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**Author:** Thakoersing, Varsha Sakina  
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SUMMARY AND PERSPECTIVES
SUMMARY

The skin forms an effective permeability barrier against penetration of foreign substances into the body and excessive water loss from the body. To generate this barrier the keratinocytes in the basal layer of the epidermis undergo a complex maturation process that finally results in the formation of the stratum corneum (SC), the outermost layer of the epidermis. The SC is the main permeability barrier of the skin and consists of terminally differentiated dead keratinocytes, called corneocytes, which are embedded in a lipid-rich extracellular environment. Due to the rather impermeable character of the cornified envelope surrounding the corneocytes, the main penetration pathway of most substances is suggested to proceed via the SC lipid domains. This is the reason why the SC lipids are considered to play a crucial role in the barrier function of the skin. The SC lipids are primarily composed of cholesterol, free fatty acids and ceramides in an approximately equimolar ratio. The lipids are organized in lipid layers (referred to as lamellae) stacked on top of each other approximately parallel to the skin surface. In human SC two lamellar phases are formed with repeat distances of approximately 13 and 6 nm, referred to as the long and short periodicity phase (LPP and SPP), respectively. The LPP is characteristic for the SC lipid organization and is considered to be important for a competent skin barrier function. The lipids within the lamellae are predominantly arranged in the dense orthorhombic packing, while a subpopulation of lipids also forms the less dense hexagonal or liquid organization. In order to determine whether active compounds hold potential for (trans)dermal application, their penetration through the skin has to be investigated. Ex vivo human or animal skin has extensively been used for this purpose. However, the use of human skin is limited due to its scarce availability. Furthermore, animal skin shows differences in e.g. dermal and epidermal thickness, hair density and SC lipid composition and therefore in skin barrier function. Additionally, from March 2013 a complete ban on animal testing of cosmetic products and their ingredients will be adopted within the EU. This
means that there is an urgent need for a suitable replacement for human or animal skin, which may be provided by three-dimensional human skin equivalents (HSEs). When HSEs were initially developed, keratinocytes were seeded in culture vessels and grown under submerged conditions. This resulted in an incomplete differentiation of the keratinocytes and consequently an epithelium that did not fully resemble native human skin. A major step forward in the development of HSEs was achieved by generating HSEs at the air-liquid interface. Due to this and many other modifications of the culture conditions HSEs nowadays mimic many aspects of native human skin and have improved SC barrier properties. They show great resemblance in tissue morphology and have an almost similar expression of early and late differentiation markers as native human skin. Furthermore, the SC barrier lipids of native human skin are also detected in the SC of HSEs.

It is more than a decade ago that the SC lipid composition and organization of HSEs were reported extensively. The published reports demonstrated that the SC lipid composition and organization of HSEs differed to some extent from native human SC. Due to the differences in SC lipid properties, HSEs have a decreased SC barrier function compared to native human SC. They generally overestimate compound penetration and therefore do not provide a reliable in vitro - in vivo correlation with regard to permeation studies. Additionally, the desquamation process, which regulates the shedding of the superficial SC layers, is impaired in HSEs. This leads to gradual thickening of the SC as the culture period is prolonged, which may also influence the outcome of permeation studies. This indicates that HSEs can be improved further to achieve an even higher resemblance to native human skin. During the last few years the culture conditions have been further optimized and two novel in-house HSEs have been developed in Leiden. The novel fibroblast-derived matrix model (FDM) and Leiden epidermal model (LEM) were generated together with the full thickness collagen model.
Chapter 8

(FTM). These models were used to answer the main questions of this thesis, namely:
1. Can fully differentiated HSEs be generated under submerged conditions and how does this affect the epidermal differentiation and lipid composition?
2. Do the SC barrier properties of our novel in-house HSEs resemble the SC barrier properties of native human skin?
3. How does the SC lipid composition of the in-house HSEs relate to the SC lipid organization?
4. To what extent do the culture conditions influence the SC barrier properties of HSEs?
5. How can the SC barrier properties of HSEs be improved?

Submerged versus air-exposed HSEs

In *chapter 2* studies are reported in which HSEs were generated under submerged and air-exposed conditions. The aim of the study was to determine whether the difference in micro-environment during keratinocyte differentiation affects skin architecture of HSEs. HSEs generated under submerged conditions have a similar tissue morphology, expression of several differentiation markers and show the presence of all SC barrier lipids as their air-exposed counterparts. However, they contain less fatty acids and one of the ceramide subclasses with a very long fatty acid chain (referred to as acylceramides) than the air-exposed HSEs. Additionally, the results demonstrate that the SC of FTMs generated under air-exposed conditions contain more natural moisturizing factors than FTMs generated under submerged conditions, but nevertheless has a reduced SC hydration level. The results presented in *chapter 2* show for the first time that HSEs can form a well-organized epidermis, including a SC, when generated under submerged conditions.
**SC barrier properties of the novel HSEs**

In *chapter 3* the SC barrier function, SC lipid composition and organization of FDM, LEM and FTM are presented. The HSEs have a similar morphology and expression of several early and late differentiation markers as native human skin, except for the observed expression of keratin 16 and premature expression of involucrin. To compare the SC barrier function of FDM, LEM, FTM and native human skin permeation studies, using benzocaine as a model drug, were performed. The SC of FDM and LEM showed a three times higher permeability, while the SC of FTM showed a five time higher permeability for benzocaine compared to native human SC. These results indicate that all HSEs have a decreased barrier function compared to native human SC. Therefore, the SC lipid organization and composition of the HSEs was investigated. All HSEs showed the presence of the LPP, which is considered to be important for a competent skin barrier. However, no indication of the presence of the SPP was observed. Furthermore, all HSEs have a mainly hexagonal arrangement of the SC lipids, a less dense packing than the orthorhombic packing observed in human SC. Additionally, the HSEs have an increased lipid disordering and form a liquid phase at a lower temperature compared to native human SC. All HSEs showed the presence of cholesterol, free fatty acids and eight ceramide subclasses that can be distinguished using high performance thin layer chromatography (HPTLC), similarly as human SC. Although the lipid composition was not determined in a quantitative manner, the results strongly indicate that the HSEs have a reduced free fatty acid content and an increased level of some acylceramides compared to human SC.

**The effect of culture conditions on the SC barrier properties of HSEs**

After establishing that the HSEs show differences in their SC barrier properties compared to native human SC, we examined whether these differences are caused by culture conditions or by the isolation procedure used to obtain single cells. The
latter involves enzymatic digestion of native human skin and is required to generate HSEs. The experiments conducted to answer this question are described in chapter 4. Intact full-thickness native human skin explants and epidermal sheets were expanded on fibroblast-populated collagen matrices. The morphology, differentiation process and barrier properties of the explants and outgrowths were examined. The outgrowths from the skin explants have a similar morphology and expression of several differentiation markers, except for an increased expression of involucrin and observed expression of keratin 16, as native human skin. The presence of keratin 16 indicates that the keratinocytes in the outgrowths are activated. Interestingly, the SC of the explants increased during the culture period, exceeding the number of SC layers found in vivo. This indicates that the desquamation process is impaired when native human skin is cultured in vitro. HPTLC results show that the SC lipid profiles of the outgrowths are very similar to that of HSEs. They also have a reduced free fatty acid content in their SC compared to native human skin. All ceramide classes are present in the outgrowths of these cultures, but an increase in the very long chain acylceramides is observed compared to native human SC. The SC lipid organization of the outgrowths is also similar to that of HSEs, which includes a predominant presence of the LPP and a mainly hexagonal lateral lipid organization. In addition, there is an indication of an increased presence of a liquid phase. This unambiguously demonstrates that the observed differences in SC barrier properties between the HSEs and native human skin are not caused by isolation of primary cells, but by the culture conditions. Efforts made to improve the SC lipid properties of HSEs should therefore be focused on optimizing the culture conditions.

The correlation between the SC lipid composition and SC lipid organization of HSEs

In the studies described in chapter 3, the SC lipid composition of HSEs was examined. In these studies the lipid profiles of the HSEs showed some changes
compared to native human skin. In order to provide more insight in these differences, quantitative analysis of the SC lipid composition was performed using HPTLC. As no information was obtained concerning the chain lengths of the ceramides and free fatty acids, a novel liquid chromatography/mass spectroscopy (LC/MS) method was additionally used to further analyze the SC lipids of the HSEs. These experiments are described in chapter 5. Quantification of the SC lipids showed that all HSEs have a reduced free fatty acid content compared to human SC. Additionally, LC/MS analysis revealed that all HSEs have an increased presence of mono-unsaturated fatty acids (MUFAs) compared to human SC. Fatty acids induce the orthorhombic packing and their reduced level and altered composition are expected to be the key factors for the presence of the mainly hexagonal packing observed in the HSEs. Therefore these two changes in fatty acid profile may play an important role in the decreased permeability barrier observed for FDM, LEM and FTM. Additionally, the increased presence of MUFAs are at least partially responsible for the increased lipid disordering of the SC lipids of HSEs and the reduction in temperature at which the formation of the liquid phase occurs.

With respect to the ceramides, the LC/MS results demonstrated for the first time that the HSEs show the presence of twelve ceramide subclasses in their SC, similar to native human SC. However, the HSEs have increased levels of two ceramide subclasses with a very long fatty acid chain and ceramide species with an exceptionally short total carbon chain length. In addition, low fractions of ceramides with a mono-unsaturated acyl chain were detected. The latter ceramide species are not detected in human SC. Previous studies have shown that acylceramides induce the formation of the LPP. The increased presence of the two acylceramide subclasses in the SC of the HSEs may therefore favour the formation of the LPP over the SPP.

All HSEs also show the expression of stearoyl-CoA desaturase 1 (SCD1), the enzyme that converts saturated fatty acids into MUFAs, in the basal and
differentiated layers of the viable epidermis. In native human skin, however, the expression of SCD1 is strictly localized in the basal layer. This suggests that the HSEs may have an increased MUFA content in their SC due to increased activity of SCD1 in the suprabasal layers of the viable epidermis. The results described in chapter 5 indicate that the SC lipid properties of the HSEs can be improved by increasing the free fatty acid content in the SC and by reducing the SCD1 activity to decrease the amount of MUFAs in the SC.

**Examining ways to improve the SC barrier properties of LEMs**

After establishing that the SC barrier properties of HSEs may be improved by increasing the free fatty acid content and by reducing the MUFA content, we aimed to improve the SC lipid properties of LEM by specific media supplements. These studies are described in chapter 6. The SC fatty acid chain length distribution and saturation of LEM and human skin were quantified with LC/MS to determine to which extent they differ from each other. The results demonstrate that LEMs have a ~1.5x decreased very long chain fatty acid (fatty acids with 22-38 carbon atoms) content and a ~5x increased MUFA content compared to native human SC. The culture medium of LEM is supplemented with palmitic (C16:0), linoleic (C18:2) and arachidonic acid (C20:4). To examine the fatty acid uptake from the medium, protonated palmitic acid (C16:0) was substituted by deuterated palmitic acid, or deuterated arachidic acid (C20:0) was added to the fatty acid mixture which was supplemented to the culture medium. LC/MS analysis revealed that deuterated palmitic and arachidic acid are taken up, elongated and incorporated in the SC of LEM. However, fatty acids with a deuterated arachidic acid backbone were present in lower quantities in the SC of LEM than fatty acids with a deuterated palmitic acid backbone. In subsequent studies the level of the fatty acid mixture supplemented to the medium was increased four times or was modified by a fourfold increase in palmitic acid concentration. Furthermore, the effect of insulin on the SC MUFA content and SCD1 expression in the viable epidermis was
studied. The results demonstrate that increasing the concentration of arachidonic and linoleic acid results in a decreased content of very long chain fatty acids and an increase in MUFAs, whereas increasing the supplementation of palmitic acid had no effect on the fatty acid profile compared to the control. Additionally, modification of the insulin content in the culture medium did not affect the fatty acid profile or reduce the expression of SCD1 compared to the controls. All modifications in medium supplements did not affect the lateral or lamellar lipid organization compared to the control, except the condition wherein arachidonic and linoleic acid supplementation was increased. In the latter case the LEMs showed a reduction in the repeat distance of the LPP and the temperature at which the SC lipids are only arranged in a hexagonal packing compared to the controls. The HSEs are known to have an increased triacyl- (TAG) and diacylglyceride (DAG) content compared to native human SC. TAG and DAG are formed by linking fatty acids to a glycerol backbone. The increased presence of TAG and DAG in the SC may be a reason why the LEM has a decreased SC fatty acid content.

Chapter 6 demonstrates that the medium composition can have a considerable influence on the SC lipid properties of HSEs. However, modifications made to the culture medium so far did not lead to an improved SC barrier in LEMs. This indicates that other media modifications are required to improve the SC lipid properties of HSEs.

**Impairment of the desquamation process in vitro**

The results presented in chapter 4 indicated that the desquamation process is impaired *in vitro*. Based on these findings the expression and activity of specific desquamatory enzymes were further investigated in native human skin, HSEs and native human skin cultured for one or two weeks. These studies are described in chapter 7. The results demonstrate that the HSEs and human skin cultured for two weeks at the air-liquid interface do not desquamate. All HSEs and cultured human
skin show a similar expression of kallikrein 5, one of the important enzymes involved in the desquamation process, as native human skin. However, almost all HSEs show the expression of Lympho-epithelial Kazal type related inhibitor (LEKTI), an inhibitor of kallikreins, in more differentiated epidermal layers compared to native human skin. In FTM a decreased kallikrein 5 activity was observed in the superficial SC layers compared to native human skin. These results suggest that the impairment of the desquamation process in HSEs may partially be caused by an increased LEKTI expression.

**CONCLUSIONS**

The results presented in this thesis demonstrate that HSEs are able to form a completely stratified epidermis, including a SC, when generated under submerged conditions. However, under these conditions the HSEs have a reduced SC fatty acid and acylceramide content compared to HSEs generated at the air-liquid interface. This is an important finding since the SC is formed under submerged conditions in utero. The presented data also show that the culture conditions are of crucial importance concerning the protein expression and SC lipid properties of HSEs. Furthermore, we demonstrate that the SC lipid composition and organization of HSEs can be affected by the composition of the culture media. Novel targets (e.g. SCD1) have been identified or proposed, which can guide future research focused on improving the SC barrier function of HSEs. Optimization of the culture conditions may improve the epidermal lipid metabolism in HSEs. This may contribute to the development of a new generation of HSEs that harbor a competent SC barrier that even more closely resembles the SC lipid composition and organization of native human SC than the current HSEs.
PERSPECTIVES

Further investigation of the SC lipid properties and barrier function of HSEs

In order to achieve more insight into the correlation between the SC lipid composition, organization and barrier function of each HSE type, it would be interesting to quantify the SC fatty acid chain length distribution and saturation of FDM and FTM, just as performed for LEM. This is of importance since the fatty acid composition plays an important role in the lateral lipid organization, which is different in all HSEs compared to native human skin. Quantification of the SC fatty acid species may also provide indications as to why FTM has a higher fatty acid level, but nevertheless does not show improved SC barrier properties, and even has a decreased SC permeability barrier for benzocaine compared to FDM and LEM.

The permeation pathway of compounds through the SC is suggested to mainly proceed through the lipid domains. Therefore our studies are primarily focused on the SC lipids of HSEs to understand and improve their impaired barrier function. Whether the cornified envelope also contributes to the increased permeability across HSEs compared to native human skin, should also be investigated. This can be done by visualizing the permeation pathway of a fluorescent marker through the SC of HSEs and native human skin using confocal laser scanning microscopy. Such studies are needed to determine whether changes in the SC lipid composition are the only cause of the decreased barrier function observed for the HSEs.

The SC barrier function of the HSEs was examined with benzocaine, which has been chosen for its low molecular weight and medium lipophilicity (mw = 165.2 g/mole, LogP = 1.44), resulting in an efficient permeation through human SC. In future studies the permeation of compounds with a broad range of lipophilicity and molecular weights should be assessed as well to compare the SC permeability barrier of the HSEs with human SC further. Additionally, the SC barrier function of FDM and LEM should be compared to commercially available HSEs in order
to determine whether the novel HSEs show an improved *in vitro - in vivo* correlation when they are used for permeability studies.

*Identifying differences in epidermal lipid metabolism between HSEs and native human skin*

The results presented in this thesis demonstrate that HSEs resemble human skin to a great extent with regard to their morphology, expression of various differentiation markers and ability to synthesize all SC barrier lipids. However, the SC lipid composition of HSEs shows some differences compared to native human SC. These differences indicate that epidermal lipid metabolism of HSEs deviates in some aspects from native human skin. In order to improve the SC lipid properties of HSEs, it is essential to bring the epidermal lipid metabolism of HSEs closer to the *in vivo* situation. To achieve this, it is first important to understand how epidermal lipid metabolism of HSEs differs from native human skin. This can be done by quantifying the mRNA expression level (using RealTime PCR) or protein abundance (using gel electrophoresis or mass spectrometry) of enzymes involved in epidermal lipid metabolism and their expression/activity regulators. Peroxisome proliferator-activated receptors (PPARs) play a central role in regulation of epidermal differentiation and lipid metabolism. Although our results showed that PPARα and PPARβ/δ have a similar expression pattern in the HSEs as native human skin, they might be expressed at different levels, which may lead to disturbance of epidermal homeostasis. It would also be of particular interest to determine the expression level of enzymes involved in the fatty acid synthesis, elongation and desaturation (such as SCD1) or synthesis and degradation of DAG and TAG, since all HSEs have a reduced fatty acid content and increased MUFA, DAG and TAG level in their SC compared to human SC. These differences in SC lipid composition are partially the cause of the altered SC lipid organization observed for HSEs, which may consequently be the cause of their decreased barrier function.
Summary and perspectives

A more accurate method to assess the differences in epidermal lipid metabolism between HSEs and native human skin would be to quantify enzyme activity. However, determining enzyme activity in a 3D tissue may prove to be quite challenging. Identification of enzymes involved in epidermal lipid metabolism that are differentially expressed or active in human skin and HSEs can be considered as targets to improve the SC lipid properties of HSEs.

One potential enzymatic target could be SCD1. Immunohistochemical staining revealed that the HSEs show suprabasal expression of SCD1, whereas native human skin only shows expression of SCD1 in the basal layer. It is likely that the increased expression of SCD1 in the suprabasal layers of the viable epidermis in HSEs coincides with an increased SCD1 activity, resulting in higher SC MUFA levels compared to native human skin. SCD activity depends on various factors, such as the insulin level. However, reduction of insulin content in the culture medium of LEM did not result in changes in SC MUFA content.

Optimization of culture conditions to improve SC lipid properties of HSEs

Optimization of the culture environment and/or culture media provides an opportunity to improve epidermal lipid metabolism of HSEs and thereby their SC lipid properties. A decrease in SCD1 activity in HSEs may be achieved by addition of specific SCD inhibitors to the culture medium. This may lead to a decrease in SC MUFA content of HSEs and thereby improve their SC lipid properties. An advantage of using specific SCD inhibitors rather than general changes made to the culture medium is the absence or reduction of any off-target effects. Changes observed in HSE morphology, differentiation or SC lipid properties can therefore more easily be correlated to the activity of one enzyme.

We showed that the SC lipid properties of HSEs can differ depending on the culture conditions. HSEs generated under submerged conditions show a reduction in SC free fatty acid level compared to HSEs generated at the air-liquid interface. HSEs generated under submerged conditions develop in an environment with
100% relative humidity (RH), while air-exposed HSEs are generated at a RH of 92%. The mechanism underlying the difference in SC fatty acid level between the submerged and air-exposed HSEs is unknown. However, it is possible that a reduction in environmental humidity below 92% will further increase the SC fatty acid level in HSEs. An increase in SC fatty acid level may promote the formation of the orthorhombic packing, as shown by Bouwstra et al.\textsuperscript{21}. This may consequently lead to improved SC barrier properties in HSEs.

The described HSEs show an altered expression of involucrin and presence of keratin 16 expression, which indicates that epidermal homeostasis is not reached. This may be due to the fast maturation of keratinocytes \textit{in vitro}. The culture conditions used to generate HSEs do not reflect the \textit{in vivo} situation. An improved balance between proliferation and differentiation in HSEs may therefore be achieved by adopting culture conditions that mimic the \textit{in vivo} environment of human skin more closely. HSEs are generally cultured at 37°C, while in daily life human skin is exposed to a fluctuating environmental temperature. Additionally, the skin surface has a temperature of approximately 32°C, while the body’s interior has a temperature of 37°C. Studies performed by Gibbs \textit{et al.}\textsuperscript{26} showed that generating HSEs at 33°C instead of 37°C results in an improved balance between epidermal proliferation and differentiation. Furthermore, the oxygen level of \textit{in vivo} skin ranges from 1.5-5%, while ambient oxygen concentration is around 21% \textsuperscript{27}. It is thus also reasonable to take the environmental oxygen tension into consideration when HSEs are generated. It has been shown that low oxygen levels enhance fibroblasts growth \textsuperscript{28}. Furthermore, an oxygen level of 5% has a minor effect on keratinocyte proliferation, but impedes keratinocyte differentiation compared to keratinocytes grown at atmospheric oxygen levels \textsuperscript{29}. It is possible that fine tuning of the environmental temperature and/or oxygen level, at different stages of HSE generation, is needed to optimize fibroblast and keratinocyte growth and differentiation. This may lead to an improved epidermal homeostasis and consequently result in the formation of a competent skin barrier.
Summary and perspectives

A closer look at the in vitro desquamation process

HSEs show an increased expression of LEKTI in the suprabasal layers of the epidermis compared to native human skin. Additionally, FTM shows a reduced KLK 5 activity compared to in vivo human skin. This suggests that the impaired desquamation process in HSEs, specifically in FTM, may be caused by an increased LEKTI inhibition of KLK 5 activity. Studies described by Deraison et al. demonstrated that LEKTI inhibition occurs in a pH dependent manner. The SC of native human skin has a pH gradient that ranges from approximately pH 7.5 in the inner SC and pH 5 in the superficial SC layers. At physiological pH LEKTI binds to KLKs and thereby inhibits corneodesmolysis. At lower pH LEKTI disassociates from KLKs rendering them free to degrade corneodesmosomes. The SC pH gradient is therefore essential for a proper desquamation process. In order to investigate whether impairment of the desquamation process in HSEs is caused by an increased KLK inhibition by LEKTI, it is necessary to determine whether HSEs also have a similar pH gradient as native human SC. Accurate determination of SC pH in HSEs can be done by using a pH-sensitive fluorescent probe in conjunction with two-photon fluorescence lifetime imaging microscopy as described by Hanson et al.
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