the chemicals is required. So, for registration of chemicals and the

marketing authorization of new drugs general toxicological aspects, such as carcinogenicity or genotoxicity, and furthermore the potential risk when intentionally or unintentionally applied onto human skin need to assessed (EuropeanParliament and Council, 2006). Hence, companies have to provide data on skin irritation or corrosion, skin sensitization and skin absorption of chemical substances, pharmaceutical drugs and cosmetic products (EuropeanCommission, 1976; EuropeanParliament and Council, 2006; ICH, 2013). Due to ethical concerns and regulatory requirements the 7th amendment of the Cosmetic Directive of the European Union (EuropeanParliament and Council, 2003) bans the testing of cosmetic products and its ingredients on animals. Thus, the use of animals to conduct such studies is more and more limited. Beside regulatory requirements and ethical concerns, the benefit of animal experiments is discussed controversially (Mestas and Hughes, 2004; Pound *et al.*, 2004; Seok *et al.*, 2013; van der Worp *et al.*, 2010). Differences in the inflammatory response (Seok *et al.*, 2013), the innate and adaptive immune system (Mestas and Hughes, 2004) or the metabolism of xenobiotics (Hartung, 2008) are only some to mention. Furthermore, methodological mistakes, such as the use of to low or to high doses of the compound or the animal study group not reflecting the situation in humans, limit the transferability of the results obtained in animals onto humans (Pound *et al.*, 2004; van der Worp *et al.*, 2010).

For the evaluation of skin irritation, corrosion or absorption the use of human cadaver skin and *in vivo* studies in humans is limited as well, since ethical concerns are rising. Furthermore, major differences in the biotransformation of compounds in comparison to native human skin make results not fully reliable (Baetz *et al.*, 2012). Hence, much effort has been made to develop an *in vitro* test system which provides a reliable basis for risk assessment and which is free of any ethical concerns. Based on these requirements the establishment and characterization of reconstructed human skin or epidermis for *in vitro* risk assessment and pharmacological testing has been pursued (for review see: (Gibbs, 2009; Netzlaff *et al.*, 2005; Ponec, 2002; Van Gele *et al.*, 2011)). The development of such *in vitro* skin or epidermis models emerged from the discovery, that the differentiation of normal human keratinocytes can be achieved *in vitro* by culturing them at the air-liquid interface (Prunieras *et al.*, 1983). Subsequently, the development of reconstructed skin for the replacement in wounds, such as burns (MacNeil, 2007), and for *in vitro* risk assessment was initiated. Today, there a several different types of skin or epidermis models for *in vitro* research described in the literature. Some are in-house models developed by researchers (Carlson *et al.*, 2008), others are commercially available and used by many different groups all over the world

(e.g. EpiDerm™, MatTek, Ashland, MA, USA; EpiSkin by SkinEthic, Lyon, France; epiCS). Today, reconstructed human epidermis is approved by the Organization for Economic Cooperation and Development (OECD) for *in vitro* testing on skin corrosion and skin irritation, hence, explicit guidelines for conducting these experiments are published (OECD, 2004b, 2010). The use of reconstructed human epidermis for the assessment of skin absorption was validated in 2008 in a large study in Germany (Schäfer-Korting *et al.*, 2008). The OECD is in the process of approving this method and stated the use of reconstructed human epidermis acceptable in the case that the obtained data from reference substances are comparable to those obtained by using human skin *ex vivo* (OECD, 2004a; Van Gele *et al.*, 2011).

*In vitro* skin models are classified into two subtypes: The first is mimicking the epidermal part of human skin only and is also referred to as reconstructed human epidermis (RHE). Besides these epidermal models, so-called full-thickness *in vitro* skin models have been developed. They are composed out of a dermal equivalent on which the epidermal layer is grown (for review see: (Gibbs, 2009; Van Gele *et al.*, 2011)). The construction of the epidermal layer is based on the use of normal human keratinocytes (NHKs). For the preparation of reconstructed human epidermis NHKs are seeded onto inert filter membranes, usually composed of cellulose acetate or polycarbonate (Gibbs, 2009; Van Gele *et al.*, 2011). For the development of reconstructed human full thickness skin, the dermal layer has to be established before keratinocytes can be seeded on top. The dermal layer can be mimicked either by the use of a de-epidermized dermis obtained from human cadaver skin (Van Gele *et al.*, 2011) or by the use of a jelly-like collagen-matrix in which normal human dermal fibroblasts are embedded (Carlson *et al.*, 2008; Van Gele *et al.*, 2011). On top of the dermal equivalent NHKs are seeded. As soon as confluence of NHKs either on the filter membrane or on the artificial dermis is reached, the culture is lifted to the air-liquid interface and fed with a special medium from beneath the filter membrane or the dermal equivalent, respectively. During the next days of cultivation keratinocytes differentiate to a fully developed epidermis with all viable layers and a competent stratum corneum. Depending on the protocol, which includes the quantity of keratinocytes seeded and the mixture of differentiation medium used, the constructed *in vitro* models can be used at a special time point to conduct the desired experiment (Batheja *et al.*, 2009; Carlson *et al.*, 2008; Gibbs, 2009; Van Gele *et al.*, 2011).

Commercially available reconstructed epidermis and skin models have been characterized thoroughly in terms of morphology, lipid composition, differentiation

markers and metabolic activity (Luu-The *et al.*, 2009; Netzlaff *et al.*, 2005; Ponec *et al.*, 2002; Ponec *et al.*, 2000). Furthermore, the suitability of reconstructed human epidermis as test systems for skin irritation, skin corrosion and skin absorption has been proven (Kandarova *et al.*, 2006; Netzlaff *et al.*, 2005; Schäfer-Korting *et al.*, 2008; Schäfer-Korting *et al.*, 2006; Spielmann *et al.*, 2007; Welss *et al.*, 2004). It is clearly demonstrated, that *in vitro* models do successfully mimic the morphological structure of their *in vivo* counterpart. The major epidermal layers - stratum corneum, granulosum, spinosum and basale - are developed (Netzlaff *et al.*, 2005; Ponec, 2002; Ponec *et al.*, 2002). Main structural components, such as keratohyalin granules or lamellar bodies indicate the successful mimicking of the differentiation process of keratinocytes (for review see: (Netzlaff *et al.*, 2005; Ponec, 2002)). This is strengthened further by the detection of relevant differentiation markers, such as involucrin, loricrin, keratin 1 or keratin 10, in the skin models (Netzlaff *et al.*, 2005; Ponec, 2002). On the ultrastructural level minor differences, for example in the cellular shape or the presence of lipid droplets in some skin models, was observed (Netzlaff *et al.*, 2005; Ponec *et al.*, 2002). All major lipid classes are found in reconstructed human epidermis but all evaluated models revealed minor differences in comparison to human skin (Netzlaff *et al.*, 2005; Ponec, 2002; Ponec *et al.*, 2002). Besides, the lipids which are attached covalently to the cornified envelope in human skin *in vivo* have been demonstrated to be generated *in vitro* (Ponec, 2002), too, and thus form the scaffold for the correct alignment of the extracellular lipids into lamellae as demonstrated by evaluation of the ultrastructure (Ponec *et al.*, 2002). In conclusion, the general lipid composition is rather closely mimicking the *in vivo* situation (Netzlaff *et al.*, 2005), but there are still differences in the lipid organization *in vitro* and *in vivo* (Ponec, 2002). *In vitro* mainly the hexagonal packing has been demonstrated, whereas in vivo mainly the orthorhombic packing is found (Ponec, 2002; Thakoersing *et al.*, 2012).

However, those skin models also bear disadvantages which have to be taken into account. One major problem of reconstructed human epidermis and skin, respectively, is the limited life span of the cultures. This has improved in the past few years with the use of serum-free media and the reduction of epidermal growth factor concentration in the differentiation medium (Gibbs *et al.*, 1997; Van Gele *et al.*, 2011). Still, the ongoing contraction of the collagen matrix in full thickness models, the increased thickening of the stratum corneum and the reduced thickness of the viable epidermis with prolonged cultivation time, are restricting the use of the constructs to several days in which the epidermis is fully developed and the construct still in good condition. The thickening of the stratum corneum in *in vitro* cultures is most probably caused by the missing

desquamation process (Ponec, 2002). Another deficiency of *in vitro* skin models is the weaker barrier function compared to native human skin (Gibbs, 2009; Netzlaff *et al.*, 2005; Ponec, 2002; Van Gele *et al.*, 2011). Yet, reconstructed human epidermis is a useful tool for assessing skin corrosion, skin irritation and skin absorption. Although the permeation of xenobiotics is overestimated, the rank order of substances with diverse physiochemical properties remains the same as can be found in human skin ex vivo (Schäfer-Korting *et al.*, 2008). Thus, the permeation of xenobiotics can be evaluated by the use of reconstructed human epidermis and so the number of animal experiments can be reduced in the future. Furthermore, currently not all cell types of human skin are found in *in vitro* models. For the assessment of immunological reactions of the skin, the absence of langerhans cells as part of the immune defense has to be mentioned as the most striking disadvantage, but much effort is made to improve the situation (Laubach *et al.*, 2010). Besides missing cell types, the absence of skin appendages like hair follicles and sweat glands is another problem, which might influence results obtained in *in vitro* studies.

In conclusion, *in vitro* skin models have great similarities to human skin and are a useful tool in *in vitro* research but still lack some important characteristics of their *in vivo* counterpart. Further improvement is required, especially in the development of skin barrier function, but much effort is made and the future seems promising.

## 1.5 *In Vitro* Skin Disease Models

Beside the development of reconstructed skin resembling the healthy state of its *in vivo* counterpart, it has become more and more of interest to establish models for different skin diseases. Skin diseases, such as atopic dermatitis, are a major public health problem (for review see: (Bieber, 2010; Leung *et al.*, 2004; Schon and Boehncke, 2005)). Still, the underlying pathomechanisms are not fully unraveled and new therapeutic options are needed, since the currently available therapies are treating symptoms and do not improve the disease mechanism (Elias *et al.*, 2008; Schon and Boehncke, 2005). Studies in humans are not easy to conduct and raise ethical issues. Animal models are an option and have provided deeper insight into disease mechanisms in the past (Fallon *et al.*, 2009; Scharschmidt *et al.*, 2009), but ethical concerns are rising. Furthermore, the transferability of results obtained in animals onto the human situation is questionable (Hartung, 2008; Mestas and Hughes, 2004; Seok *et al.*, 2013; van der Worp *et al.*, 2010). Hence, i*n vitro* skin disease models are a

helpful tool, which does not raise ethical concerns and furthermore better reflects the *in vivo* situation, since they are composed of normal human skin cells (Carlson *et al.*, 2008). *In vitro* skin disease models provide not only the opportunity to test cosmetics or drugs on diseased skin, but they can give a deeper insight into the pathophysiology of the mimicked skin disease as well (for review see:(Semlin *et al.*, 2010)). Several models for varying diseases of human skin have been established and will be introduced here. E.g. the mechanism of wound healing and skin repair can be evaluated *in vitro*. Thereby, the wounds are created either with the use of a scalpel or by burning the model with a laser (O'Leary *et al.*, 2002; Vaughan *et al.*, 2004). Such wounded models can be used further for the evaluation of the influence of drugs and their carriers on the wound healing process (Küchler *et al.*, 2010; Wolf *et al.*, 2009). Another well-established mimicked disease state is the infection with *Candida albicans*. Thereby, not only the classic *in vitro* skin models but also *in vitro* models of oral and vaginal epithelia have been used to study the effects of *Candida albicans* on the tissue. The implementation of immune cells in these disease models improved the insight into the immune response of human tissue to mycosis and new therapeutically options could be evaluated (Korting *et al.*, 1998; Schaller *et al.*, 2004; Schaller *et al.*, 2006).

Since malignant skin disease are on the rise, *in vitro* models of melanoma and non-melanoma skin cancer are of great interest for researchers to learn more about the disease pathophysiology and to evaluate new therapeutically options. Such models are in an early stage of development, some of them look quite promising but have to proof their suitability in further evaluation (Gibot *et al.*, 2013; Hoeller-Obrigkeit *et al.*, 2009; Li *et al.*, 2011). Höller-Obrigkeit et al. developed an *in vitro* skin construct of squamous cell carcinoma by the use of head and neck cancer squamous cells (SCC 12) and demonstrated great similarity to the in vivo situation as shown by the typical cluster formation of SCC 12 inside the epidermis. Furthermore, they validated their model by the use of photodynamic therapy (Hoeller-Obrigkeit *et al.*, 2009). Providing a protocol for the construction of an *in vitro* melanoma skin construct Li et al. demonstrated by the use of melanoma cells of different stages of disease that the *in vivo* situation can be mimicked better than in monolayer cultures (Li *et al.*, 2011). Hence, the disease pathophysiology can be unraveled further and new therapeutic options can be evaluated by means of this skin construct. The implementation of a microvascular network into an *in vitro* skin construct by Gibot et al. provides the opportunity to evaluate the proangiogenic effect of melanoma cells (Gibot *et al.*, 2013). This allows the evaluation of the metastatic progress and the development of new therapeutic options.

The classic skin diseases are of interest as well. Some *in vitro* models for psoriasis vulgaris, a chronic skin disease with an incidence of about 2 % worldwide, have been established and characterized (Barker *et al.*, 2004; Jean *et al.*, 2009; MatTek, 2013; Tjabringa *et al.*, 2008). They are constructed either by the use of keratinocytes from the lesional skin of patients or with normal human keratinocytes which have been stimulated with psoriasis-associated cytokines (e.g. tumor necrosis factor (TNF) α or IL-1α) before. The state of the disease can be mimicked pretty well in terms of abnormal morphology of the skin and released pro-inflammatory cytokines (such as TNFα or interferon (INF) γ) through keratinocytes. Yet, the absence of immune cells is a big disadvantage and limits the use of such *in vitro* models, still (Barker *et al.*, 2004; Jean *et al.*, 2009; MatTek, 2013; Tjabringa *et al.*, 2008). An *in vitro* model mimicking hallmarks of atopic dermatitis is described by Engelhart et al.. They developed their model based on the immortalized keratinocyte cell line HaCaT in which they integrated activated T-cells. With this approach, several hallmarks of atopic skin, such as the increased levels of proinflammatory cytokines and a disturbed epidermal maturation, could be mimicked (Engelhart *et al.*, 2005). The application of dexamethasone or tacrolimus, respectively, reduced cytokine release and improved epidermal maturation (Engelhart *et al.*, 2005). One major drawback of this work is the use of HaCaTs, since HaCaTs differ from normal human keratinocytes in several aspects, such as the release of different chemokines after triggering an immune response (Kollisch *et al.*, 2005; Olaru and Jensen, 2010). Oji et al. gained better insight in the peeling skin disease by the genetic evaluation of a large family and found a loss-of-function mutation in the gene encoding for corneodesmosin to predispose for disease development (Oji *et al.*, 2010). By the generation of an *in vitro* skin construct by the use of cells from lesional skin of one patient Oji et al. demonstrated that the loss of corneodesmosin leads to disturbed epidermal maturation and impaired barrier function. Furthermore, according to the genetic constitution of the evaluated family, where peeling skin disease was associated with atopic dermatitis, loss-of-function in corneodesmosin might contribute to the development of atopic dermatitis (Oji *et al.*, 2010).

Gene silencing in skin constructs opened new ways for mimicking skin diseases (Mildner *et al.*, 2006). Today, it is possible to generate skin disease models by the use of normal cells in which a gene can be selectively knocked down and not only by the use of cells obtained from lesional skin of patients. This enables researchers to study the effects caused by one single gene and therefore provides the opportunity to learn

more about disease pathophysiology. First insights into the effects of reduced *FLG* expression in an *in vitro* model were by silencing *FLG* by the use of siRNA in normal human keratinocytes. Skin models were constructed with these cells and cultivated for seven days (Mildner *et al.*, 2010). Histological evaluation demonstrated a reduced FLG expression and less keratohyalin granules in the stratum granulosum of the *FLG* knock down skin construct. The expression of other differentiation markers was not affected, as well as the composition of skin lipids in the stratum corneum. The barrier function was harmfully affected, as demonstrated by the increased permeability of the model dye Lucifer yellow in *FLG* knock down constructs. Furthermore, the reduced expression of FLG led to a decrease of urocanic acid and consequently to a diminished defense against UV radiation (Mildner *et al.*, 2010).

## 1.6 Aim of this Thesis

The aim of this thesis was the establishment and characterization of an *in vitro* skin disease model based on *FLG* knock down, to mimic fundamental features of *FLG*-associated skin diseases, such as atopic dermatitis and ichthyosis vulgaris *in vitro*. Such a model can be used to unravel the disease pathophysiology and might serve as a tool for the development and testing of new therapeutic options under avoidance of animal studies. The first aim was to establish a stable knock down of *FLG* in the *in vitro* skin model during the whole cultivation period to allow for the evaluation of features caused by the diminished *FLG* expression. Beside a histological examination of the generated *in vitro* skin model the assessment of the barrier function by performing skin absorption studies and the evaluation of the inflammatory threshold to a local irritant was aimed for. To gain deeper insight into the influence of *FLG* on lipids of the stratum corneum, the composition and organization of the extracellular lipids should be evaluated. Following the characterization of the *FLG* knock down model, improvement of the model by the implementation of vasculature and immune cells was aimed for. Finally, the effect of drugs, which are able to increase *FLG* expression in the skin, should be evaluated in the *FLG* knock down model, to allow for a first hint onto a new treatment option of *FLG*-associated diseases. The established *in vitro FLG* knock down model should be the base for future development of a complex *in vitro* model of atopic dermatitis.

# 2. Materials

In this section those materials are listed, which are not part of the published or accepted manuscripts.

## 2.1 Technical Equipment

| | |
|---|---|
| Autoclave | Systec, Wettenberg |
| Centrifuge Eppendorf 5415D | Eppendorf, Hamburg |
| Centrifuge Megafuge 1.0R | Heraeus, Hanau |
| $CO_2$-Incubator Heracell 240i | Thermo Fisher Scientific, Waltham, USA |
| Easypet Pipettor | Eppendorf, Hamburg |
| Fluorescence micrsocope BZ-8000 | Keyence, Neu-Isenburg |
| Freeze Microtome CM1510S | Leica Microsystems, Wetzlar |
| Incubator Heraeus Function Line Typ B6 | Thermo Fisher Scientific, Waltham, USA |
| Heraeus Pico 17 Centrifuge | Thermo Fisher Scientific, Waltham, USA |
| LaminAir HB 2472 | Heraeus, Hanau |
| Magnetic stirrer RO 10 power IKAMAG | IKA Werke GmbH, Staufen |
| Microplate reader FLUOstar Optima | BMG Labtech, Offenburg |
| Microtom Hyrax M40 | Zeiss, Jena |
| Nalgene Cryo 1°C Freezing Container | Nalge Nunc International Corporation, Rochester, NY, USA |
| Neubauer Counting Chamber | Zeiss, Jena |
| Paraffinization station Microm EC350 | Zeiss, Jena |
| Paraffin stretching water bath GFL 1052 | Zeiss, Jena |

| | |
|---|---|
| Phase contrast microscope Axiovert 135 | Zeiss, Jena |
| pH meter 766 Calimatic | Knick, Nürnberg |
| Pipets Eppendorf Reference | Eppendorf, Hamburg |
| Real Time PCR System LightCycler 480 | Roche, Mannheim |
| Scintillation Counter 1450 Microbeta TM Plus | Wallac, Freiburg |
| SG-Labostar 2-DI/-UV high purity water system | SG Wasseraufbereitung und Regenerierstation GmbH, Barsbüttel |
| Skin Punch, Ø 15 mm | Bauhaus, Berlin |
| Ultrasonic bath Sonorex RK 100 | Bandelin, Berlin |
| Water bath DC3/W26 | Thermo Haake, Karlsruhe |
| Vortex Genie 2 | Bender Hobein, Zürich, Switzerland |

## 2.2 Reagents and Consumable Supplies

| | |
|---|---|
| 1,2,6,7-$^{3}$H-testosterone (100 Ci/mmol) | Amersham, Freiburg |
| 1-methyl-$^{14}$C-caffeine (51.20 Ci/mmol) | ARC Inc., St. Louis, USA |
| 2-phospho-L-ascorbic acid trisodium salt | Sigma-Aldrich, Munich |
| 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) | Sigma-Aldrich, Munich |
| 6-well companion plates for cell culture insert | BD, Heidelberg |
| 6-well plates | TPP, Trasadingen |
| 96-well ELISA microplates | GreinerBioOne, Frickenhausen |
| Acetic acid | Sigma-Aldrich, Munich |
| Acetone | VWR, Darmstadt |
| Albumine, Fraktion V, protease free (BSA) | Carl Roth, Karlsruhe |
| Buffy coats | DRK Blutspendedienst, Berlin |

| | |
|---|---|
| Cell culture flasks 75 and 150 cm² | TPP, Trasadingen, Switzerland |
| Cell culture inserts for 6-well plates (pore size 3 µm, translucent PET membrane, growth area 4.22 cm²) | BD, Heidelberg |
| Cell strainer (sterile, pore size 40 µm) | VWR, Darmstadt |
| CellTracker Green CMFDA | Invitrogen, Darmstadt |
| Centrifuge tubes (15 ml, 50 ml) | Sarstedt, Nürnbrecht |
| cis-4,7,10,13,16,19-docosahexaenoic acid | Sigma-Aldrich, Munich |
| Clofibrate | Sigma-Aldrich, Munich |
| Cover slips for microscopy slides | Carl Roth, Karlsruhe |
| Cryo tubes 2 ml | VWR, Darmstadt |
| DAPI Antifading Mounting Medium | Dianova, Hamburg |
| Dispase II | Roche, Mannheim |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich, Munich |
| Disodium ethylenediaminetetraacetic acid dihydrate | Sigma-Aldrich, Munich |
| Disodium hydrogenphosphate | Merck, Darmstadt |
| Dulbecco's Modified Eagle's Medium (DMEM) | Sigma-Aldrich, Munich |
| Dulbecco's Phosphate Buffered Saline (PBS) w/o $Ca^{2+}$, $Mg^{2+}$ | PAA Laboratories, Cöbe |
| Endothelial cell base medium 2 (EBM-2) | Lonza, Walkersville, USA |
| Eosin G solution | Carl Roth, Karlsruhe |
| Eppendorf safe-lock tubes (1.5 ml, 2 ml) | Eppendorf, Hamburg |
| Ethanol abs. (EtOH) | VWR, Darmstadt |
| Ethylenediaminetetraacetic acid (EDTA) | Sigma-Aldrich, Munich |

| | |
|---|---|
| EZ-DeWax solution | Biogenex, Fremont, USA |
| Fetal Calves Serum (FCS I) | Biochrom, Berlin |
| Fetal Calves Serum (FCS II) | Invitrogen, Darmstadt |
| Ficoll | Sigma-Aldrich, Munich |
| Filaggrin specific Stealth siRNA (HSS177192) | Invitrogen, Darmstadt |
| Goat serum | Dianova, Hamburg |
| Goat-anti-Mouse DyLight 488 Antibody | Dianova, Hamburg |
| Goat-anti-Rabbit DyLight 594 Antibody | Dianova, Hamburg |
| HiPerfect Transfection Reagent | Qiagen, Hilden |
| Human epidermal growth factor (hEGF) | Sigma-Aldrich |
| Hydrochloric acid conc. | VWR, Darmstadt |
| Hydrogen peroxide 30% | Carl Roth, Darmstadt |
| Isopropanol | VWR, Darmstadt |
| Keratinocyte basal medium (KBM) | Lonza, Walkersville, USA |
| L-glutamine | Biochrom, Berlin |
| Magnetic stir bar | VWR, Darmstadt |
| Mayer's haematoxylin solution | Carl Roth, Karlsruhe |
| Methanol (MeOH) | Sigma-Aldrich, Munich |
| Microscopy slides Superfrost Ultra Plus | Menzel-Gläser, Braunschweig |
| Mouse-anti-FLG Antibody | Dianova, Hamburg |
| Mouse-anti-PECAM1 Antibody | DAKO, Hamburg |
| Multiwell plates for PCR | Roche, Mannheim |
| Nescofilm | Carl Roth, Karlsruhe |
| Optiphase supermix szintillation cocktail | Perkin Elmer, Rodgau |

| | |
|---|---|
| PCR stripes | Carl Roth, Karlsruhe |
| Penicillin / streptomycin (liquid) (10,000 U/ml / 10,000 µg/ml) | Biochrom, Berlin |
| Petri dish for cell culture | TPP, Trasadingen |
| Phosphoric acid | Sigma-Aldrich, Munich |
| Pipette tips | Sarstedt, Nürnbrecht |
| Potassium chloride | Sigma-Aldrich, Munich |
| Potassium dihydrogenphosphate | Carl Roth, Karlsruhe |
| Potassium hydroxide | Merck, Darmstadt |
| Purecol bovine collagen I (3 mg/ml) | Nutacon, Leimuiden, Netherlands |
| Primer for RT-PCR | Tib Molbiol, Berlin |
| Rabbit-anti-vWf Antibody | DAKO, Hamburg |
| Red blood cell lysis solution 10x | Miltenyi Biotec, Bergisch Gladbach |
| Roti Histofix | Carl Roth, Karlsruhe |
| Roti Histol | Carl Roth, Karlsruhe |
| Roti Histokitt | Carl Roth, Karlsruhe |
| RPMI 1640 medium | Sigma-Aldrich, Munich |
| Scalpel blades | VWR, Darmstadt |
| Sealing foil for multiwell plate | Roche, Mannheim |
| Serological pipets (5 ml, 10 ml, 25 ml) | Sarstedt, Nürnbrecht |
| Sodium azide | Carl Roth, Darmstadt |
| Sodium chloride | Sigma-Aldrich, Munich |
| Sodium dodecyl sulfate (SDS) | Sigma-Aldrich, Munich |
| Sodium hydroxide | Sigma-Aldrich, Munich |

| | |
|---|---|
| Stainless steel beads (Ø 5 mm) | Qiagen, Hilden |
| Supplements for keratinocyte growth medium | Lonza, Walkersville, USA |
| Supplements for endothelial growth medium | Lonza, Walkersville, USA |
| SYBR Green Master I | Roche, Mannheim |
| Tetramethylbenzidine (TMB) | Sigma-Aldrich, Munich |
| Tissue freezing medium | Leica, Nussloch |
| Tris base | Sigma-Aldrich, Munich |
| Tris hydrochloride | Sigma-Aldrich, Munich |
| Trisodium citrate dihydrate | Carl Roth, Karlsruhe |
| Trypsin | Biochrom, Berlin |
| Tumor necrosis factor α (TNFα) | R&D Systems, Wiesbaden |
| Tween 20 | Carl Roth, Karlsruhe |
| Water, sterile, pyrogen-free | Carl Roth, Karlsruhe |

## 2.3 Culture Media and Solutions

### 2.3.1 Media and Solutions for Cell Culture

All Media and solutions for the cell culture experiments were handled under sterile conditions and stored at 4°C. Self-prepared solutions were either autoclaved or sterile filtered before use.

Keratinocyte Growth Medium (KGM)

| | |
|---|---|
| KBM supplemented with: | bovine prurity extract (BPE)<br>insulin<br>human epidermal growth factor (hEGF)<br>hydrocortisone<br>gentamicin<br>amphotericin B |

The concentrations are not shared by the company (Lonza, Walkersville, USA).

Fibroblasts Growth Medium (FGM)

| | |
|---|---|
| DMEM supplemented with: | 2 mM L-glutamine<br>100 I.U./ml penicillin<br>100 µg/ml streptomycin<br>10 % FCS I |

Endothelial Cell Growth Medium (EGM-2)

| | |
|---|---|
| EBM-2 supplemented with: | hydrocortisone<br>human epidermal growth factor (hEGF)<br>fetal bovine serum (FBS)<br>vascular endothelial growth factor (VEGF)<br>human fibroblast growth factor B (hFGF-B)<br>insulin-like growth factor 1 (R3-IGF-1)<br>ascorbic acid<br>heparin<br>gentamicin<br>amphotericin B |

The concentrations are not shared by the company (Lonza, Walkersville, USA).

Peripheral Blood Mononuclear Cell (PBMC) Culture Medium

| | |
|---|---|
| RPMI-1640 supplemented with: | 10% heat-inactivated FCS I<br>2 mM L-glutamine<br>100 I.U./ml penicillin<br>100 µg/ml streptomycin |

Transport Medium

| | |
|---|---|
| DMEM I supplemented with: | 100 I.U./ml penicillin<br>100 µg/ml streptomycin |

Stop Medium

| | |
|---|---|
| DMEM I supplemented with: | 10% FCS I<br>100 I.U./ml penicillin<br>100 µg/ml streptomycin |

Cell Freezing Medium

| | |
|---|---|
| DMEM I supplemented with: | 10% DMSO<br>10% FCS I |

Phosphate Buffered Saline (PBS) pH 7.4

| | |
|---|---|
| Aqua bidest. supplemented with: | 0.2 g/l KCl<br>8.0 g/l NaCl<br>0.2 g/l $KH_2PO_4$<br>1.44 g/l $Na_2HPO_4$ |

PBS/EDTA

| | |
|---|---|
| PBS (w/o $Ca^{2+}$, $Mg^{2+}$) supplemented with: | 2 mM EDTA |

| | |
|---|---|
| Red Blood Cell Lysis Solution (1x) | 5.0 ml red blood cell lysis solution(10x)<br>45.0 ml water, sterile, pyrogen-free |

The solution was always freshly prepared. Unused solution was discarded at the end of the day.

Trypsin/EDTA

| | |
|---|---|
| PBS supplemented with: | 1.67 mg/ml trypsin<br>0.67 mg/ml EDTA |

Dispase Stock Solution

| | |
|---|---|
| PBS supplemented with: | 10 µg/ml Dispase II |

The stock solution was stored at -20°C and diluted 1:10 with PBS before use.

### 2.3.2 Buffers for Immunohistochemistry Experiments

| | |
|---|---|
| PBS based washing buffer | 5.0 g BSA<br>125 µl Tween 20<br>in 500.0 ml PBS |

The buffer was always freshly prepared. The washing buffer was used for all fluorescence-based staining procedures except for the detection of filaggrin. Here, the buffer was prepared without Tween 20. The buffer was also used do dilute the primary and secondary antibodies.

### 2.3.3 Solutions for Supplementation with Docosahexaenoic Acid or Clofibrate

| | |
|---|---|
| DHA Stock Solution (100 mM) | 25 mg DHA (= 7.61 $x10^{-5}$ mol)<br>ad 760 µl sterile filtered DMSO<br><br>The solution was aliquoted in safe lock tubes (1.5 ml) and stored at -20°C. |
| CLF Stock Solution (1 M) | 250 mg CLF (= 0.001 mol)<br>ad 1000 µl sterile filtered DMSO<br><br>The solution was aliquoted in safe lock tubes (1.5 ml) and stored at 4°C. |

## 2.4 Cells

normal human keratinocytes (NHKs)

isolated from juvenile foreskin obtained from cooperating medical practices and hospitals with ethical permission, used passages 2-3

normal human dermal fibroblasts (NHDFs)

isolated from juvenile foreskin obtained from cooperating medical practices and hospitals with ethical permission, used passages 2-4

human umbilical vein cord endothelial cells (HUVECs)

Charité, Berlin, used passages 3-9

peripheral blood mononuclear cells (PBMCs)

isolated from buffy coats, obtained from DRK Blutspendedienst, Berlin

# 3. Methods

In this chapter those methods are described that are not part of the published or accepted manuscripts.

## 3.1 Isolation and Cultivation of Normal Human Keratinocytes (NHKs)

For isolation of NHKs juvenile foreskin was obtained from circumcision surgeries in cooperating medical practices and hospitals (Vogler *et al.*, 2003). The skin was delivered in transport medium at 4°C and immediately after arrival washed with PBS. Subsequently, the skin was placed into a petri dish filled with 5 ml PBS and cut into small pieces (about 4 x 4 mm). 600 µl dispase stock solution were added and the skin was incubated over night at 4°C. The next day, epidermis was pulled off, placed in a 15 ml centrifuge tube and incubated for two min with trypsin/EDTA (diluted 1:1 with PBS) at 37°C. The migration of cells out of the epidermis was checked under the microscope and 8 ml stop medium were added to interrupt the trypsin digestion. After removal of leftover epidermis, the cell suspension was centrifuged (1000 rpm, 5 min). The supernatant was removed and the cells were washed with PBS, centrifuged again, resuspended in 10 ml KGM and seeded into a 75 cm² cell culture flask. The next day, medium was changed to get rid of non-adherent, dead cells.

Every 2 - 3 days medium was changed and when confluence of about 70 % was reached the culture was split in a ratio of 1 : 2 up to 1 : 4. Therefore the culture was washed with PBS and incubated with 2 ml trypsin/EDTA for 5 min at 37°C, 5 % $CO_2$. The detachment of cells was checked under the microscope and 6 ml stop medium were added. The cell suspension was transferred into a 50 ml centrifuge tube, centrifuged (1000 rpm, 5 min) and the supernatant was thrown away. After washing with PBS, the cells were resuspended in an appropriate amount of KGM and seeded into 75 cm² cell culture flasks.

For all experiments NHKs of passage two or three were used.

## 3.2 Isolation and Cultivation of Normal Human Dermal Fibroblasts (NHDFs)

The isolation of NHDFs was performed as published elsewhere (Hoeller *et al.*, 2001). Therefore, the leftover pieces of dermis from NHK isolation were used. The pieces of

dermis were placed into the wells of a 6-well plate and incubated for 20 min (37°C, 5 % $CO_{2)}$ without medium. Subsequently, 1 ml fibroblast growth medium (FGM) was added carefully and the dermis was cultivated for one week (37°C, 5 % $CO_2$). Meanwhile, the migration of NHDFs out of the dermis was checked every day under the microscope. Every other day 0.5 ml FGM was added. After one week, the dermis was removed and the NHDF culture was washed carefully with PBS and fed with 1 ml fresh FGM. The cells were cultivated (37°C, 5 % $CO_2$) with a change of medium twice a week until they reached a confluence of about 70 %, then they were split and seeded into 75 cm² cell culture flasks in the same way as NHKs.

For all experiments NDHFs of passages two to four were used.

## 3.3 Cultivation of Human Umbilical Vein Cord Endothelial Cells (HUVECs)

The endothelial cell line HUVEC was cultivated with EGM-2 in 75 cm² cell culture flasks (37°C, 5 % $CO_2$). Medium was changed every 2 - 3 days. When the cells reached a confluence of about 70 % they were split in the same way as NHKs except for a shortening of the trypsin digestion step to 1 min.

For all experiments HUVECs of passages three to nine were used.

## 3.4 Isolation, Cultivation and Cell Tracker Staining of Human PBMCs

Human PBMCs were isolated from fresh buffy coats. These were obtained from the German Red Cross and immediately after delivery the isolation was started. The buffy coat was diluted (1:3 - 1:5) with PBS/EDTA. Subsequently, 35 ml of the diluted buffy coat were layered on top of 15 ml Ficoll in a 50 ml centrifuge tube. After centrifugation (40 min, 1400 rpm, brake turned off) the white leukocytes-layer was transferred into a new 50 ml centrifuge tube, filled up to 50 ml with PBS/EDTA and centrifuged (1200 rpm, 10 min). Subsequently, the supernatant was removed and the cell pellet was resuspended in red blood cell lysis solution (1x) to destroy leftover erythrocytes. After incubation (12 min, room temperature), the cell suspension was centrifuged (1200 rpm, 5 min) and washed 3 times with PBS/EDTA. The centrifugation between the washing steps was performed at 1000 rpm for 15 min. With this condition thrombocytes remain in the supernatant and hence can be washed out. The purified PBMCs were

resuspended in PBS/EDTA, homogenized with a cell strainer, centrifuged (1200 rpm, 10 min) and finally resuspended in PBMC culture medium.

Immediately after isolation PBMCs were counted and suspended in RPMI-1640 ($1x10^6$ cells/ml) for staining with cell tracker. For each ml of the cell suspension 1 µl CellTracker™ Green CMFDA (Invitrogen, Darmstadt, Germany) (5 µg/ml in DMSO) was added and the suspension was incubated for 45 min at 37°C under constant movement. Subsequently, the cells were washed twice with PBS/EDTA, counted and resuspended in PBMC culture medium ($1 \times 10^6$ cells / ml). The uptake of the fluorescence labeled cell tracker by PBMCs was controlled under the fluorescence microscope. Immediately after staining PBMCs were used for the migration experiments.

## 3.5 Knock Down of *FLG* in NHKs

24 h before the cultivation of the *in vitro* skin constructs was started, NHKs were transfected with *FLG* specific siRNA (sequence: 5´-CAG CUC CAG ACA AUC AGG CAC UCA U-3´; NM_002016). For each confluent NHK a stock solution of KGM, transfection reagent Hiperfect and FLG specific siRNA was prepared according to the manufacturer's instructions. Subsequently, the confluent NHK culture was fed with fresh KGM and the siRNA stock solution was added. The NHKs were incubated for 24 h (37°C, 5 % $CO_2$).

## 3.6 Cultivation of the *In Vitro* Skin Construct

The *in vitro* skin constructs were prepared as published elsewhere (Eckl *et al.*, 2011). Briefly, for building up the collagen-fibroblast-matrix collagen I was mixed with buffer and brought no neutral pH. Subsequently, fibroblasts ($2.5 \times 10^5$ / construct) in FCS II were added. The jellylike mixture was mixed carefully and 2.5 ml were poured into a cell culture insert. After gelatinization of the matrix, KGM was added and the incubation was continued for 2 h at 37°C, 5% $CO_2$. Finally, KGM was removed and replaced by NHKs in KGM ($4.2 \times 10^6$ / construct, with or without *FLG* knock down). 24 h postincubation (37°C, 5% $CO_2$) the airlift was performed, therefore the medium was removed from the top of the collagen-fibroblast-matrix and from inside the well beneath the cell culture insert and a proprietary differentiation medium was added. The top of

the skin construct stayed dry from this time on. Skin constructs were cultivated (37°C, 5% $CO_2$) for 7 or 14 days, respectively. Medium was changed every other day.

## 3.7 Implementation of an Endothelial Cell Layer in the *In Vitro* Skin Construct

For the implementation of an endothelial cell layer in the *in vitro* skin construct the preparation was adjusted slightly. First, the collagen-fibroblast-matrix was prepared inside of a cell culture insert as described under 3.6. After solidification, the cell culture insert with the collagen-fibroblast-matrix was turned upside-down and $1x10^6$ HUVECs in 1 ml EGM were seeded onto the basal side of the insert membrane. After incubation for one hour (37°C, 5% $CO_2$) the insert was turned around and placed into a 6-well companion plate containing EGM. KGM was added on top of the collagen-fibroblast-matrix and after incubation for two hours (37°C, 5% $CO_2$) replaced by NHK cell suspension ($4.2x10^6$ / construct). The following day, the airlift was performed and the constructs were put on differentiation medium. Therefore, the proprietary differentiation medium was mixed (1:1) with EGM and supplemented with 1.36 mM 2-phospho-L-ascorbic acid (Sorrell *et al.*, 2007). Constructs were kept in culture for 7 - 14 days with a change of medium every other day.

## 3.8 Immunofluorescence Analysis of vWf, PECAM-1 and FLG

For the detection of the endothelial cell markers von Willebrand factor (vWf) and platelet endothelial cell adhesion molecule (PECAM-1) the whole skin construct with implemented endothelial cell layer was fixed in Roti Histofix for 15 min. After several washing steps the protein structures were blocked with normal goat serum (1:20 in PBS). Subsequently, the constructs were incubated with primary antibody (rabbit-anti-vWf 1:200 or mouse-anti-PECAM-1 1:20, respectively) for 30 min at room temperature. Following several washing steps, the constructs were incubated with secondary antibody (goat-anti-rabbit DyLight 594 1:400 or goat-anti-mouse DyLight 488 1:400, respectively) for 60 min at room temperature in the dark. Finally, the constructs were washed again and placed in a 6-well companion plate onto washing buffer containing 3 drops of DAPI Antifading Mounting Medium and immediately analyzed by fluorescence microscopy.

For evaluation of FLG expression, skin constructs (normal and *FLG* knock down, with or w/o DHA, respectively) were embedded in tissue freezing medium and frozen using

liquid nitrogen. After overnight storage at -80°C, the constructs were cut to vertical slices (5 µm) with a freeze microtome. The sections were fixed using ice-cold acetone, washed with washing buffer and blocked with normal goat serum (1:20 in PBS). The sections were incubated overnight at 4°C with primary mouse-anti-FLG antibody (1:50) and subsequently with goat-anti-mouse DyLight 488 antibody (1:400). After washing, the sections were embedded in DAPI antifading mounting medium and analyzed with a fluorescence microscope.

### 3.9 Migration of Human PBMCs into the *In Vitro* Skin Construct

To study the migration of PBMCs into the *in vitro* skin construct, four different experimental set-ups haven been performed.

In the first approach, a skin construct with endothelial cell layer (14 days in culture) has been damaged with SDS (1% in PBS) by topical application for 15 min. This approach has been shown before to trigger an inflammatory reaction in the skin constructs and to lead to increased levels of IL-6 and -8 (see 4.1 skin irritation test for details) (Küchler *et al.*, 2011). Skin constructs with PBS or without impairment served as controls. Following the damage, the skin construct was placed onto 2 ml PBMC culture medium containing $5x10^5$ PBMCs/ml and incubated for 24 h (37°C, 5% $CO_2$). Thereafter, they were embedded in paraffin and stained with H&E.

In the second approach, the impairment of the skin construct with endothelial cell layer (14 days in culture) was changed. According to published procedures (Weindl *et al.*, 2011) tumor necrosis factor α (TNFα) solution (10 µg/ml in PBS) was applied topically (10 µl/cm²) for one hour. Subsequently, the skin construct was put on PBMC culture medium containing $1x10^6$ PBMCs/ml (stained with cell tracker) and incubated for 24 h (37°C, 5% $CO_2$). Skin constructs without topical impairment and without PBMCs served as control. Then they were embedded in paraffin, cut into 5 µm vertical slices and analyzed with normal and fluorescence light microscopy.

In the third approach, the impairment of the skin construct (without endothelial cell layer, 9 days in culture) was done systemically by adding TNFα to the differentiation medium (final concentration 50 ng/ml) (Huh *et al.*, 2010) for 4 hours. Subsequently, skin constructs were incubated for 24 h (37°C, 5% $CO_2$) with PBMC culture medium containing $1x10^6$ PBMCs/ml (stained with cell tracker). A construct without impairment and without the addition of PBMCs served as control. Thereafter, skin constructs were

embedded in tissue freezing medium, cut to 5 µm vertical slices and analyzed by light and fluorescence microscopy. The embedding procedure was changed from paraffin to tissue freezing medium, since tissue freezing is less time consuming and the constructs could be analyzed faster.

In the last approach, the TNFα (final concentration 50 ng/ml) was added to the differentiation medium of the skin constructs (14 days in culture, with and without endothelial cell layer, respectively) 2 days before the migration experiment was performed. To enhance the migration of the PBMCs, two skin constructs (one with and one without endothelial cell layer, respectively) were turned upside down just before adding the PBMCs (Schaller *et al.*, 2006) and $1x10^6$ cell tracker stained PBMCs in 1 ml PBMC culture medium were applied to the basal side of the cell culture insert membrane. 24 h post incubation in the upside down position (with a constant feeding with fresh medium), the constructs were embedded in tissue freezing medium, cut into 5 µm vertical slices and analyzed by light and fluorescence microscopy.

### 3.10 Supplementation with Docosahexaenoic Acid (DHA) or Clofibrate (CLF)

The skin constructs (normal and *FLG* knock down) have been generated according to the standard procedure. At the same time as the airlift was performed the supplementation with 100 µM DHA or 1 mM CLF, respectively, was started according to a previously published method (Zhang *et al.*, 2012). 1 µl of the DHA or CLF stock solution, respectively, was added to 1 ml differentiation medium. The constructs were cultivated for 14 days with a change of medium every other day. DHA or CLF, respectively, was supplemented with every change of medium. Untreated skin constructs served as control. Subsequently, RNA isolation, cDNA synthesis, rtRCR and H&E staining were performed as described in section 4.1.

# 4. Results

In this section the results are presented as published or accepted manuscripts or as unpublished data.

## 4.1 Establishment of an *In Vitro FLG* Knock Down Skin Construct

An *in vitro* skin construct based on *FLG* knock down has been established and the effects of reduced expression of *FLG* on skin differentiation, the inflammatory response to a local irritant and the percutaneous absorption of topically applied drugs have been evaluated. The epidermal maturation was obviously disturbed in the *FLG* knock down skin construct as demonstrated by histological examination. The skin irritation test revealed a significant decrease of the inflammatory threshold to a local irritant in the *FLG* knock down model as demonstrated by increased levels of the pro-inflammatory cytokines IL-6 and IL-8. The barrier function was assessed by performing skin absorption studies with a hydrophilic (caffeine) and a lipophilic (testosterone) model drug. Only the permeation of the lipophilic drug was affected by the reduced *FLG* expression. The *FLG* knock down skin construct is therefore a helpful tool in studying the effects of the reduced *FLG* expression on skin maturation, barrier function and inflammatory threshold. Furthermore, the *in vitro* construct will allow for assessment of other *FLG*-associated effects in the skin.

These results are presented as published manuscript under 4.1.2.

### 4.1.1 Personal Contribution

Together with Dr. Sarah Küchler (Institute for Pharmacy, Freie Universität Berlin, Germany), the *in vitro* skin construct was established in our laboratory. Several batches have been cultivated by the use of cells from different donors and the knock down efficiency of *FLG* was evaluated in every batch to ensure a stable knock down over the whole cultivation period. Since the equipment for paraffinization was not available in our laboratory, the histological examination by H&E staining was performed in collaboration with Prof. Dr. Johanna Plendl (Institute for Veterinary Anatomy, Freie Universität Berlin, Germany). The histological evaluation revealed a disturbed formation of the stratum corneum in the *FLG* knock down models, indicating a disturbed barrier. Therefore, the barrier function was assessed by me by performing

skin absorption studies according to OECD approved guidelines. Thereby, the evaluation of testosterone permeation was performed by me alone, whereas for the evaluation of caffeine permeation the help of Katharina Ackermann (Institute for Pharmacy, Freie Universität Berlin, Germany) is gratefully acknowledged. For further assessment of *FLG* associated features, which are usually found in atopic skin, I performed the skin irritation test with the local irritant sodium dodecylsulfate to evaluate the inflammatory threshold in the *FLG* knock down model. Thereby, the conduction of the skin irritation test and the subsequent data analysis, including the determination of cell viability via MTT dye reduction test and the quantification of the released pro-inflammatory cytokines IL-1alpha, IL-6 and IL-8, was performed by me. The evaluation of lactate dehydrogenase release was performed in collaboration with Prof. Dr. Hans Christian Korting (Department of Dermatology and Allergology, Ludwig-Maximilians University Munich, Germany). The manuscript was written in collaboration with Dr. Sarah Küchler (Institute for Pharmacy, Freie Universität Berlin, Germany).

### 4.1.2 Published Manuscript

S. Küchler, **D. Henkes**, K.M. Eckl, K. Ackermann, J. Plendl, H.C. Korting, H.C. Hennies, M. Schäfer-Korting: Hallmarks of Atopic Skin Mimicked *In Vitro* by Means of a Skin Disease Model Based on *FLG* Knock-down. ATLA 39, 471-480, 2011

## 4.2 Characterization of *In Vitro* Skin Constructs in Terms of Lipid Profile and Skin Acidification

In this part the impact of the reduced expression of *FLG* on the skin lipid order, skin lipid composition and skin acidification was evaluated in the established *FLG* knock down skin construct. Although the amount of urocanic acid and pyrrolidone-5-carboxylic acid were significantly reduced, we found a physiological skin surface pH in the *in vitro* skin constructs. Hence, the sodium/hydrogen antiporter (NHE-1) and the secretory phospholipase $A_2$ ($sPLA_2$) acidification pathways were evaluated on mRNA and protein level. NHE-1 and the $sPLA_2$ isoform IIA were found to be upregulated in the *FLG* knock down skin construct. Coincidently with the upregulated $sPLA_2$-IIA, we found an increased amount of free fatty acids in the *FLG* knock down skin construct. The ceramide profile was not affected by the reduced *FLG* expression, whereas a disorganization of the lipid lamellae could be found. We could demonstrate a feedback mechanism between *FLG* and acidification pathways of the skin and revealed that the influence of *FLG* on skin lipid composition is marginal.

These results are presented as accepted manuscript under 4.2.2.

### 4.2.1 Personal Contribution

To evaluate the lipid composition and organization in the stratum corneum of the *in vitro* skin construct collaboration with Prof. Dr. Kateřina Vávrová (Faculty of Pharmacy, Charles University in Prague, Czech Republic) was started. The skin constructs with or without *FLG* knock down, respectively, were cultivated and the stratum corneum was isolated by me. The evaluation of knock down efficiency was performed by me in every batch, to ensure that the observed features were associated with a reduced *FLG* expression. Since the analysis of the lipid profile revealed and increased level of free fatty acids in the *FLG* knock down construct, I aimed for assessing the amount of several isoforms of $sPLA_2$ on mRNA and protein level on day 14 of cultivation of the skin constructs. Therefore, PCR studies and immunofluorescence experiments have been performed by me. In parallel, the expression of the sodium/hydrogen antiporter (NHE-1) was evaluated on mRNA and protein level on day 14 of cultivation by me. The manuscript was written in collaboration with Dr. Sarah Küchler (Institute for Pharmacy, Freie Universität Berlin, Germany) and Prof. Dr. Kateřina Vávrová (Faculty of Pharmacy, Charles University in Prague, Czech Republic).

### 4.2.2 Accepted Manuscript

K. Vávrová, **D. Henkes**, K. Strüver, M. Sochorová, B. Školová, M. Witting, W. Friess, S. Schreml, R.J. Meier, M. Schäfer-Korting, J.W. Fluhr, S. Küchler: Filaggrin Deficiency Leads to Impaired Lipid Profile and Altered Acidification Pathways in a 3 D Skin Construct. – *Accepted of Publication in the Journal of Investigative Dermatology* doi:10.1038/jid.2013.402

## 4.3 PPAR Modulators Influence FLG Expression in a *FLG* Knock Down Skin Construct

Evaluation of FLG Expression and Histological Examination

Treating skin constructs (normal or *FLG* knock down, respectively) with the peroxisome proliferator-activated receptor (PPAR) α modulator docosahexaenoic acid (DHA) significantly increased the mRNA expression of *FLG* in the constructs. The relative mRNA expression of *FLG* was increased about 18.96 ± 9.27 fold (mean ± SEM, n = 4) in normal and about 2.69 ± 1.67 fold (mean ± SEM, n = 4) in *FLG* knock down constructs when compared to normal, untreated tissue, where the expression is 1 by definition (Fig. 3). The knock down efficiency was 75.25 ± 2.69 % (mean ± SEM, n = 4). These results were confirmed on the protein level by immunofluorescence experiments (Fig. 4).

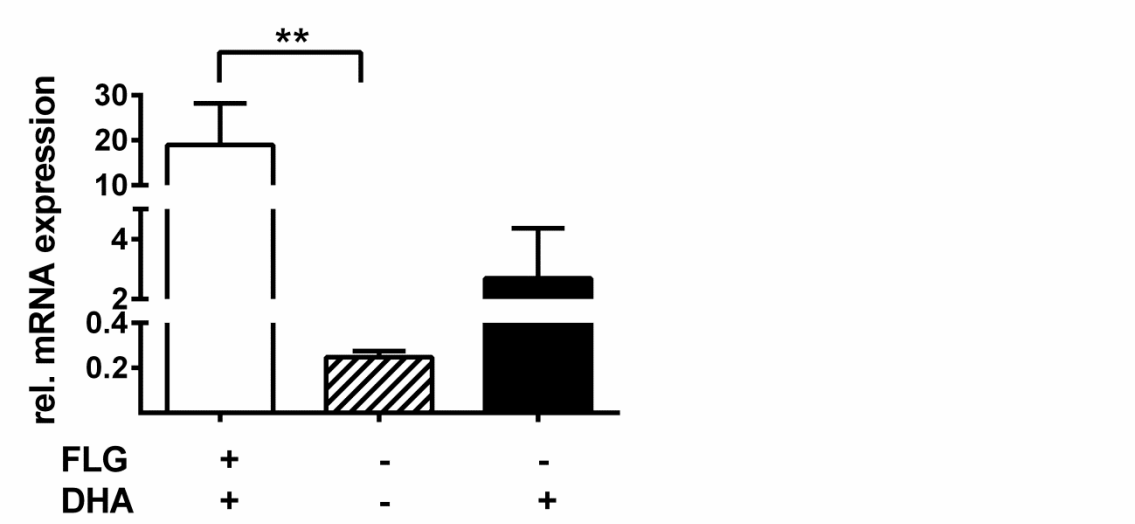


**Fig. 3 *FLG* expression after DHA incubation was determined on mRNA level by RT-PCR. The expression was strongest in normal (FLG+) skin constructs treated with DHA (+DHA) (18.96 ± 9.27) and lowest in *FLG* knock down (FLG-) constructs without DHA treatment (FLG-) (0.25 ± 0.03). In *FLG* knock down constructs the expression on mRNA level was increased about 2.69 ± 1.67 fold. FLG expression in normal, untreated constructs is 1 by definition. Data are presented as mean ± SEM, n = 4, *p < 0.05.**

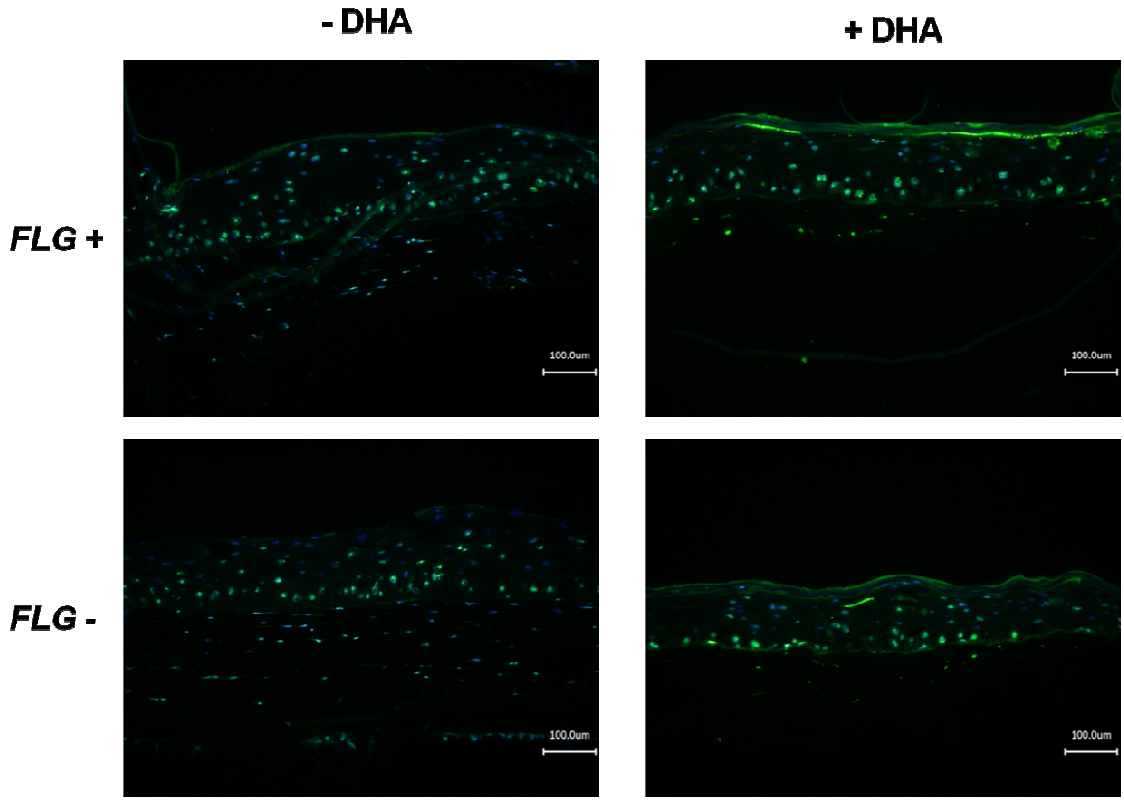


**Fig. 4 Immunofluorescence staining of FLG after DHA incubation. Green fluorescence indicating FLG was strongest in normal (*FLG*+) skin constructs treated with DHA (DHA+) and lowest in *FLG* knock down skin constructs (*FLG*-) without DHA (DHA-). A significant upregulation of FLG is observed in the FLG- constructs treated with DHA. Nuclei were stained with DAPI (blue fluorescence), magnification 10x, bar = 100µm.**

The histological evaluation of DHA treated and untreated constructs revealed some minor differences in epidermal maturation (Fig. 5). The thickness of the stratum corneum is obviously increased indicating the DHA stimulating keratinocyte differentiation. Some disturbances, such as spongiosis and disturbed erection of the keratinocytes, of the basal layer were observed, as well.

The supplementation with the PPARα modulator clofibrate (CLF) increased *FLG* mRNA expression about 14.42 fold in one batch of normal skin constructs. In the *FLG* knock down construct of the same batch treated with CLF an increase of about 50 % of FLG expression was detected, when compared to untreated *FLG* knock down construct. These results could not be confirmed with skin constructs cultivated with cells derived from different donors.

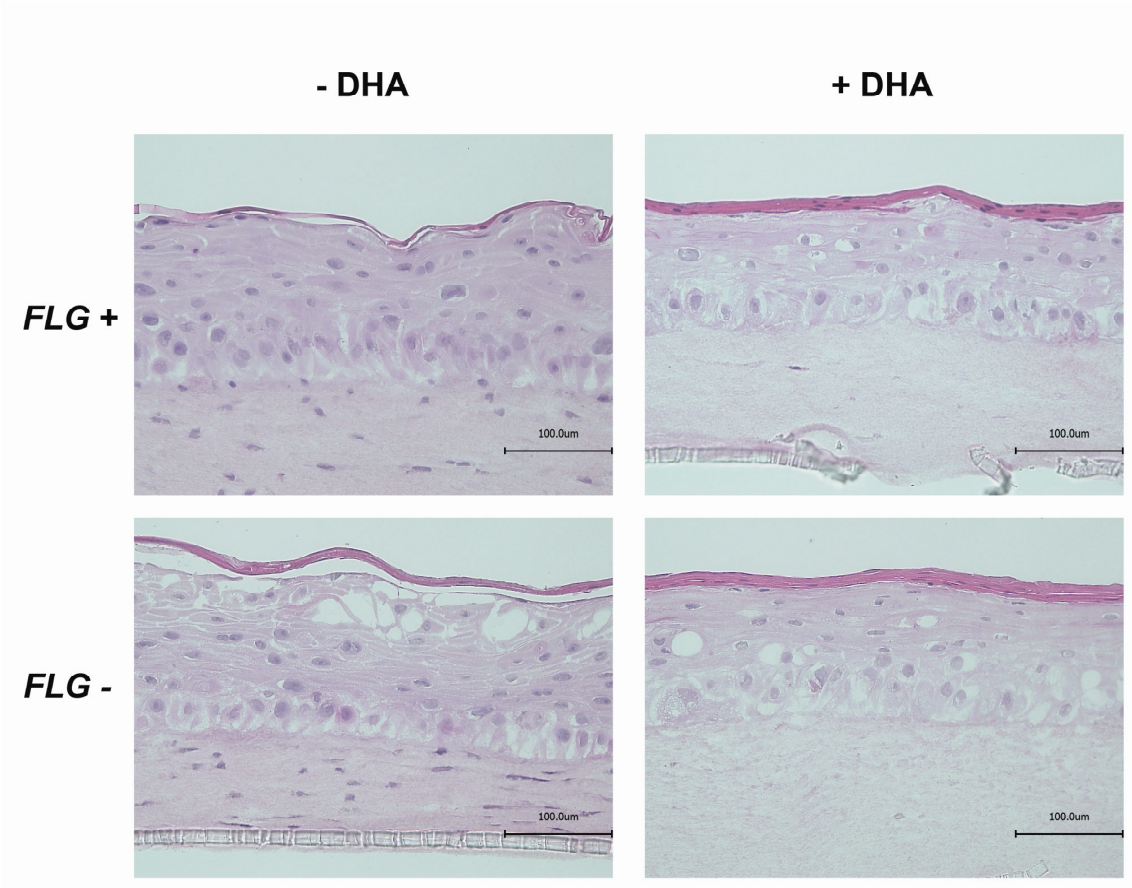


**Fig. 5 Histological evaluation of skin constructs after treatment with DHA revealed only minor differences in the epidermal maturation. Obviously, the thickness of the stratum corneum is increased following DHA treatment. Furthermore a slight disturbance of the formation of the stratum basale and spongiosis is visible. Pictures are representative from one batch, magnification 20x, bar = 100 µm.**

## 4.4 Implementation of an Endothelial Monolayer in the *In Vitro* Skin Construct

In preliminary studies for vascularization of the *in vitro* skin construct, the endothelial cells were embedded into the collagen-fibroblast-matrix. However, the incorperation of endothelial cells into the collagen-fibroblast-matrix of the *in vitro* skin construct was not successful, since the endothelial cells phagocytized the collagen and long-term cultivation was not possible due to shrinking of the collagen-matrix. Thus, the method was adjusted and the endothelial cells were seeded onto the basal side of the cell culture insert. This approach was successful and the results obtained with are presented here.

### Histological Examination

The histological analysis revealed a successful implementation of endothelial cells on the basal side of the cell culture insert. The HUVECs stick nicely to the basal side of the cell culture insert and built up a confluent monolayer (Fig. 6). To assess the viability and functionality of the implemented endothelial cell monolayer, the presence of the two specific endothelial cell markers von Willebrand factor (vWf) and platelet endothelial cell adhesion molecule (PECAM-1) have been evaluated by immunostaining. Both markers were detected, indicating the viability and functionality of the endothelial cell monolayer (Fig. 7).

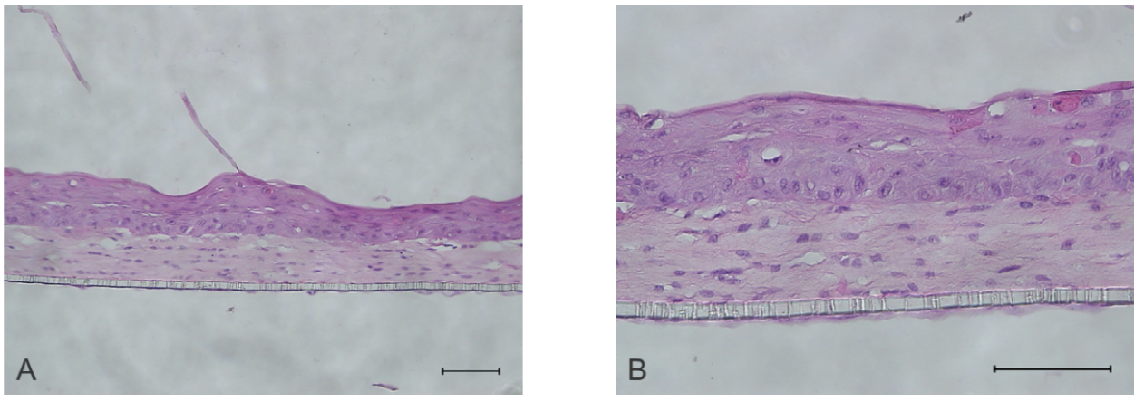


**Fig. 6 Histological pictures of the *in vitro* skin constructs with HUVECs. 1x10$^6$ HUVECs were seeded onto the basal of the cell culture insert, they streched out and built up a confluent monolayer, which sticks nicely to the basal side of the cell culture insert. Constructs were cultivated for 14 days, embedded in paraffin, cut into 5 µm vertical slices and stained with H&E (A: magnification 10x, bar = 100 µm; B: magnification 20x, bar = 100 µm).**

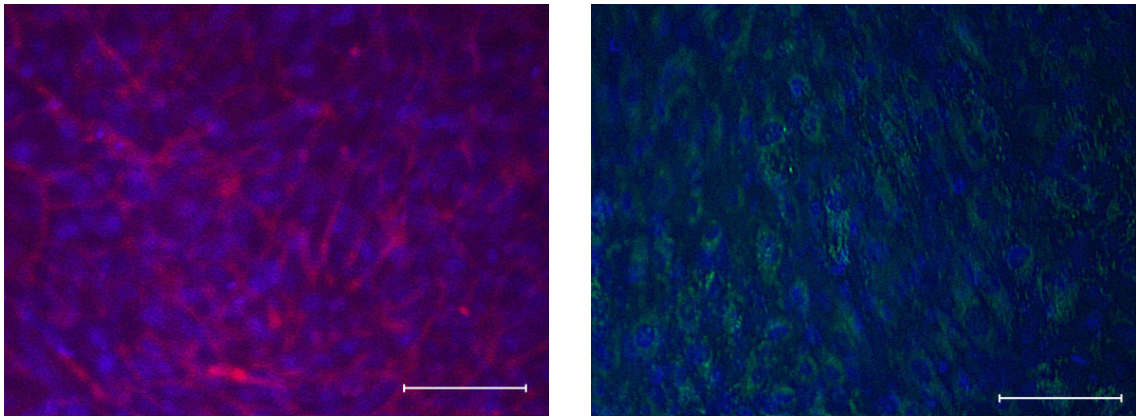

**Fig. 7 Immunofluorescence staining of the endothelial cell monolayer of the skin construct at day 14. Left: Red fluorescence indicating endothelial cell marker PECAM-1 Right: Green fluorescence indicating endothelial cell marker vWf. The presence of both specific endothelial cell markers demonstrates the viability and functionality of the endothelial cell monolyer.Nuclei were stained with DAPI (blue fluorescence) (magnification 20x, bars = 100µm).**

Yet, the implementation of the endothelial cell monolayer seemed to disturb the maturation of the skin construct: The fibroblast in the collagen matrix did not stretch out, the erection of the keratinocytes in the stratum basale seemed to be disturbed resulting in incomplete epidermal maturation with less well developed stratum corneum (Fig. 6). The disturbances in epidermal maturation might be caused by the change of differentiation medium. For implementation of the endothelial monolayer, the differentiation medium was mixed with endothelial cell medium and supplemented with ascorbate. This change might have reduced the concentrations of supplements required for epidermal maturation. If these histological effects have an impact on skin barrier function needs to be assessed in future experiments.

## 4.5 Migration Behavior of Human PBMCs into the *In Vitro* Skin Construct

To study the migration of human PBMCs into the *in vitro* skin constructs and to evaluate a potential difference of PBMC migration between the normal and *FLG* knock down construct several experimental setups have been performed. The experiments were performed only with skin constructs prepared with untransfected normal human keratinocytes. Initially, inflammation of the *in vitro* skin constructs was induced by the application of the local irritant sodium dodecylsulfate (SDS). SDS increases the release on inflammatory cytokines IL-6 and -8 significantly (Küchler *et al.*, 2011). For analysis the skin constructs have been embedded in paraffin and stained with H&E. Next, the procedure was adjusted slightly. Since I aimed for inducing a stronger inflammation in the *in vitro* skin construct, I decided for the topical application of TNFα, according to published procedures (Weindl *et al.*, 2011). Furthermore, for the improvement of the analysis of PBMC migration the PBMCs were fed with a cell tracking dye. Since this second approach was not successful, I decided to induce the inflammation by systemic application of TNFα in a third experimental setup (Huh *et al.*, 2010). The cytokine was added to induce a strong inflammatory reaction. Freezing of the skin constructs instead of paraffinization was chosen in order to exclude a potential loss of fluorescence of the stained PBMCs by a more rapid procedure. In the last experimental setup, I wanted to ensure, that the migration of PBMCs into the *in vitro* skin construct is not hampered by the distance, which the PBMCs have to cross, when given into the well underneath the *in vitro* skin construct. Therefore, the skin construct has been turned upside down and the PBMCs were applied directly onto the basal side of the cell culture insert. Unfortunately, none of the tested experimental setups allowed for studying the migration of human PBMCs into the skin constructs. In all approaches no PBMC migration was detected.

# 5. Final Discussion

The overall aim of this thesis was the establishment on an *in vitro* skin model, which mimics major characteristics of *FLG* associated skin diseases and can be used to study the impact of low or lacking *FLG* expression on skin differentiation and barrier function. Furthermore, the underlying pathomechanism of *FLG* associated skin diseases should be investigable.

## 5.1 Impact of Reduced *FLG* Expression on Skin Differentiation and Development of Barrier Function

Atopic dermatitis (AD) and ichthyosis vulgaris (IV), skin diseases associated with loss-of-function mutations in *FLG,* are major public health problems. AD is mainly a problem in the industrialized world and its prevalence is rising (for review see: (Brown and McLean, 2012; Irvine *et al.*, 2011; Sandilands *et al.*, 2009)). It is known, that FLG mutation lead to disturbances of the normal epidermal development. The skin of ft/ft mice, which lack FLG, exhibits a defective barrier and an increased allergen ingress, which induces inflammation (Fallon *et al.*, 2009; Scharschmidt *et al.*, 2009). Still, the pathophysiology of AD and the impact of *FLG* mutations on it, is not understood in full and further research is required (Elias *et al.*, 2008; Sandilands *et al.*, 2009). Since *in vivo* studies in humans and animals raise ethical concerns and are expensive, new *in vitro* test systems are urgently required. *In vitro* skin models have become of major interest in the past years and have been proven to be suitable for assessing skin irritation, skin corrosion and skin absorption studies (for review see: (Netzlaff *et al.*, 2005)). Based on this, the development of models mimicking varying diseases of human skin has been pursued (for review see (Semlin *et al.*, 2010)), but the development of models for complex diseases such as AD or IV have not been accomplished yet (Engelhart *et al.*, 2005; Mildner *et al.*, 2010; Simonsen and Fullerton, 2007).

In this study, an *in vitro* skin construct based on *FLG* knock down using siRNA was established and thoroughly characterized aiming for more insight on the impact of FLG in skin barrier development and skin diseases and to establish a base which can be used in future studies to establish an *in vitro* model for AD. The knock down with siRNA was stable over the culturing time of 14 days and its efficiency determined by RT-PCR was always > 75 % (Küchler *et al.*, 2011), which is an excellent result (Ren *et al.*,

2006). The specificity of siRNA was good as shown by the use of non-coding siRNA for reference. The impact of *FLG* knock down on FLG protein expression in the skin construct was verified by immunofluorescence experiments, which revealed notable reduction of fluorescence staining intensity in *FLG* knock down constructs in comparison to normal constructs. H&E staining demonstrated, that the skin construct grown out of normal human keratinocytes without *FLG* knock down develops a fully differentiated epidermal compartment with all viable layers and functional stratum corneum. In contrast, the knock down of *FLG* obviously leads to disturbed maturation of the epidermal compartment (Küchler *et al.*, 2011). Morphological hallmarks like intercellular spongiosis or impaired SC development, which can be found in skin of patients suffering from AD (Kerl *et al.*, 2003) were presented *in vitro* following *FLG* knock down. In contrast, an *in vitro FLG* knock down model grown for seven days did not reveal any abnormalities in epidermal maturation and SC development (Mildner *et al.*, 2010). Indicating, that the impact of decreased *FLG* expression becomes visible only after longer (14 days) cultivation of our skin construct. In unpublished, preliminary experiments we did not find a significant difference in skin morphology after seven days in culture, too.

To assess the barrier function of the *in vitro* skin construct further, skin absorption studies have been performed. Such studies can reveal the barrier property of the *in vitro* skin construct in general, when comparing to permeation data published for human skin or other *in vitro* skin models, and, furthermore, can unravel the impact of reduced *FLG* expression on percutaneous uptake of applied substances. The studies have been performed according to validated and OECD adopted experimental procedures for *in vitro* skin models with the OECD-approved test substances testosterone (lipophilic, logP = 3.47) and caffeine (hydrophilic, logP = -0.08) (OECD, 2004a; Schäfer-Korting *et al.*, 2008). Permeation data of topically applied substances on skin with impaired barrier function and especially on lesional AD or IV skin, respectively, is rare. Based on the well-known diminished barrier in patients, however, one could believe that the permeability must be higher than through intact skin (for review see: (Chiang *et al.*, 2012; Proksch *et al.*, 2009)). For the development of new drugs for the treatment of AD or IV, respectively, skin absorption studies are needed. Besides ethical concerns, animal experiments on percutaneous absorption might lead to false results since human and rodent skin differs in fundamental features. Animal skin is composed of other cell types and shows differences in lipid composition. Most importantly, the density of hair follicles is much higher and hair follicles are a major shunt pathway for skin absorption. All these differences affect skin permeability (for

review see: (Van Gele *et al.*, 2011)). An *in vitro* test system, which is easy to handle, cost-effective and has a high reliability is therefore of great interest. In this study it was demonstrated, that the generated *in vitro* skin construct can be used for skin absorption studies. On day 14 of cultivation, the percutaneous uptake of the hydrophilic substance caffeine differed only marginally between normal and *FLG* knock down construct, whereas the permeation of the lipophilic testosterone was increased in *FLG* knock down compared to normal constructs (Küchler *et al.*, 2011). The lack of statistical significance can be attributed to the great variability of donors used for the construction of the skin construct, since in every batch another donor was used. Furthermore, these results are well in accordance with the differences in lipid organization, where a disturbed intercellular lipid lamellae organization has been found on day 14 of cultivation (Vávrová *et al.*, 2013). The correlation between permeability and intercellular lipid lamellae organization is described in the literature (Potts and Francoeur, 1990)

## 5.2 Impact of FLG on Skin Lipids Organization / Composition and Skin Acidification

The skin from AD patients shows among other features abnormalities in the intercellular lipid lamellae of the stratum corneum. The overall concentration of the ceramides is significantly decreased (Choi and Maibach, 2005; Holleran *et al.*, 2006; Jakasa *et al.*, 2011; Jungersted *et al.*, 2010; Proksch *et al.*, 2003). Furthermore, the lipid organization is obviously disturbed, showing a more hexagonal instead of orthorhombic packing (Groen *et al.*, 2011; Pilgram *et al.*, 2001). Different pathologic features have been suggested to cause the disturbances, such as a defect in lamellar body synthesis and secretion (Gruber *et al.*, 2011) or changes in the activity of the sphingomyelinase (Jensen *et al.*, 2004), but studies, which evaluate the impact of *FLG* mutations on extracellular lipid composition or organization are rare (Jungersted *et al.*, 2010; Mildner *et al.*, 2010). The developed *in vitro FLG* knock down construct allows for the evaluation of a potential correlation between decreased *FLG* expression and skin lipid disturbances. Therefore, the lipid organization and composition of the *in vitro* skin construct was evaluated in collaboration with Prof. Dr. Kateřina Vávrová (Faculty of Pharmacy, Charles University in Prague, Czech Republic). We found more disordered intercellular lipid lamellae in the *FLG* knock down skin construct on day 14 of cultivation, while no difference to the normal construct was found on day 7. The lipid organization of the normal construct seems to improve with the cultivation time, coming closer to the lipid organization of human skin ex vivo (Vávrová *et al.*, 2013). The

differences in lipid organization are well in accordance with the results obtained in the skin absorption studies: there, a difference in permeability was found on day 14 of cultivation, but not on day 7 (Küchler *et al.*, 2011; Vávrová *et al.*, 2013). Since our *in vitro* skin construct did not form the orthorhombic packing of lipids, we were not able to evaluate the impact of *FLG* expression on the formation of the lipid lattice (Vávrová *et al.*, 2013). The disability of skin constructs to form a hexagonal lipid lattice was demonstrated by others as well (Thakoersing *et al.*, 2012). Based on the finding of a disturbed lipid organization we assumed to find a difference in the lipid composition of the skin constructs. Surprisingly, the ceramide, cholesterol and cholesterol sulfate levels did not differ between the *FLG* knock down and the normal skin construct (Vávrová *et al.*, 2013). So, the reduced expression of *FLG* is most probably not provoking the reduced ceramide levels, which are quite often found in AD skin (Imokawa *et al.*, 1991; Ishikawa *et al.*, 2010). Our results are well in accordance with previously conducted studies by others, which did not find a correlation between *FLG* mutations and decreased ceramide levels in skin from AD patients with known *FLG* mutations (Jungersted *et al.*, 2010) or in organotypic *FLG* knock down skin models (Mildner *et al.*, 2010). Our findings indicate to other pathologic mechanisms in AD skin, which explain the decreased ceramides levels. In terms of the other extracellular lipids the most striking difference, which we found between the normal and the *FLG* knock down skin construct, was a significant increase of the amount of free fatty acids in the *FLG* knock down construct on day 14 of cultivation (Vávrová *et al.*, 2013). Based on these findings, we aimed for analyzing the expression of the secretory phospholipase $A_2$ ($sPLA_2$). The $sPLA_2$ is mainly responsible for the cleavage of phospholipids to free fatty acids at the stratum granulosum - stratum corneum - interface (Mao-Qiang *et al.*, 1995). Several isoforms of the $sPLA_2$ exist in human skin and currently it is unclear which one is the most important (for review see: (Valentin and Lambeau, 2000)). Hence, the expression of the isoforms IB, IIA, IID, IIF, V, X and XIIA was evaluated on mRNA level via RT-PCR. As sPLA appears to be involved in the skin acidification, additionally the expression of the sodium/hydrogen antiporter (NHE-1) was analyzed. On day 14 of cultivation a significant upregulation of the $sPLA_2$-IIA and NHE-1 was found in the *FLG* knock down construct. This result was verified on protein level by the use of immunofluorescence experiments (Vávrová *et al.*, 2013). The expression of all other sPLA isoforms did not differ between normal and *FLG* knock down construct. The $sPLA_2$-IIA and NHE-1 have just recently been demonstrated to be upregulated in skin from *FLG* deficient ft/ft mice (Fluhr *et al.*, 2010). $sPLA_2$ and the NHE-1 are involved in acidification of the skin (for review see: (Chan and Mauro, 2011)). An acidic skin

surface pH is required for the formation of ceramides, since the two most important ceramide generating enzymes, the sphingomyelinase and the β-glucocerebrosidase have their optimal activity at a pH of about 5.5 (for review see: (Choi and Maibach, 2005; Feingold, 2007; Holleran *et al.*, 2006)). The degradation products of FLG, urocanic and pyrrolidone-5-carboxylic acid, are under discussion to have an influence on skin surface pH as well (Fluhr *et al.*, 2010; Krien and Kermici, 2000; Sandilands *et al.*, 2009). Based on the upregulation of two acidification pathways NHE-1 and $sPLA_2$ in the *FLG* knock down skin construct, we hypothesize, that the acidification pathways probably have a feedback among each other to warrant the acidic skin surface pH, when one is compromised. The *in vitro FLG* knock down construct cultivated for 14 days allows for basic research and unbiased investigations of selectively the lack of FLG and its impact on skin acidification. Furthermore, contributing acidification pathways can be analyzed in parallel and the studies can easily be conducted in a time dependent manner. With the *FLG* knock down constructs deeper insight into skin acidification was gained. The impact of FLG on AD pathophysiology in terms of disturbed skin lipid organization and composition was unraveled further.

## 5.3 Reduced FLG Expression Diminishes Inflammatory Threshold

In recent years a new idea of the underlying pathophysiology in AD emerged: Elias P.M. et al. substantiated the idea of an outside-to-inside mechanism instead of inside-to-outside hypothesis (for review see: (Elias *et al.*, 2008)). Based on the findings, that loss-of-function mutations in *FLG* are a major predisposing factor leading to AD, thes hypothesized, that in affected skin the first symptom arising is a defect in the barrier function and that the inflammation follows thereafter, due to the uptake of potential allergens/haptens through the impaired barrier. For assessment of the inflammatory threshold in FLG deficient skin, we evaluated the effects of the skin irritant sodium dodecylsulfate in normal and *FLG* knock down skin constructs. The evaluation was conducted based on the validated OECD guideline for *in vitro* skin irritation testing by the use of reconstructed human epidermis (OECD, 2010). The method was slightly adjusted (decreased concentration of SDS, shortened incubation time) to perform better with the *FLG* knock down construct. We found increased levels of the pro-inflammatory cytokines IL-6 and -8, already when applying the control phosphate buffered saline and even more when applying the local irritant, in *FLG* knock down in comparison to normal constructs (Küchler *et al.*, 2011). These results are not surprising, since water is a known irritant in AD skin (for review see: (Tsai and

Maibach, 1999)). Furthermore, we assessed cell viability following the application of sodium dodecylsulfate and found a significant decrease in the *FLG* knock down constructs (Küchler *et al.*, 2011). The results gained from the skin irritation test indicate that the impaired barrier due to the reduced expression of *FLG* results in increased susceptibility for skin irritation. The applied substances penetrate more easily and cause a stronger inflammatory reaction, indicating a smaller threshold of the disease construct to local irritants. Our findings are well in accordance with the situation found in ft/ft mice, the animal model of *FLG* mutations, where the application of potential allergens results in an increased uptake through the paracellular route and consequentially to a stronger inflammatory reaction (Fallon *et al.*, 2009; Scharschmidt *et al.*, 2009).

## 5.4 PPAR Modulators Increase the Expression of FLG. Can They Provide a New Treatment Option?

Current therapeutically options for the treatment of AD and IV do not target the underlying pathologic mechanisms, but rather ease the arising symptoms (for review see: (Bieber, 2010; Elias, 2010)). Specific treatment strategies may be superior. Besides, the development on an *in vitro* test system to study safety and effectiveness of potential new drugs is of interest (Basketter *et al.*, 2012; Van Gele *et al.*, 2011). The presence of PPARα receptors and the PPARα agonist induced upregulation of *FLG* expression have been demonstrated in human skin and *in vitro* skin constructs (Michalik and Wahli, 2007; Rivier *et al.*, 2000; Schmuth *et al.*, 2008; Zhang *et al.*, 2012). Beside the upregulation of FLG expression, PPARα receptor modulators have been demonstrated to exhibit several other beneficial effects on skin, like the improvement of permeability barrier homeostasis and SC integrity or the acceleration of postnatal SC acidification (Fluhr *et al.*, 2009; Schmuth *et al.*, 2008). An increase in the inflammatory threshold by the application of a PPARα modulator has been demonstrated in mouse models of allergic and irritant contact dermatitis (Sheu *et al.*, 2002). Furthermore, PPARα modulators induce the synthesis of colesterol, free fatty acids and ceramides (Man *et al.*, 2006; Rivier *et al.*, 2000; Schmuth *et al.*, 2008). All these impacts on the skin, might be beneficial for patients suffering from AD or IV, since they have an impaired skin barrier function, decreased inflammatory threshold and a decreased level of ceramides (for review see: (Bieber, 2010; Elias *et al.*, 2008; Leung *et al.*, 2004)). For the first time we demonstrated that the upregulation of *FLG* expression is feasible in skin constructs with a diminished expression of FLG and that

these skin constructs could serve as a first test systems for preclinical testing of new compounds. The supplementation with docosahexaenoic acid (DHA) increased the *FLG* expression significantly. The *FLG* expression in knock down constructs after DHA stimulation even exceeded the *FLG* expression of normal skin construct. Thus, the treatment with a PPARα modulator increases *FLG* expression in *FLG* deficient skin and might therefore improve the features of *FLG* deficiency in the skin. Exposure to clofibrate induced the *FLG* expression only in one studied batch. This result could not be repeated with skin constructs grown with cells from other donors. Since other authors have demonstrated a positive effect on *FLG* expression by clofibrate (Rivier *et al.*, 2000; Zhang *et al.*, 2012), experimental problems need to be assessed in future experiments. Since a reduced *FLG* expression is thought to be causing impaired barrier function in the skin of *FLG* mutation carriers and that this barrier defect is the first step in the development of AD (for review see: (Elias *et al.*, 2008)), an upregulation of *FLG* expression might be an option for AD treatment addressing the underlying pathomechanism and not just treating symptoms. Besides, it has been shown that even in AD patients not suffering from mutation in *FLG* have an reduced expression of FLG (Howell *et al.*, 2007). So, AD patients might have benefit from the treatment with a PPARα modulator. Clofibrate has been demonstrated to normalize the ceramide profile in skin constructs when applied in combination with ascorbic acid (Batheja *et al.*, 2009). PPAR modulators might provide a new option in the treatment of AD or IV, respectively, due to the prevention of glucocorticoid-induced side effects, when these drug classes are administered in combination (Demerjian *et al.*, 2009; Hatano *et al.*, 2011).

Taken togehter, I could demonstrate that the established *FLG* knock down construct is suitable for the evaluation of new treatment strategies for AD. Especially, to develop therapeutic options interfering with the underlying pathomechanism.

## 5.5 Generation of More Complex *In Vitro* Skin Constructs

The implementation of further cells into *in vitro* skin models is of great interest to develop a more complex model, since a further assimilation to native human skin is desired. Thereby, the vascularization and the implementation of immune cells are pursued to generate more complex *in vitro* skin constructs, which allow for a deeper insight into the skin physiology and skin disease pathophysiology. With the

implementation of immune cells, the evaluation of immune reactions in the skin will be possible.

For vascularization of *in vitro* skin constructs several setups for co-culturing of dermal fibroblasts and endothelial cells are described in the literature (Hudon *et al.*, 2003; Sorrell *et al.*, 2007). I aimed for culturing of the endothelial cells together with the fibroblast in the collagen matrix of the *in vitro* skin construct. However, embedding of endothelial cells in the collagen was not successful, since the endothelial cells started to phagocytize the collagen. The phagocytosis of collagen by endothelial cells was also observed previously (Meyer *et al.*, 1997; Smedsrod *et al.*, 1995). Hence, the setup for endothelial cell implementation was changed. The cultivation of an endothelial monolayer on inert filter membranes has been demonstrated and a confluent endothelial cell monolayer is sufficient to study the migration of immune cells across the wall of blood vessels *in vitro* (Huh *et al.*, 2010). This approach could be adjusted to our *in vitro* skin construct and a confluent endothelial monolayer was implemented on the basal side of the cell culture insert in which the *in vitro* skin construct was cultivated. Histological examination revealed an intact, viable layer of endothelial cells, but, furthermore, demonstrated the impairment of epidermal maturation of the skin construct. One reason for the impaired epidermal maturation might be the change of the differentiation medium. For better growth of the endothelial cells, the medium was mixed with endothelial cell medium and supplemented with ascorbate-phosphate. This approach has been proven to be beneficial in a fibroblast-endothelial-cell co-culture system (Sorrell *et al.*, 2007). Obviously, this change of the differentiation medium cannot easily be transferred onto complete *in vitro* skin models, important supplements for epidermal maturation probably need to be added. If these histological effects have an impact on skin barrier function, needs to be assessed in future experiments. Nevertheless, the implementation of a confluent endothelial cell monolayer into the *in vitro* skin construct was successful. The viability and functionality of the endothelial cell monolayer was demonstrated by the presence of the two specific endothelial cell markers von Willebrand factor and platelet endothelial cell adhesion molecule 1 (PECAM-1). PECAM-1 has been proclaimed to be important for angiogenesis, since blocking of PECAM-1 with specific antibodies inhibits the elongation and migration of endothelial cells. The formation of cell-cell-interactions is inhibited (Yang *et al.*, 1999). Thus, the presence of the endothelial cell marker PECAM-1 reveals intact cell-cell-interactions in the endothelial cell monolayer of the *in vitro* skin construct. von Willebrand factor is synthesized in human endothelial cells in vivo and is released upon stimulation (such as injury of the blood vessel) to maintain normal blood hemostasis

(Powazniak *et al.*, 2011; Turner *et al.*, 2009). With the detection of von Willebrand factor in the endothelial cell monolayer of the complex *in vitro* skin construct the functionality of the cells is demonstrated. Hence, the implementation of an endothelial cell monolayer into the *in vitro* construct by principle is possible and therefore the data presented here provide a base for further studies.

Human PBMCs are a mixture of monocytes and lymphocytes (Rajamaran *et al.*, 1977). These cells, mainly activated T cells, are often found as infiltrate of the lesional skin of AD patients (for review see: (Kerstan *et al.*, 2009; Leung *et al.*, 2004). I aimed for mimicking the migration by means of a complex *in vitro* skin construct. PBMCs were isolated from human peripheral blood and different experimental setups have been evaluated, but unfortunately, no setup could be established by which the migration of human PBMCs could be studied. So, at the moment, the role of immune cells cannot be studied with the established skin construct and further work is needed to develop a more complex skin model. Many different parameters might have influenced the migration negatively. First of all, I only used normal skin constructs to perform these experiments. The inflammatory response to a local irritant is significantly stronger in the *FLG* knock down constructs (Küchler *et al.*, 2011), the stimulation might be sub-threshold in the normal construct. In future experiments, the use of *FLG* knock down constructs instead of normal constructs should be pursued. Furthermore, the stimuli used in the experiments might have been below the trigger of PBMC migration. In future experimental setups, this should be carefully investigated. Currently, it is not clarified, which mechanistic features initiate the migration of T-Lymphocytes into the lesional skin of atopic patients (for review see: (Kerstan *et al.*, 2009)). Yet, the infiltration with T-cells or monocytes depends on the pattern of released cytokines by keratinocytes (Barker *et al.*, 1991; Goebeler *et al.*, 2001). Therefore, the induction of the inflammation in the *in vitro* skin construct might be crucial for the migration of immune cells and should be studied in future experiments. In acute, lesional skin of AD patients increased levels of the Th2 interleukins 4, 5 and 13 along with an infiltrate of activated T cells have been demonstrated (for review see: (Leung *et al.*, 2004)), so these interleukins might provide a new basis for future studies. Additionally, features, which influence the migration negatively, need to be assessed. It was demonstrated, that the expression of chemokine receptors on leukocytes is changed after isolation (Nieto *et al.*, 2012). Since the receptor profile of PBMCs was not assessed in this study, it is possible, that the PBMCs lost the respective receptor needed for recruitment. Another aspect for the failing of the evaluated approaches, might be the adhesion behavior of PBMCs. Since the monocytes adhere on glass and plastic

surface (Rajamaran *et al.*, 1977), it is possible that they stuck to the cell culture plastic ware. A constant movement of the cell suspension might be required to induce the adhesion of PBMCs to the endothelial cells (Huh *et al.*, 2010). Additionally the analysis of the migration should be improved. The observation of the migration via confocal microscopy of the complete *in vitro* skin construct (Huh *et al.*, 2010) might provide deeper insight into the behavior of human PBMCs when added to the skin construct. Nevertheless, *in vitro* constructs have been demonstrated to be suitable to study the migration of immune cells (Schaller *et al.*, 2004). Hence, the adjustment of the experimental setup with our *in vitro* skin construct can make the evaluation of the migration of immune cells possible.

# 6. Prospects

In this thesis an *in vitro FLG* knock down skin construct was established by the use of siRNA and intensively characterized in terms of skin morphology, barrier function and skin lipid composition. Thereby, it has been demonstrated that pathological mechanisms can be unraveled further by means of this skin construct. Furthermore, the suitability as a test system for new treatment strategies has been demonstrated.

The established *in vitro* skin construct allows for studying of the acidification pathways of human skin intensively. Thereby, the role of sodium/hydrogen antiporter (NHE-1) and secretory phospholipase $A_2$ ($sPLA_2$) needs more detailed evaluation. In future experiments their impact can be assessed in studies where these genes are silenced by the use of siRNA in the *in vitro* skin construct. Hence, the impact of each single pathway can be unraveled. Furthermore, the effects of the supplementation with PPAR modulators should be studied more intensively. Thereby, mainly the impact on lipid organization and composition is of interest. Besides, the migration of immune cells into the *in vitro* skin construct should be studied more intensively.

With the established *in vitro FLG* knock down skin construct a good base has been generated to develop a complex model of FLG associated skin diseases such as AD. For the development of such an AD model, immunocompetent cells, such as dendritic cells, need to be integrated into the *in vitro* skin construct in order to reflect the inflammatory feature of AD. Since loss-of-function mutations in *FLG* are only one major predisposing factor leading to the development of AD, the influence of other genes and environmental factors needs to be assessed carefully and then must be implemented in the model. Thereby, the use of cells derived from lesional skin of AD patients for the construction of the *in vitro* model might be one step to gain better insight.

# 7. Summary

Although loss-of-function mutations of *FLG* are a well-known major predisposing factor for atopic dermatitis (AD) and ichthyosis vulgaris (IV), respectively, only little is known about the effects caused by the reduced expression of this protein. Experiments in patients are expensive and bring the disadvantage that the features of one single gene effect cannot be observed. Often used animal models - like the flaky tail mouse - raise ethical concerns and bring the disadvantage of species related differences in the physiology beside a different pathomechanism. *In vitro* skin constructs are a helpful tool to overcome these disadvantages. It is possible to study the effect of the loss-of-function in just one single gene, ethical concerns are not arising and species related differences are not given since the constructs are made from human material.

In this study an *in vitro* skin construct based on *FLG* knock down cultivated for 14 days was established and characterized. Morphological abnormalities like the intra- and intercellular spongiosis or the disturbed stratum corneum development could be linked to the diminished expression of *FLG*. Furthermore, the impact on lipid organization and composition was evaluated. In terms of lipid composition, the most striking difference between normal and *FLG* knock down construct was the increase in free fatty acids, whereas only minor differences in ceramide, cholesterol and cholesterol sulfate composition were found. This is well in accordance with the evaluated increase of secretory phospholipase $A_2$-IIA ($sPLA_2$-IIA) expression found in the *FLG* knock down construct, which results in increased levels of free fatty acids via the "phospholipids - to - free fatty acids pathway". Moreover, a significantly increased expression of the sodium/hydrogen antiporter (NHE-1) has been found in the *FLG* knock down construct. Based on these findings and on the fact, that the "FLG-to-urocanic acid/pyrrolidone carboxylic acid" acidification pathway is restricted in the *FLG* knock down construct, a feedback mechanism between these pathways can be hypothesized: When FLG expression is reduced and therefore lower amounts of the acidifying amino acids urocanic acid and pyrrolidone carboxylic acid are produced, the NHE-1 and / or $sPLA_2$ becomes upregulated to ensure the most possible acidic skin surface pH. For further assessment of the barrier function, skin absorption studies have been performed. Permeation of the hydrophilic model substance caffeine did not differ in the normal or *FLG* knock down skin construct, respectively, whereas the permeation of the lipophilic model drug testosterone was enhanced in the disease construct, indicating a disturbed barrier. This is in accordance with the disturbed lipid organization in the *FLG* knock

down constructs, which most probably is mainly affecting the permeation of lipophilic compounds.

Besides the disturbed barrier, a diminished inflammatory threshold in the *FLG* knock down construct to the standard local irritant SDS was demonstrated. Hence, the inflammatory feature of AD skin was mimicked by means of the *in vitro* skin construct as well. Furthermore, the suitability of the skin construct as *in vitro* pharmacological testing tool for the assessment of new therapeutically option has been demonstrated by the enhanced *FLG* expression following a supplementation with the PPARα modulator docosahexaenoic acid.

By the implementation of an endothelial cell layer, a further assimilation of the *in vitro* skin construct to the *in vivo* situation could be achieved. Still, further work is needed since at the current state of development the endothelial cells seem to disturb the epidermal maturation.

In conclusion, the established *in vitro* skin construct is a helpful tool in basic research, offers new insights into skin physiology, disease pathophysiology and offers the opportunity to evaluate new treatment options. Therefore, the established *in vitro FLG* knock down construct is a relevant step in the generation of a complex *in vitro* model of atopic dermatitis.

# 8. Zusammenfassung

Funktionsverlustmutationen im Filaggrin-Gen sind bekannte Risikofaktoren für die Entwicklung einer atopischen Dermatitis (AD) oder einer Ichthyosis vulgaris (IV). Trotzdem sind die Auswirkungen, die die reduzierte Bildung des Proteins mit sich bringt, weitgehend unbekannt. Untersuchungen an Patienten direkt sind kostspielig und haben den großen Nachteil, dass andere Faktoren das Ergebnis beeinflussen und nicht die Auswirkungen eines einzelnen Gendefekts beurteilt werden können. Versuche in Tiermodellen, wie der flaky tail Maus, sind ethisch nicht immer vertretbar und haben darüber hinaus den großen Nachteil, dass zum einen die Physiologie von Tier und Mensch doch sehr unterschiedlich ist, zum anderen unterscheidet sich auch die Pathophysiologie der Krankheit. Die Entwicklung von *in vitro* Hautmodellen hat die Forschung deshalb einen großen Schritt weiter gebracht, da wesentliche Nachteile, die im Patienten- oder Tierversuch entstehen, überwunden sind. Mit Hilfe von Hautmodellen ist es möglich die Auswirkungen eines Funktionsverlusts in einem einzelnen Gen zu untersuchen ohne dass ethische Regeln verletzt werden. Da *in vitro* Hautmodelle aus humanem Material generiert werden, liegen auch keine Spezies bedingten Unterschiede vor.

In der vorliegenden Arbeit wurde ein *in vitro* Hautmodell, in dem spezifisch das Filaggrin-Gen ausgeschaltet wurde und das für 14 Tage kultiviert wurde, etabliert und charakterisiert. Die reduzierte Expression von Filaggrin führt zu morphologischen Veränderungen, wie z.B. einer intra- und interzellulären Ödembildung sowie einer gestörten Entwicklung des Stratum corneum. Des Weiteren wurde die Organisation und Zusammensetzung der Hautlipide untersucht. Dabei wurde als größter Unterschied zwischen normalen und *FLG* knock down Modellen eine signifikant größere Menge an freien Fettsäuren in dem krankhaft veränderten Modell gefunden. Die Zusammensetzung der anderen Hautlipide, Ceramide, Cholesterol und Cholesterolsulfat, war dagegen nahezu gleich. Die größere Menge an freien Fettsäuren geht mit einer erhöhten Expression der sekretorischen Phospholipase $A_2$-IIA ($sPLA_2$-IIA) einher. Die $sPLA_2$ sind die für die Bildung von freien Fettsäuren aus Phospholipiden wesentlichen Enzyme im Stratum corneum. Ein signifikanter Anstieg der Expression des Natrium-/Protonen-Antiporters NHE-1 in den *FLG* knock down in Verbindung mit der Tatsache, dass in dem *FLG* knock down Modell die Azidifizierung des Stratum corneums nicht über die Bildung von Urocanin- und Pyrrolidoncarbonsäure aus Filaggrin ablaufen kann, führt zu der Hypothese einer Rückkoppelung zwischen den verschiedenen Azidifizierungsmechanismen: Reduzierte

Expression des Filaggrin und damit geringere Bildung von Urocanin- und Pyrrolidoncarbonsäure aus Histidin führt zu einer Hochregulation von NHE-1 und / oder $sPLA_2$, um den physiologisch sauren Hautoberflächen pH sowit möglich aufrecht zu erhalten. Die Barrierefunktion des Hautmodells wurde anhand der Durchführung von Hautabsorptionsstudien weiter charakterisiert. Dabei wurde für die hydrophile Modellsubstanz Coffein kein Unterschied in der Permeation des normalen oder des *FLG* knock down Modells gefunden. Für die lipophile Modellsubstanz Testosteron hingegen wurde ein deutlicher Anstieg der Permeation durch das *FLG* knock down Modell verzeichnet, was ein deutlicher Hinweis auf eine gestörte Hautbarriere liefert. Die erhöhte Permeation einer lipophilen Substanz ist höchstwahrscheinlich auf die gestörte Lipidorganisation des *FLG* knock down Modells zurückzuführen.

Neben der gestörten Hautbarriere wurde in dem *FLG* knock down Modell weiterhin eine geringere Schwelle gegenüber dem lokal applizierten Standardirritans SDS nachgewiesen. Folglich kann mit dem Hautmodell auch die entzündliche Komponente der atopischen Dermatitis erfolgreich dargestellt werden. Des Weiteren wurde gezeigt, dass das etablierte *in vitro* Hautmodell für die pharmakologische Testung geeignet ist und neue therapeutische Optionen mit Hilfe des Modells entwickelt werden können. Dies wurde anhand des PPARα Modulators Docosahexaensäure und dem daraus resultierenden signifikanten Anstieg der FLG Expression verdeutlicht. Die Anwendung von PPARα Modulatoren könnte eine neue Strategie in der Behandlung der atopischen Dermatitis sein.

Die Implementierung einer Endothelzellschicht in das etablierte *in vitro* Hautmodell brachte eine noch größere Annäherung des Modells an die in vivo Situation mit sich. Nichtsdestotrotz, ist hier eine Weiterentwicklung von Nöten, da bislang die Implementierung der Endothelzellen eine Verschlechterung der epidermalen Reifung des Hautmodells mit sich bringt.

Das hier etablierte *in vitro FLG* knock down Hautmodell ist eine erfolgreiche Grundlage für die Forschung. Mit Hilfe des Modells ist es möglich tiefere Einblicke in die Physiologie der Haut und die pathophysiologischen Zustände von Hautkrankheiten zu bekommen und neue Strategien für die Behandlung von Hautkrankheiten zu entwickeln. Das *FLG* knock down Modell bildet ferner den ersten Schritt in der Entwicklung eines komplexen Modells der atopischen Dermatitis.

# 9. References

Artik S, Ruzicka T (2003) Complementary therapy for atopic eczema and other allergic skin diseases. *Dermatol Ther* 16:150-63.

Baetz FM, Klipper W, Korting HC*, et al.* (2012) Esterase activity in excised and reconstructed human skin - Biotransformation of prednicarbate and the model dye fluorescein diacetate. *Eur J Pharm Biopharm*.

Barker CL, McHale MT, Gillies AK*, et al.* (2004) The development and characterization of an in vitro model of psoriasis. *The Journal of investigative dermatology* 123:892-901.

Barker JN, Mitra RS, Griffiths CE*, et al.* (1991) Keratinocytes as initiators of inflammation. *Lancet* 337:211-4.

Barker JN, Palmer CN, Zhao Y*, et al.* (2007) Null mutations in the filaggrin gene (FLG) determine major susceptibility to early-onset atopic dermatitis that persists into adulthood. *The Journal of investigative dermatology* 127:564-7.

Basketter DA, Clewell H, Kimber I*, et al.* (2012) A roadmap for the development of alternative (non-animal) methods for systemic toxicity testing - t4 report*. *ALTEX* 29:3-91.

Batheja P, Song Y, Wertz P*, et al.* (2009) Effects of growth conditions on the barrier properties of a human skin equivalent. *Pharm Res* 26:1689-700.

Bieber T (2010) Atopic dermatitis. *Ann Dermatol* 22:125-37.

Bitoun E, Micheloni A, Lamant L*, et al.* (2003) LEKTI proteolytic processing in human primary keratinocytes, tissue distribution and defective expression in Netherton syndrome. *Hum Mol Genet* 12:2417-30.

Blume-Peytavi U, Metz M (2012) Atopic dermatitis in children: management of pruritus. *J Eur Acad Dermatol Venereol* 26 Suppl 6:2-8.

Brown SJ, McLean WH (2012) One remarkable molecule: filaggrin. *The Journal of investigative dermatology* 132:751-62.

Candi E, Schmidt R, Melino G (2005) The cornified envelope: a model of cell death in the skin. *Nat Rev Mol Cell Biol* 6:328-40.

Carlson MW, Alt-Holland A, Egles C*, et al.* (2008) Three-dimensional tissue models of normal and diseased skin. *Curr Protoc Cell Biol* Chapter 19:Unit 19 9.

Chamlin SL, Kao J, Frieden IJ*, et al.* (2002) Ceramide-dominant barrier repair lipids alleviate childhood atopic dermatitis: changes in barrier function provide a sensitive indicator of disease activity. *J Am Acad Dermatol* 47:198-208.

Chan A, Mauro T (2011) Acidification in the epidermis and the role of secretory phospholipases. *Dermatoendocrinol* 3:84-90.

Chiang A, Tudela E, Maibach HI (2012) Percutaneous absorption in diseased skin: an overview. *J Appl Toxicol* 32:537-63.

Choi MJ, Maibach HI (2005) Role of ceramides in barrier function of healthy and diseased skin. *Am J Clin Dermatol* 6:215-23.

Cork MJ, Danby SG, Vasilopoulos Y*, et al.* (2009) Epidermal Barrier Dysfunction in Atopic Dermatitis. *The Journal of investigative dermatology* 129:1892-908.

Dale BA (1977) Purification and characterization of a basic protein from the stratum corneum of mammalian epidermis. *Biochim Biophys Acta* 491:193-204.

Demerjian M, Choi EH, Man MQ*, et al.* (2009) Activators of PPARs and LXR decrease the adverse effects of exogenous glucocorticoids on the epidermis. *Exp Dermatol* 18:643-9.

Descargues P, Deraison C, Bonnart C*, et al.* (2005) Spink5-deficient mice mimic Netherton syndrome through degradation of desmoglein 1 by epidermal protease hyperactivity. *Nat Genet* 37:56-65.

Eckl KM, Alef T, Torres S*, et al.* (2011) Full-thickness human skin models for congenital ichthyosis and related keratinization disorders. *The Journal of investigative dermatology* 131:1938-42.

Elias PM (1983) Epidermal lipids, barrier function, and desquamation. *The Journal of investigative dermatology* 80:44s-9s.

Elias PM (2010) Therapeutic Implications of a Barrier-based Pathogenesis of Atopic Dermatitis. *Ann Dermatol* 22:245-54.

Elias PM, Hatano Y, Williams ML (2008) Basis for the barrier abnormality in atopic dermatitis: outside-inside-outside pathogenic mechanisms. *J Allergy Clin Immunol* 121:1337-43.

Engelhart K, El Hindi T, Biesalski HK*, et al.* (2005) In vitro reproduction of clinical hallmarks of eczematous dermatitis in organotypic skin models. *Arch Dermatol Res* 297:1-9.

EuropeanCommission (1976) Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products. *Offical Journal of the European Communities* L262:169-200.

EuropeanParliament, Council (2006) Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. *Offical Journal of the European Communities*.

EuropeanParliament, Council EU (2003) Directive 2003/15/EC of the European Parliament and of the Council of 27 February 2003 amending Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products. *Offical Journal of the European Union* L 66:26-35.

Fallon PG, Sasaki T, Sandilands A*, et al.* (2009) A homozygous frameshift mutation in the mouse Flg gene facilitates enhanced percutaneous allergen priming. *Nat Genet* 41:602-8.

FDA (2006) Guidance for Industry Nonclinical Safety Evaluation of Drug or Biologic Combinations available at http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm. Accessed 08.05.2013.

Feingold KR (2007) Thematic review series: skin lipids. The role of epidermal lipids in cutaneous permeability barrier homeostasis. *J Lipid Res* 48:2531-46.

Fleckman P, Brumbaugh S (2002) Absence of the granular layer and keratohyalin define a morphologically distinct subset of individuals with ichthyosis vulgaris. *Exp Dermatol* 11:327-36.

Fleckman P, Dale BA, Holbrook KA (1985) Profilaggrin, a high-molecular-weight precursor of filaggrin in human epidermis and cultured keratinocytes. *The Journal of investigative dermatology* 85:507-12.

Fluhr JW, Behne MJ, Brown BE*, et al.* (2004) Stratum corneum acidification in neonatal skin: secretory phospholipase A2 and the sodium/hydrogen antiporter-1 acidify neonatal rat stratum corneum. *The Journal of investigative dermatology* 122:320-9.

Fluhr JW, Elias PM, Man MQ*, et al.* (2010) Is the filaggrin-histidine-urocanic acid pathway essential for stratum corneum acidification? *The Journal of investigative dermatology* 130:2141-4.

Fluhr JW, Man MQ, Hachem JP*, et al.* (2009) Topical peroxisome proliferator activated receptor activators accelerate postnatal stratum corneum acidification. *The Journal of investigative dermatology* 129:365-74.

Folster-Holst R, Stoll M, Koch WA*, et al.* (2005) Lack of association of SPINK5 polymorphisms with nonsyndromic atopic dermatitis in the population of Northern Germany. *Br J Dermatol* 152:1365-7.

Gan SQ, McBride OW, Idler WW*, et al.* (1990) Organization, structure, and polymorphisms of the human profilaggrin gene. *Biochemistry* 29:9432-40.

Garg A, Chren MM, Sands LP*, et al.* (2001) Psychological stress perturbs epidermal permeability barrier homeostasis: implications for the pathogenesis of stress-associated skin disorders. *Arch Dermatol* 137:53-9.

Gibbs S (2009) In vitro irritation models and immune reactions. *Skin Pharmacol Physiol* 22:103-13.

Gibbs S, Vicanova J, Bouwstra J*, et al.* (1997) Culture of reconstructed epidermis in a defined medium at 33 degrees C shows a delayed epidermal maturation, prolonged lifespan and improved stratum corneum. *Arch Dermatol Res* 289:585-95.

Gibot L, Galbraith T, Huot J*, et al.* (2013) Development of a tridimensional microvascularized human skin substitute to study melanoma biology. *Clin Exp Metastasis* 30:83-90.

Goebeler M, Trautmann A, Voss A*, et al.* (2001) Differential and sequential expression of multiple chemokines during elicitation of allergic contact hypersensitivity. *Am J Pathol* 158:431-40.

Groen D, Poole DS, Gooris GS*, et al.* (2011) Is an orthorhombic lateral packing and a proper lamellar organization important for the skin barrier function? *Biochim Biophys Acta* 1808:1529-37.

Gruber R, Elias PM, Crumrine D*, et al.* (2011) Filaggrin genotype in ichthyosis vulgaris predicts abnormalities in epidermal structure and function. *Am J Pathol* 178:2252-63.

Hachem JP, Crumrine D, Fluhr J*, et al.* (2003) pH directly regulates epidermal permeability barrier homeostasis, and stratum corneum integrity/cohesion. *The Journal of investigative dermatology* 121:345-53.

Hartung T (2008) Food for thought... on animal tests. *ALTEX* 25:3-16.

Hatano Y, Elias PM, Crumrine D*, et al.* (2011) Efficacy of combined peroxisome proliferator-activated receptor-alpha ligand and glucocorticoid therapy in a murine model of atopic dermatitis. *The Journal of investigative dermatology* 131:1845-52.

Hatano Y, Terashi H, Arakawa S*, et al.* (2005) Interleukin-4 suppresses the enhancement of ceramide synthesis and cutaneous permeability barrier functions induced by tumor necrosis factor-alpha and interferon-gamma in human epidermis. *The Journal of investigative dermatology* 124:786-92.

Heimall J, Spergel JM (2012) Filaggrin mutations and atopy: consequences for future therapeutics. *Expert Rev Clin Immunol* 8:189-97.

Hirota T, Takahashi A, Kubo M*, et al.* (2012) Genome-wide association study identifies eight new susceptibility loci for atopic dermatitis in the Japanese population. *Nat Genet* 44:1222-6.

Hoeller-Obrigkeit D, Jugert FK, Beermann T*, et al.* (2009) Effects of photodynamic therapy evaluated in a novel three-dimensional squamous cell carcinoma organ construct of the skin. *Photochem Photobiol* 85:272-8.

Hoeller D, Huppertz B, Roos TC*, et al.* (2001) An improved and rapid method to construct skin equivalents from human hair follicles and fibroblasts. *Exp Dermatol* 10:264-71.

Hoffjan S, Stemmler S (2007) On the role of the epidermal differentiation complex in ichthyosis vulgaris, atopic dermatitis and psoriasis. *Br J Dermatol* 157:441-9.

Holleran WM, Takagi Y, Uchida Y (2006) Epidermal sphingolipids: metabolism, function, and roles in skin disorders. *FEBS Lett* 580:5456-66.

Hoste E, Kemperman P, Devos M*, et al.* (2011) Caspase-14 is required for filaggrin degradation to natural moisturizing factors in the skin. *The Journal of investigative dermatology* 131:2233-41.

Howell MD, Kim BE, Gao P*, et al.* (2007) Cytokine modulation of atopic dermatitis filaggrin skin expression. *J Allergy Clin Immunol* 120:150-5.

Hudon V, Berthod F, Black AF*, et al.* (2003) A tissue-engineered endothelialized dermis to study the modulation of angiogenic and angiostatic molecules on capillary-like tube formation in vitro. *Br J Dermatol* 148:1094-104.

Huh D, Matthews BD, Mammoto A*, et al.* (2010) Reconstituting organ-level lung functions on a chip. *Science* 328:1662-8.

ICH (2013) International Conference on Harmonisation Safety Guidelines available at http://www.ich.org/products/guidelines/safety/article/safety-guidelines.html. Accessed 08.05.2013.

Imokawa G, Abe A, Jin K*, et al.* (1991) Decreased level of ceramides in stratum corneum of atopic dermatitis: an etiologic factor in atopic dry skin? *The Journal of investigative dermatology* 96:523-6.

Irvine AD, McLean WH, Leung DY (2011) Filaggrin mutations associated with skin and allergic diseases. *N Engl J Med* 365:1315-27.

Ishikawa J, Narita H, Kondo N*, et al.* (2010) Changes in the ceramide profile of atopic dermatitis patients. *The Journal of investigative dermatology* 130:2511-4.

Jakasa I, Koster ES, Calkoen F*, et al.* (2011) Skin barrier function in healthy subjects and patients with atopic dermatitis in relation to filaggrin loss-of-function mutations. *The Journal of investigative dermatology* 131:540-2.

Jean J, Lapointe M, Soucy J*, et al.* (2009) Development of an in vitro psoriatic skin model by tissue engineering. *J Dermatol Sci* 53:19-25.

Jensen JM, Folster-Holst R, Baranowsky A*, et al.* (2004) Impaired sphingomyelinase activity and epidermal differentiation in atopic dermatitis. *The Journal of investigative dermatology* 122:1423-31.

Johansson SG, Bieber T, Dahl R*, et al.* (2004) Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *J Allergy Clin Immunol* 113:832-6.

Jungersted JM, Scheer H, Mempel M*, et al.* (2010) Stratum corneum lipids, skin barrier function and filaggrin mutations in patients with atopic eczema. *Allergy* 65:911-8.

Kalinin A, Marekov LN, Steinert PM (2001) Assembly of the epidermal cornified cell envelope. *J Cell Sci* 114:3069-70.

Kalinin AE, Kajava AV, Steinert PM (2002) Epithelial barrier function: assembly and structural features of the cornified cell envelope. *Bioessays* 24:789-800.

Kandarova H, Liebsch M, Spielmann H*, et al.* (2006) Assessment of the human epidermis model SkinEthic RHE for in vitro skin corrosion testing of chemicals according to new OECD TG 431. *Toxicol In Vitro* 20:547-59.

Kaplan DH (2010) In vivo function of Langerhans cells and dermal dendritic cells. *Trends Immunol* 31:446-51.

Kato A, Fukai K, Oiso N*, et al.* (2003) Association of SPINK5 gene polymorphisms with atopic dermatitis in the Japanese population. *Br J Dermatol* 148:665-9.

Katoh N (2009) Future perspectives in the treatment of atopic dermatitis. *J Dermatol* 36:367-76.

Kerl H, Garbe C, Cerroni L*, et al.* (2003) Histopathologie der Haut. (Berlin: Springer Verlag, 970pp.

Kerstan A, Leverkus M, Trautmann A (2009) Effector pathways during eczematous dermatitis: where inflammation meets cell death. *Exp Dermatol* 18:893-9.

Kezic S, Kemperman PM, Koster ES*, et al.* (2008) Loss-of-function mutations in the filaggrin gene lead to reduced level of natural moisturizing factor in the stratum corneum. *The Journal of investigative dermatology* 128:2117-9.

Kezic S, O'Regan GM, Yau N*, et al.* (2011) Levels of filaggrin degradation products are influenced by both filaggrin genotype and atopic dermatitis severity. *Allergy* 66:934-40.

Kollisch G, Kalali BN, Voelcker V*, et al.* (2005) Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. *Immunology* 114:531-41.

Komatsu N, Takata M, Otsuki N*, et al.* (2002) Elevated stratum corneum hydrolytic activity in Netherton syndrome suggests an inhibitory regulation of desquamation by SPINK5-derived peptides. *The Journal of investigative dermatology* 118:436-43.

Korting HC, Patzak U, Schaller M*, et al.* (1998) A model of human cutaneous candidosis based on reconstructed human epidermis for the light and electron microscopic study of pathogenesis and treatment. *J Infect* 36:259-67.

Krien PM, Kermici M (2000) Evidence for the existence of a self-regulated enzymatic process within the human stratum corneum -an unexpected role for urocanic acid. *The Journal of investigative dermatology* 115:414-20.

Krug M, Oji V, Traupe H*, et al.* (2009) Ichthyoses - Part 1: Differential diagnosis of vulgar ichthyoses and therapeutic options. *J Dtsch Dermatol Ges* 7:511-9.

Küchler S, Henkes D, Eckl KM*, et al.* (2011) Hallmarks of atopic skin mimicked in vitro by means of a skin disease model based on FLG knock-down. *Altern Lab Anim* 39:471-80.

Küchler S, Wolf NB, Heilmann S*, et al.* (2010) 3D-wound healing model: influence of morphine and solid lipid nanoparticles. *J Biotechnol* 148:24-30.

Laubach V, Zoller N, Rossberg M*, et al.* (2010) Integration of Langerhans-like cells into a human skin equivalent. *Arch Dermatol Res* 303:135-9.

Leung DY (2003) Infection in atopic dermatitis. *Curr Opin Pediatr* 15:399-404.

Leung DY, Boguniewicz M, Howell MD*, et al.* (2004) New insights into atopic dermatitis. *J Clin Invest* 113:651-7.

Li L, Fukunaga-Kalabis M, Herlyn M (2011) The three-dimensional human skin reconstruct model: a tool to study normal skin and melanoma progression. *J Vis Exp*.

Liu FT, Goodarzi H, Chen HY (2011) IgE, mast cells, and eosinophils in atopic dermatitis. *Clin Rev Allergy Immunol* 41:298-310.

Luu-The V, Duche D, Ferraris C*, et al.* (2009) Expression profiles of phases 1 and 2 metabolizing enzymes in human skin and the reconstructed skin models Episkin and full thickness model from Episkin. *J Steroid Biochem Mol Biol* 116:178-86.

MacNeil S (2007) Progress and opportunities for tissue-engineered skin. *Nature* 445:874-80.

Man MQ, Choi EH, Schmuth M*, et al.* (2006) Basis for improved permeability barrier homeostasis induced by PPAR and LXR activators: liposensors stimulate lipid synthesis, lamellar body secretion, and post-secretory lipid processing. *The Journal of investigative dermatology* 126:386-92.

Mao-Qiang M, Feingold KR, Jain M*, et al.* (1995) Extracellular processing of phospholipids is required for permeability barrier homeostasis. *J Lipid Res* 36:1925-35.

Marenholz I, Nickel R, Ruschendorf F*, et al.* (2006) Filaggrin loss-of-function mutations predispose to phenotypes involved in the atopic march. *J Allergy Clin Immunol* 118:866-71.

MatTek C (2013) Psoriasis Tissue. Available at: http://www.mattek.com/pages/products/psoriasis/. Accessed 2013/05/01.

Menon GK, Cleary GW, Lane ME (2012) The structure and function of the stratum corneum. *Int J Pharm* 435:3-9.

Mestas J, Hughes CC (2004) Of mice and not men: differences between mouse and human immunology. *J Immunol* 172:2731-8.

Meyer GT, Matthias LJ, Noack L*, et al.* (1997) Lumen formation during angiogenesis in vitro involves phagocytic activity, formation and secretion of vacuoles, cell death, and capillary tube remodelling by different populations of endothelial cells. *Anat Rec* 249:327-40.

Michalik L, Wahli W (2007) Peroxisome proliferator-activated receptors (PPARs) in skin health, repair and disease. *Biochim Biophys Acta* 1771:991-8.

Mildner M, Ballaun C, Stichenwirth M*, et al.* (2006) Gene silencing in a human organotypic skin model. *Biochem Biophys Res Commun* 348:76-82.

Mildner M, Jin J, Eckhart L*, et al.* (2010) Knockdown of filaggrin impairs diffusion barrier function and increases UV sensitivity in a human skin model. *The Journal of investigative dermatology* 130:2286-94.

Neis MM, Peters B, Dreuw A*, et al.* (2006) Enhanced expression levels of IL-31 correlate with IL-4 and IL-13 in atopic and allergic contact dermatitis. *J Allergy Clin Immunol* 118:930-7.

Nemes Z, Steinert PM (1999) Bricks and mortar of the epidermal barrier. *Exp Mol Med* 31:5-19.

Netzlaff F, Lehr CM, Wertz PW*, et al.* (2005) The human epidermis models EpiSkin, SkinEthic and EpiDerm: an evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport. *Eur J Pharm Biopharm* 60:167-78.

Nieto JC, Canto E, Zamora C*, et al.* (2012) Selective loss of chemokine receptor expression on leukocytes after cell isolation. *PLoS One* 7:e31297.

O'Leary R, Arrowsmith M, Wood EJ (2002) Characterization of the living skin equivalent as a model of cutaneous re-epithelialization. *Cell Biochem Funct* 20:129-41.

O'Regan GM, Irvine AD (2008) The role of filaggrin loss-of-function mutations in atopic dermatitis. *Curr Opin Allergy Clin Immunol* 8:406-10.

O'Regan GM, Kemperman PM, Sandilands A*, et al.* (2010) Raman profiles of the stratum corneum define 3 filaggrin genotype-determined atopic dermatitis endophenotypes. *J Allergy Clin Immunol* 126:574-80 e1.

OECD (2004a) Guidance Document No.28 for the Conduct of Skin Absorption Studies. *OECD Environmental Health and Safety Publications Series on Testing and Assessment*.

OECD (2004b) Test No. 431: In Vitro Skin Corrosion: Human Skin Model Test. *OECD Guidelines for the Testing of Chemicals* Section 4.

OECD (2010) Test No. 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method. *OECD Guidelines for the Testing of Chemicals* Section 4.

Oji V, Eckl KM, Aufenvenne K*, et al.* (2010) Loss of corneodesmosin leads to severe skin barrier defect, pruritus, and atopy: unraveling the peeling skin disease. *Am J Hum Genet* 87:274-81.

Okulicz JF, Schwartz RA (2003) Hereditary and acquired ichthyosis vulgaris. *Int J Dermatol* 42:95-8.

Olaru F, Jensen LE (2010) Chemokine expression by human keratinocyte cell lines after activation of Toll-like receptors. *Exp Dermatol* 19:e314-6.

Palmer CN, Irvine AD, Terron-Kwiatkowski A*, et al.* (2006) Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 38:441-6.

Paternoster L, Standl M, Chen CM*, et al.* (2011) Meta-analysis of genome-wide association studies identifies three new risk loci for atopic dermatitis. *Nat Genet* 44:187-92.

Pilgram GS, Vissers DC, van der Meulen H*, et al.* (2001) Aberrant lipid organization in stratum corneum of patients with atopic dermatitis and lamellar ichthyosis. *The Journal of investigative dermatology* 117:710-7.

Ponec M (2002) Skin constructs for replacement of skin tissues for in vitro testing. *Adv Drug Deliv Rev* 54 Suppl 1:S19-30.

Ponec M, Boelsma E, Gibbs S*, et al.* (2002) Characterization of reconstructed skin models. *Skin Pharmacol Appl Skin Physiol* 15 Suppl 1:4-17.

Ponec M, Boelsma E, Weerheim A*, et al.* (2000) Lipid and ultrastructural characterization of reconstructed skin models. *Int J Pharm* 203:211-25.

Potts RO, Francoeur ML (1990) Lipid biophysics of water loss through the skin. *Proc Natl Acad Sci U S A* 87:3871-3.

Pound P, Ebrahim S, Sandercock P*, et al.* (2004) Where is the evidence that animal research benefits humans? *BMJ* 328:514-7.

Powazniak Y, Kempfer AC, Pereyra JC*, et al.* (2011) VWF and ADAMTS13 behavior in estradiol-treated HUVEC. *Eur J Haematol* 86:140-7.

Presland RB, Haydock PV, Fleckman P*, et al.* (1992) Characterization of the human epidermal profilaggrin gene. Genomic organization and identification of an S-100-like calcium binding domain at the amino terminus. *J Biol Chem* 267:23772-81.

Proksch E, Brandner JM, Jensen JM (2008) The skin: an indispensable barrier. *Exp Dermatol* 17:1063-72.

Proksch E, Folster-Holst R, Brautigam M*, et al.* (2009) Role of the epidermal barrier in atopic dermatitis. *J Dtsch Dermatol Ges* 7:899-910.

Proksch E, Jensen JM, Elias PM (2003) Skin lipids and epidermal differentiation in atopic dermatitis. *Clin Dermatol* 21:134-44.

Prunieras M, Regnier M, Woodley D (1983) Methods for cultivation of keratinocytes with an air-liquid interface. *The Journal of investigative dermatology* 81:28s-33s.

Rajamaran R, Fox R, Vethamany V*, et al.* (1977) Adhesion and spreading behaviour of human peripheral blood mononuclear cells (PBMC) in vitro. *Experimental Cell Reserach* 107:179-90.

Rawlings AV, Harding CR (2004) Moisturization and skin barrier function. *Dermatol Ther* 17 Suppl 1:43-8.

Ren Y, Gong W, Xu Q*, et al.* (2006) siRecords: an extensive database of mammalian siRNAs with efficacy ratings. *Bioinformatics* 22:1027-8.

Resing KA, Thulin C, Whiting K*, et al.* (1995) Characterization of profilaggrin endoproteinase 1. A regulated cytoplasmic endoproteinase of epidermis. *J Biol Chem* 270:28193-8.

Ring J, Alomar A, Bieber T*, et al.* (2012a) Guidelines for treatment of atopic eczema (atopic dermatitis) part I. *J Eur Acad Dermatol Venereol* 26:1045-60.

Ring J, Alomar A, Bieber T*, et al.* (2012b) Guidelines for treatment of atopic eczema (atopic dermatitis) Part II. *J Eur Acad Dermatol Venereol* 26:1176-93.

Rivier M, Castiel I, Safonova I*, et al.* (2000) Peroxisome proliferator-activated receptor-alpha enhances lipid metabolism in a skin equivalent model. *The Journal of investigative dermatology* 114:681-7.

Sandilands A, O'Regan GM, Liao H*, et al.* (2006) Prevalent and rare mutations in the gene encoding filaggrin cause ichthyosis vulgaris and predispose individuals to atopic dermatitis. *The Journal of investigative dermatology* 126:1770-5.

Sandilands A, Sutherland C, Irvine AD*, et al.* (2009) Filaggrin in the frontline: role in skin barrier function and disease. *J Cell Sci* 122:1285-94.

Sandilands A, Terron-Kwiatkowski A, Hull PR*, et al.* (2007) Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. *Nat Genet* 39:650-4.

Schäfer-Korting M, Bock U, Diembeck W*, et al.* (2008) The use of reconstructed human epidermis for skin absorption testing: Results of the validation study. *Altern Lab Anim* 36:161-87.

Schäfer-Korting M, Bock U, Gamer A*, et al.* (2006) Reconstructed human epidermis for skin absorption testing: results of the German prevalidation study. *Altern Lab Anim* 34:283-94.

Schaller M, Boeld U, Oberbauer S*, et al.* (2004) Polymorphonuclear leukocytes (PMNs) induce protective Th1-type cytokine epithelial responses in an in vitro model of oral candidosis. *Microbiology* 150:2807-13.

Schaller M, Zakikhany K, Naglik JR*, et al.* (2006) Models of oral and vaginal candidiasis based on in vitro reconstituted human epithelia. *Nat Protoc* 1:2767-73.

Scharschmidt TC, Man MQ, Hatano Y*, et al.* (2009) Filaggrin deficiency confers a paracellular barrier abnormality that reduces inflammatory thresholds to irritants and haptens. *J Allergy Clin Immunol* 124:496-506, e1-6.

Schmid-Wendtner MH, Korting HC (2006) The pH of the skin surface and its impact on the barrier function. *Skin Pharmacol Physiol* 19:296-302.

Schmuth M, Gruber R, Elias PM*, et al.* (2007) Ichthyosis update: towards a function-driven model of pathogenesis of the disorders of cornification and the role of corneocyte proteins in these disorders. *Adv Dermatol* 23:231-56.

Schmuth M, Jiang YJ, Dubrac S*, et al.* (2008) Thematic review series: skin lipids. Peroxisome proliferator-activated receptors and liver X receptors in epidermal biology. *J Lipid Res* 49:499-509.

Schon MP, Boehncke WH (2005) Psoriasis. *N Engl J Med* 352:1899-912.

Semlin L, Schafer-Korting M, Borelli C*, et al.* (2010) In vitro models for human skin disease. *Drug Discov Today* 16:132-9.

Seok J, Warren HS, Cuenca AG*, et al.* (2013) Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A* 110:3507-12.

Sheu MY, Fowler AJ, Kao J*, et al.* (2002) Topical peroxisome proliferator activated receptor-alpha activators reduce inflammation in irritant and allergic contact dermatitis models. *The Journal of investigative dermatology* 118:94-101.

Simonsen L, Fullerton A (2007) Development of an in vitro skin permeation model simulating atopic dermatitis skin for the evaluation of dermatological products. *Skin Pharmacol Physiol* 20:230-6.

Simpson CL, Patel DM, Green KJ (2011) Deconstructing the skin: cytoarchitectural determinants of epidermal morphogenesis. *Nat Rev Mol Cell Biol* 12:565-80.

Smedsrod B, Olsen R, Sveinbjornsson B (1995) Circulating collagen is catabolized by endocytosis mainly by endothelial cells of endocardium in cod (Gadus morhua). *Cell Tissue Res* 280:39-48.

Smith FJ, Irvine AD, Terron-Kwiatkowski A*, et al.* (2006) Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris. *Nat Genet* 38:337-42.

Sorrell JM, Baber MA, Caplan AI (2007) A self-assembled fibroblast-endothelial cell co-culture system that supports in vitro vasculogenesis by both human umbilical vein endothelial cells and human dermal microvascular endothelial cells. *Cells Tissues Organs* 186:157-68.

Soumelis V, Reche PA, Kanzler H*, et al.* (2002) Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 3:673-80.

Spergel JM, Paller AS (2003) Atopic dermatitis and the atopic march. *J Allergy Clin Immunol* 112:S118-27.

Spielmann H, Hoffmann S, Liebsch M*, et al.* (2007) The ECVAM international validation study on in vitro tests for acute skin irritation: report on the validity of the EPISKIN and EpiDerm assays and on the Skin Integrity Function Test. *Altern Lab Anim* 35:559-601.

Sprecher E, Chavanas S, DiGiovanna JJ*, et al.* (2001) The spectrum of pathogenic mutations in SPINK5 in 19 families with Netherton syndrome: implications for mutation detection and first case of prenatal diagnosis. *The Journal of investigative dermatology* 117:179-87.

Steinert PM, Cantieri JS, Teller DC*, et al.* (1981) Characterization of a class of cationic proteins that specifically interact with intermediate filaments. *Proc Natl Acad Sci U S A* 78:4097-101.

Sugarman JL, Fluhr JW, Fowler AJ*, et al.* (2003) The objective severity assessment of atopic dermatitis score: an objective measure using permeability barrier function and stratum corneum hydration with computer-assisted estimates for extent of disease. *Arch Dermatol* 139:1417-22.

Thakoersing VS, Gooris GS, Mulder A*, et al.* (2012) Unraveling barrier properties of three different in-house human skin equivalents. *Tissue Eng Part C Methods* 18:1-11.

Tjabringa G, Bergers M, van Rens D*, et al.* (2008) Development and validation of human psoriatic skin equivalents. *Am J Pathol* 173:815-23.

Tsai TF, Maibach HI (1999) How irritant is water? An overview. *Contact Dermatitis* 41:311-4.

Turner NA, Nolasco L, Ruggeri ZM*, et al.* (2009) Endothelial cell ADAMTS-13 and VWF: production, release, and VWF string cleavage. *Blood* 114:5102-11.

Vahlquist A, Ganemo A, Virtanen M (2008) Congenital ichthyosis: an overview of current and emerging therapies. *Acta Derm Venereol* 88:4-14.

Valentin E, Lambeau G (2000) Increasing molecular diversity of secreted phospholipases A(2) and their receptors and binding proteins. *Biochim Biophys Acta* 1488:59-70.

van der Worp HB, Howells DW, Sena ES*, et al.* (2010) Can animal models of disease reliably inform human studies? *PLoS Med* 7:e1000245.

Van Gele M, Geusens B, Brochez L*, et al.* (2011) Three-dimensional skin models as tools for transdermal drug delivery: challenges and limitations. *Expert Opin Drug Deliv* 8:705-20.

Vasilopoulos Y, Cork MJ, Murphy R*, et al.* (2004) Genetic association between an AACC insertion in the 3'UTR of the stratum corneum chymotryptic enzyme gene and atopic dermatitis. *The Journal of investigative dermatology* 123:62-6.

Vaughan MB, Ramirez RD, Brown SA*, et al.* (2004) A reproducible laser-wounded skin equivalent model to study the effects of aging in vitro. *Rejuvenation Res* 7:99-110.

Vávrová K, Henkes D, Strüver K*, et al.* (2013) Filaggrin Deficiency Leads to Impaired Lipid Profile and Altered Acidification Pathways in a 3 D Skin Construct. *accepted for publication in the Journal of Investigative Dermatology*.

Vogler R, Sauer B, Kim DS*, et al.* (2003) Sphingosine-1-phosphate and its potentially paradoxical effects on critical parameters of cutaneous wound healing. *The Journal of investigative dermatology* 120:693-700.

Walters KA, Roberts MS (2002) The Structure and Function of the Skin. In: *Dermatological and Transdermal Formulations* (Walters KA, ed), New York, Basel: Marcel Dekker Inc.

Weidinger S, Baurecht H, Wagenpfeil S*, et al.* (2008a) Analysis of the individual and aggregate genetic contributions of previously identified serine peptidase inhibitor Kazal type 5 (SPINK5), kallikrein-related peptidase 7 (KLK7), and filaggrin (FLG) polymorphisms to eczema risk. *J Allergy Clin Immunol* 122:560-8 e4.

Weidinger S, Illig T, Baurecht H*, et al.* (2006) Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations. *J Allergy Clin Immunol* 118:214-9.

Weidinger S, O'Sullivan M, Illig T*, et al.* (2008b) Filaggrin mutations, atopic eczema, hay fever, and asthma in children. *J Allergy Clin Immunol* 121:1203-9 e1.

Weindl G, Castello F, Schafer-Korting M (2011) Evaluation of anti-inflammatory and atrophogenic effects of glucocorticoids on reconstructed human skin. *Altern Lab Anim* 39:173-87.

Wells RS, Kerr CB (1966) Clinical features of autosomal dominant and sex-linked ichthyosis in an English population. *British medical journal* 1:947-50.

Welss T, Basketter DA, Schroder KR (2004) In vitro skin irritation: facts and future. State of the art review of mechanisms and models. *Toxicol In Vitro* 18:231-43.

Wolf NB, Küchler S, Radowski MR*, et al.* (2009) Influences of opioids and nanoparticles on in vitro wound healing models. *Eur J Pharm Biopharm* 73:34-42.

Yang S, Graham J, Kahn JW*, et al.* (1999) Functional roles for PECAM-1 (CD31) and VE-cadherin (CD144) in tube assembly and lumen formation in three-dimensional collagen gels. *Am J Pathol* 155:887-95.

Yoo J, Omori M, Gyarmati D*, et al.* (2005) Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. *J Exp Med* 202:541-9.

Zhang C, Gurevich I, Aneskievich BJ (2012) Organotypic Modeling of Human Keratinocyte Response to Peroxisome Proliferators. *Cells Tissues Organs*.

Ziegler SF, Artis D (2010) Sensing the outside world: TSLP regulates barrier immunity. *Nat Immunol* 11:289-93.

## Selbstständigkeitserklärung

Hiermit erkläre ich, Dominika Henkes, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Verwendung anderer als der angegebenen Hilfsmittel angefertigt habe.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Mainz, den 24.08.2013
Dominika Henkes

## Curriculum Vitae

Der Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt.