CHAPTER 10

SUMMARY AND DISCUSSION

The epidermis forms the organism's outermost barrier to the environment and protects the body from dehydration, from injuries due to chemical and physicial stress and the invasion of pathogens (McGrath et al., 2004). Normal cornification and development of the epidermal barrier is essential for terrestrial life and alterations of these processes may results in skin diseases or even death. Elucidation of mechanisms underlying epidermal barrier formation is therefore an area of great interest for medical research. Establishment and refinement of both *in vivo* and *in vitro* experimental systems over the past two decades have largely contributed to our understanding of this process and has resulted in a better knowledge of the pathomechanisms of various skin diseases (Auxenfans et al., 2009; Hutton et al., 2008; Hickerson et al., 2011; Commandeur et al., 2009; El Ghalbzouri et al., 2003).

The aim of this thesis was to develop a robust and reproducible human *in vitro* skin model, suited to silence individual genes and to study their impact on the epidermal differentiation process. This approach was also chosen with the perspective to establish a complement and possibly an alternative to current animal models. Our approach has allowed us to provide new insights into the roles of several differentiation-associated gene products in epidermal barrier formation.

In particular we studied the functions of matriptase-1 (Chapter 3 and 4), DNase1L2 (Chapter 5), DNase 2 (Chapter 6), filaggrin and its degradation products (Chapter 7), as well as histidase (Chapter 8) in epidermal barrier formation. We showed that loss of matriptase-1 is associated with features of defective barriers i.e. parakeratosis, found in skin disorders such as psoriasis. We demonstrated that human DNase1L2 plays a role in degradation of nuclei in stratum corneum and, furthermore that the silencing of the filaggrin gene leads to skin barrier defects independently of the presence of inflammatory cells. We extended our study from the skin to investigate an organotypic vaginal epithelial model, in which we studied innate antimicrobial defense mechanisms (Chapter 9). The results of these studies were published in peer-reviewed scientific journals.

10.1. The organotypic knock-down skin model as an alternative to animal experiments

Organotypic skin models are used in dermatological research since decades (Auxenfans et al., 2009). They can easily be generated, are highly reproducible and reflect many features of human skin *in vivo*. Therefore, these models became a standard technique in dermatological research, especially for investigations on mechanisms and molecules involved in terminal KC differentiation and barrier formation. One of our major goals was the development of a human knock-down skin model, which would also allow studies on gene deletions in an *in vitro* model.

In Chapter 3 we analyzed knock-down skin cultures of two genes which have been investigated in knock-out mouse models before. We have chosen knock-downs for VEGF, which showed no epidermal phenotype in the mouse (Rossiter et al., 2004), and matriptase-1 (List et al., 2002; List et al., 2003), which showed a severe phenotype in the mouse model. When we compared the *in vitro* organotypic knock-down skin with the knock-out mouse models, we found many similarities i.e. no alterations in the epidermal development by inhibiting VEGF expression and defects in KC differentiation and stratum corneum formation by the inhibition of matriptase-1 expression. Furthermore, also biological mechanisms, such as the lack of filaggrin processing in matriptase-1 deficient mice, were detectable in the *in vitro* model. These findings suggest that the *in vitro* organotypic knock-down skin model is a powerful new method for the investigation of genes and their contribution to epidermal development and formation of a functional barrier.

Compared to transgenic animal models, the *in vitro* organotypic knock-down skin model has several advantages as it i) is much less time consuming, ii) allows the screening for many different genes within a short time period, as the establishment of an animal model takes at least several months with uncertain outcome, iii) is less expensive, iv) allows investigations on the direct involvement of target genes in KC-differentiation, without the influence of any other cell type, and v) is a human model. Especially the mouse skin differs from human skin and many genes have different functions in these two species (Harding and Scott, 1983). However, in an experimental setting, where interaction of KC with other cells is requested, our model

is limited. The addition of single epidermal cell types like Langerhans cells or melanocytes has been shown to be feasible, even in knock-down models (Van Gele et al., 2011; Li et al., 2011; Auxenfans et al., 2009). More complex systems are very difficult to generate and the outcome of such models is questionable. Nevertheless, over the last years, the organotypic knock-down skin model has become a standard method, especially for investigations on epidermal differentiation and barrier formation. In addition, this alternative technique strongly reduces the necessity of animal experiments in dermatological research for certain questions.

10.2. Matriptase-1 and its contribution to epidermal barrier formation in healthy and diseased skin

Our studies on matriptase-1 knock-down in the organotypic skin model confirmed for the first time its role in stratum corneum formation in the human system (Chapter 3). Comparable to what has been observed in the knock-out mouse, matriptase-1 knock-down in our *in vitro* model led to hyperkeratosis, which was comparable to what is observed in certain forms of ichthyosis. In the meantime it had been demonstrated that loss of matriptase-1 function appears to be a central event in the pathogenesis of autosomal recessive Ichthyosis with hypotrychosis (Basel-Vanagaite et al., 2007; Avrahami et al., 2008; Alef et al., 2009), a rare skin disorder which is characterized by thickened, grayish, scaly skin and slow growing curly, sparse and fragile hair. In contrast to the phenotype of the mouse model, we also found strong parakeratosis, characterized by the retention of nuclei in the stratum corneum. Since parakeratosis is a hallmark of the histological picture of psoriasis we investigated matriptase-1 expression in psoriatic lesions, and found an almost complete loss of its expression and activity in lesional skin only (Chapter 3). In contrast to autosomal recessive Ichthyosis with hypotrychosis where a mutation of the gene coding for matriptase-1 is causative, the central pathogenic event in psoriasis is thought to be the deregulation of inflammatory cytokines, in particular a strong up-regulation of TNFα production (de Kerkhof, 2007; Lowes et al., 2007). When we stimulated KC with TNFα, we indeed found a strong reduction of matriptase-1 expression, and moreover could demonstrate that the IKK2/NFκB pathway is crucial for this process. In addition,

treatment of organotypic skin cultures with TNFα led to hyper- and parakeratosis which was accompanied with a complete loss of matriptase-1 expression. Together, these studies unraveled the importance of matriptase-1 for proper KC differentiation and the formation of a functional epidermal barrier in humans. In addition, they suggest a contribution of this serine protease in the pathogenesis of inflammatory skin diseases such as psoriasis as consequence of its modulation by inflammatory cytokines.

10.3. The function and importance of DNases in the epidermis

An essential step in skin barrier formation is the degradation of nuclei and nuclear DNA. However, the exact mechanisms underlying this process, as well as the enzymes involved, have not been investigated in detail before. Therefore, we tried to unravel the contribution of DNases to KC differentiation in KC in monolayer cultures, organotypic skin cultures as well as in mouse models. By analyzing the transcription levels of several DNases we found that DNase1L2 was the only known DNAse which was strongly up-regulated in the late steps of KC differentiation. DNase activity assays of stratum corneum samples mainly identified activities displaying DNase 2 like properties. Thus, we analyzed these two DNases and their contribution to stratum corneum formation in more detail. Knock-down of DNase 2 in our organotypic skin model did not lead to alterations in epidermal development but strong reduction in DNase activity of the stratum corneum. By contrast, knock-down of DNase1L2 resulted in retention of nuclei in the stratum corneum, suggesting that DNase1L2 plays a key role in the breakdown of nuclear DNA during KC differentiation. In addition, DNase1L2 expression was also strongly down-regulated in skin diseases with parakeratosis, such as psoriasis and Bowen´s disease. In contrast to what we observed in the human organotypic culture system DNase1L2 deficient mice, which were created in our laboratory (Fischer et al., 2011), had a normal stratum corneum and did not show parakeratosis of the interfollicular epidermis. However, nuclear remnants were abundantly present in hair and nails, the scales on the tail as well as on the surface of the tongue and the esophagus. This finding was surprising, however, immune-staining of mouse skin with DNase1L2 antibodies showed

dramatically reduced expression levels as compared to human skin, suggesting a different role of this enzyme in mouse skin compared to human skin. In addition, generation of an organotypic skin culture, with KC derived from the DNase1L2 deficient mice, showed no parakeratosis, excluding an *in vitro* artifact. Our results demonstrate that important differences exist between mouse skin and human skin also in basic processes such as KC differentiation and underline the importance of high quality human model systems as a complement to animal models.

10.4. Novel insights in the function of filaggrin

Many studies on the role of filaggrin in skin barrier function have used the “flaky tail” mouse, a spontaneous recessive mouse mutant which arose in 1958 on the background of an existing recessive hair phenotype and is characterized by dry and flaky skin and neonatal abnormalities in the tail and paws (Presland et al., 2000). These mice express a truncated profilaggrin that cannot be further processed into filaggrin monomers (Presland et al., 2000) and the epidermal barrier function is impaired (Fallon et al., 2009; Scharschmidt et al., 2009). However, considering the species-specific properties of filaggrin (Harding and Scott, 1983), caution should be taken when interpreting and comparing the function of filaggrin in animal models with that of human filaggrin. In addition these mice contain an additional mutation (Chapter 1.3.3.) which could play a confounding role and which makes data difficult to interpret. Furthermore, since impairment of the skin barrier leads to inflammation which in turn can affect KC differentiation, it is difficult in an *in vivo* model to delineate the cell autonomous effects of the filaggrin mutation from the effects secondary to inflammation. We therefore established a filaggrin deficient human *in vitro* organotypic skin model to analyze the direct contribution of this protein to epidermal development and barrier function (Chapter 7). Morphologically, the filaggrin knock-down model is characterized by a reduced number and size of keratohyalin granules, which function as a storage place for filaggrin. Whereas stratum corneum formation seems to be normal, the formation and extrusion of lamellar bodies is disturbed, suggesting a defect in the formation of the lamellar lipid bilayer. Analysis of the total lipid content in our model reveals no change after filaggrin knock-down. This finding

is in accordance with two recent studies, showing an impaired extracellular lipid organization in filaggrin deficiency (Scharschmidt et al., 2009; Gruber et al., 2011). It is still unclear how filaggrin impairs lamellar bodies and the lipid organization in the stratum corneum.

Studies involving keratins and filaggrin purified from skin suggested that filaggrin can interact with different types of intermediate filaments thereby leading to their aggregation into macrofibrils (Dale et al., 1978). In addition, transient over-expression of human filaggrin *in vitro* results in the collapse of the intermediate filament-network (Dale et al., 1997; Presland et al., 2001). When we analyzed our knock-down model, we found that keratin aggregation is still taking place, even in the absence of filaggrin. Similarly, in Ichthyosis vulgaris patients with filaggrin null mutations keratin aggregation was also found, suggesting that in the human system filaggrin is dispensable for keratin aggregation (Sybert et al., 1985; Weidenthaler et al., 1993).

When studying skin barrier function in humans suffering from atopic dermatitis or in flaky tail mice it is difficult to rule out confounding factors such as the impact of inflammatory cytokines on barrier function. Our finding that the hydrophilic dye Lucifer yellow was able to penetrate the stratum corneum in the filaggrin deficient organotypic skin suggests that filaggrin deficiency alone is sufficient to impair the function of the epidermal barrier and therefore represents a direct link between these two situations. Possible mechanisms involved in the dramatic effect on the barrier function could be the impaired secretion of lamellar bodies as well as altered stratum corneum extracellular membranes (Scharschmidt et al., 2009; Yoneda et al., 2012). However, further investigations are necessary to identify the mechanism by which filaggrin deficiency leads to impaired lamellar body formation and barrier function.

In the stratum corneum, filaggrin is degraded into amino acids and other components of the natural moisturizing factors and thereby contributes to the retention of water within corneocytes (McGrath and Uitto, 2008). One important degradation product of filaggrin is UCA. UCA is derived from the amino acid histidine, which is converted by the enzyme histidase (Chapter 6). Our studies on histidase showed that this enzyme is also abundantly expressed in the granular layer of the skin, and its expression in monolayer KC cultures is strongly induced by differentiation. When we analyzed UCA

and histidase in our filaggrin knock-down skin samples, we found a massive decrease of UCA production, whereas histidase was not regulated, suggesting a reduction of histamine due to the lack of filaggrin. Using the Peruvian mouse model, we could previously show that lack of histidase, leading to reduced UCA levels, strongly increased the UV-sensitivity after UVB-irradiation (Barresi et al., 2011). Similarly, when we irradiated the organotypic skin model after filaggrin knock down we observed increased UV-sensitivity most likely as a consequence of the absence of UCA.

Together our studies on filaggrin knock-down in an organotypic skin culture, led to the discovery of new functions of filaggrin in epidermal barrier formation. We found that i) filaggrin is dispensable for keratin aggregation, ii) reduced filaggrin expression alters lamellar body production and extrusion, iii) filaggrin knock-down leads to strongly reduced UCA levels and increased photosensitivity in filaggrin deficient skin samples and iv) lack of filaggrin expression alone is sufficient to impair the epidermal diffusion barrier function.

10.5. The use of other models to study epithelial mechanisms

Epithelial *in vitro* models have already been described for many different organs, such as lung, eye and vagina (Ayehunie et al., 2006; Lin et al., 2011; Meloni et al., 2011; Schleh et al., 2012). Although the organotypic skin culture is still the most prominent model system, more and more research in the field of pulmonology, ophthalmology or gynecology fall back on such model systems. From a general point of view it might be very interesting to identify common signaling pathways and mechanisms in epithelial tissues comparing different epithelial *in vitro* models. Therefore, we investigated in our study (Chapter 9) the production and action of the anti-microbial peptide psoriasin (S100A7) in a vaginal epithelial model. From our previous studies on psoriasin (Abtin et al., 2008), and studies form others (Gläser et al., 2005), it had become apparent that this protein is one of the major *E.coli*-cidal factors in the skin, both *in vivo* and *in vitro*. We could demonstrate for the first time that S100A7 is by far the most abundant *E.coli*-cidal factor in vaginal fluids *in vitro*, and that it is strongly expressed in the vaginal epithelial model. The establishment of

an *in vitro* knock-down vaginal epithelial model will be one of our major goals in future studies. Such models would extend the scope of possibilities to study epithelial components of tissue homeostasis in the genital tract.

CONCLUDING REMARKS

Formation of the epidermal barrier is a complex process involving the tight regulation of the expression of structural proteins, enzymes and cytokines, implementing different signaling pathways depending on the differentiation stage of KC. Alterations of skin barrier formation occur as consequence of many skin diseases but can also be the primary event leading to skin diseases. Organotypic skin models have greatly contributed to delineate molecular mechanisms underlying the epidermal differentiation process. In this thesis we have adapted a human organotypic skin model to knock-down individual genes and study their impact on the differentiation of epidermal KC *in vitro*. Using this model we have provided new insights in the formation of the skin barrier of human skin.

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