A human organotypic knock-down skin model to study molecules involved in epidermal keratinocyte differentiation. An alternative to knock-out animal experiments in dermatological research

Mildner, M.

CHAPTER 1

GENERAL INTRODUCTION

1. Function of the skin

The skin is the largest organ of the mammalian body. It protects the organism against environmental aggressions and microbial pathogens and forms an inside out barrier preventing fluid loss (Elias 2005). The skin is also a sensorial organ containing a dense network of nerve endings specialized end organs sensing heat and cold, vibration, pressure, touch and pain, thus, allowing the organism to react to environmental cues. Due to its location at the interface between the organism and the environment, skin also plays an important role both as a site of initiation as well as a target for an immune response (Schmidt et al., 2000). Sensitization to foreign antigens can be initiated in the skin by specialized antigen-presenting cells such as epidermal Langerhans cells, and immune reactions within the skin may manifest as severe inflammatory diseases such as atopic dermatitis and psoriasis. In addition, also non-immune skin-cells are able to produce cytokines, chemokines and growth factors thereby modulating inflammatory reactions (Bolognia et al., 2008).

2. Structure of the skin

Human skin consists of 3 compartments: the epidermis, the underlying dermis and the subcutis (Figure 1).

The epidermis, a stratified squamous epithelium which is constantly renewing, forms the outermost part of the skin (McGrath et al., 2004). Depending on the body-site, 4-5 layers which represent different stages of epithelial differentiation can be distinguished within the epidermis (Figure 2). Layers from the inside to the outside are the basal or germinative layer (*stratum basale* or *stratum germinativum*), the spinous layer (*stratum spinosum*), the granular layer (*stratum granulosum*), the clear

or translucent layer (*stratum lucidum)* which is present in the epidermis of in palms and soles only and the cornified layer (*stratum corneum*) (Fuchs 1990).

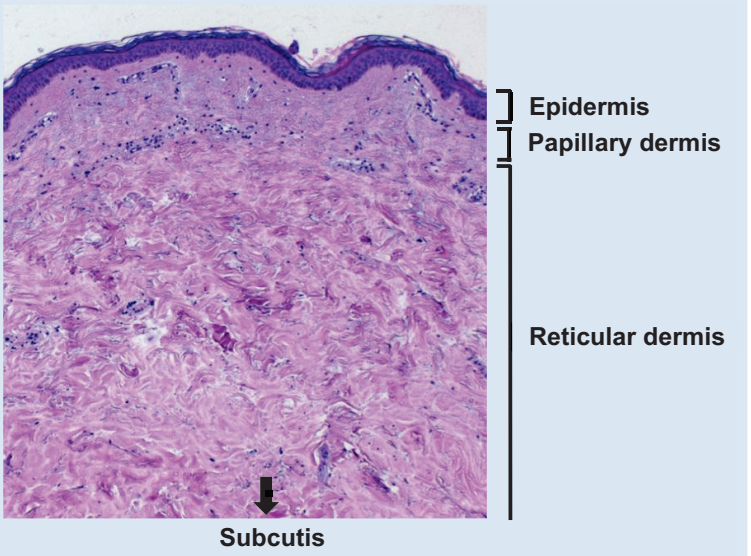

Figure 1: Structure of the skin. H&E-staining of normal breast skin is shown. The cell-rich epidermis is anchored to the papillary dermis which contains a network of thin, unarranged collagen fibers. In the reticular layer these fibers become thicker and more organized. The subcutis is located underneath the reticular dermis.

More than 95% of all cells in the epidermis are keratinocytes (KC), which are tightly interconnected by desmosomes (Proksch et al., 2004). Beside KC, other cell types such as melanocytes (MC), Merkel cells, Langerhans cells and T cells are regular present within the human epidermis (Figure 3). The epidermis itself is not vascularized but exclusively nourished from the highly vascularized dermis by diffusion. The different epidermal layers are formed through terminal differentiation of KC (Chapter 1, 3.1.). The epidermis is tightly anchored to the dermis *via* the basal membrane (*lamina basalis*), which is at the interface between basal KC and dermis. The basal membrane consists of specialized glycoproteins, produced and secreted by both epidermal KC and dermal fibroblasts (FB) (Alitalo et al., 1984; McDonald, 1988).

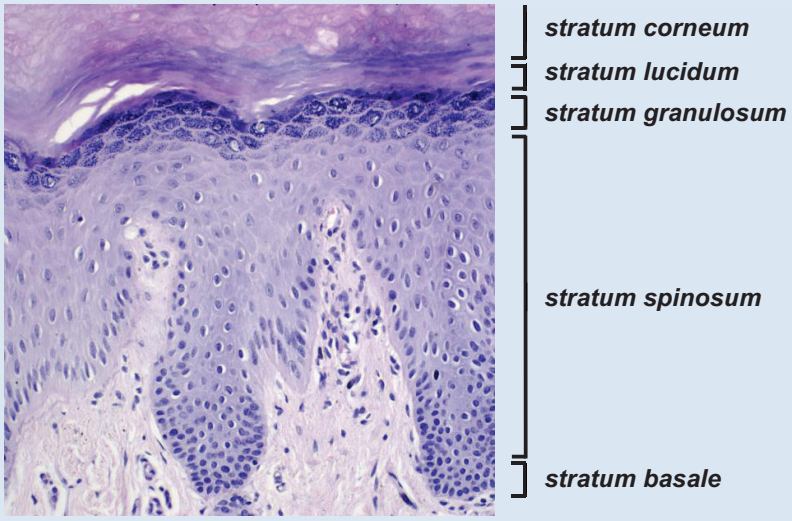

Figure 2: Structure of the epidermis. H&E-staining of normal skin from the sole is shown. The *stratum basale* (basal layer) is attached to the basement membrane and consists of proliferating KC. In the suprabasal layer, KC start to differentiate, giving rise to the *stratum spinosum* (spinous layer), the *stratum granulosum* (granular layer) and the *stratum corneum* (cornified layer). In thicker skin such as skin from the palm and sole, the *stratum lucidum* is visible between the *stratum granulosum* and the *stratum corneum*.

The dermis can be subdivided into the papillary and the reticular layer. The subepidermal, papillary layer contains a network of thin, unarranged collagen fibers, which become thicker and more organized in the reticular layer. The cell density within the dermis is much lower than in the epidermis since its main volume is formed by extracellular matrix components primarily collagens, elastin and glycosaminoglycans (McDonald, 1988). These structural proteins provide stability and elasticity to the skin. FB are the main producers of the different components of the extracellular matrix. In addition, they interact with epidermal KC and other skin cells by the production of cytokines and growth factors (Nolte, 2008). In contrast to the epidermis, the dermis contains dense networks of blood capillaries and nerves. Several types of immune cells are either resident within the dermis such as mast cells, dermal dendritic cells, macrophages and T cells, or home to the dermis in the course of inflammatory reactions of the skin (Hentzer and Kobayasi, 1980) (Figure 3). Also adnex organs i.e. hair follicles, sebaceous glands and sweat glands are located

within the dermis and extend through the reticular layers into the underlying subcutaneous fat tissue.

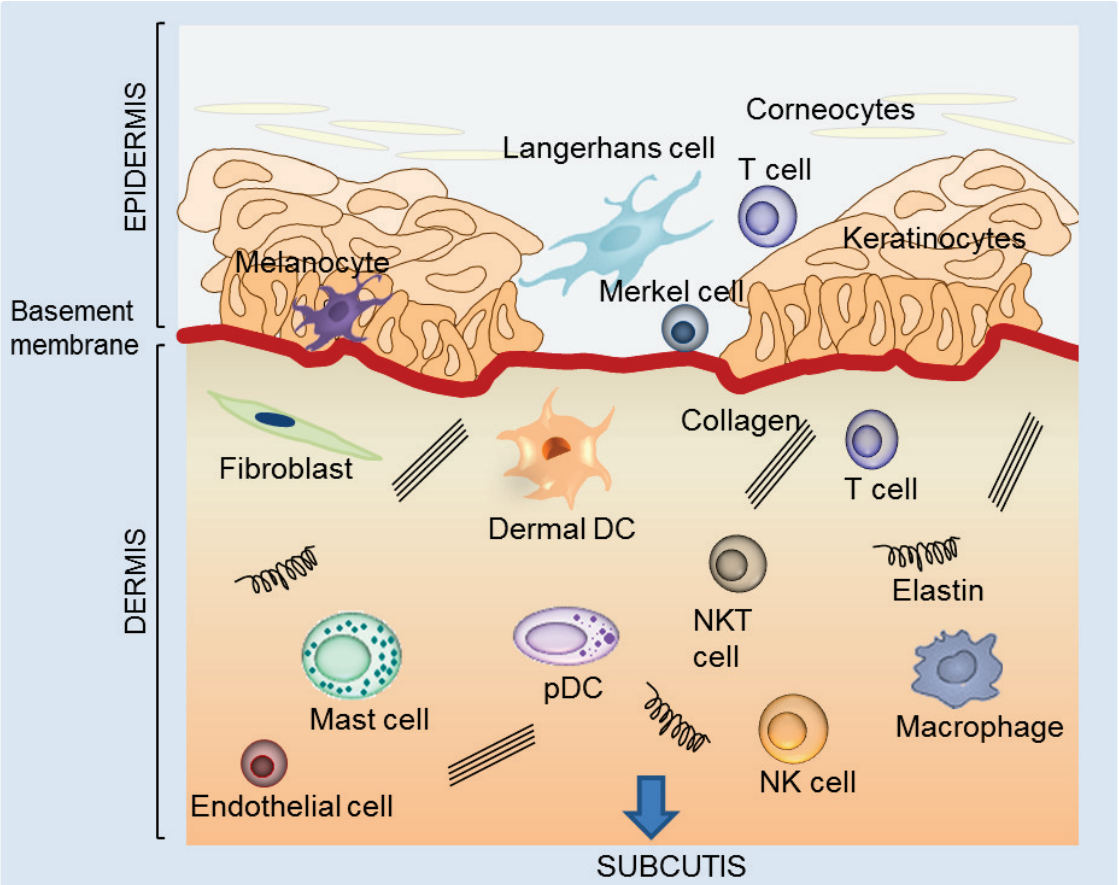

Figure 3: Scheme of the skin – the different layers and cell types. The major structures and cell types in the epidermis and the dermis is shown in the scheme. The epidermis is separated from the dermis by the basement membrane. The subcutis is located underneath the dermis. Adapted from *Elbe-Bürger, 2012*

The innermost layer of the skin, the subcutis, consists of highly vascularized loose connective tissue and adipose tissue which serve both as a storage for energy and as an insulator against low temperatures. Fibrous bands are anchoring the skin to the deep fascia and the overlaying skin layers.

3. Epidermal keratinocyte differentiation

3.1. Regulation of the epidermal differentiation program

Keratinocyte terminal differentiation refers to the conversion of viable KC into corneocytes, which are anuclear cellular ghosts (Haake and Holbrook, 1999). KC stem cells are present both in the basal layer of interfollicular epidermis and the bulge region of the hair follicles (Alonso, 2003). They give rise to transient amplifying KC, which after a limited number of cell divisions leave the basal layer start to differentiate (Alonso, 2003) ultimately forming corneocytes. The corneocytes remain linked by corneodesmosomes and together with intercellular lamellar lipids form the outer skin barrier, the stratum corneum (Haake and Holbrook K, 1999). When reaching the skin surface the intercellular bridges between corneocytes are cleaved by serine proteases and the individual corneocytes are shed from the surface (Pierard, 2000). The differentiation of basal KC into corneocytes is a continuously ongoing process, which in humans takes about 30 days (Dover, 1994). To maintain the epidermal homeostasis, the number of epidermal cells has to remain constant, i.e. the number of newly generated cells must compensate the number of cells shed from the surface. This complex process is tightly regulated and aberrations are observed in skin diseases such as psoriasis, which is characterized by a hyperproliferative and hyperkeratotic epidermal phenotype (see Chapter 3).

During terminal differentiation, epidermal KC undergo several characteristic morphological changes which can be observed by conventional light microscopy (Figure 2) (Haake and Holbrook, 1999). In the basal layer KC are well organized and vertically oriented. In the lower spinous layer KC enlarge their volume and form many desmosomal cell-cell interactions, conferring the typical spinous shape due to the shrinking of the cell body after fixation, while the intercellular contacts remain intact. In the upper spinous layer and in the granular layer KC become more and more flattened and horizontally oriented. In the granular layer the differentiation process is already advanced and many basophil keratohyalin granules (Weiss and Zelickson, 1995) consisting mainly of filaggrin (see Chapter 1, 4.1) are formed. Furthermore, so called “lamellar bodies” are produced in this layer (Norlen, 2001). They contain lipids and enzymes which are secreted into the intercellular spaces between corneocytes.

In the outermost living layer of the stratum granulosum the transition from viable KC to dead corneocyte takes place. This process, referred to as “terminal KC differentiation”, involves removal of all cellular organelles including mitochondria and nuclei (Haake and Holbrook, 1999).

Whereas the morphological changes of KC during their differentiation process have been well studied, the complex molecular and biochemical mechanism regulating this process are still not fully understood. Once KC enter the differentiation program at the transition from the basal layer to the spinous layer they switch the expression profile of structural proteins and enzymes (O'Guin et al., 1987; Fuchs and Green, 1980). Expression of keratins (KRT) 5 and 14, which are only expressed in basal KC in situ and by proliferating KC in monolayer culture, are switched off and simultaneously the expression of KRT 1 and 10 is switched on (Bowden et al., 1987; Morley and Lane, 1994; Fuchs, 1990). The latter KRT are able to form a tighter network which is linked to the intercellular desmosomes. In the granular epidermal layer other structural proteins such as involucrin, loricrin and small proline-rich proteins are synthesized which are necessary to build the cornified envelope (Simon and Green, 1985; Mehrel, et al., 1990). These protein layers are further cross-linked by epidermal transglutaminases (TG) (Eckert, 2003). An additional feature readily distinguishable by histology are keratohyalin granules which were name-givers for the stratum granulosum and which contain high amounts of pro-filaggrin (Weiss and Zelickson, 1995). After proteolytic maturation, this high molecular weight protein is involved in aggregating the KRT-filaments into tightly aligned KRT-bundles, thereby inducing the collapse of the cells leading to their flattening (see Chapter 1, 4.4.). All these differentiation associated processes are tightly regulated by a variety of different signaling pathways. Recently, a new class of transcriptional regulators, namely microRNAs (miRNAs) (Ambros, 2001) have been shown to play a crucial role in KC-differentiation (Yi et al., 2006). These small molecules can bind to specific target mRNAs, leading to their degradation or inactivation. For example miR-203 is a miRNA exclusively expressed in the suprabasal layers of the epidermis. Expression of miR-203 in the basal KC induces premature KC differentiation. On the other hand, removal of miR-203 from suprabasal KC leads to hyper-proliferation, which is no longer restricted to the basal layer (Lena et al., 2008). But not only on the

transcriptional level but also on the translational or even more importantly on the post-translational level many regulatory processes take place during KC differentiation. Key-players in post-translational modifications are proteases, which are involved in multiple steps of KC differentiation (Zeeuwen, 2004) as also discussed below (Chapter 1, 3.4.).

3.2. Keratinocyte cornification and epidermal barrier formation

The most important part of the epidermal barrier function lies within the outermost layer of the epidermis, the stratum corneum. It consists of 15-20 layers of corneocytes, which are cellular ghosts that lack nuclei and organelles and which are interconnected by corneodesmosomes (Haake and Holbrook, 1999). The intercellular spaces between the corneocytes contain lipids in a lamellar arrangement (Wertz, 1992; Proksch, 2003; Elias, 2008). These lipids are produced in the granular layer of the epidermis and are packed into lamellar granules. The formation of the cornified envelope is a crucial step in the establishment of a functional barrier. Its assembly is a multi-step process, which is initialized by the expression of several differentiation associated proteins, such as involucrin, envoplakin and periplakin, which then associate with keratin filaments and desmosomes (Candi, 2005). After crosslinking of these proteins by TG-1, they build a layer along the inner plasma membrane, thereby forming a scaffold. In parallel, lamellar granules fuse with the apical plasma membrane of KC of the last living layer of the stratum granulosum, and extrude their lipid content together with lipid modifying enzymes into the intercellular spaces (Elias, 1992), which surrounds the cornified envelope. Finally, the late differentiation proteins loricrin and small proline rich proteins are crosslinked by TG-1 and TG-3 to the scaffold to strengthen its structure. The complete cornified envelope, consisting of the protein and the lipid envelopes together with the intercellular lipid lamellae, provide a formidabel barrier function to the outside as well as to the inside (Candi, 2005). When reaching the surface of the stratum corneum, corneocytes are shed after cleavage of the corneodesmosomes by the serine proteases kallikrein-5 and -7, the aspartic protease cathepsin D and the cysteine protease cathepsin V (Caubet et al., 2004; Horikoshi et al., 1999; Igarashi et al., 2004; Borgoño, 2007). Formation of

the cornified envelope and desquamation are both key events for skin homeostasis. Defects in any of these events either results in or is associated with the vast majority of skin diseases.

3.3. Filaggrin and the natural moisturizing factor (NMF)

One key protein for the development of a fully functional epidermal barrier is filaggrin (Chapter 8) (Steinert et al., 1981; Brown et al., 2011). This large polyprotein of >400 kDa is synthesized in the granular layer of the epidermis. Structurally, human filaggrin is composed of one S100-like calcium binding A-domain, one B-domain, two imperfect filaggrin monomers and 10-12 almost identical filaggrin monomers of 37 kDa (Figure 4). In contrast, in mice the number of filaggrin repeats varies from 12 and 20, depending on the strain (Rothnagel and Steinert, 1990; Zhang, et al., 2002; Fallon, et al., 2009; Sandilands et al., 2009). In the stratum granulosum profilaggrin is highly phorsphorylated and retained in the keratohyalin granules.

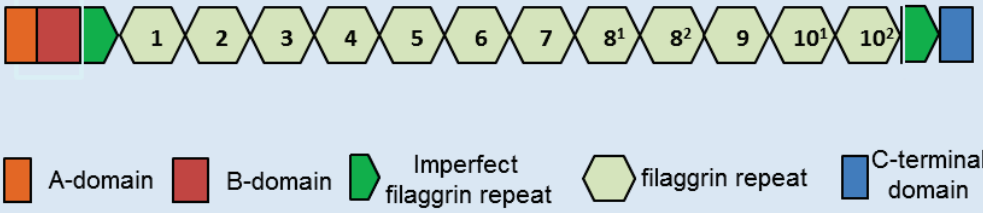

Figure 4: Structure of human profilaggrin. Human profilaggrin consists of one A- and one B-domain, two imperfect filaggrin repeats, 10-12 filaggrin repeats and C-terminal domain.

During terminal differentiation of KC, filaggrin is proteolytically processed to mature filaggrin monomers. The initial step of filaggrin processing is the dephosphorylation of the protein by phosphatases such as PP2A (Kam et al., 1993; Lonsdale-Eccles et al., 1982; Resing et al., 1985; Ovaere et al., 2009). This leads to the release of profilaggrin from the keratohyalin granules into the cytoplasm. The proteases calpain-1 and furin have been shown to cleave the linker region and the N terminus of pro-

filaggrin. Also matriptase-1 (Chapters 2 and 3) and prostasin have been shown to be involved in this process (List et al., 2003; Netzel-Arnett et al., 2006). All cleavage products of pro-filaggrin contribute to the cornification process of KC. The linker region has been shown to become a part of the cornified envelope, and the filaggrin monomers are thought to be important molecules for keratin aggregation, leading to the flattening of the corneocytes. Ultimately, in the stratum corneum filaggrin monomers are degraded into amino acids and their derivates. This step is initiated by deimination of filaggrin by deiminases (Tarcsa et al., 1996; Méchin et al., 2007), followed by further degradation by other, still unknown enzymes and Caspase-14 (Denecker et al., 2007; Hoste et al., 2011). This step prepares filaggrin monomers for efficient degradation into amino acids. Finally, filaggrin fragments are degraded to free amino acids by bleomycin hydrolase and calpain-1 (Kamata et al., 2009), and some of the free amino acids are further modified to several derivatives such as urocanic acid (UCA) and pyrrolidone-5-carboxylic acid (PCA). All these degradation products are part of the "the natural moisturizing factor" (NMF), which is important for stratum corneum hydration (Rawlings and Harding, 2004) (Figure 5). Recently, *filaggrin* mutations which are associated with the pathogenesis of ichthyosis vulgaris and atopic dermatitis have been described. Up to now more than 40 *filaggrin* null mutations have been described which result in the absence of this protein from the epidermis. The most prominent in the European population are R501X, 2282del4 and R2447X (McGrath and Uitto, 2008; Palmer et al., 2006; Smith et al., 2006; Sandilands et al., 2007; Sandilands et al., 2009.) Patients with *filaggrin* null mutations display a reduced stratum corneum hydration, an increased transepidermal water loss (TEWL) and a decreased level of total NMF as well as the filaggrin degradation products UCA and PCA (Kezic et al., 2008; Kezic et al.2011). The importance of filaggrin for barrier formation was also investigated in the flaky tail mouse, a mouse model of mutated *filaggrin* (Presland et al., 2000). These mice show impaired lipid organization in the stratum corneum due to abnormalities in lamellar body formation and extrusion (Fallon et al., 2009; Scharschmidt et al., 2009). Furthermore, enhanced percutaneous allergen priming is detected, which strengthen the finding of a non-functional epidermal barrier. This mutant mouse however, is actually a double homozygous of flaky tail and another mutation, called matted. The matted phenotype begins to be visible during the second hair cycle as the hair coat reaches its full

growth. Phenotypically, hairs stick together in clumps, hence the name matted (Jarrett and Spearman, 1957; Searle and Spearman, 1957). Due to this further mutation interpretation of the data obtained with this model is difficult, and therefore the exact contribution of the filaggrin mutation itself to the phenotype is not clear (Presland et al., 2000). In addition due to species-specific properties of filaggrin, caution should be taken when interpreting and comparing the function of filaggrin in animal models with that of human filaggrin (Harding and Scott, 1983).

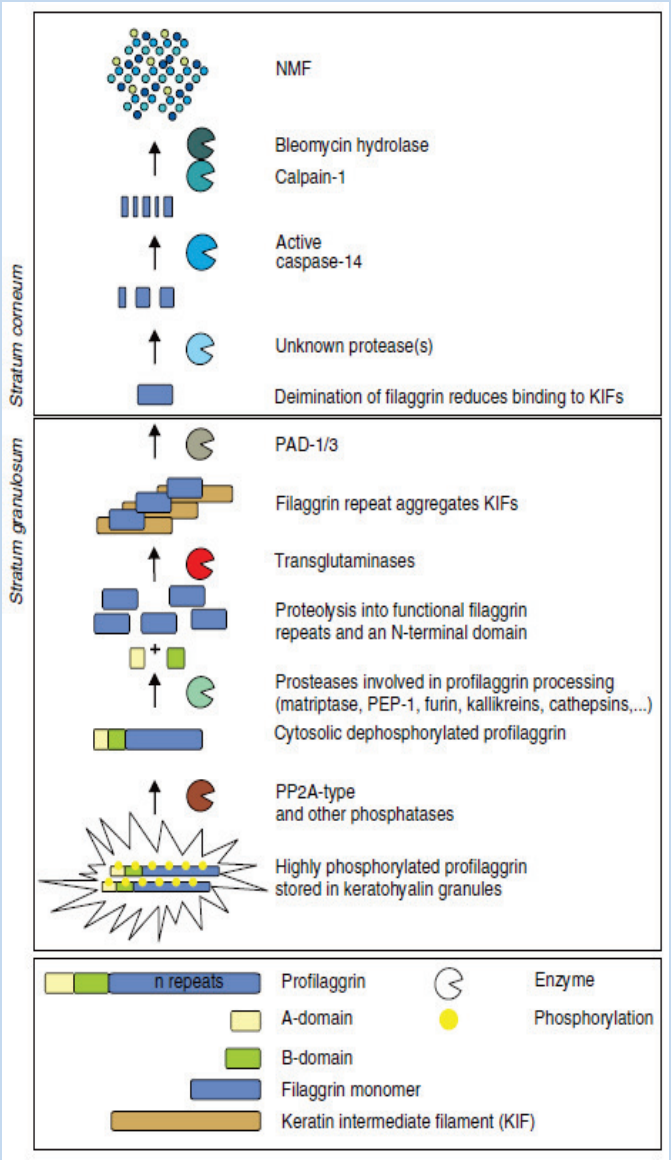

Figure 5: Hypothetical over-view of the pathway involved in filaggrin degradation.
Upon dephosphorylation, profilaggrin is released and proteolyzed into filaggrin repeats. Filaggrin binds and strengthens keratin inter-mediate filaments (KIFs), thereby promoting cell flattening. Degradation of the filaggrin monomer starts in the deeper SC layers by partial proteolysis by one or more unknown proteases. Next, caspase-14-mediated cleavage prepares these fragments for efficient degradation into free aminoacids, which contribute to the natural moisturizing factors (NMFs). Calpain1 and bleomycinhydrolase also participate in this process. Modified from *Hoste et al., 2011*

3.4. Enzymes involved in terminal keratinocyte differentiation

KC express a variety of different enzymes which play important roles during different steps of differentiation and formation of a functional barrier (see Chapter 1, 3.2.). Our research focused on two types of enzymes: proteases and nucleases.

In the next two paragraphs specific enzymes, which have been shown to participate in terminal KC differentiation i.e. matriptase-1 and DNases, representing a major part of this thesis, will be described in more detail.

3.4.1. Matriptase-1

Matriptase-1 is a transmembrane serine protease which is auto-proteolytically activated, thereby leading to the initiation of other proteolytic cascades (Chapters 3 and 4) (Oberst et al., 2003). Several substrates including pro-urokinase, plasminogen activator, hepatocyte growth factor, protease-activated receptor-2, matrix metalloproteinase-3 and pro-kallikrein have been described as target for proteolytic activities of matriptase-1 (Takeuchi et al., 2000; Lee et al., 2000; Jin et al., 2006; Sales et al., 2010). Deletion of the matriptase-1 gene in a mouse model results in a lethal phenotype (List et al., 2002). These mice are dying shortly after birth due to severe skin problems. They show defects in lipid matrix formation, in cornified envelope morphogenesis and in stratum corneum desquamation (List et al., 2002). At the molecular level matriptase-1-deficient mice lack mature filaggrin, whereas pro-filaggrin accumulates (List et al., 2003). Skin transplanted from these mice onto SCID-mice develops an ichthyosis like phenotype, characterized by thickened scaly epidermis and hair loss (List et al., 2003). In addition to epidermal KC, matriptase-1 is also expressed in simple, stratified, transitional and pseudo-stratified epithelia, which do not undergo cornification or express filaggrin, suggesting additional roles of this important protease in epithelial homeostasis, which still await elucidation.

3.4.2. DNases

The formation of functional corneocytes involves several major changes, including the degradation of nuclei and cellular DNA (Santoianni and Rothman, 1961). When KC convert from living cells of the stratum granulosum to dead corneocytes, nuclei are broken down and nuclear DNA is degraded (McCall and Cohen, 1991; Lippens et al., 2005; Kroemer et al., 2009). If this step of KC differentiation is impaired it results in the retention of nuclei in corneocytes as it is observed in parakeratotis which is a hallmark of psoriasis. The enzymes responsible for DNA-degradation are called DNases. In addition to their role for the degradation of cellular DNA, DNases are also important for the removal of exogenous DNA such as DNA derived from colonizing or invading microorganisms (Eckhart et al., 2007). Several DNases are expressed in the epidermis including DNase1L2 (Chapter 5) and DNase 2 (Chapter 6).

We have shown that in human epidermis DNase1L2 is expressed in differentiated KC of the granular layer (Fischer et al., 2007; Fischer et al., 2011). Degradation of DNA by DNase1L2 results in DNA fragments which are detectable by the terminal deoxynucleotidyl transferase dUTP nick end labeling reaction (TUNEL). DNase1L2 is most active at pH 5.6. *In vitro*, inflammatory cytokines such as TNFα and IL-1β have been shown to upregulate DNase1L2 expression *via* the NF-κB signaling pathway. The contribution of DNase1L2 to DNA degradation in epidermal KC was investigated in a DNase1L2 deficient mouse (Fischer et al., 2011). DNase1L2 deficiency neither affects viability and health of the mice nor epidermal development. Nuclear remnants however, are still present in hair and nails, the scales on the tail as well as on the surface of the tongue and the esophagus (Fischer et al., 2011).

DNase 2 is an acid DNase present in the lysosomes of virtually all cell types. DNase 2 generates DNA fragments that are not detectable by the TUNEL reaction. The pH optimum of DNase 2 is in the range of pH 4.5 to pH 5.0. Its main function is the degradation of phagocytized DNA in the lysosomes (Nagata, 2005) and the degradation of DNA in nuclei expelled from erythroblasts in the course of differentiation to erythrocytes (Kawane et al., 2001). Deficiency of DNase 2 in mice is lethal (Krieser et al., 2002) due to a massive interferon response and autoimmunity against undigested DNA (Yoshida et al., 2005).

3.5. Histidase – Urocanic Acid

Histidase, also known as L-histidine ammonia lyase is the enzyme which transforms histamine to UCA (Chapters 7 and 8). It is expressed in the liver and the epidermis. Histidase mutations have been identified as the cause of histidinemia, a benign metabolic disorder (Lam et al., 1996). These mutations lead to an increased concentration of histidine and a decreased concentration of UCA in blood, urine and epidermis (Baden et al., 1969; Lam et al., 1996). In the Peruvian mouse, a mouse model with mutated histidase, leading to a loss of histidase activity, the contribution of this enzyme to the epidermal barrier function has been investigated. Whereas skin morphology and barrier function was normal in these mice, the content of UCA was strongly reduced, which led to a dramatically increase in photo-sensitivity.

UCA has been suggested to be an important UV absorbing factor of the skin already many years ago (Zenisek et al., 1955; Tabachnik, 1957). Indeed topical application of UCA onto the human skin leads to increased photoprotection (Baden and Pathak, 1967; de Fine Olivarius et al., 1996). In addition to direct UVB absorption, UCA is an anti-oxidant, shown by the ability to scavenge hydroxyl radicals (Kammeyer et al., 1999; Kammeyer et al. 2001). Due to its immunosuppressive properties, the use in sunscreen products has been contested and is forbidden in some countries (DeFabo and Noonan, 1983; Andersen, 1995).

4. Models to study epidermal keratinocyte differentiation *in vitro*

KC differentiation involves a complex cellular program that has been extensively studied during the past decades. Apart from the use of knockout or transgenic animal models *in vitro,* differentiation models have been shown to be most practical for investigation of mechanisms involved in stratum corneum biogenesis. These *in vitro* models range from simple monolayer cultures to complex reconstructed organotypic skin-equivalent cultures. The use of such *in vitro* skin models has helped to reduce the use of experimental animals for both basic research and testing of compounds with supposed activities on skin cells. For our studies we have developed a highly reproducible method to specifically knock-down desired target genes in KC in an

organotypic skin model (Chapter 1, 4.3.). This approach and the results from studies on the epidermal differentiation process represent a major part of this thesis.

4.1. Monolayer keratinocyte culture

The monolayer culture is the simplest way to investigate KC biology *in vitro*. KC are isolated from skin samples by a two-step enzymatic digestion. First, the epidermis is separated from the dermis by the protease dispase (Kitano and Okada et al., 1983). Subsequently, the epidermis is digested with trypsin, generating single cell suspension of KC, which are then propagated in KC-specific media containing KC growth factors. These special media are selective for KC growth and therefore other cell-types which are present in the epidermis, such as melanocytes and Langerhans cells, are absent from the cultures after a few days of cultivation (Watt, 1989). The remaining KC are highly proliferative and keep their undifferentiated state for up to 20 doublings. Rheinwald and Green in 1975 were the first who showed that KC in monolayer culture can, to a certain degree, differentiate and form multilayered epithelium like structures (Rheinwald and Green, 1975; Rheinwald and Green, 1977). This can be triggered by increasing the calcium concentration or, closer to the *in vivo* situation, by growing KC to 100% confluence and keeping them confluent for several days (Rendl et al., 2002). However, by using such an approach the investigation of KC differentiation is limited. Especially processes, taking place during the last steps of KC differentiation such as the transition from KC to corneocytes and the formation of the epidermal barrier, cannot be properly analyzed in monolayer cultures. For example pro-filaggrin, an important protein for epidermal barrier formation (Chapter 1, 3.3.), although expressed in monolayer cultures after several days of confluency, is not processed to mature filaggrin (Rendl et al., 2002). This suggests that in contrast to normal epidermis, important proteases are either not present or not active in monolayer cultures. This is indeed the case for caspase-14, an enzyme which is involved in the processing of mature filaggrin into single amino acids (Figure 5) (Hoste et al., 2011). In normal skin caspase-14 is cleaved within the last steps of KC differentiation and thereby activated. In monolayer KC cultures, caspase-14 is only present as a non-activated pro-caspase (Rendl et al., 2002). This example shows

that, especially for studies on terminal KC differentiation, monolayer cultures are not sufficient and more complex *in vitro* models are needed.

4.2. *Ex vivo* culture (skin explants)

Skin explants are cultures of viable skin, collected by surgery or biopsy. After disinfection and removal of the subcutaneous fat, the skin samples are placed in culture dishes in appropriate culture media. Explant cultures can be maintained for several weeks and can be exposed to drugs *in vitro.* Effects on proliferation, differentiation and cell survival (toxicity) can be investigated this way. An advantage of skin explants is the fact that the full spectrum of cell types present in skin can be investigated. For example the migration of dendritic cells within the skin and out of the skin has been analyzed this way (Stoitzner et al., 1999; Lukas et al., 1996). In addition, also explants from diseased skin can be studied. Skin explants from healthy and diseased skin can also be expanded on a FB-seeded collagen matrix, as it was shown for squamous cell carcinoma (Commandeur et al., 2009) and epidermolysis bullosa simplex (El Ghalbzouri et al., 2003). For studies on epidermal differentiation however, this model has the limit that the epidermis is already fully established and therefore lacks the dynamics which would allow dissecting the different differentiation steps.

4.3. Organotypic skin culture

Organotypic skin cultures also called “skin equivalents” are *in vitro* generated epidermal or full thickness artificial skin samples reflecting many features of normal skin. Skin equivalents are generated from primary monolayer cell cultures, which under defined conditions are able to form functional epidermis (Figure 6, for a detailed protocol see Chapter 2). The most basic model consists of KC alone growing on a filter membrane (Ponec et al., 1997; Boelsma et al., 2000). Since *in vivo* stromal cells, mainly FB, strongly influence epidermal development and function (Commandeur et al., 2011), the reconstitution of both the epithelial and mesenchymal compartments *in vitro* is desirable. A recent publication describes the evolution of

these models (Auxenfans et al., 2009). The dermal compartment is either a de-epidermized dermis or a dermal equivalent consisting of a collagen

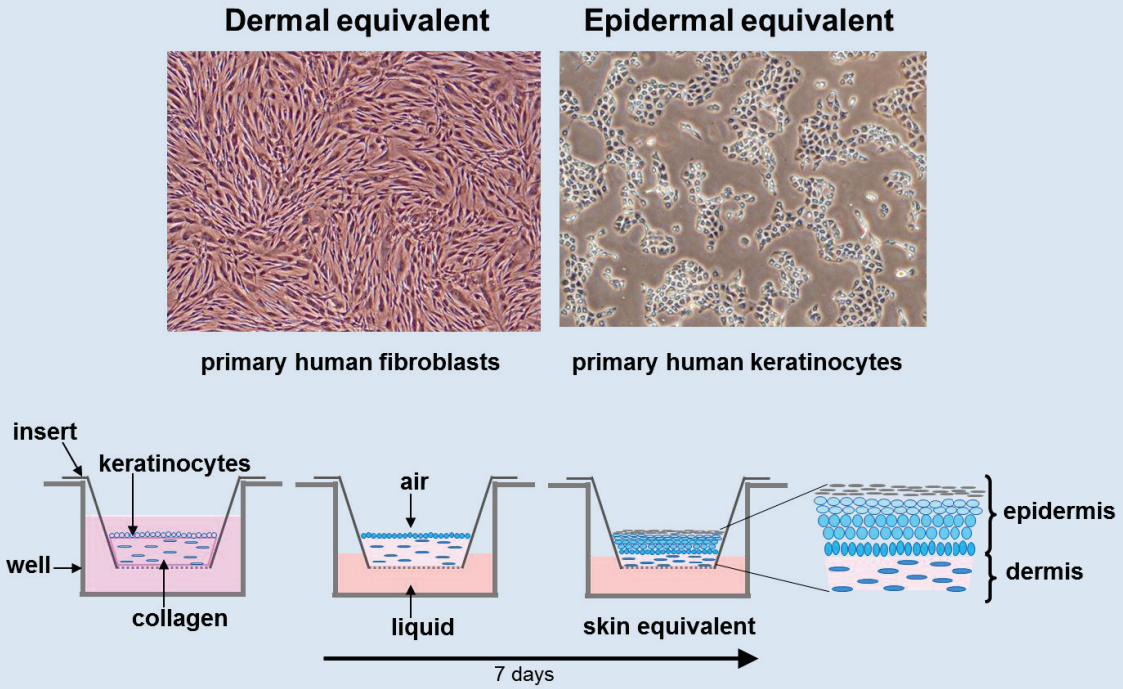

Figure 6: Generation of a full-thickness organotypic skin. For the establishment of a full-thickness skin equivalent, human primary fibroblasts and keratinocytes are used in passages 2-4. After assembling the fibroblast containing collagen matrix, keratinocytes were seeded onto the matrix and cultured submerged for 24-48 hours. After exposing keratinocytes to air, the equivalents are further cultured for 6-7 days.

matrix seeded with FB. We use the latter approach. On top of the dermal equivalent, primary KC are seeded and allowed to differentiate for several days (Figure 6). The differentiation process is initiated by lifting KC to the air and changing the culture medium to a defined serum free KC differentiation medium containing a high Ca^{2+} concentration (Figure 6). Figure 7 shows kinetics of epidermal development using this model. The different steps of KC differentiation, as well as alterations induced by any kind of treatment at any time point, in the differentiation program can be investigated. The fully developed model is often used for cytotoxicity assays induced for instance by chemicals or UV-radiation. Since the organotypic skin is fully differentiated, investigations especially on the versatile effects of UV-radiation on KC are favorable in this model, compared to monolayer cultures. In recent years the full-

thickness skin equivalent has been constantly improved. The epidermal equivalent for instance can be completed by the addition of primary melanocytes (MC) (Van Gele et al., 2011). This allows studies on MC-KC interactions or on pigmentation. Instead of MC also melanoma cells can be implemented in this model facilitating studies on melanoma growth and metastasis (Li et al., 2011). The dermal compartment has been further improved by adding endothelial cells forming blood vessel like structures or nerve cells (Gingras et al., 2003; Gingras et al., 2008; Herman et al., 2009). Even a model containing additionally a subcutis, made up of pre-adipocytes and mature adipocytes, cultured underneath the dermal compartment has been developed (Keck et al., 2011). These models allow investigations on a variety of skin-specific cell types and their interactions with other skin cells.

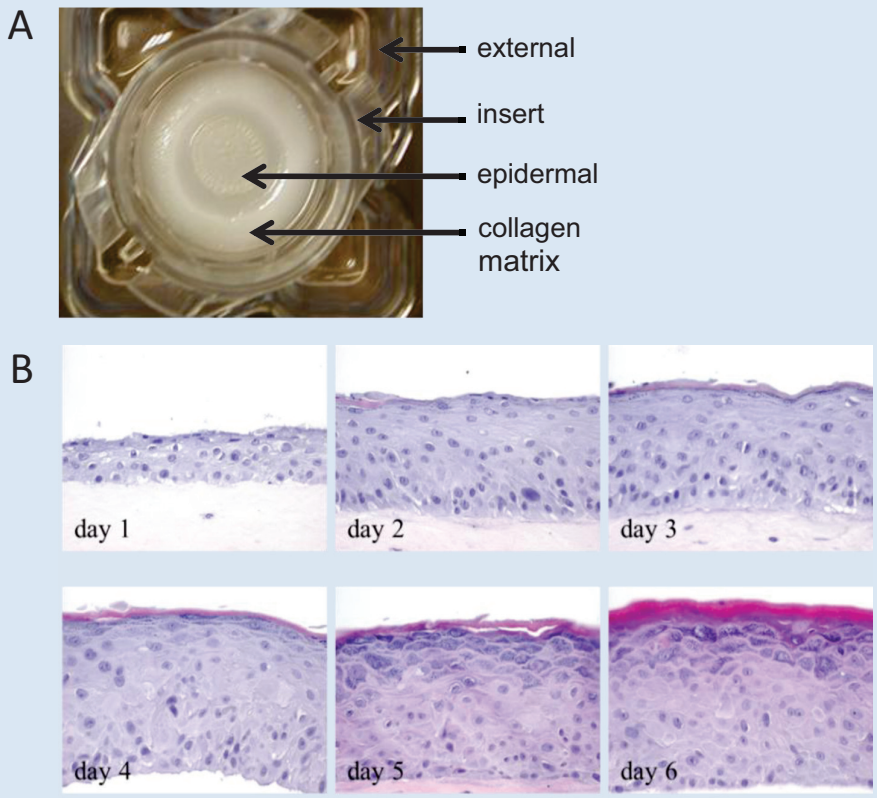

Figure 7: Epidermal development in an organotypic skin culture (A) A photograph of a 6-day old organotypic skin culture is shown. The air exposed epidermal equivalent is attached to the collagen matrix. **(B)** H&E-staining of organotypic skin samples at the indicated time points is shown. Between days 3 and 4 the granular layer and the cornified layer starts to form. At day 6 the organotypic skin is fully developed.

4.4. Organotypic knock-down skin culture

In an attempt to better study the contribution of individual proteins to skin barrier formation we have developed a method to study the effect of the deletion of individual genes in an organotypic skin model (Chapters 2 and 3). In this model knock-down of target genes is achieved by using siRNA technology. siRNAs are small double-stranded RNAs of 21 nucleotides which in a sequence-specific manner bind to the target gene, the target sequence is inactivated/degraded, ultimately leading to the inhibition of protein synthesis (Elbashir et al., 2001). The respective siRNAs against desired target genes are transfected into primary KC in monolayer culture before they are seeded on the collagen matrix (for a detailed protocol see Chapter 2).

To generate an organotypic knock-down skin culture, the siRNA transfected KC are seeded on the collagen matrix 24 to 48 hours after transfection, and cultured further as described above (Chapter 1, 4.3.). Remarkably, transient transfection of siRNA oligos with Lipofectamine 2000, which we are using in our model, is sufficient to significantly inhibit gene expression for the whole culture period. This allows a high throughput screening for genes involved in epidermal development and KC differentiation. Using this model we were able to knock-down and investigate a variety of genes including matriptase-1 (Chapter 3), VEGF (Chapter 3), filaggrin (Chapter 8), several DNases (Chapter 5 and 6), caspase-14 (unpublished data) as well as transcription factors such as the AP-1 members c-Jun and JunB (Ballaun et al., 2008). A recent publication by Hickerson et al. used a similar model to specifically inhibit the mutated keratin-6 allele in KC from pachyonychia congenita patients (Hickerson et al., 2011). Interestingly, the knock-down was performed with “self-delivery” siRNAs, which is a big step forward into the use of such “drugs” for the treatment of skin diseases. These siRNAs have been modified to enhance cellular uptake. Although siRNA-based therapeutics are currently in clinical trials for a variety of organs such as lung, kidney, liver and skin (Vaishnaw et al., 2010), there are still many hurdles to take to achieve sufficient and specific delivery of the siRNAs into the target organ. The use of the *in vitro* gene silencing model is therefore important not only to discover new gene functions and molecular mechanisms, but also to further improve siRNA technology and develop new strategies of gene-therapy.

4.5. Organotypic models of other epithelia

Although the skin is the most prominent organ for which organotypic models are available, there are several other epithelial cells used to establish three dimensional models. In Chapter 9 a human organotypic vaginal model will be introduced. This model reflects several features of the female vagina including highly glycogen rich superficial cell layers, which are indicative for vaginal epithelium. Besides biological studies this model is widely used for irritation studies of spermicides, microbicides and feminine care products (Ayehunie et al., 2006). In addition, scientific questions on sexual transmitted diseases can be partially investigated using the vaginal epithelial model (Lin et al., 2011).

Other models such as corneal epithelium (Meloni et al., 2011) and bronchus epithelium (Schleh et al., 2012) are also available and widely used for cytotoxicity assay and basic research questions.

All these models are acceptable alternatives to animal models, having the big advantage of being established with human primary cells, thereby preventing species related misinterpretation of results.

AIMS OF THIS THESIS

The use of organotypic skin models in human dermatological research has been an accepted *in vitro* method for several decades. Nevertheless, gene deletion experiments are still performed in animals. However, knockout or transgenic animal experiments are very time consuming and interpretation of the data, especially in the skin, is often quite difficult. Secondary effects, induced by cells of the immune system or other cell types present in the skin, may interfere with a putative differentiation related phenotype or barrier defect. Therefore, the use of a human *in vitro* model would allow to directly studying the effects of specific gene knock-downs on KC differentiation and epidermal development. In addition, comparing epithelial development and function in the skin to other epithelia, could lead to the investigation of important common and unique mechanisms in epidermal development.

We therefore wanted to:

i) establish a human organotypic knock-down skin model which is able to reproduce known phenotypes previously described in animal experiments

ii) study the expression of the serine-protease matriptase-1 and its function in epidermal development and stratum corneum formation, as well as in skin diseases

iii) test the contribution of DNases to KC differentiation as well as epidermal development and skin homeostasis

iv) study the expression and regulation of histidase in KC

v) investigate whether filaggrin and/or its degradation products have a direct function in epidermal development and the establishment of a functional epidermal barrier

vi) address whether other organotypic epithelial models, such as an organotypic vaginal epithelial model, are also useful to study general mechanisms relevant for multilayered epithelia

REFERENCES

1. Alitalo K, Kuismanen E, Myllylä R, Kiistala U, Asko-Seljavaara S, Vaheri A. J Cell Biol. 1982 Sep;94(3):497-505. Extracellular matrix proteins of human epidermal keratinocytes and feeder 3T3 cells.
2. Alonso L, Fuchs E. Stem cells of the skin epithelium. Proc Natl Acad Sci U S A. 2003 Sep 30;100 Suppl 1:11830-5. Epub 2003 Aug 11. Review.
3. Andersen FA (1995) Final report on the safety assessment of urocanic acid. J Am Coll Toxicol 14:386–423
4. Ambros V. Cell. 2001 Dec 28;107(7):823-6. microRNAs: tiny regulators with great potential.
5. Auxenfans C, Fradette J, Lequeux C, Germain L, Kinikoglu B, Bechetoille N, Braye F, Auger FA, Damour O. Evolution of three dimensional skin equivalent models reconstructed in vitro by tissue engineering. Eur J Dermatol. 2009 Mar-Apr;19(2):107-13.
6. Ayehunie S, Cannon C, Lamore S, Kubilus J, Anderson DJ, Pudney J, Klausner M. Organotypic human vaginal-ectocervical tissue model for irritation studies of spermicides, microbicides, and feminine-care products. Toxicol In Vitro. 2006 Aug;20(5):689-98
7. Baden HP, Hori Y, Pathak MA et al. (1969) Epidermis in histidinemia. Arch Dermatol 100:432–435
8. Baden HP, Pathak MA (1967) The metabolism and function of urocanic acid in skin. J Invest Dermatol 48:11–17
9. Ballaun C, Karner S, Mrass P, Mildner M, Buchberger M, Bach J, Ban J, Harant H, Tschachler E, Eckhart L. Transcription of the caspase-14 gene in human epidermal keratinocytes requires AP-1 and NFkappaB. Biochem Biophys Res Commun. 2008 Jun 27;371(2):261-6.
10. Brown SJ, McLean WH. One remarkable molecule: filaggrin. J Invest Dermatol. 2012 Mar;132(3 Pt 2):751-62.
11. Boelsma E, Gibbs S, Faller C, Ponec M. Characterization and comparison of reconstructed skin models: morphological and immunohistochemical evaluation. Acta Derm Venereol. 2000 Mar-Apr;80(2):82-8.

12. Bolognia J.L., Jorizzo J.L. and Rapini R.P. (2008).Dermatology. Mosby.
13. Borgoño CA, Michael IP, Komatsu N, Jayakumar A, Kapadia R, Clayman GL, Sotiropoulou G, Diamandis EP.J Biol Chem. 2007 Feb 9;282(6):3640-52. Epub 2006 Dec 11.A potential role for multiple tissue kallikrein serine proteases in epidermal desquamation.
14. Bowden, P. E., Stark, H. J., Breitkreutz, D., and Fusenig, N. E. (1987). Expression and modification of keratins during terminal differentiation of mammalian epidermis. Curr Top Dev Biol 22, 35-68.
15. Candi E, Schmidt R, Melino G. Nat Rev Mol Cell Biol. 2005 Apr;6(4):328-40. The cornified envelope: a model of cell death in the skin.
16. Caubet C, Jonca N, Brattsand M, Guerrin M, Bernard D, Schmidt R, Egelrud T, Simon M, Serre G. Degradation of corneodesmosome proteins by two serine proteases of the kallikrein family, SCTE/KLK5/hK5 and SCCE/KLK7/hK7. J Invest Dermatol. 2004 May;122(5):1235-44.
17. Caulfield RH, Tyler MP, Austyn JM, Dziewulski P, McGrouther DA.Burns.The relationship between protease/anti-protease profile, angiogenesis and re-epithelialisation in acute burn wounds. 2008 Jun;34(4):474-86.
18. Commandeur S, Ho SH, de Gruijl FR, Willemze R, Tensen CP, El Ghalbzouri A. Functional characterization of cancer-associated fibroblasts of human cutaneous squamous cell carcinoma. Exp Dermatol. 2011 Sep;20(9):737-42
19. Commandeur S, de Gruijl FR, Willemze R, Tensen CP, El Ghalbzouri A. An in vitro three-dimensional model of primary human cutaneous squamous cell carcinoma. Exp Dermatol. 2009 Oct;18(10):849-56.
20. DeFabo EC, Noonan FP (1983) Mechanism of immune suppression by ultraviolet irradiation in vivo. I. Evidence for the existence of a unique photoreceptor in skin and its role in photoimmunology. J Exp Med 158:84–98
21. de Fine Olivarius F, Wulf HC, Crosby J et al. (1996) The sunscreening effect of urocanic acid. Photodermatol Photoimmunol Photomed 12:95–99
22. Denecker G, Hoste E, Gilbert B, Hochepied T, Ovaere P, Lippens S, Van den Broecke C, Van Damme P, D'Herde K, Hachem JP, Borgonie G, Presland RB, Schoonjans L, Libert C, Vandekerckhove J, Gevaert K, Vandenabeele P, Declercq W. Caspase-14 protects against epidermal UVB photodamage and water loss. Nat Cell Biol. 2007 Jun;9(6):666-74.

23. Dover R. (1994) Cell kinetics of keratinocytes. In: The Keratinocyte Handbook. (Leigh I.M.; Lane E.B.; Watt F.M.; Eds.) Cambridge: Cambridge University Press; 203-21.
24. Eckert RL, Sturniolo MT, Broome AM, Ruse M, Rorke EA. Transglutaminase function in epidermis. J Invest Dermatol. 2005 Mar;124(3):481-92.
25. Eckhart L, Fischer H, Barken KB, Tolker-Nielsen T, Tschachler E. DNase1L2 suppresses biofilm formation by Pseudomonas aeruginosa and Staphylococcus aureus. Br J Dermatol. 2007 Jun;156(6):1342-5. Epub 2007 Apr 25.
26. Elbe-Bürger A: Skin architecture and function. In: Handbook of Burns: Reconstruction and Rehabilitation; Eds: Kamolz L.P., Jeschke M.G., Horch R.E., Küntscher M., Brychta P.; Springer–Verlag, Wien, New York, pp 29-46, Vol 2, 2012.
27. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 2001 May 24;411(6836):494-8.
28. El Ghalbzouri A, Jonkman M, Kempenaar J, Ponec M. Recessive epidermolysis bullosa simplex phenotype reproduced in vitro: ablation of keratin 14 is partially compensated by keratin 17. Am J Pathol. 2003 Nov;163(5):1771-9.
29. Elias PM, Feingold KR.Lipids and the epidermal water barrier: metabolism, regulation, and pathophysiology.Semin Dermatol. 1992 Jun;11(2):176-82.
30. Elias PM, Williams ML, Holleran WM, Jiang YJ, Schmuth M.Pathogenesis of permeability barrier abnormalities in the ichthyoses: inherited disorders of lipid metabolism.J Lipid Res. 2008 Apr;49(4):697-714.
31. Elias, P. M. (2005). Stratum corneum defensive functions: an integrated view. J Invest Dermatol 125, 183-200.
32. 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
33. Fischer H, Eckhart L, Mildner M, Jaeger K, Buchberger M, Ghannadan M, Tschachler E. DNase1L2 degrades nuclear DNA during corneocyte formation. J Invest Dermatol. 2007 Jan;127(1):24-30.

34. Fischer H, Szabo S, Scherz J, Jaeger K, Rossiter H, Buchberger M, Ghannadan M, Hermann M, Theussl Essential role of the keratinocyte-specific endonuclease DNase1L2 in the removal of nuclear DNA from hair and nails. HC, Tobin DJ, Wagner EF, Tschachler E, Eckhart L. J Invest Dermatol. 2011 Jun;131(6):1208-15
35. Fuchs, E. (1990a). Epidermal differentiation.Curr Opin Cell Biol 2, 1028-1035.
36. Fuchs, E. (1990b). Epidermal differentiation: the bare essentials. J Cell Biol 111, 2807-2814.
37. Fuchs, E. (1993). Epidermal differentiation and keratin gene expression. J Cell Sci Suppl 17, 197-208.
38. Fuchs, E., and Green, H. (1980).Changes in keratin gene expression during terminal differentiation of the keratinocyte. Cell 19, 1033-1042.
39. Gingras M, Bergeron J, Déry J, Durham HD, Berthod F. In vitro development of a tissue-engineered model of peripheral nerve regeneration to study neurite growth. FASEB J. 2003 Nov;17(14):2124-6
40. Gingras M, Beaulieu MM, Gagnon V, Durham HD, Berthod F. In vitro study of axonal migration and myelination of motor neurons in a three-dimensional tissue-engineered model. Glia. 2008 Feb;56(3):354-64.
41. Haake, AR, Holbrook, K: The structure and development of skin: In: Freedberg IM, Eisen AZ, Wolff K, Austen KF, Goldsmith LA, Katz SI, Fitzpatrik, TB, eds. Dermatology in General Medicine. 1999 New York: McGraw-Hill, pp 70–114,
42. Harding CR, Scott IR (1983). Histidine-rich proteins (filaggrins): structural and functional heterogeneity during epidermal differentiation. J Mol Biol 170:651-73.
43. Hentzer, B., and Kobayasi, T. (1980). Adult human skin maintained in organ culture. II. The ultrastructure of the cellular compartment of connective tissue. Acta Derm Venereol 60, 465-476.
44. Herman IM, Leung A. Creation of human skin equivalents for the in vitro study of angiogenesis in wound healing. Methods Mol Biol. 2009;467:241-8
45. Hickerson RP, Flores MA, Leake D, Lara MF, Contag CH, Leachman SA, Kaspar RL. Use of self-delivery siRNAs to inhibit gene expression in an organotypic pachyonychia congenita model. J Invest Dermatol. 2011 May;131(5):1037-44. Epub 2011 Jan 20.

46. Horikoshi T, Igarashi S, Uchiwa H, Brysk H, Brysk MM. Role of endogenous cathepsin D-like and chymotrypsin-like proteolysis in human epidermal desquamation.Br J Dermatol. 1999 Sep;141(3):453-9.
47. Hoste E, Kemperman P, Devos M, Denecker G, Kezic S, Yau N, Gilbert B, Lippens S, De Groote P, Roelandt R, Van Damme P, Gevaert K, Presland RB, Takahara H, Puppels G, Caspers P, Vandenabeele P, Declercq W. Caspase-14 is required for filaggrin degradation to natural moisturizing factors in the skin. J Invest Dermatol. 2011 Nov;131(11):2233-41
48. Igarashi S, Takizawa T, Takizawa T, Yasuda Y, Uchiwa H, Hayashi S, Brysk H, Robinson JM, Yamamoto K, Brysk MM, Horikoshi T. Cathepsin D, but not cathepsin E, degrades desmosomes during epidermal desquamation. Br J Dermatol. 2004 Aug;151(2):355-61.
49. Jarrett, A, Spearman, RI: The keratin defect and hair-cycle of a new mutant (matted) in the house mouse. J Embryol Exp Morphol 1957 5: 103–110
50. Jin XL, Yagi M, Akiyama N, Hirosaki T, Higashi S, Lin CY et al. (2006). Matriptase activates stromelysin (MMP-3) and promotes tumor growth and angiogenesis. Cancer Science 97:1327-1334.
51. Kähäri VM, Saarialho-Kere U.Matrix metalloproteinases in skin.Exp Dermatol. 1997 Oct;6(5):199-213.
52. Kam E, Resing KA, Lim SK, Dale BA. J Cell Sci. 1993 Sep;106 (Pt 1):219-26. Identification of rat epidermal profilaggrin phosphatase as a member of the protein phosphatase 2A family.
53. Kamata Y, Taniguchi A, Yamamoto M, Nomura J, Ishihara K, Takahara H, Hibino T, Takeda A. Neutral cysteine protease bleomycin hydrolase is essential for the breakdown of deiminated filaggrin into amino acids. J Biol Chem. 2009 May 8;284(19):12829-36.
54. Kammeyer A, Eggelte TA, Bos JD et al. (1999) Urocanic acid isomers are good hydroxyl radical scavengers: a comparative study with structural analogues and with uric acid. Biochim Biophys Acta 1428:117–120
55. Kammeyer A, Eggelte TA, Overmars H et al. (2001) Oxidative breakdown and conversion of urocanic acid isomers by hydroxyl radical generating systems. Biochim Biophys Acta 1526:277–285

56. Kawane K, Fukuyama H, Kondoh G, Takeda J, Ohsawa Y, Uchiyama Y, Nagata S. Science. 2001 May 25;292(5521):1546-9. Requirement of DNase II for definitive erythropoiesis in the mouse fetal liver.

57. Keck M, Haluza D, Selig HF, Jahl M, Lumenta DB, Kamolz LP, Frey M. Adipose tissue engineering: three different approaches to seed preadipocytes on a collagen-elastin matrix. Ann Plast Surg. 2011 Nov;67(5):484-8.

58. Kezic S, Kemperman PM, Koster ES, de Jongh CM, Thio HB, Campbell LE, Irvine AD, McLean WH, Puppels GJ, Caspers PJ.Loss-of-function mutations in the filaggrin gene lead to reduced level of natural moisturizing factor in the stratum corneum. J Invest Dermatol. 2008 Aug;128(8):2117-9

59. Kezic S, O'Regan GM, Yau N, Sandilands A, Chen H, Campbell LE, Kroboth K, Watson R, Rowland M, McLean WH, Irvine AD. Levels of filaggrin degradation products are influenced by both filaggrin genotype and atopic dermatitis severity. Allergy. 2011 Jul;66(7):934-40.

60. Khatib AM, Bassi D, Siegfried G, Klein-Szanto AJ, Ouafik L.Endo/exo-proteolysis in neoplastic progression and metastasis.J Mol Med (Berl). 2005 Nov;83(11):856-64.

61. Kitano Y, Okada N. Separation of the epidermal sheet by dispase.Br J Dermatol. 1983 May;108(5):555-60.

62. Krieser RJ, MacLea KS, Longnecker DS, Fields JL, Fiering S, Eastman A. Deoxyribonuclease IIalpha is required during the phagocytic phase of apoptosis and its loss causes perinatal lethality. Cell Death Differ. 2002 Sep;9(9):956-62.

63. Kroemer G, Galluzzi L, Vandenabeele P et al. (2009) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 16:3–11

64. Lam WK, Cleary MA, Wraith JE et al. (1996) Histidinaemia: a benign metabolic disorder. Arch Dis Child 74:343–346 | Article | PubMed | ChemPort |

65. Lena AM, Shalom-Feuerstein R, Rivetti di Val Cervo P, Aberdam D, Knight RA, Melino G, Candi E.miR-203 represses 'stemness' by repressing DeltaNp63. Cell Death Differ. 2008 Jul;15(7):1187-95. Epub 2008 May 16.

66. Lee SL, Dickson RB, Lin CY (2000). Activation of hepatocyte growth factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease. Journal of Biological Chemistry 275: 36720-36725.
67. Lonsdale-Eccles JD, Teller DC, Dale BA.Biochemistry. 1982 Nov 9;21(23):5940-8. Characterization of a phosphorylated form of the intermediate filament-aggregating protein filaggrin.
68. Li L, Fukunaga-Kalabis M, Herlyn M. The three-dimensional human skin reconstruct model: a tool to study normal skin and melanoma progression. J Vis Exp. 2011 Aug 3;(54).
69. Lin X, Paskaleva EE, Chang W, Shekhtman A, Canki M. Inhibition of HIV-1 infection in ex vivo cervical tissue model of human vagina by palmitic acid; implications for a microbicide development. PLoS One. 2011;6(9):e24803.
70. Lippens S, Denecker G, Ovaere P et al. (2005) Death penalty for keratinocytes: apoptosis versus cornification. Cell Death Differ 12:1497–1508
71. List K, Haudenschild CC, Szabo R, Chen WJ, Wahl SM, Swaim W et al. (2002). Matriptase/MT-SP1 is required for postnatal survival, epidermal barrier function, hair follicle development, and thymic homeostasis. Oncogene 21: 3765-3779.
72. List K, Szabo R, Wertz PW, Segre J, Haudenschild CC, Kim SY et al. (2003). Loss of proteolytically processed filaggrin caused by epidermal deletion of matriptase/MT-SP1. Journal of Cell Biology 163: 901-910.
73. Liu W, Spero DM.Cysteine protease cathepsin S as a key step in antigen presentation.Drug News Perspect. 2004 Jul-Aug;17(6):357-63.
74. Lukas M, Stössel H, Hefel L, Imamura S, Fritsch P, Sepp NT, Schuler G, Romani N. Human cutaneous dendritic cells migrate through dermal lymphatic vessels in a skin organ culture model. J Invest Dermatol. 1996 Jun;106(6):1293-9.
75. Martin P.Wound healing--aiming for perfect skin regeneration.Science. 1997 Apr 4;276(5309):75-81.
76. McCall CA, Cohen JJ (1991) Programmed cell death in terminally differentiating keratinocytes: role of endogenous endonuclease. J Invest Dermatol 97:111–114

77. McDonald JA., Annu Rev Cell Biol. 1988;4:183-207. Extracellular matrix assembly.
78. McGrath J.A., Eady R.A., Pope F.M. (2004). Rook's Textbook of Dermatology (7th ed.). Blackwell Publishing. pp. 3.1–3.6. ISBN 9780632064298.
79. McGrath JA, Uitto J (2008). The filaggrin story: novel insights into skin-barrier function and disease. Trends Mol Med 14:20-7.
80. Méchin MC, Sebbag M, Arnaud J, Nachat R, Foulquier C, Adoue V, Coudane F, Duplan H, Schmitt AM, Chavanas S, Guerrin M, Serre G, Simon M. Update on peptidylarginine deiminases and deimination in skin physiology and severe human diseases. Int J Cosmet Sci. 2007 Jun;29(3):147-68.
81. Meloni M, De Servi B, Marasco D, Del Prete S. Molecular mechanism of ocular surface damage: application to an in vitro dry eye model on human corneal epithelium. Mol Vis. 2011 Jan 12;17:113-26.
82. Meyer-Hoffert U.Reddish, scaly, and itchy: how proteases and their inhibitors contribute to inflammatory skin diseases.Arch Immunol Ther Exp (Warsz). 2009 Sep-Oct;57(5):345-54.
83. Mehrel, T., Hohl, D., Rothnagel, J. A., Longley, M. A., Bundman, D., Cheng, C., Lichti, U., Bisher, M. E., Steven, A. C., Steinert, P. M., and et al. (1990). Identification of a major keratinocyte cell envelope protein, loricrin. Cell 61, 1103-1112.
84. Morley S. M., Lane E. B. (1994) The keratinocyte cytoskeleton. The Keratinocyte Handbook.(Leigh I.M.; Lane E.B.; Watt F.M.; Eds.) Cambridge: Cambridge University Press; 293-322.
85. Nagata S. DNA degradation in development and programmed cell death.Annu Rev Immunol. 2005;23:853-75.
86. Netzel-Arnett S, Currie BM, Szabo R, Lin CY, Chen LM, Chai KX, Antalis TM, Bugge TH, List K. Evidence for a matriptase-prostasin proteolytic cascade regulating terminal epidermal differentiation. J Biol Chem. 2006 Nov 3;281(44):32941-5
87. Nolte SV, Xu W, Rennekampff HO, Rodemann HP. Diversity of fibroblasts--a review on implications for skin tissue engineering. Cells Tissues Organs. 2008;187(3):165-76.

88. Norlen, L. (2001) Skin barrier formation: the membrane folding model. J. Investig. Dermatol. 117, 823– 829
89. Oberst MD, Williams CA, Dickson RB, Johnson MD, Lin CY.The activation of matriptase requires its noncatalytic domains, serine protease domain, and its cognate inhibitor.J Biol Chem. 2003 Jul 18;278(29):26773-9.
90. O'Guin, W. M., Galvin, S., Schermer, A., and Sun, T. T. (1987). Patterns of keratin expression define distinct pathways of epithelial development and differentiation. Curr Top Dev Biol 22, 97-125.
91. Ovaere P, Lippens S, Vandenabeele P et al. (2009) The emerging roles of serine protease cascades in the epidermis. Trends Biochem Sci 34:453–63
92. 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.
93. Pierard GE, Goffin V, Hermanns-Le T, Pierard-Franchimont C., Int J Mol Med. 2000 Aug;6(2):217-21. Corneocyte desquamation.
94. Ponec M, Weerheim A, Kempenaar J, Mulder A, Gooris GS, Bouwstra J, Mommaas AM. The formation of competent barrier lipids in reconstructed human epidermis requires the presence of vitamin C. J Invest Dermatol. 1997 Sep;109(3):348-55.
95. Presland RB, Boggess D, Lewis SP, Hull C, Fleckman P, Sundberg JP. Loss of normal profilaggrin and filaggrin in flaky tail (ft/ft) mice: an animal model for the filaggrin-deficient skin disease ichthyosis vulgaris. J Invest Dermatol. 2000 Dec;115(6):1072-81.
96. Proksch E, Brandner JM, Jensen JM. (2008).The skin: an indispensable barrier. Exp Dermatol. 17(12):1063-72
97. Proksch E, Jensen JM, Elias PM.Skin lipids and epidermal differentiation in atopic dermatitis. Clin Dermatol. 2003 Mar-Apr;21(2):134-44
98. Rawlings AV, Harding CR.Dermatol Ther. 2004;17 Suppl 1:43-8.Moisturization and skin barrier function.
99. Rendl M, Ban J, Mrass P, Mayer C, Lengauer B, Eckhart L, Declerq W, Tschachler E. Caspase-14 expression by epidermal keratinocytes is regulated by retinoids in a differentiation-associated manner. J Invest Dermatol. 2002 Nov;119(5):1150-5.

100. Resing KA, Dale BA, Walsh KA. Multiple copies of phosphorylated filaggrin in epidermal profilaggrin demonstrated by analysis of tryptic peptides. Biochemistry. 1985 Jul 16;24(15):4167-75.
101. Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell. 1975 Nov;6(3):331-43.
102. Rheinwald JG, Green H. Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. Nature.1977 Feb 3;265(5593):421-4.
103. Stoitzner P, Zanella M, Ortner U, Lukas M, Tagwerker A, Janke K, Lutz MB, Schuler G, Echtenacher B, Ryffel B, Koch F, Romani N. Migration of langerhans cells and dermal dendritic cells in skin organ cultures: augmentation by TNF-alpha and IL-1beta. J Leukoc Biol. 1999 Sep;66(3):462-70.
104. Rothnagel JA, Steinert PM (1990) The structure of the gene for mouse filaggrinand a comparison of the repeating units. J Biol Chem 265:1862–5
105. 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
106. 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 39:650-54.
107. Santoianni P, Rothman S (1961) Nucleic acid-splitting enzymes in human epidermis and their possible role in keratinization. J Invest Dermatol 37: 489–495.
108. Sales KU, Masedunskas A, Bey AL, Rasmussen AL, Weigert R, List K, Szabo R, Overbeek PA, Bugge TH.Matriptase initiates activation of epidermal pro-kallikrein and disease onset in a mouse model of Netherton syndrome. Nat Genet. 2010 Aug;42(8):676-83.
109. 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.
110. Schleh C, Rothen-Rutishauser BM, Blank F, Lauenstein HD, Nassimi M, Krug N, Braun A, Erpenbeck VJ, Gehr P, Hohlfeld JM. Surfactant Protein D

modulates allergen particle uptake and inflammatory response in a human epithelial airway model. Respir Res. 2012 Feb 1;13(1):8

111. Schmidt RF, Thews G, Lang F (Hrsg.): Physiologie des Menschen. Springer, Berlin. 2000. 28. Auflage. ISBN 3-540-66733-4
112. Searle, AG, Spearman, RI: "Matted", a new hair-mutant in the house-mouse: genetics and morphology. J Embryol Exp Morphol 1957 5: 93–102
113. Simon, M., and Green, H. (1984).Participation of membrane-associated proteins in the formation of the cross-linked envelope of the keratinocyte. Cell 36, 827-834.
114. 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.
115. Steinert PM, Cantieri JS, Teller DC, Lonsdale-Eccles JD, Dale BA.Characterization of a class of cationic proteins that specifically interact with intermediate filaments.Proc Natl Acad Sci U S A. 1981 Jul;78(7):4097-101.
116. Tabachnik J (1957) Urocanic acid, the major acid soluble UV absorbing compound in guinea pig epidermis. Arch Biochem Biophys 70:295–297
117. Takeuchi T, Harris JL, Huang W, Yan KW, Coughlin SR, Craik CS (2000). Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. Journal of Biological Chemistry 275: 26333-26342.
118. Tarcsa E, Marekov LN, Mei G, Melino G, Lee SC, Steinert PM. J Biol Chem. 1996 Nov 29;271(48):30709-16. Protein unfolding by peptidylarginine deiminase.Substrate specificity and structural relationships of the natural substrates trichohyalin and filaggrin.
119. Ulukaya E, Acilan C, Yilmaz Y.Cell Biochem Funct. 2011 Aug;29(6):468-80. doi: 10.1002/cbf.1774. Epub 2011 Jul 19.Apoptosis: why and how does it occur in biology?
120. Van Gele M, Geusens B, Speeckaert R, Dynoodt P, Vanhoecke B, Van Den Bossche K, Lambert J. Development of a 3D pigmented skin model to evaluate RNAi-induced depigmentation. Exp Dermatol. 2011 Sep;20(9):773-5.
121. Watt, E M. (1989) Terminal differentiation of epidermal keratinocytes.Curr.Opin. Cell Biol. 1, 1107-1115

122. Weiss, L. W., and Zelickson, A. S. (1975). Embryology of the epidermis: ultrastructural aspects. II. Period of differentiation in the mouse with mammalian comparisons. Acta Derm Venereol 55, 321-329.
123. Wertz PW. Epidermal lipids.Semin Dermatol. 1992 Jun;11(2):106-13.
124. Yi R, O'Carroll D, Pasolli HA, Zhang Z, Dietrich FS, Tarakhovsky A, Fuchs E. Morphogenesis in skin is governed by discrete sets of differentially expressed microRNAs. Nat Genet. 2006 Mar;38(3):356-62. Epub 2006 Feb 5.
125. Yoshida H, Okabe Y, Kawane K, Fukuyama H, Nagata S. Lethal anemia caused by interferon-beta produced in mouse embryos carrying undigested DNA. Nat Immunol. 2005 Jan;6(1):49-56. Epub 2004 Nov 28.
126. Zenisek A, Kral JA, Hais IM (1955) Sun-screening effect of urocanic acid. Biochim Biophys Acta 18:589–591
126. Zhang D, Karunaratne S, Kessler M et al. (2002) Characterization of mouse profilaggrin: evidence for nuclear engulfment and translocation of the profilaggrin B-domain during epidermal differentiation. J Invest Dermatol 119:905–12
127. Zeeuwen PL. Epidermal differentiation: the role of proteases and their inhibitors. Eur J Cell Biol. 2004 Dec;83(11-12):761-73.