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Author: Drongelen, Vincent van
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General Introduction
1. Human skin

1.1 Structure of human skin

Human skin is composed of two layers: the dermis and the epidermis (figure 1). The underlying dermis is populated with fibroblasts which produce collagens and elastins, and supports the outermost layer, the epidermis. The epidermis mainly consists of keratinocytes and serves as an boundary between the external environment and the internal body. The epidermis is composed of four layers (Figure 1). In the innermost layer, the stratum basale (SB), the keratinocytes proliferate. Thereafter, the cells escape from this layer and start to migrate and differentiate through the stratum spinosum (SS) towards the stratum granulosum (SG). During this process, lamellar bodies are formed in the SS and more extensively in the SG. These vesicles contain various molecules involved in the formation of the outermost epidermal layer the stratum corneum (SC), including lipids and lipid precursors, various enzymes and antimicrobial peptides (AMPs). At the SG-SC interface, when the keratinocytes undergo terminal differentiation, the contents of the lamellar bodies is released during a process that is called lamellar body extrusion. During terminal differentiation the keratinocytes loose their nucleus and their cell membrane is replaced by a cornified envelope, after which they are referred to as corneocytes. The corneocytes are surrounded by a hydrophobic extracellular lipid matrix and together these form the SC. The structure of the SC is often compared to a “brick-and-mortar” structure, in which the corneocytes represent the bricks, and the surrounding lipid matrix the mortar. This lipid matrix mainly consists of three lipid classes; ceramides (CERs), free fatty acids (FFAs) and cholesterol. The corneocytes are interconnected through “linker”-proteins, the so-called corneodesmosomes. Kallikreins degrade these corneodesmosomes in the outer layers of the SC, which allows shedding of the outer SC, a process that is referred to as desquamation and is required for proper SC turnover. Besides keratinocytes, the epidermis contains various other cell types including dendritic cells (langerhans cells) and melanocytes.

1.2 The skin barrier and its components

Human skin is continuously in contact with the external environment and encounters many potential pathogens such as bacteria, fungi, viruses and parasites as well as harmful and/or toxic substances. To protect against such external influences, the epidermis provides a barrier in three different ways. As a physical barrier the SC prevents the penetration of harmful pathogens, allergens and toxic exogenous chemicals or substances into the viable epidermis. In addition, the SC prevents dehydration of the body by regulating the transepidermal water loss (TEWL). The lipid matrix in the SC is highly organized into lipid layers, which compose the lipid lamella. These lamellae are regularly stacked on top of each other, parallel to the corneocytes (Figure 1b). In human SC, two coexisting lamellar phases have been identified with repeat distances of ~13 nm (for the long periodicity phase, LPP) and ~6 nm (the short periodicity phase, SPP). Furthermore, within the lipid lamellae the lipids are packed in a
certain density. The lateral packing in human SC is mainly orthorhombic (very dense), although a subpopulation of lipids are organized into a hexagonal lateral packing (less dense, Figure 1b)\textsuperscript{11}. Furthermore, the keratinocytes present in the upper layers of the SG are interconnected by tight junctions which provides an additional physical barrier\textsuperscript{12}.

The epidermis also acts as a chemical barrier, in which various AMPs defend against invading pathogens. AMPs exhibit broad-spectrum activity against bacteria, fungi and viruses. Synthesis of AMPs primarily occurs in the keratinocytes in the SG, after which there are packaged into lamellar bodies and transported to the SC where they are released into the intercellular regions\textsuperscript{13}. Finally, there is the immunological barrier, in which the keratinocytes are part of the constitutively active innate immune response that provides an immediate but fairly nonspecific response, while skin-homing T cells that are part of the adaptive immunity provide a specific and long-lasting response\textsuperscript{14-16}. The immune response will be discussed in more detail in chapter 2.2.

**Figure 1:** schematic overview of human skin and the stratum corneum. (a) Human skin is composed of a dermal compartment that consists mainly of collagen fibers, elastins and fibroblasts. The epidermis is divided into four layers (strata): the stratum basale, stratum spinosum, stratum granulosum and the stratum corneum. The epidermis contains mainly keratinocytes, but also melanocytes and langerhans cells are present. (b) The outermost layer of the skin is the stratum corneum, which is composed of corneocytes that are surrounded by lipids. These lipids are mainly cholesterol, free fatty acids and ceramides. The lipids are organized into stacked lipid lamella forming two lamellar phases with a repeat distance (\(z\)) of 13 nm (the LPP) or 6 nm (the SPP). In addition, within the lipid lamellae the lipids are organized with a certain density which is referred to as the lateral packing. The lateral packing SC can be orthorhombic (very dense), hexagonal (dense) or liquid (loose).
2. Atopic dermatitis

2.1 Clinical features of atopic dermatitis
Atopic dermatitis (AD), also frequently termed atopic eczema, is one of the most common inflammatory skin diseases. The first written description of AD dates back to the early 19th century. In 1925 the term “atopy” was introduced by Coca, to signify the tendency to develop allergies to food and inhalant substances which subsequently manifested as eczema, asthma and hay fever on a hereditary basis. In 1933, Wise and Sulzberger provided the first detailed diagnosis of AD. AD is clinically characterized by a broad spectrum of manifestations. Clinical features that are regularly present are dryness of the skin (xerosis), itch (pruritis), redness of the skin (erythema) and chronic or relapsing eczematous lesions. Although AD can potentially affect any part of the body, it most commonly affects the flexures and the face (Figure 2).

Over the past three decades, the lifetime prevalence of AD has increased 2-to-3 fold and is currently affecting 15-30% in children and 2-10% in adults. Over 50% of the children with AD eventually progress into the development of hay fever (allergic rhinitis) and/or asthma; the so-called atopic march. The physical discomfort from the highly pruritic lesions in children with moderate-to-severe AD results in sleep deprivation and subsequently cause reduced functioning of the patients and their close relatives, as well as distress, anxiety, embarrassment, poor self-esteem and lack of self-confidence.

Figure 2: an example of the clinical presentation of atopic dermatitis. In general, non-lesional AD skin appears normal, whereas lesional skin of AD patients appears as red (erythema) and is very dry (xerosis) as well as very itchy (pruritis).

2.2 What is first, barrier dysfunction or inflammation?
The cause of AD is complex and it is the result of the interaction between susceptibility genes and the host environment. These gene-environment interactions result in two principle
characteristics of AD; (1) impaired skin barrier function and (2) immunological abnormalities. Which one of these two characteristics is the initiating factor in AD development is still a topic of extensive research, but traditionally, two competing hypothesis are presented to explain the pathogenesis of AD:

1) The inside-out hypothesis, which suggests that an intrinsic immunological defect predisposes individuals to atopy and that this IgE-mediated sensitization will result in AD\textsuperscript{17,25-28}.

2) The outside-in hypothesis, which suggests that disruption of the skin barrier, either resulting from a genetic defect in skin barrier formation or as a result of changes in the environment, would lead to sensitization and subsequently to AD\textsuperscript{29-32}.

Experimental data from literature provides support for both hypothesis. However, accumulating evidence shows that there is an interplay between the immune system and the skin barrier. Examples of such interactions will be discussed in 2.3.3.

2.2.1. Epidermal barrier dysfunction in AD

As one of the principle characteristics of AD, the epidermal barrier (dys-)function has been subject to extensive research. Both non-lesional and to a larger extent lesional skin of AD patients display several barrier abnormalities, including increased TEWL, reduced SC hydration and increased SC permeability for irritants such as SDS\textsuperscript{33-37}.

2.2.2. The SC lipids and their role in the skin barrier in AD

Based on the observations of delayed and possibly incomplete lamellar body extrusion in the skin from AD patients, it was hypothesized that lipid synthesis at the SG-SC interface in AD skin was impaired, which could explain the impaired skin barrier function in AD\textsuperscript{38,39}. Since then, the SC lipid composition of AD skin has been subject to much additional research. Early studies revealed a reduction in total levels of the SC lipids as well as decreased CER levels in both non-lesional and lesional skin of AD patients\textsuperscript{40,41}. More detailed analysis revealed that a specific CER subclass, CER [EOS], was drastically reduced in AD skin, whereas other subclasses, CER[NS], [AS] and [AP] showed a relative increase\textsuperscript{40,42}. The CER nomenclature is explained in Figure 3.

Whereas most studies have been focussing on the CER subclass composition in AD skin, a recent study has characterized the complete CER profile of AD patients, including the 12 CER subclasses and their chain length distribution of each subclass\textsuperscript{43}. Subsequent studies showed a relative increase in the levels of short chain CERs and a reduction in the levels of CERs with very long acyl chains, so-called acyl-CERs, in non-lesional and more extensively in lesional skin\textsuperscript{44}. The changes in CER chain length were reflected in a reduced skin barrier function, which was determined through the assessment of the TEWL\textsuperscript{44}. A following study revealed that parallel to the changes in CER composition, the FFA composition was also affected, i.e. reduced FFA chain length and increased levels of unsaturated FFAs\textsuperscript{44}.
Although the enzymatic pathways involved in SC lipid synthesis are still not fully elucidated, several enzymes are promising candidates for the changes in SC lipid composition in AD. Two enzymes that are involved in CER processing, β-glucocerebrosidase (Gcase) and acid sphingomyelinase (aSmase), are reported to be affected in AD\textsuperscript{45,46}. Both these enzymes convert precursor-CERs into CERs, and alterations in their expression and/or activity may result in changes in SC CER composition and in the total level of CER in the SC. In addition, since the levels of acyl-CERs was reduced, ceramide synthase 3 (CerS3), which is involved in the synthesis of acyl-CERs, might also be a potential candidate. The discovery of shorter FFA chain lengths and increased levels of unsaturated FFAs indicate that other candidate enzymes might also play a role. Especially those enzymes that are involved in the elongation and desaturation of FFAs, e.g. the elongases (ELOVLs) and Stearyl-CoA desaturase (SCD), respectively, might be altered in AD. Using mouse models, two ELOVLs (ELOVL1 and ELOVL4) that are involved in the elongation of the very long FFAs, were shown to be important for proper barrier formation\textsuperscript{47,48}. Furthermore, \textit{in vitro} studies have shown that the increased levels of unsaturated FFAs in human skin equivalents was accompanied by increased epidermal expression of SCD and that increasing levels of unsaturated FFAs negatively affect the SC barrier function\textsuperscript{49,50}.
2.2.3. Filaggrin and its role in skin barrier function and AD

Besides SC lipids, structural proteins such as tight junction proteins, are important contributors to the skin barrier and skin barrier function\textsuperscript{51}. Various skin barrier-related proteins are being investigated for their role in AD or their contribution to AD. However, the strong association between loss-of-function mutations in filaggrin (FLG) and AD is one of the most robust and reproducible association that is observed in complex human disorders. Since this discovery, FLG has been subject to extensive research\textsuperscript{52-54}. The FLG protein was already discovered in 1977 as a highly insoluble, histidine-rich protein that was present in epidermal extracts. This protein has later been shown to condense and align keratin intermediate filaments \textit{in vitro}, and was therefore named filaggrin (for filament aggregating protein)\textsuperscript{55,56}. Due to the repetitive nature of the \textit{FLG} gene, advanced sequencing technology and some innovative polymerase chain reaction (PCR) protocols were necessary for comprehensive analysis of the \textit{FLG} gene prior to the discovery of the first pathogenic FLG mutations. Initially, FLG mutations were discovered to be the underlying cause for ichthyosis vulgaris (IV), a skin disease that is characterized by a dry and scaly skin and that is often associated with AD\textsuperscript{57}. Within several months after this discovery, two FLG mutations (R501X and 2282del4) were found to be a major predisposing factor for development of AD\textsuperscript{52}. Since this breakthrough, over 45 FLG mutations have been identified in AD patients, which were shown to have a specific distribution between European and Asian populations\textsuperscript{52,58-67}.

The \textit{FLG} gene is located on chromosome 1q21 in the so-called epidermal differentiation complex (EDC), which is a large chromosomal region that contains over 70 genes encoding proteins that are involved in terminal differentiation of keratinocytes. Besides the genes for FLG, this region also include genes encoding loricrin, involucrin, late cornified envelop (LCE) proteins and many others. The \textit{FLG} gene encodes a precursor protein, profilaggrin, which during keratinocyte differentiation is cleaved into 10-12 nearly identical FLG monomers by multiple proteases including caspase 14\textsuperscript{57,68}. In the corneocytes, FLG is finally degraded into free amino acids and their derivatives, which are part of the natural moisturizing factor (NMF), a process that involves caspase 14 as well as other enzymes\textsuperscript{69}. The FLG degradation products include histidine and glutamine, which are further degraded into pyrrolidone carboxylic acid (PCA) and urocanic acid (UCA)\textsuperscript{70}. PCA and UCA have been shown to have several functions, including water retention, protection against ultraviolet (UV) radiation and modulation of the immune function(Figure 4)\textsuperscript{71,72}.
Figure 4: overview of filaggrin processing and (hypothetical) function in skin barrier Profilaggrin is expressed in the terminally differentiating keratinocytes in the stratum granulosum and is the major constituent of the keratohyalin granules. In general, profilaggrin is composed of: a calcium (Ca\textsuperscript{2+}) binding N-terminal domain that contains an A and B domain, 10-12 FLG repeats and a unique C-terminal domain. Terminal differentiation depends on an increase in the calcium (Ca\textsuperscript{2+}) concentration. An increase in Ca\textsuperscript{2+} results in dephosphorylation of profilaggrin allowing multiple proteases, to cleave the FLG monomers. The FLG monomers bind to keratins which assists in the formation of the cornified envelope, thereby providing structure rigidity. Subsequently, various enzymes including caspase 14 degrade FLG into UCA, PCA and free amino acids, which are part of the natural moisturizing factor (NMF). The NMF functions in water retention, protection against ultraviolet (UV) radiation, and modulation of the immune function. Figure based on McAleer and Irvine\textsuperscript{73}.

Although it is widely accepted that FLG mutations lead to a barrier defect, the underlying mechanisms of how FLG as an intracellular protein affects the paracellular barrier are still to be elucidated\textsuperscript{74}. Based on the various functions of (pro)-FLG and the FLG breakdown products, FLG loss-of-function mutations might cause skin barrier defects through various mechanisms:

1. FLG is part of the cornified envelope and aggregates keratins, thereby contributing to the structure and function of the SC. Absence or reduced FLG might therefore lead to a defective SC.
2. FLG breakdown products PCA and UCA are part of the NMF, which regulates skin hydration. Various studies have shown that FLG mutations are major determinants of the reduced NMF levels and the level of these breakdown products have been shown to be influenced by FLG genotype, as well as by AD severity and might contribute to the dryness of the skin in AD\textsuperscript{75-78}.
3. PCA and UCA regulate the SC pH, which is important for the regulation of the activity of various enzymes that are involved in SC barrier homeostasis, including serine proteases and enzymes involved in lipid synthesis\textsuperscript{79}. In normal skin, the pH is 5.5 which is required for the optimal activity for such enzymes. An increase in pH might affect enzyme activity and could therefore result in a defective barrier. However, protein analysis for several enzymes involved in FFA and CER synthesis revealed that such enzymes are mainly expressed in the SG of the epidermis, making it unlikely that changes in SC pH affect the activity of these enzymes\textsuperscript{80}. 
The presence of FLG mutations are undeniably the foremost genetic risk factor for AD development. However, up to 50% of the AD patients do not have a FLG mutation or are heterozygous\textsuperscript{52,67,81}. In addition, there is currently no convincing data that shows a clear relationship between FLG mutations and reduced skin barrier function in AD\textsuperscript{82-85}. A recent study with IV patients that were homozygous or compound heterozygous for FLG mutations displayed only moderate changes in epidermal permeability function\textsuperscript{86}, demonstrating that the high TEWL values observed in AD patients cannot be ascribed to FLG mutations only\textsuperscript{87}.

\subsection*{2.2.4. External factors that influence skin barrier and AD}

Besides intrinsic factors, such as FLG mutations, which might disturb the skin barrier homeostasis in AD, external influences like scratching in response to the itch that is present in the skin of AD patients cause skin barrier disruption and therefore plays an important role. The itching and the rash in AD can be triggered by various environmental factors. These factors include allergens (e.g. house dust mite (HDM), pollen and animal dander), irritants (e.g. use of soaps, crèmes, cosmetics or wool), climate (a dry environment or dry cold air results in dry skin and thereby cause itch) and bacterial toxins\textsuperscript{26,88,89}. In particular toxins secreted by \textit{Staphylococcus aureus}, an opportunistic pathogen, that frequently colonizes skin from AD patients have been shown to influence disease severity\textsuperscript{90,91}. While \textit{S. aureus} skin colonization is found in only 5% of healthy individuals, 76\% to 100\% of the AD patients show \textit{S. aureus} colonization on non-lesional and lesional skin, respectively\textsuperscript{92-94}. Furthermore, recent studies have shown that in lesional AD skin, \textit{S. aureus} forms biofilms, rendering these bacteria less susceptible for treatment and allows worsening of the AD skin lesions\textsuperscript{95}. In addition, \textit{S. aureus} itself can be an important trigger for AD, since \textit{S. aureus} antigens and superantigens were shown to evoke a T cell mediated immune response\textsuperscript{26}.

\section*{2.3 The immune response and immune dysregulation in AD}

The immune system can be divided into the innate immune response and the adaptive immune response. While the innate immune response with the keratinocytes, langerhans cells and various leukocytes such as natural killer (NK) cells, is nonspecific and short-term, it provides a quick defence against a broad range of pathogens. Conversely, the adaptive immune response with T cells and B cells is highly specific and long-lasting, but rather slow. AD is characterized by abnormalities in both the innate and the adaptive immune response.

\subsection{2.3.1 Innate immunity abnormalities in AD}

As part of the innate immune response, keratinocytes play an important role. They express various receptors that are important for detection of pathogens, e.g. Toll-like receptors (TLRs). In addition, in response to pathogenic stimuli, keratinocytes produce chemokines and pro-inflammatory cytokines such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), interleukin (IL-)1 and in particular thymic stromal lymphopoietin (TSLP). The latter cytokine was shown to be a key cytokine for the initiation of AD\textsuperscript{96-98}. TSLP promotes a Th2 response, either directly through
the induction of Th2 cytokine-expressing cells or indirectly through polarization of dendritic cells\textsuperscript{99,100}. Furthermore, keratinocytes produce anti-microbial molecules including RNases and AMPs. These AMPs are chemicals present in the epidermis and have antimicrobial activity against various pathogens, such as bacteria, viruses and fungi. These AMPs include LL-37 and β-defensins (human β-defensins, hBDs)\textsuperscript{101}. In healthy skin, small amounts of hBD-2 and hBD-3 are present, but their expression is elevated in lesional AD skin\textsuperscript{102,103}. However, compared to psoriasis, another immune-mediated skin disorder in which the expression of hBD-2 and hBD-3 is drastically increased, both non-lesional and lesional AD skin showed decreased expression of both AMPs\textsuperscript{104,105}.

2.3.2 Adaptive immunity abnormalities in AD

Lesional AD skin shows the presence of skin infiltrating T-cells, which are predominantly Th2 cells in the acute phase and Th1 cells in the chronic phase. During the acute phase, Th2 cells produce a large array of cytokines such as IL-4, IL-5, IL-10, IL-13 and IL-31 which are important for the cutaneous immune response\textsuperscript{98}. Because of their functional overlap, most \textit{in vitro} studies evaluate the effects of both IL-4 and IL-13. IL-4 is a key Th2 cytokine and is found to be critical for the differentiation of naïve T cells into Th2 cells as well as for IgE production, eosinophil recruitment and other functions. IL-13 is mainly secreted by Th2 cells and is a central mediator of allergic inflammation. Expression of IL-13 is found in both acute and chronic lesions of AD\textsuperscript{106}. Expression of IL-4 and IL-13 in the epidermis of mice was shown to induce dermatitis, indicating their important role in AD\textsuperscript{107,108}.

IL-31 is a more recently identified cytokine which is primarily produced by Th2 cells\textsuperscript{109}. The expression levels of IL-31 have been reported to be higher in lesional AD skin compared to non-lesional skin and correlate with the IL-4 and IL-13 levels in the skin of AD patients\textsuperscript{110}. Much functional evidence comes from IL-31 transgenic mice, which developed spontaneous pruritus and skin lesions, both hallmarks of AD. In addition, in a mouse model that spontaneously develops AD, IL-31 levels correlated with scratching behaviour, indicating that IL-31 is particularly important for the induction of the itching in the skin\textsuperscript{111}. Mouse models and their usage for studying AD pathogenesis will be discussed in 3.1.

2.3.3. So? Which one is first, barrier dysfunction or inflammation?

Although the skin barrier with proteins and SC lipids on one hand, and the immune response with T cells and cytokines on the other hand, appear to be two separate defence mechanisms, recent studies suggest that there is an interplay between the two. The AD related cytokines have been found to affect the skin barrier on various levels.

The observation that AD patients without FLG mutations showed reduced FLG expression, implied a role for other factors. \textit{In vitro} and \textit{in vivo} experiments have shown that IL-4 and IL-13, two cytokines that are abundantly present in lesional AD skin, reduce the expression
of FLG as well as that of loricrin and involucrin in keratinocytes indicating that these cytokines have a broad effect on keratinocyte differentiation\textsuperscript{112,113}. Besides IL-4 and IL-13, also IL-31 downregulates FLG expression\textsuperscript{114}. In addition to their effects on keratinocyte differentiation, IL-4 and IL-13 were demonstrated to downregulate the expression of caspase 14, an important enzyme for the degradation of FLG into NMF and thereby plays a role in regulation of the skin hydration\textsuperscript{115}. Furthermore, AD related cytokines have been shown to affect epidermal adhesion molecules and tight junctions in keratinocytes\textsuperscript{116-118}. Besides their effects on the epidermal keratinocytes, Th2 cytokines have also been found to affect the SC lipid composition, i.e. IL-4 downregulated Gcase and aSmase expression that consequently resulted in reduced SC CER levels \textit{in vitro}\textsuperscript{119}. These findings add another dimension to the discussion which entity is the initiating factor for the AD phenotype.
3. Models to study AD pathogenesis

3.1 In vivo models to study AD pathogenesis

Much of our understanding about AD pathogenesis is based on studies using mouse models. Since the description of the NC/Nga mouse in 1997 as the first mouse model for AD, a growing number of mouse models has been developed\textsuperscript{120}. These can be categorized into three groups: (1) mice that spontaneously develop skin lesions that closely resemble AD, (2) genetically engineered mice (transgenic mice), and (3) mice in which AD features are induced by allergens, haptens or epicutaneous application of sensitizers\textsuperscript{121,122}.

Examples of mice that develop spontaneous skin lesions include the NC/Nga mouse and the flaky tail (ft) mouse\textsuperscript{120,123}. The ft mice have been widely used as a model of heritable skin barrier deficiencies and spontaneous dermatitis as well as a model for FLG deficiency that was associated with AD pathogenesis in patients\textsuperscript{124-128}. Ft mice have a frameshift mutation in the mouse filaggrin (Flg) gene which results in absence of the FLG protein. This mouse model has therefore frequently been used as a model to study the role of FLG in skin homeostasis. However, recent studies have shown that an additional mutation in Matt (Tmem79) was the cause of the barrier defects in these mice rather than the Flg mutation\textsuperscript{123,129-131}. Transgenic mouse models for AD are generally mice lacking or overexpressing proteins, in particular cytokines involved in AD pathogenesis, e.g. mice with skin-specific overexpression of IL-4, IL-13 or TSLP, as well as the previously mentioned mice that overexpress IL-31\textsuperscript{107-109,132}.

AD features in mice can also be induced through skin injury, e.g. repeated tape stripping or scratching induced by epicutaneous sensitization, or through multiple challenges with so-called haptens, e.g. oxazolone (OXZ) and trinitrochlorobenzene (TNCB). Application of such haptens has been shown to result in a Th2-dominated inflammatory response similar to human AD\textsuperscript{133,134}.

All these mouse models display different characteristics of AD, comparable to those seen in AD patients and collectively, they have significantly contributed to our understanding of the pathogenesis of this complex skin disease.

3.2 In vitro human skin equivalents

As illustrated above, mouse models are excellent tools for studying the role of particular genes or proteins, such as cytokines, through the generation of transgenic mice or for studying the influences of environmental factors on AD pathogenesis in vivo. However, their usage for studies that focus on the skin barrier and other epidermal features is limited for several reasons, including differences in skin morphology and in barrier function. Mouse skin contains many hair follicles, is usually thin (3 viable cell layers, versus 6-10 viable cell layers in human skin) provides less of a water barrier and displays higher percutaneous absorption, which limits their use for topical drug-delivery studies\textsuperscript{135}. Moreover, findings from toxicological and
inflammatory studies using mouse models do not always correlate with human responses\textsuperscript{136,137}. Furthermore, both social pressure from animal welfare groups and the public opinion, as well as political pressure from the European Union. Since 2009, the European Union wants to ban animal testing for cosmetic and chemical means, requires development for alternatives for the screening of newly compounds, e.g. for the treatment of AD.

As an alternative, \textit{in vitro} human skin equivalents (HSEs) can be used for studying various aspects of skin biology. \textit{In vitro} three-dimensional HSEs recapitulate many features of native human skin, such as similarities in morphology, expression of differentiation and proliferation markers and various SC barrier properties. The SC from HSEs established with primary keratinocytes contain all CER subclasses. Despite the presence of a competent skin barrier in terms of the presence of the LPP, SC permeability studies have shown that HSEs have a decreased barrier function compared to native skin, as well as a less ordered lateral lipid organization when compared to native SC\textsuperscript{130,138}. HSEs are generally established by using primary keratinocytes. Seeding of primary keratinocytes onto a fibroblast populated dermal substrate, i.e. rat tail collagen or de-epidermized dermis results in a full thickness HSE (FT-HSE) whereas seeding onto an artificial matrix, i.e. an inert filter, results in an epidermal skin equivalent (Figure 5)\textsuperscript{139-141}. These keratinocytes are usually obtained from surplus skin, most commonly juvenile foreskin or after mammary or abdominal surgery. In addition to in-house HSEs, various HSEs that are established with primary keratinocytes are commercially available. These have been characterized for their permeability and absorption properties and have been shown to be applicable for \textit{in vitro} permeation studies and toxicity screenings, e.g. Episkin (SkinEthic) or Epiderm (MatTek)\textsuperscript{142-144}. Although usage of primary keratinocytes results in HSEs that mimic native skin to a large extent, they have several drawbacks, including a limited \textit{in vitro} lifespan, a large donor-to-donor variation and limited availability.

\subsection*{3.2.1 Cell lines as an alternative to primary keratinocytes to establish human skin equivalents}

In order to overcome the limitations of primary keratinocytes, immortalized keratinocyte cell lines can be used. Such cell lines provide an unlimited source of cells that allow the generation of reproducible and consistent HSEs, reducing intra- and inter-laboratory variations. One of the most frequently used keratinocyte cell line is the spontaneously immortalized HaCaT, which has been shown to remain non-tumorigenic during long- term culture\textsuperscript{145,146}. When used for generating HSEs using various dermal substrates, HaCaT cells displayed a disordered tissue structure with abnormally shaped nuclei and a limited ability for the generation of an organized mature cornified epithelium with a proper SC barrier function\textsuperscript{147}. Advancements in culture methods have resulted in HSEs with HaCaT cells that display an fairly organized epidermis when cultured on a human fibroblast populated dermal substitute\textsuperscript{148-150}. Despite the inability to form a proper and functional SC, reconstructed epidermis with HaCaT cells is often considered functional and frequently used as a model for unravelling molecular
mechanisms regulating keratinocyte growth and differentiation\textsuperscript{148-150}. Nevertheless, when topical application, investigation of underlying biological mechanisms or mimicking skin diseases such as AD are the main focus, the inability to generate a proper SC must be considered.

More recently, another keratinocyte cell line has been described, the Near-Diploid Immortal KeratinocyteS, NIKS\textsuperscript{\textregistered}. These NIKS\textsuperscript{\textregistered} are spontaneously immortalized keratinocytes which were accidently found to proliferate beyond the lifespan of normal primary keratinocytes\textsuperscript{151}. These cells have been successfully used for the generation of HSEs, StrataTest\textsuperscript{\textregistered}, which are now commercially available for toxicity screening\textsuperscript{152}. In addition to spontaneous immortalization, keratinocyte cell lines can be obtained by genetic engineering of keratinocytes to bypass certain cell cycle checkpoints, thereby preventing growth arrest and cell death. In absence of non-pathogenic telomere shortening or DNA damage, bypassing of cell cycle checkpoints

\textbf{Figure 5:} Schematic overview of establishing in vitro human skin equivalents (HSEs) HSEs can be established with primary keratinocytes and fibroblasts which are isolated from native human skin, or with keratinocytes and fibroblasts obtained from immortalized cell lines. Depending on the aim of the study, different HSEs can be generated. Full thickness HSEs can be established by seeding keratinocytes (primary or immortalized) onto a fibroblast-populated dermal collagen matrix. By placing a skin biopsy onto the dermal matrix an explant HSE can be generated. To establish epidermal skin equivalents, keratinocytes can be seeded onto an inert acellular filter. Air exposed culturing of the HSEs allow the development of a fully stratified epidermis.
results in the generation of a non-oncogenic cell line. For example overexpression of a protein cyclin-dependent kinase 4 (Cdk4) results in a bypass of telomere dependent replicative senescence without losing the ability to differentiate normally\textsuperscript{133}.

### 3.3 Modulation of skin models to mimic AD \textit{in vitro}

Currently, no cure for AD is available. In many cases, the disease manifestations cease or improve remarkably by the age of five. However, persisting AD is often found to precede the atopic march, a concept to describe the progression of AD into allergic rhinitis and asthma. Treatment of AD is currently focused on suppression of the immune response, restoring skin barrier function through (re-)hydration of the skin, suppression of the itch, in order to reduce scratching and mechanical injury, and anti-microbial treatment to reduce/prevent bacterial, viral and fungal infections. Other measures include avoidance of triggers and usage of appropriate anti-infective agents and anti-histamines. Due to their limited efficacy and their adverse effects, there is much room for improvement in AD therapy.

Because of the aforementioned arguments, in particular the difficulties in the translation of results obtained with mouse models towards humans, newly developed \textit{in vitro} three dimensional HSEs that mimic AD as closely as possible can be useful for biological and pharmacological intervention studies for AD\textsuperscript{135}. To mimic the filaggrin mutations as seen in AD patients, RNA interference (RNAi) using small interfering RNA (siRNA) or short hairpin RNA (shRNA) can be delivered to keratinocytes in order to reduce filaggrin mRNA and protein expression (so-called knockdown). Initial studies have used lipofectamine-based transfection in primary keratinocytes with synthetic siRNA to knockdown filaggrin (FLG-KD) in HSEs. However, studies using these FLG-KD have generated controversial results. One study showed a defective barrier function as illustrated by an increased epidermal uptake for a fluorescent dye, whereas another showed that there were no changes in the surface pH\textsuperscript{154,155}. Cytokine supplementation to the medium of HSEs to mimic the inflammation as seen in lesional AD skin, resulted in various morphological and molecular characteristics of AD\textsuperscript{156}. 
Aim and outline of this thesis

The aim of the research described in this thesis was to develop a new in vitro human skin equivalent (HSE) for human atopic dermatitis (AD), with the focus on the role of filaggrin. The ultimate goal was to establish reproducible HSEs to eventually could be used for screening and testing purposes of new therapeutic compounds, which are desired due to the rapid increase in prevalence of AD.

In an attempt to establish reproducible HSEs, we have established and characterized a novel full thickness HSE using the N/TERT cell line, so-called N/TERT based human skin equivalents (N-HSEs). Of these N-HSEs, the SC lipid properties have been characterized in detail, which are described in chapter 2. Using the N/TERT cell line and RNAi technology, a secondary cell line was generated in which filaggrin knockdown (FLG-KD) was present. These cells were subsequently used for the generation of full thickness HSEs, so-called FLG-KD HSEs. To unravel the role of FLG in SC barrier properties as seen in AD, these FLG-KD HSEs were characterized for their epidermal differentiation and SC barrier lipid properties, which are described in chapter 3.

Because of the limited material that is generally available from diseased skin, an explant HSE was used to expand small amounts of starting material, by passaging small fragments of the outgrowth area. Chapter 4 describes this method using healthy skin, as well as the consequences of subsequent passaging outgrowth on epidermal morphogenesis, SC lipid organization and lipid composition. This approach was also used in chapter 5, in which primary material from AD patients with or without FLG mutations was used. The possibility of this method and the possible role of FLG in epidermal morphogenesis are described in this chapter as well.

In chapter 6, a FLG deficient N/TERT based epidermal skin model (NEM) was used, by means of FLG-KD or through IL-31 supplementation, to evaluate the role of FLG in epidermal S. aureus colonization. In addition, the subsequent epidermal response was studied as well. To evaluate whether we can establish a HSE that mimic features of lesional AD skin, a full thickness HSE with primary keratinocytes was established in the presence of a mixture of AD related cytokines, which was compared to lesional AD skin. The results of this study are described in chapter 7.

A summary, discussion and the future perspectives of the results described in this thesis is presented in chapter 8.
General introduction

References


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