A novel formulation for skin barrier repair : from ex vivo assessment towards clinical studies

Berkers, T.

Chapter 3

Degree of skin barrier disruption affects lipid organization in regenerated stratum corneum

Tineke Berkers[1], Dani Visscher[1], Gert S. Gooris[1], Joke A. Bouwstra[1]

[1] Department of Drug Delivery Technology, Cluster BioTherapeutics, Leiden Academic Centre for Drug Research, Leiden University, Leiden, 2333 CC, The Netherlands

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Abbreviations

CER	Ceramide
FA	Fatty acid
FTIR	Fourier transform infrared spectroscopy
HE	Haematoxylin and eosin
LPP	Long periodicity phase
MUFA	Mono-unsaturated fatty acid
SAXD	Small angle X-ray diffraction
SC	Stratum corneum
SkinBaR	Skin barrier repair
SPP	Short periodicity phase

Keywords

Skin barrier repair, parakeratosis, lateral lipid organization, lamellar lipid organization, stratum corneum, skin model

Abstract

Previously, a skin barrier repair model was developed to examine the effect of formulations on the lipid properties of compromised skin. In this model, the lipid organization mimics that of several skin diseases with an impaired skin barrier and a less dense lateral lipid organization. Additionally, parakeratosis was occasionally observed. In the present study, we investigated if the extent of initial barrier disruption affects lipid organization and parakeratosis in regenerated SC.

After barrier disruption and SC regeneration the fraction of lipids adopting a less dense lateral organization gradually increased with increasing degree of barrier disruption. Only when 75% of the SC was removed, parakeratosis and a change in lamellar organization were observed. This demonstrates the possibility to use the skin barrier repair model to study the effects of formulations on compromised skin in which the presence of parakeratosis and lipid organization can be modified by the extent of barrier disruption.

Introduction

The skin barrier function is located in the uppermost layer of the epidermis, the stratum corneum (SC). This layer consists of corneocytes embedded in a lipid matrix.[1] The lipids form two crystalline lamellar phases with repeat distances of 13 nm (long periodicity phase, LPP) and 6 nm (short periodicity phase, SPP), see Figure 1.[2-4] Within the lipid lamellae, the lipids mainly adopt a dense orthorhombic lateral packing in healthy skin.[5-8] In several skin diseases, the barrier function is affected, e.g. atopic dermatitis, lamellar ichthyosis, Netherton syndrome, and psoriasis.[9] The reduced barrier function is characterized by e.g. a higher fraction of SC lipids that adopts a hexagonal lateral packing compared to healthy skin.[10] The change in barrier function in several skin diseases depends on the severity of the disease.[9-11]

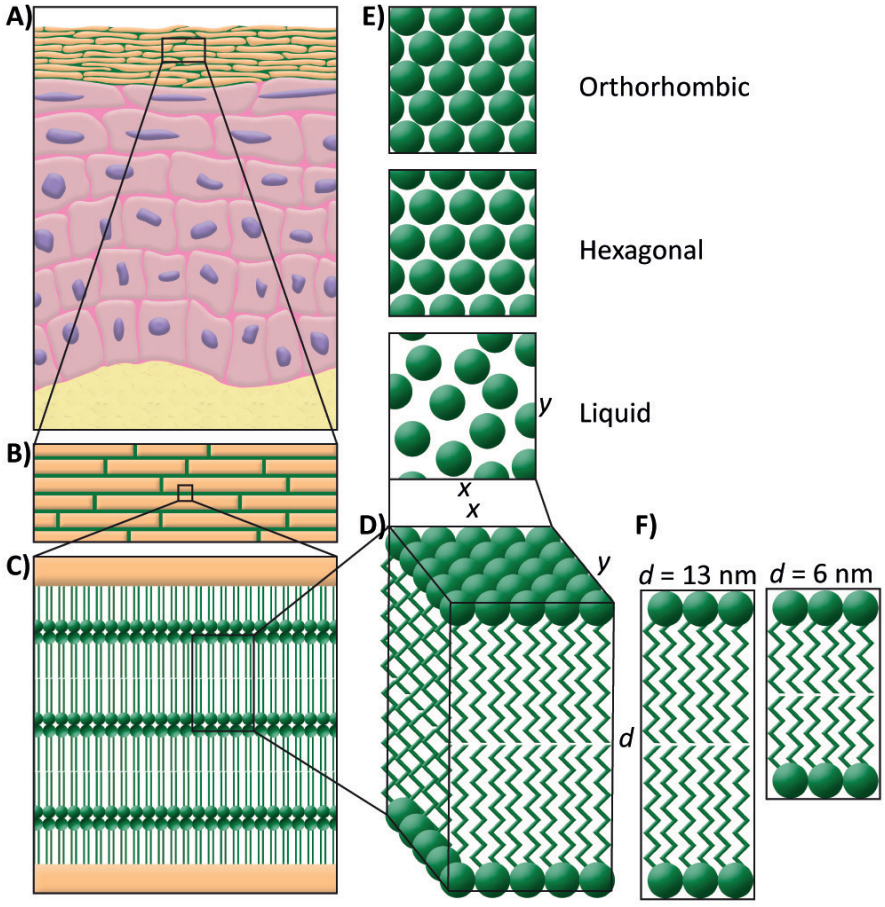

Figure 1. Lipid organization in the SC. A) Schematic overview of the skin, **B)** A brick-and-mortar structure is mimicking the corneocytes embedded in the lipid matrix, **C)** In between the corneocytes, the lipids are stacked in lamellae, **D)** Detail of the lipid lamellae, **E)** Perpendicular to the lamellae, the lipids are organized in a lateral packing. This can be either orthorhombic, hexagonal, or liquid (top view), **F)** The lipid lamellae are stacked on top of each other with a repeat distance (d) of either 13 nm (LPP) or 6 nm (SPP).

Recently, a human skin barrier repair model was developed which can be used to study the interactions of topical formulations with the SC during skin barrier repair.[12] This model is referred to as the SkinBaR model and exhibits several characteristics of the lipid composition and organization in SC of diseased skin, such as Netherton syndrome, lamellar ichthyosis, and atopic dermatitis. In this model, the SC is removed by stripping *ex vivo* human skin and regenerated during a culture period of 8 days. In our previous studies, occasionally nuclei were observed in the regenerated SC after culturing, which is known as parakeratosis. Parakeratosis is also observed in several skin diseases with a disturbed barrier[13], wound healing[14], and after tape stripping *in vivo*.[15] It is caused by abnormal keratinocyte maturation. We hypothesize that the degree of barrier disruption influences the rate at which the barrier is repaired and may therefore be associated with the presence of parakeratosis and the extent the lipid organization in the SC lipid matrix changes, mimicking more closely the lipid organization in SC of diseased skin. If this is the case, the SkinBaR model could be used to study the effects of barrier repair formulations on various levels of altered lipid organization, mimicking several aspects of the lipid organization in diseased skin. The formulations can be applied during or after barrier regeneration.[16]
The aim of this study was to induce several degrees of barrier disruption by stripping *ex vivo* human skin and regenerating the SC during culture. We intended to mimic multiple levels of skin disease severity as analyzed by the morphology and lateral and lamellar lipid organization which are indicative for the skin barrier function.

Methods

Chemicals

Cyanoacrylate (Bison, Goes, the Netherlands) was bought in a local shop. Xylene was purchased from Biosolve (Valkenswaard, the Netherlands), 4% buffered formaldehyde was acquired from Added Pharma (Oss, the Netherlands), paraffin, haematoxylin, and eosin were obtained from Klinipath (Duiven, the Netherlands). DMEM, Ham's F12, and penicillin/streptomycin were purchased from Fisher Scientific (Waltham, Massachusetts, USA). Bovine serum albumin, sodium bromide, ethanol, acetone, trypsin, trypsin inhibitor, selenious acid, hydrocortisone, isoproterenol, L-carnitine, L-serine, insulin, α-tocopherol acetate, vitamin C, arachidonic acid, linoleic acid, and palmitic acid were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands).

Stripping and culturing of ex vivo skin

Human abdomen or mamma skin from three donors (age 20, 30, and 67) was obtained after abdominoplasty or breast reduction, from a local hospital, after written informed consent, and was used within 12 hours after cosmetic surgery. The subcutaneous fat was removed with a scalpel, and the skin was wiped with 70% ethanol and Millipore water. Subsequently, the skin was dermatomed to a thickness of 600 µm (D80 Dermatome, Humeca, Borne, the Netherlands), punched in circles (ø=26 mm), clamped in a custom made device, and stripped with preheated cyanoacrylate on

a preheated metal cylinder as described before.[12] SC was stripped by removing the cylinder in one stroke. The stripping procedure was repeated to remove either 25%, 50%, or 75% of the SC. After 75% of the SC was removed the skin appeared shiny. Typically, 6-8 strips were needed to reach a shiny appearance of the whole sample, varying between skin donors. Cultured non-stripped (0%) skin and native skin (e.g. non-stripped and not cultured) were used as control. Per donor, the different conditions were cultured in duplo.
The stripped *ex vivo* skin was cultured at the air-liquid interface for 8 days in a deep 6-well culturing plate (Organogenesis, Canton, MA, USA), as previously described.[12] The samples were either cryofixed, paraffin embedded, or SC was isolated.

Isolation of SC

SC was isolated from the (cultured) skin by trypsin digestion. The skin was placed in 0.1% trypsin solution in PBS and kept overnight at 4˚C, followed by one hour at 37˚C. Trypsin is a proteolytic enzyme which is used to separate SC from the epidermis.[17] SC was peeled off, washed in 0.1% trypsin inhibitor, and twice in Millipore water. SC sheets were stored over silica gel under argon until use. SC was used for either infrared spectroscopy measurements or X-ray diffraction measurements.

Safranin-O red and heamatoxylin and eosin staining

To examine the number of corneocyte layers 5 µm cryofixed sections were stained with 1% (w/v) Safranin-O solution for 1 minute followed by 20 minutes incubation in 2% (w/v) KOH solution. Haematoxylin and Eosin (HE) staining was performed on 5 µm paraffin sections to examine the morphology. After Safranin-O and HE staining, at least 3 light microscopy images were taken per culture condition at 20x and 63x magnification.

Fourier transformed infrared spectroscopy

Fourier transformed infrared (FTIR) spectroscopy was used to examine the lateral lipid packing of isolated SC samples as a function of temperature (0-90˚C). The samples were placed over a 27% NaBr solution in D_2O for 24 hours at room temperature, reaching a final hydration level of around 20% in the SC, and sandwiched between two AgBr-windows. Spectra were obtained using a Varian 670-IR FTIR spectrometer (Agilent Technologies, Santa Clara, USA), as described before.[16]
The onset temperature of the ordered-disordered transition was obtained from the peak positions of the CH_2 symmetric stretching vibrations in the FTIR spectra. Peak positions were plotted as a function of temperature. The intercept of two regression lines fitted to the linear parts of the graph was calculated which describes the onset transition temperature, as described before.[16]

Small angle X-ray diffraction

Small angle X-ray diffraction (SAXD) was employed to examine the lamellar organization of the SC lipids. Measurements were performed at the European Synchrotron Radiation Facility (Grenoble, France) using the Dutch-Belgian beamline (station BM26B). 24 hours prior to the measurements, SC samples were hydrated over a 27% NaBr solution at room temperature reaching a hydration level of about 20%. A custom made sample holder was used to orientate the samples parallel to the X-ray beam. Diffraction data was collected at room temperature on a Pilatus 1M detector for a period of 5 or 10 minutes, as described earlier.[18] The scattering vector (q) was calculated using the scattering angle (θ) and the wavelength (λ) by $q=4\pi \sin\theta/\lambda$. The spacing of the lamellar phase can be calculated from the position of the peak maxima (q) using $2\pi/q$.

Statistical analysis

One-way ANOVA with post hoc correction for multiple comparisons or with a post-hoc trend test was used to analyze the data using GraphPad Prism 7 (San Diego, CA, USA). All differences are described relative to native human skin.

Results

Human skin cultured after various degrees of barrier disruption results in complete SC regeneration

After staining with safranin-O, the number of corneocyte layers was determined for at least 3 different spots per image and at least 3 images were taken per culturing condition for each skin donor. Per condition, the results of the three donors were pooled. Native human skin showed 11.0 ± 4.0 (mean ± SD) corneocyte layers, whereas after removing of the SC until the skin surface appeared shiny 3.1 ± 2.8 SC cell layers remained on the viable epidermis, meaning that about 75% of the corneocyte layers was removed. The number of strips needed to remove 75% of the SC was reduced to ¾ and ½ in order to obtain less stripped skin. This resulted in skin with either 5.0 ± 3.4 (=50% stripped) or 7.7 ± 4.4 (=25% stripped) remaining corneocyte layers on the viable epidermis, see Figure 2A. All stripped samples had a significant lower number of corneocyte layers than the native SC ($p<0.001$). A post-hoc test showed that there is a linear trend with decreasing number of corneocyte layers for the samples prior to culture (slope: -2.6, $p<0.0001$). After the 8-day culturing period, the number of corneocyte layers in the regenerated SC is significantly increased to 10.8 ± 3.6, 10.3 ± 2.6, and 11.2 ± 3.1, for 75%, 50%, and 25% stripped SC, respectively, which is comparable to the number of layers in native human skin before stripping.

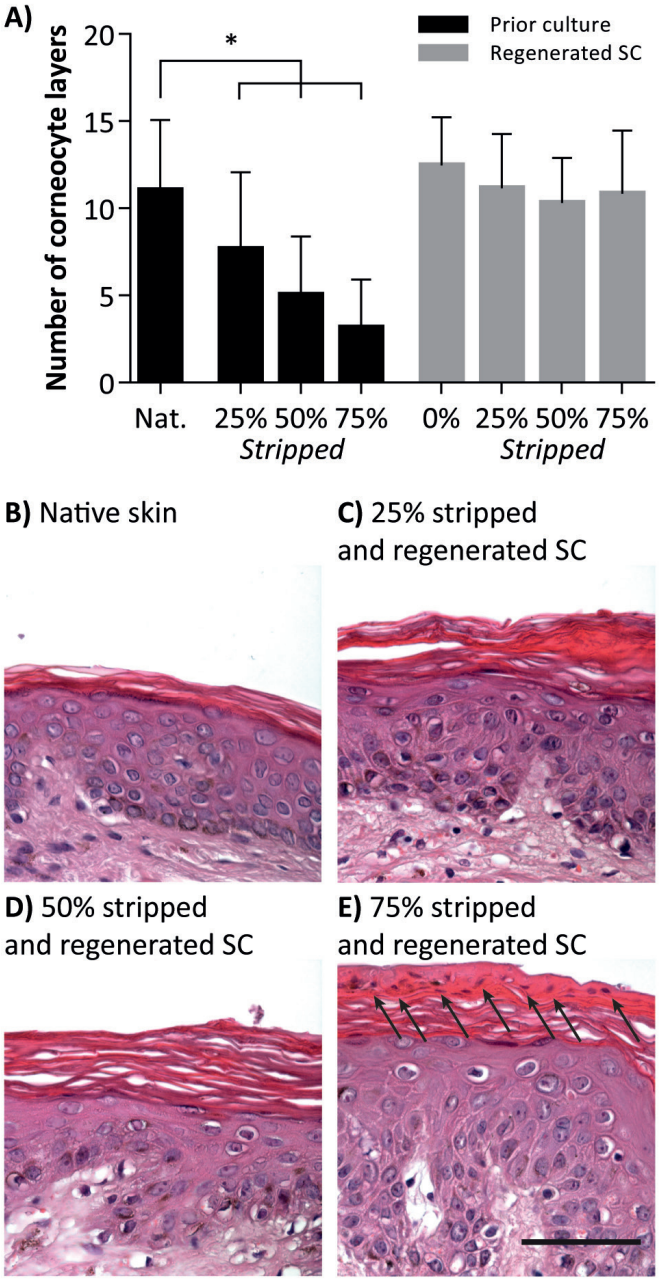

Figure 2. Number of corneocyte layers and morphology. A) The number of layers in native SC (Nat.), stripped SC, and regenerated SC after culturing. Bars represent Mean ± SD. Morphology of **B)** Native skin, and **C-E)** Stripped and cultured skin with regenerated SC. SC was stripped away for 25%, 50%, and 75%, respectively. * $p<0.001$ (ANOVA), arrows: parakeratosis, scale bar: 50 µm.

Parakeratosis is visible when the majority of the SC is removed

The morphology of the cultured skin sections was visualized using HE staining. The staining revealed that after removing 75% of the SC and subsequent culturing, prominent parakeratosis is observed (see arrows in Figure 2E), mainly in the top layers of the regenerated SC. Furthermore, spongiosis with an irregular keratinocyte distribution in the epidermis was observed in skin with SC that was regenerated after 75% of the SC was removed. After 50% or 25% of the SC was removed, the regenerated SC did not exhibit parakeratosis or major differences in morphology.

Lateral lipid organization changes gradually with number of removed corneocyte layers

The lipid organization of the stripped and regenerated SC was examined and compared to native SC. The lateral lipid packing was examined using the CH_2 rocking vibrations in the FTIR spectra in a temperature range between 0°C and 90°C. A hexagonal lateral lipid packing is characterized by a single contour positioned at a wavenumber of 719 cm^{-1}, while an orthorhombic packing is characterized by a doublet positioned at 719 and 730 cm^{-1}. All spectra were scaled at the difference between the absorption at 719 cm^{-1} (peak position) and the absorption at 715 cm^{-1} (base). This difference in absorption was kept constant in all spectra. In this way relative peak heights of the peak at around 730 cm^{-1} could be compared between samples.
Figure 3 shows representative FTIR spectra of one donor. As shown in Figure 3A, in native SC two strong contours were observed at 719 and 730 cm^{-1} at 0°C, indicating that the lipids adopt an orthorhombic lateral lipid packing. The contour positioned at 730 cm^{-1} started to decrease in intensity at around 34°C and disappeared at around 48°C, indicating a phase transition from an orthorhombic to a hexagonal lateral lipid packing. The intensity of the peak at 730 cm^{-1} at 0°C gradually decreased when 25% to 50% and 75% of SC was removed before culturing. When increasing the temperature, the peak at 730 cm^{-1} disappeared between 30°C and 48°C (25% of SC was stripped), and 20°C and 36°C (50% of SC was stripped) in the FTIR spectra of regenerated SC. After removing of 75% of the corneocyte layers and regeneration of the SC, the orthorhombic to hexagonal transition occurred between 8°C and 30°C as monitored by the disappearance of the 730 cm^{-1} in the corresponding FTIR spectrum.

Lipid ordering only affected when significant amount of SC is removed

The conformational ordering of the lipids was examined using the thermotropic behavior of the CH_2 symmetric stretching vibrations in the FTIR spectrum in a temperature range of 0-90°C. Fully extended lipid chains show a high conformational ordering, which is characterized by a CH_2 stretching vibration peak position at wavenumbers below 2850 cm^{-1}. When lipids have a high conformational disordering, e.g. when lipids are in liquid state, the peak position is shifted to wavenumbers higher than 2852 cm^{-1}. The onset transition temperature was determined as described above.
The CH_2 symmetric stretching vibrations obtained from representative data of one donor are plotted as a function of temperature in Figure 3E. The wavenumber at 10°C and the onset transition temperatures are mean values of the spectra of the three donors.

At 10˚C, a wavenumber of 2848.81 ± 0.01 cm^{-1} (mean ± SD) was observed in native SC. The wavenumbers of cultured control skin, and after regeneration of 25% and 50% of the SC were comparable to native SC (p=0.74, p=0.35, and p=0.39, respectively). Only when 75% of the SC was removed, the wavenumber at 10˚C was increased to 2849.38 ± 0.25 cm^{-1} (p=0.04). This indicates that, even at low temperatures, the lipid ordering of the regenerated SC is affected.
Furthermore, the stretching vibrations are used to compare the onset transition temperatures of the ordered-disordered phase transition. For native SC the onset transition temperature was 72.5 ± 3.6˚C (mean ± SD). Furthermore, at around 30-40˚C, a small shift in wavenumber is observed, indicating an orthorhombic to hexagonal transition. Lipids in the SC of cultured control skin (0% of SC was stripped) showed similar temperature dependence as native SC with a transition temperature of 68.9 ± 4.7˚C. After regeneration of the SC after 25% or 50% of the SC was stripped, the onset temperatures of the ordered-disordered phase transition were 68.9 ± 2.9˚C and 67.9 ± 1.3˚C, respectively. These onset transition temperatures were not statistically significant different from that of native SC. However, SC regeneration after 75% of the SC was removed, resulted in a different temperature dependence of the CH_2 stretching vibrations. The onset of the ordered-disordered phase transition took place at a statistically significant lower temperature of 62.9 ± 2.1˚C (p=0.01). This is apparent from the line in Figure 3E which is shifted to the left and increases to higher wavenumbers at lower temperatures. Furthermore, the small shift in wavenumber at around 30-40˚C was not observed in regenerated SC after 50% or 75% of the SC was stripped.

The lamellar repeat distance decreases only after removing most of the SC

The lamellar organization in the lipid matrix was examined using SAXD. Figure 4 shows SAXD patterns of a representative donor of native SC and SC that was regenerated after stripping and culturing. Two weak diffraction peaks (indicated by I and III) and one strong peak (indicated by II) can be observed. The LPP contributes to all three peaks, whereas the SPP only contributes to peak II. The peak corresponding to phase separated cholesterol is marked by an asterisk. The peak position (q) of the main peak (II) was used to examine whether a difference in lamellar spacing was induced by SC disruption. The spacing of the lipids in the stripped and regenerated SC was compared to the spacing of native SC of the same donor. In this way, a mean difference was obtained. The spacing in native SC was 6.3 ± 0.1 nm (mean ± SD). The difference in spacing was 0.05 nm for the SC of the cultured control sample (0% of SC was stripped) and for regenerated SC after 25% or 50% of the SC was stripped this difference was -0.06 nm. None of these changes were statistically different. However, when SC was regenerated after 75% of the SC was removed, the difference in spacing was -0.23 nm, which was statistically significant different from native SC (p=0.001). Furthermore, the intensity of diffraction peaks I and III decreased substantially, indicating a reduction in the presence of the LPP. Additionally, a shoulder appears close to the position of peak II at a q-value of around 1.3 nm^{-1}.

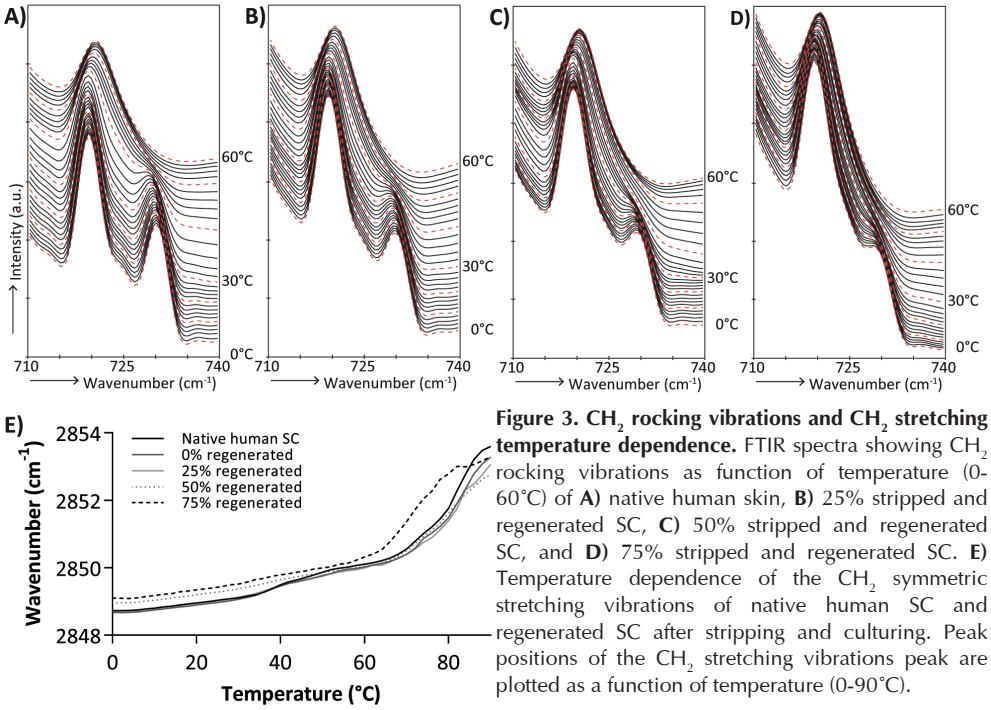

Figure 3. CH_2 rocking vibrations and CH_2 stretching temperature dependence. FTIR spectra showing CH_2 rocking vibrations as function of temperature (0-60˚C) of **A)** native human skin, **B)** 25% stripped and regenerated SC, **C)** 50% stripped and regenerated SC, and **D)** 75% stripped and regenerated SC. **E)** Temperature dependence of the CH_2 symmetric stretching vibrations of native human SC and regenerated SC after stripping and culturing. Peak positions of the CH_2 stretching vibrations peak are plotted as a function of temperature (0-90˚C).

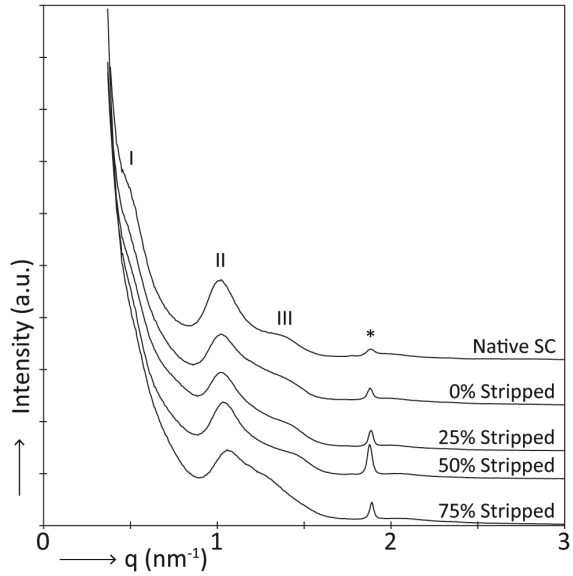

Figure 4. A typical example of SAXD patterns of native SC and regenerated SC after stripping and culturing. The peak positions (q) are indicative for the spacing of the lipid lamellae. The 1st, 2nd, and 3rd order peaks of the LPP are labeled with I, II, and III, respectively. The first order of the SPP also contributes to peak II. Phase separated cholesterol is labeled with *.

Discussion

In this study we showed that in regenerated SC of the SkinBaR model we can on demand gradually decrease the fraction of lipids adopting a dense orthorhombic lateral packing by varying the initial degree of barrier disruption. The obtained changes in lipid lateral packing compared to native SC are very similar to that observed in SC of several inflammatory skin diseases.[9-11] These results indicate that the SkinBaR model can be used to study skin barrier repair formulations that aim to normalize the SC lipid organization in diseased skin. The degree of disruption also influenced the presence of parakeratosis, as we showed that parakeratosis was only present in the examined models when 75% of the SC was removed, resulting in 3 remaining corneocyte layers before the skin started to regenerate the SC. Additionally, parakeratosis was predominantly observed in the upper corneocyte layers. This strongly indicates that only during generation of the initial corneocyte layers parakeratosis is induced, probably as a fast response to repair the skin barrier after disruption. Independent of the degree of barrier disruption, the number of corneocyte layers after 8 days of regeneration was comparable to that in SC of native skin. This indicates that the degree of disruption affects the rate of regeneration, and that the skin is still viable. Previous observations have shown that the proliferation rate, as examined by fraction of Ki-67 positively stained nuclei, immediately after SC removal was higher than the proliferation rate after a longer recovery period.[12,15] It is known that cultured skin does not desquamate and that the corneocyte layers accumulate during culture.[19] However, this is believed to be of little influence on the SkinBaR model since the non-stripped cultured SC showed a non-significant increase of only 1.4 corneocyte layers during culturing indicating that the formation of the SC is inhibited.

When focusing on the lateral lipid organization, the lipid density, which is the fraction of lipids forming an orthorhombic lateral packing, of the regenerated SC decreased when the degree of barrier disruption was increased. At skin temperature (32˚C), in the FTIR spectrum no contour at 730 cm^{-1} was observed when 75% of the SC was regenerated, demonstrating that at this temperature the lipids had adopted a hexagonal packing. However, when removing less corneocyte layers, the contour at 730 cm^{-1} was visible as a shoulder (after 50% of SC was stripped) or as a peak (native or after 25% of SC was stripped) at 32˚C. An indication of the lipid ordering is provided by the CH_2 stretching vibrations. When comparing these stretching vibrations of regenerated SC after 75% of SC was stripped to that of regenerated SC after less intensive disruption of SC, the onset temperature of the ordered-disordered phase transition was significantly lower and the position of the CH_2 vibrations at 32˚C were at higher frequency. These observations suggest a less ordered organization of the lipids in the regenerated SC after 75% SC removal, even at low temperatures. Also the lamellar organization was only affected in regenerated SC after 75% of the SC was removed. These results indicate that the lateral lipid packing is more responsive to barrier disruption than the conformational ordering and the lamellar organization.

Previously, a more abundant presence of lipids adopting a hexagonal packing has been related to a higher level of mono-unsaturated fatty acids (MUFAs)[20], and shorter fatty acid (FA) and ceramide (CER) chain length.[21] In the SkinBaR model, both an increased level of short chain CER and unsaturated CER were observed.[22] Mono-unsaturated

CERs are derived from a chemical linkage of a MUFA to a sphingoid base.[23] The enzyme stearoyl-CoA desaturase catalyzes the conversion of saturated FAs to MUFAs. In the SkinBaR model, we have demonstrated an extended stearoyl-CoA desaturase expression compared to native human skin, suggesting also an increased level of MUFAs.[12]

Similar changes in lipid organization and composition have been observed in atopic dermatitis skin. In lesional and non-lesional skin of atopic dermatitis patients a higher fraction of lipids adopts a hexagonal lipid organization, which coincides with a higher fraction of MUFAs and a reduced chain length of FAs and CERs.[10,24,25] These changes are more pronounced in lesional skin than in non-lesional skin.[10] Besides atopic dermatitis, several other skin diseases are characterized by a disrupted skin barrier.[9,11] Again, the extent of barrier disruption may vary depending on the severity of the disease. This may be of influence on the effect of topically applied barrier repair formulations.

The results in this study show that the degree of barrier disruption of the SkinBaR model can be controlled and that the changes in lipid organization and the level of parakeratosis depend on the initial extent of barrier disruption. This means that if less corneocyte layers remain before regeneration is initiated, the deviations in lateral and lamellar lipid organization compared to native SC are more substantial. This variation in the degree of deviation of the lipid organization in the SkinBaR model can be used in studying the effect of barrier repair formulations on the lipid organization. In this way the SkinBaR model can serve as a model for the lipid organization in several skin diseases of which the lipid organization deviates in a similar manner from that in native human skin. Previously, other skin models (the human skin equivalents) have been used to test topical formulations.[26] However, the 8-day culturing period of the SkinBaR model is less time-consuming than a culturing period of 2-3 weeks often used to generate human skin equivalents.[27-29] Furthermore, it is a challenge to influence the lateral packing of the skin equivalents by modifying the culture conditions[27,30,31], while the lateral packing of the SkinBaR model can be adjusted on demand. Unlike these human skin equivalents, the SkinBaR model offers the possibility to apply topical barrier repair formulations directly after several degrees of barrier disruption and study the lamellar and lateral lipid organization of regenerated SC.

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