Human skin equivalents: impaired barrier function in relation to the lipid and protein properties of the stratum corneum

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Human skin equivalents: Impaired barrier function in relation to the lipid and protein properties of the stratum corneum

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ABSTRACT

To advance drug development representative reliable skin models are indispensable. Animal skin as test model for human skin delivery is restricted as their properties greatly differ from human skin. In vitro 3D-human skin equivalents (HSEs) are valuable tools as they recapitulate important aspects of the human skin. However, HSEs still lack the full barrier functionality as observed in native human skin, resulting in suboptimal screening outcome. In this review we provide an overview of established in-house and commercially available HSEs and discuss in more detail to what extent their skin barrier biology is mimicked *in vitro* focusing on the lipid properties and cornified envelope. Further, we will illustrate how underlying factors, such as culture medium improvements and environmental factors affect the barrier lipids. Lastly, potential improvements in skin barrier function will be proposed aiming at a new generation of HSEs that may replace animal skin delivery studies fully.

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Abbreviations: 3D, three dimensional; CHOL, cholesterol; CERs, ceramides; CerS3, ceramide synthase 3; DED, de-epidermized-dermis; FFAs, free fatty acids; ECM, extracellular matrix; DEJ, dermal-epidermal junction; FTM, full thickness model; HPTLC, high performance thin layer chromatography; HSE, human skin equivalent; LC/MS, liquid chromatography combined with mass spectrometry; LXR, liver X receptor; LPP, long periodicity phase; SC, stratum corneum; SCD-1, stearoyl-CoA desaturase; SG, stratum granulosum; SPP, short periodicity phase; SPRR, small proline rich proteins; SS, stratumspinosum.

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1. Introduction

In current practice, animal models are extensively used by the pharmaceutical industry for drug development. A series of *in silico*, *in vitro* and *in vivo* studies are required before new developed drug may be administered to humans. For skin delivery studies, rodent skin (e.g. mice, porcine, rat, guinea pigs) and snake skin are mostly used as test-systems in percutaneous absorption studies due to their easy handling, availability and small size. [1] However, the reliability of rodent skin as test systems for human skin delivery is limited, as rodent, but also snake skin, greatly differs from human skin in structure and function. [2] The golden standard for *ex-vivo* percutaneous absorption studies is *ex vivo* human skin, mostly either abdominal or mammae skin. It has been shown that the amount of free fatty acids (FFAs), ceramides (CERs) and triglycerides, the thickness of the viable epidermis and stratum corneum (SC) and the density of hair follicles are crucial factors causing differences between the skin barriers among these species. [3] This poor correlation of animal data to humans may result in compound attrition in later stages of costly clinical trials. To minimize the number of failed lead compounds and late-stage clinical products, improving the preclinical screening process is paramount to pharmaceutical industry. Since the availability of human skin is limited, more representative, reliable and cost-effective replacement techniques are needed in industrial skin delivery drug screening programs. In recent years there is increasing opposition in the society against the use of animals to assess skin safety and permeability, as this has been considered inhumane and unnecessary. In 2009 the European Union effectuated the ban on animal testing for the cosmetic industry. This accelerated the development of *in-vitro* models that are currently used to test skin toxicity, skin corrosion and skin penetration of raw materials, ingredients and finished end products. [4] Hence, novel *in vitro* technologies mimicking several aspects of the human skin barrier are urgently needed for better interpretation and extrapolation of the data towards the human skin condition. In this review we will focus on the most sophisticated skin models, three dimensional (3D)-cultures of human skin. After a brief summary of the human skin structure and lipid properties, the main focus is the barrier function of human skin equivalents (HSEs) and how this is related to the lipid properties in SC.

2. The human skin structure

2.1. Dermis and epidermis structure

The dermis is a thick fibrous layer that consists of two sublayers, the papillary and reticular dermis. The papillary fibroblasts are situated in the extracellular matrix (ECM) just below the epidermis, while the reticular fibroblasts are found in the lower part of the dermal compartment. [5] Both fibroblast subtypes exhibit a different gene expression profile and have a distinct role in dermal and epidermal morphogenesis. [5–7] Both layers contain ECM components such as elastin, collagens and glycosaminoglycans, which gives structure and elasticity to the skin. The main functions of the dermis are regulation of temperature, hydration, and being an insulator and mechano-sensor. It contains several skin appendages, such as hair roots, sebaceous glands, sweat glands, nerves, and a vascular structure that is responsible for the supply of the required nutrients for the dermis and epidermis. The epidermal basal cells are attached to the basement membrane (BM) by hemidesmosomes, collagen XVII, laminin 332 and integrin $\alpha 6\beta 4$. The BM has multiple functions. It i) allows exchange of molecules and cells between the epidermis and dermal compartments, ii) regulates adhesion, migration, and proliferation of keratinocytes and fibroblasts and iii) provides structural support for the epidermis, while keeping the dermis and epidermis together. In addition, the BM plays a fundamental role in the re-epithelialisation process during wound healing. [8] Once the basal cells are attached to the BM, they are programmed to execute the early, late and terminal differentiation programmes on their way to the surface to become a corneocyte. During this journey, which normally takes around 28 days in human skin, the keratinocytes change in morphology and start to produce different keratins, growth factors, cytokines and complement factors. The process of cornification will be further explained in more details in the next paragraph.

2.2. Cornification of the skin

The epidermis is a very exceptional tissue renewing itself continuously and therefore it requires a tight regulation of epidermal proliferation and differentiation. [9–11] A schematic cross-section of the epidermis is provided in Fig. 1. In the stratum basale (SB) the keratinocytes proliferate. As soon as the cells escape from SB into the SS, keratinocyte differentiation is initiated. The keratinocytes move gradually from the stratum spinosum (SS) in the direction of the skin surface during which the function and content of the keratinocytes changes. In the SS the biosynthesis of precursor barrier lipids and several keratin fibres start. When keratinocytes enter the stratum granulosum (SG) the cornification proteins including (pro-)filaggrin, proline-rich proteins, involucrin, and loricrin are synthesized and are encapsulated in keratohyalin granules. [12,13] The synthesis of precursor barrier lipids, that is phospholipids (precursors of free fatty acids), and glucosylceramides and sphingomyelin (both precursors of CERs), see Fig. 2, is intensified and together with the lipid enzymes the precursor barrier lipids are stored in lamellar bodies. During the formation of the final differentiation product, the SC, the keratinocytes transform into the keratin-containing corneocytes. Simultaneously the densely cross-linked cornified envelope is formed by sequential deposition of the various proteins, such as small proline rich proteins, involucrin, loricrin and filaggrin. [14,15] All these proteins are coordinately regulated: a reduction in one protein may be compensated by an up regulation of another protein. [16] This makes the skin barrier function highly adaptive and flexible. To the cornified envelope a monolayer of non-polar lipids is esterified, referred to as the bound lipids, the precursor lipids of which are also glucosylceramides. Involucrin is reported to be the linking protein. [17,18] As the affinity of the cornified envelope proteins for binding the lipids might not be equal, a modulation of the cornified envelope composition may also affect the bound lipid composition. Besides the formation of the corneocytes, an intercellular lipid matrix is formed composed of non-polar free lipids. Both the bound and free lipids are made available by extruding the lamellar bodies containing the precursor lipids together with lipid enzymes, see Fig. 1. [19,20] During this process there is a high metabolic activity and the lipid composition changes dramatically. The bound lipid monolayer serves as a template for the free lipids that form lipid lamellae oriented approximately parallel to the corneocytes. The cornified envelope in concert with the monolayer of bound lipids is considered to act as a barrier for compounds to enter the dead

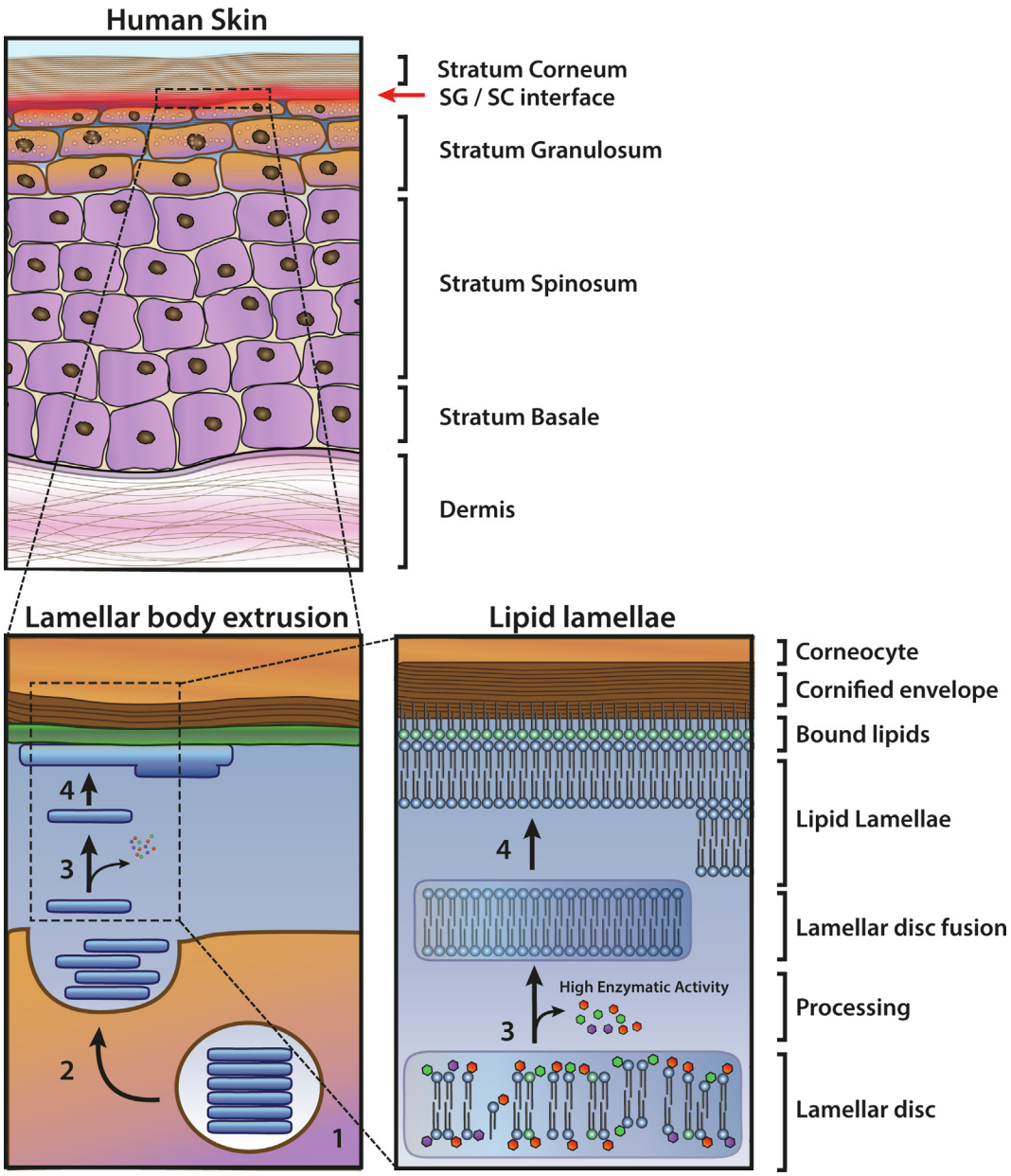

Fig. 1. Schematic view of the epidermal cross section and the lamellar body extrusion process. The epidermis consists of a stratum basal, stratum spinosum, stratum spinosum and stratum corneum. In stratum spinosum there is an increased synthesis of precursors of barrier lipids that are stored in lamellar bodies. The lamellar bodies are extruded at the interface between stratum spinosum and stratum corneum and release their content into the intercellular space. During this extrusion process the phospholipids are converted to fatty acids, while glucosylceramides and sphingomyelin are converted into ceramides. Enzymes catalysing these chemical modifications are also stored in the lamellar bodies. Simultaneously to the chemical changes, the lamellar disk fuse together and form the lipid lamellae. Most probably the lipids bound to the cornified envelope serve as a template for this fusion process.

cells. Therefore, an important penetration route is along the intercellular tortuous pathway, which makes the lipid matrix crucial for the skin barrier function. Barrier lipids form two lamellar phases in human SC, these are the long periodicity phase (LPP) with a repeat distance of approximately 13 nm and the short periodicity phase (SPP) with a repeat distance of around 6 nm, see Fig. 3. [21] Within the lipid lamellae, the lipids are mainly arranged in a dense crystalline orthorhombic lateral packing, although a subpopulation of lipids also forms a hexagonal packing and a minor lipid fraction even adopts to a liquid phase. [22,23] This predominant crystalline nature and the presence of a proper lamellar phase limits the diffusion of compounds travelling along the lipid matrix. Perturbations in the lipid composition and organisation will have a profound impact on the diffusion process as this will facilitate intercellular penetration and as such reduces the skin barrier. [23–25] *Consequently, both the crystalline lipid lamellae and the densely cross-linked cornified envelope with bound lipids are key players in a proper skin barrier function.*

2.3. Details on the lipid composition in SC

As the SC lipids form mainly crystalline lipid lamellae, which are not present in any other biological membrane, the lipid composition should be very exceptional. The main lipid classes are ceramides (CERs), cholesterol (CHOL) and FFAs. [26] No phospholipids are present in the SC. Of these lipid classes, the most intriguing lipids are the twin-chained CERs. The CERs consist of an acyl chain and a sphingoid base. Nowadays a high number of CER subclasses

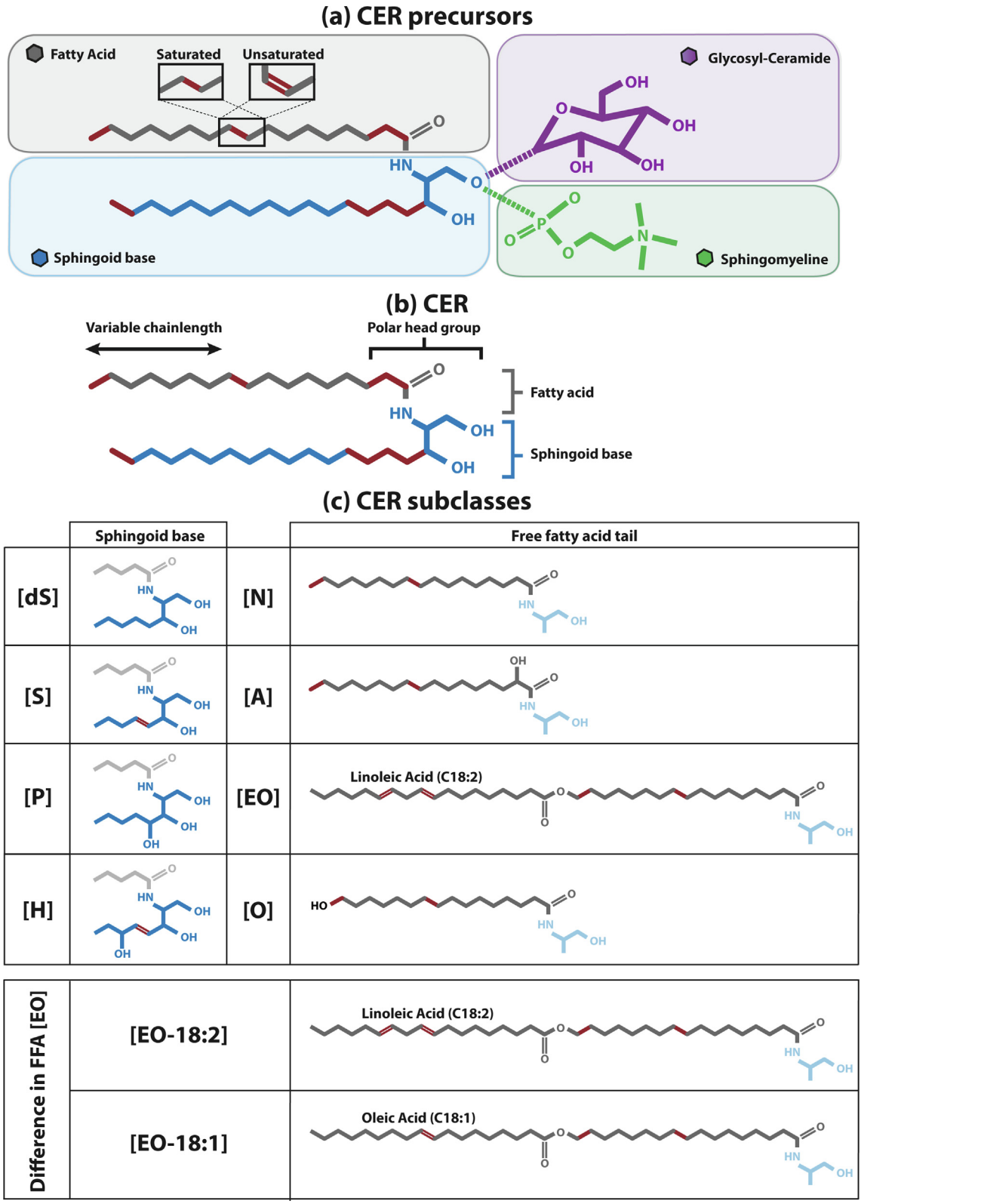

Fig. 2. The molecular structure of the CER subclasses and their precursors. The precursors of CERs are glucosylceramides and sphingomyelin. The CERs are composed of a large number of subclasses. The CERs consist of an acyl chain: non-hydroxy (N), α-hydroxy (A), ω-hydroxy (O) or linoleic acid esterfied to an ω-hydroxy acyl chain (EO). The acyl-chain is linked to a sphingoid base: dihydrosphingosine (dS), sphingosine (S), phytosphingsine (P) or 6-hydroxysphingosine (H). There are three CER classes i) CERs with subclasses NdS, NS, NP, NH, AdS, AS, AP, and AH, ii) CER EO with subclasses EOdS, EOS, EOP, and EOH and iii) CER O with subclasses OdS, OS, OP, and OH. In each subclass, CERs vary in chain length and can be saturated or monounsaturated. In addition, CER EO structure containing an esterified-oleic acid rather than linoleic acid is also identified. The CER O are those CERs bound to the CE. With some modifications this figure is adapted from [32].

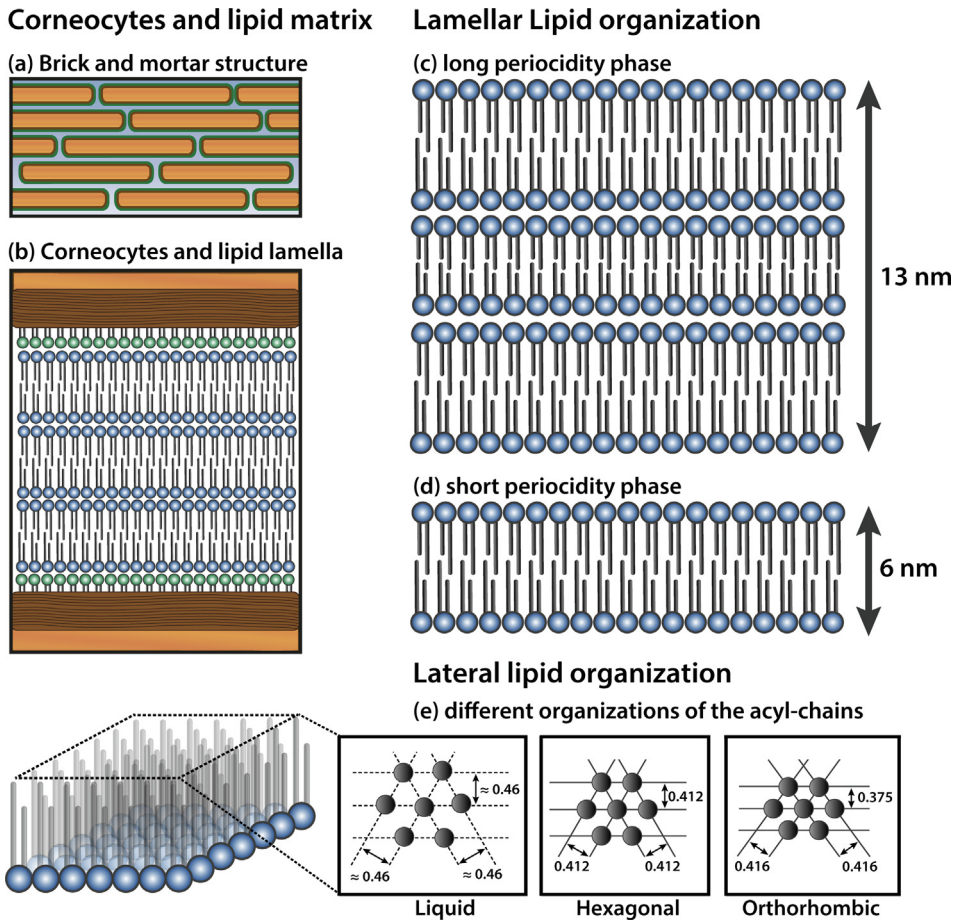

Fig. 3. Schematic presentation of lipid matrix organization in SC. (a) The SC is composed of corneocytes surrounded by a lipid matrix, referred to as the brick and mortar structure. (b) The corneocytes are covered by a cornified envelope. A monolayer of lipids (bound lipids) is attached to this cornified envelope. The free lipids adopt two lamellae phases, the long periodicity phase (LPP) shown in (c), and the short periodicity phase (SPP) shown in (d). The lipids within the lamellae assemble in (e) either a liquid (loose packing), a hexagonal (dense packing) or an orthorhombic packing (very dense packing). This figure is adapted with modifications from [32]

have been identified all differing in acyl chain and/or sphingoid base architecture. In Fig. 2 the 16 most prevalent CER subclasses are provided together with the nomenclature. [27] In each of the subclasses there is a wide acyl chain length distribution, while this is to a lesser extent also encountered in the sphingoid base. This results in a number of SC CER species exceeding easily 3000. [28]

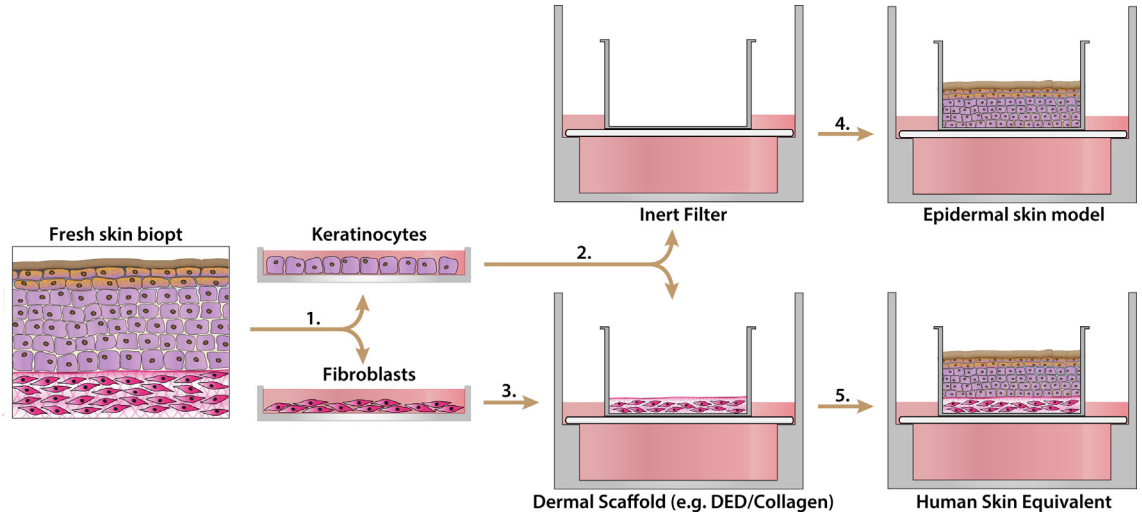

Fig. 4. Basic principles of generating human skin equivalents (HSEs). 1) cells are isolated from fresh human skin tissue and cultured until use; 2) fibroblasts are embedded in dermal scaffold (de-epidermized dermis (DED) or collagen matrix) and cultured under submerged condition; 3) keratinocytes are seeded onto the fibroblast-populated dermal scaffold, or onto an inert filter membrane; 4) the keratinocyte-seeded dermal matrix is cultured under specific conditions at the air–liquid until full stratification is reached.

Out of these subclasses, 4 subclasses have an exceptional structure with an unsaturated acyl chain ester linked to an ω-hydroxy acyl chain. [29] These CER subclasses, referred to as CER EO (also referred to as acylCERs) are particularly important as they play a prominent role in the formation of the LPP. [30,31] With respect to unsaturation in human skin, only a small fraction of the CERs have an acyl chain with an unsaturated bonding in the central part of the acyl chain. [32] With respect to the FFAs the chain length distribution is also very wide ranging typically between 14 and 34 carbon atoms. [33] In native human skin only a minority of the FFAs is unsaturated or hydroxylated. Besides the free lipids, there are also lipids chemically linked to the cornified envelope. The lipids (primarily ω-hydroxy CERs (CER-O)) do have the same precursors as the CER EO, but are esterfied with the ω-hydroxy acyl chain to involucrin. [34] Four bound CER subclasses have been identified, see Fig. 2.

3. Human skin equivalents

3.1. Human skin equivalents, a brief history

Cultivation of adult mammalian skin epithelium *in vitro* was described for the first time in 1948 [35] and was subsequently adapted by other groups. [36] It was demonstrated that cell suspensions obtained from slices of human epidermis after trypsin digestion can undergo long-term culture. However, the epithelium-like cells showed more resemblance with HeLa cells than keratinocytes. [37] In 1960 it was shown that isolated keratinocytes from adult guinea pig skin were able to form colonies in culture. This occurred when seeded at high density and in the absence of a dermal support. Surprisingly when seeded at low density and cultured under submerged conditions the cells had the tendency to differentiate. [36] In 1975 it were Rheinwald and Green who reported that lethally irradiated 3T3 fibroblasts could be used as feeder layers to generate cultures of human keratinocyte colonies that originated from a single keratinocyte. [38] In a follow up study a big step forward in the development of HSEs was obtained when lethally irradiated 3T3 fibroblasts were used as feeder layers to generate cultures of human keratinocyte colonies. [38] This scientific breakthrough allowed scientists to use large quantities of keratinocytes for tissue engineering purposes, which resulted in cultures of multi-layered keratinocyte-structures. [39] This paved the way for the development of the first *in vitro* HSE in 1976. [40] This HSE was further improved by mimicking physiological condition, such as the use of fibroblasts embedded in collagen or de-epidermized-dermis (DED) as support and culturing at the air–liquid interface, see Fig. 4. [41–44] These so called full thickness models (FTMs) were further improved and resulted in the generation of 3D cultures with a higher degree of differentiation, a better morphology and the presence of lamellar bodies that extruded their content at the interface between SG and SC. [44] Further optimization conditions, such as the use of serum free medium, growth factors, supplementation of vitamins and lipids, improved the morphology, lipid composition, organization and barrier function of HSEs, as described more detailed below. With respect to lipid composition and barrier function improvement, a major step was made by supplementation the culture medium with vitamin C (ascorbic acid) to the culture medium, which improved the lipid properties of the HSEs significantly. [45] Nowadays, many types of HSEs exist under a different nomenclature (e.g. skin models, raft-cultures, cultured skin substitutes, living skin equivalent and organotypic skin cultures), that include next to keratinocytes and different subsets of fibroblasts other cell types, like melanocytes, dendritic cells, Langerhans cells and T-cells. [46–49]. Many HSEs show similar epidermal morphology as native human skin. In line with this the expression of early and late differentiation markers, such as keratin 10 in the SB and the late differentiation markers, filaggrin and loricrin in SG, is very similar to that in *ex vivo* human skin. However, a closer look reveals that in *ex vivo* native skin the late differentiation marker involucrin is expressed only in the SG, while in HSEs it is already expressed in the SS. [50] Furthermore, most (if not all) HSEs harbour an activated epidermis, as expressed by the hyperproliferation-associated markers such as keratin 6, 16 and 17. This suggests that epidermal homeostasis has not been reached in most of the HSEs.

Nowadays several epidermal and FTMs are commercially available, the most important being Epiderm™ (MatTek, USA), SkinEthic™ RHE (L'Oreal, France) and EpiCS® (SkinInVitro GmbH, Germany). While FTMs are mostly used for research purposes, epidermal models are often used for screening and safety testing. [51,52] Therefore epidermal models have been validated according to European (EU) guidelines and implemented into the EU and Organisation for Economic Co-operation and Development (OECD) guidelines for testing dangerous ingredients for the skin. [51,53–56] Current fast developing technologies, such as, 3D-printing and skin-on-a-chip accelerated the development of novel high-throughput HSEs. However, it remains to be seen if these novel-technologies will render skin tissue with optimal skin architecture, functionality and barrier properties. First we will review the barrier properties of the HSEs. Many publications report on the barrier properties based on the localization and intensity of a fluorescent dye in the HSE. These measurements are often carried out as a

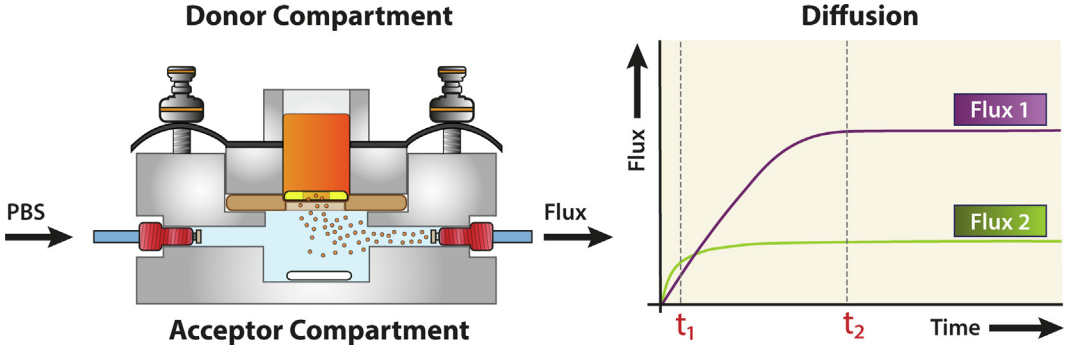

Fig. 5. Flux profiles into skin tissues with different barrier function. On the left-hand side a flow through diffusion cell is depicted. On the right-hand side two flux profiles of the amount of model drug transported *into* tissue 1 (flux 1) or *into* tissue 2 (flux 2). When determining the amount of model drug after a time period t_1 in each tissue, most of the drug accumulated in tissue 2. This often is interpreted as tissue 2 has an impaired barrier compared to tissue 1. However, when determining the accumulated amount of model drug after period t_2, most of the model drug accumulated in tissue 1. This is often interpreted as tissue 1 has an impaired barrier function compared to tissue 2. This demonstrates why measuring the accumulation of a fluorescently labelled compound after a single time point does not provide information about differences in skin barrier.

Table 1
An overview is provided about the permeation studies performed with HSEs together with the type of control skin used. The model drugs or water used for the permeation studies is also provided. In addition, information is provided about the lipid characteristics that are measured together with the references to the papers. Abbreviations not used elsewhere: lam. = lamellar phases examined with either small angle X-ray diffraction (SAXD) or electron microscopy (EM). Pack = packing of lipids; NHS = native human skin; HCS = human cadaver skin; HMS = hairless mouse skin; HSC = human stratum corneum; TEWL = trans epidermal water loss.

Ref	Drug(s)	HSE	control	lipid properties analysed				Remark: study focussed on
				Profile***	Lam.	Pack.	Bound CERs	
Schmook [57]	terbinafine clotrimazole hydrocortisone salicylic acid	SkinEthic grafSkin LSE	NHS Pig skin					
Lotte [58]	lauric acid caffeine mannitol	SkinEthic EpiSkin EpiDerm						Reproducibility of models studied
Roy [69] Nolte [87]	p-amino-benzoates	FTM (LSE)	NHS	x	x(EM)		x	
Michel [70]	benzoic acid	FTM	NHS					
Michel [71,90]	hydrocortisone sucrose caffeine	FDM**	HMS	x				
Mak [68]	Water	FTM-DED	NHS					
Kennedy [86]				x	x(EM)	x	x	
Asbill [73]	hydrocortisone caffeine tamoxifen		HCS EpiDerm HMS	x				
Batheja [74]	caffeine hydrocortisone ketoprofen DEET malathion paraoxon		NHS EpiDermFT	x				Medium addition Vit C, PPAR-α agonist, Lipids. Improved lipid profle
Dreher [60]	caffeine	EpiSkin	NHS					Various formulations
Boelsma [100] Ponec [96]	α-tocopherol	EpiDerm		x	x (EM, SAXD)		x	Used
Hammel [61]	naltrexone naltrexon-derivatives	EpiDerm	NHS					Metabolism studies
Netzlaff [64] Schreiber [62] Schafer-Korting [63] Schafer-Korting [65]	caffeine testosterone	Episkin	Full thickness NHS Spit thickness NHS HSC NHS epidermis	x				
Pasonen-Seppanen [75] Suhonen [77] Pappinen [103] Kuntsche [103,107]	TEWL corticosterone 18 compounds*	Rat FTM	NHS	x x	x (EM) x(SAXD)		 x	Study medium addition Vit C
Ackermann [66]	testosterone caffeine benzoic acid nicotine	Phenion	Pig skin					
Thakoersing [50] Thakoersing [108]	Para-ethyl amino benzoate	FTM Epidermal FDM**	NHS	x x	X (EM, SAXD)	X		
Mieremet [78]	TEWL	FTM		x	x(SXAD)			Chitosan added to collagen
Helder [32] Boiten [34]	TEWL	FTM		x	x(SAXD)	X	 x	Medium addition Liver X-receptor agonist/antagonist
Akagi [84]	aminopyrine antipyrine flurbiprofen isosorbide-5-mononitrate	FTM	NHS					Layer by layer approach to prepare the dermis

*raffinose, sucrose, mannitol,methylglucose, salicylic acid, glucerol, atenolol, sotalol, nadolol, pindolol, metoprolol, timolol, alprenolol, aldosterone, propranolol, corsticosterone, testosterone, β-esteradiol; **Fibroblast derived model; ***profile: either CER or barrier lipid profile or both.

one time-point measurement. As these are no kinetic studies, they cannot provide information on the barrier function. A further explanation why this is not correct is provided in Fig. 5. Therefore in this review we will only focus on reports describing permeation studies using diffusion cells.

3.2. Penetration studies using HSEs

3.2.1. Penetration studies using commercial HSEs

Several permeation studies were employed using commercial HSE models. In Table 1 an overview is presented of the commercial

as well as in-house HSEs used in penetration studies together with the lipid properties that were analysed. One of the first studies was performed by Schmook et al. [57] They compared SkinEthic™ and grafSkin™ LSE™ with pig skin and human skin. In all studies using the model drugs terbinafine, clotrimazole, hydrocortisone and dalicylic acid the HSEs were much more permeable than rat, pig and human skin, albeit that the difference for salicylic acid was less pronounced. Lotte et al. studied the reproducibility of the SkinEthic™, EpiDerm™ and Episkin™ models and observed a higher reproducibility for lipophilic drugs than for hydrophilic drugs. [58] In 2002 Dreher and co-workers compared the epidermal models EpidDerm™ and EpiSkin™ to native human skin using various vehicles and testosterone and caffeine as test compounds. [59,60] The rank order in permeability of the two commercial models were the same as found in human skin, but the permeability was much higher than human skin. A similar observation was reported by Hammell et al. [61] The group of Schaefer-Korting reported a pre-validation study in which again caffeine and testosterone were used in 3 different HSEs: SkinEthic™, EpiDerm™ and EpiSkin™, and observed for caffeine a much higher permeability compared to human and pig skin, while the permeability for testosterone was less different, but still around 4–10 times higher. [62,63] An extensive study was published by Netzclaff and colleagues, in which EpiSkin™ was compared with human full thickness skin, dermatomed skin, heat separated epidermis and trypsin isolated SC using the same model compounds. Consistent with earlier studies the permeability was higher for EpiSkin™ compared to the permeability of the differently prepared human skin membranes that actually had very similar barrier properties. [64] Another study reported permeability studies using hydroxycortisone (P = 1.43) and testosterone (P = 3.39) formulated in different vehicles. [65] They compared the permeability of SkinEthic™ (epidermal model) and EpiDermFT™ (FTM) with that of full thickness and dermatomed human skin. Both models were more permeable than native human skin. [65] A factor 2–4 difference in permeability was observed. In addition, a new full-thickness model (Phenion™) was studied and compared with pig skin. These data showed that the Phenion™ model exhibits for some permeants a high permeability and thus an impaired skin barrier. [66] In 2014 Fleischli et al. compared SkinEthic™ epidermal model with human skin *in vivo* using confocal Raman spectroscopy. [67] This enabled them to measure drug gradients after removal of the formulation. They used compounds with a large variation in P value (varying between −0.07 (caffeine) and 5.91 (octyl methoxy cinnamate). Independent of the P-values, in all cases the drug gradient in the SkinEthic™ model was much less steep than in the *in vivo* situation.

These studies consistently showed that commercial HSE are more permeable than native human or pig skin. During the last 6–7 years, no further development focusing on improving and testing commercial HSE models were reported with respect to barrier function and therefore, the use of these models for drug permeation studies cannot be recommended. Whether this is also the case for in-house HSE models is provided next.

3.2.2. Penetration studies using in-house HSEs

Mak et al. studied the permeability of an HSE cultured using an in house air-exposed 3D-DED cultures (FTM). [68] They used tritiated water and showed that the permeability of their FTM model was much higher than human skin. A reduction in humidity during culture and supplementation of vitamin D3 increased the skin barrier of HSE to some extent. Organogenesis, a biotech company focussing on wound care, published a permeability study using a FTM (in that publication referred to as living skin equivalent), in which methyl, ethyl and butyl *para*-aminobenzoate were used as model drugs. [69] Compared with human cadaver skin, the permeability of the FTM was much higher varying between a factor of 2.5 (most lipophilic compound) to around 10 (most hydrophilic compound). Despite the large differences in permeability, the ranking of the three model drugs was the same: the most lipophilic test compound had the lowest flux, the most hydrophilic test compound had the highest flux. This study showed that the models needed to be further optimized. It took several years before other permeability studies of in-house HSEs were reported.

Michel et al. introduced an engineered skin model. [70] Permeability studies were performed using benzoic acid, hydrocortisone, sucrose and caffeine. In all permeation studies the FTM had a significant barrier, but it was much lower than that of hairless mouse skin and human skin. [71] A few years later the group reported a FTM using fibroblasts that produced their own collagen on to which keratinocytes were seeded. The model was enriched with pilosebaceous glands, which improved the skin barrier, but the engineered skin was more permeable for hydrocortisone than the native human skin, but less permeable than mouse skin. [72] The group of Michniak reported an in-house FTM, which they compared to human cadaver skin and EpiDerm™. [73] In this study they used tamoxifen (lipophilic), hydrocortisone (medium lipophilic) and caffeine (hydrophilic) as test drugs. The in-house model provided an improved barrier compared to EpiDerm™ using tamoxifen and caffeine, but not for hydrocortisone. In addition, the in-house model was 2–5 times more permeable than human cadaver skin, showed a poor morphology as it did not show all distinct viable epidermal layers. No further characterization of differentiation properties were reported. In 2009 the morphology and the lipid composition of this in-house model was improved by supplementing the culture medium with vitamin C, fatty acids and a PPARα agonist: Hydrocortisone and caffeine flux was lowest when the 3 different supplements were added to the culture medium. [74] Despite this improvement, when comparing the barrier function using 6 drugs, the barrier of the in-house model was less efficient than that of native human skin and similar to that of EpiDerm™.

A series of interesting studies were reported by the group of Urtti. They developed an in-house epidermal model based on a rat keratinocyte cell-line. In their first publication, the flux of corticosterone with or without vitamin C supplemented to the culture medium was measured. [75] They observed that a longer culture period (resulting in an increase in the SC thickness) increased the difference in permeability between the two FTMs and vitamin C supplementation reduced the permeability substantially. In a subsequent study the permeability of 18 compounds varying in log (octanol/water concentration), referred to as P, between −4.3 and 3.9 were examined and compared with native human skin. [76,77] They observed a reasonable comparison with their rat epidermal model being more permeable by a factor 0.3 to 5.2 than human skin. Although all findings reported above were not directly compared with other HSEs in a single study, this rat epidermal model seems to enclose an improved barrier compared to the commercial models available in 2003, the year these data was published. A note for discussion is that the rat epidermal model was cultured air-exposed during three weeks resulting in a very thick SC of around 35 μm and this reduces the permeability.

Thakoersing et al. reported ethyl-para aminobenzoate fluxes of three in-house models in Leiden: an FTM model, an epidermal model (Leiden epidermal model) and a fibroblast-derived model, in which the fibroblasts produced their own ECM. [50] The composition of the culture medium in these three models was very similar. The FTM was most permeable (around a factor 4–5 more permeable than human skin), while the other models showed around 3-times higher flux than native human skin, see Fig. 6. Using the FTM a series of papers were published describing studies to improve the barrier function properties by adjusting the culture medium (supplementation of vitamin D3 or Liver X-receptor (LXR) antagonist), adjusting the environmental conditions (reduced

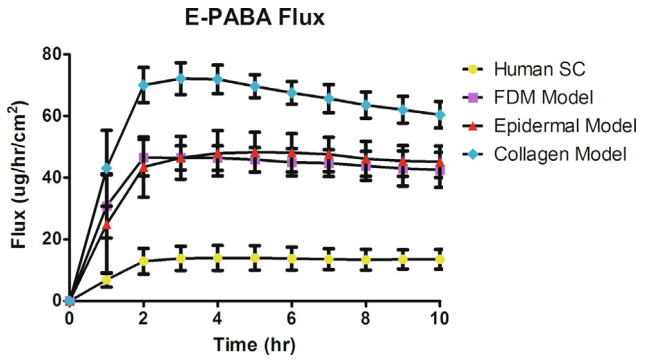

Fig. 6. Para-ethyl aminobenzoate diffusion profile. Para-ethyl aminobenzoate (Ethyl-PABA) was used as a model drug to investigate the permeability of SC of three HSEs, the FDM (fibroblast derived model), LEM (Leiden epidermal model), the FTM (full thickness model) and stratum corneum (SC) of native human skin. The data represent the mean and standarddeviation of at least four measurements. Reproduced with permission. [50]

humidity, culture temperature or oxygen level) or altering the composition of the dermal compartment [32,78–82]. Although optimizing the conditions improved the lipid barrier properties, the FTM remained more permeable than native human skin: In a very recent study by combining supplementation of a LXR antagonist in the culture medium and chitosan in the dermal compartment, the barrier function improved (Helder et al, unpublished results), but the skin barrier was not yet at the level of native human skin. In another study it was shown that supplementation of α-linoleic acid resulted in a slightly improved barrier for testosterone, but not for benzoic acid. [83]

Akagi reported HSEs engineered using a completely different method to generate HSEs, namely a layer by layer assembled extracellular matrix approach. [84] The HSEs showed an excellent morphology, and the dermal compartment contained also capillaries. Permeability measurements were performed in HSEs without the capillaries. Diffusion studies with HSEs were performed with compounds varying in P values between −1.55 and 1.61. The permeability of the tested drugs was a factor 5–10 higher in the in-house model compared to human skin. This shows that a completely other method of generation of HSEs did not result in an improved barrier.

In conclusion, the barrier function of most in-house models has not yet reached a level similar to the native human skin, although major improvements were made. However, for certain safety testing protocols HSEs are perfectly suitable, such as for predicting of corrosive effect/potential of compounds/chemicals. In this assay the entire barrier and underlying cells are being destroyed as long the compounds penetrates sufficiently through the skin. This can be very well measured in most epidermal models. However, for drug delivery studies, the prediction of drug penetration that are topically applied should be more accurate as for most drugs a specific permeation rate is required to achieve the desired therapeutic effect. If skin models are used for permeation studies one may argue that this may cause inaccurate prediction and compound attrition. Similar false prediction will also occur for formulations and for penetration enhancers if tested in models with an impaired barrier function. Information about the underlying factors of this reduced barrier function are important for finding ways to further improve this barrier. This will be reviewed in the next paragraph.

3.3. Underlying factors of the impaired barrier function

3.3.1. Lipid composition and organization in HSEs: Early studies

The first studies were mainly reporting differences between submerged and air exposed cultures and compared the results with native human skin, as shown in Table 2 providing a list of changes in the HSE culture conditions that resulted in an improved skin barrier. In 1988 the first studies focused on the lipid composition of keratinocytes that were grown on a DED air-exposed and submerged. [44] Air-exposure resulted in drastically increased lipid fraction of CERs (especially the level of CER EO) and in addition the longer chain FFAs compared to submerged conditions. Air-exposure is thus essential for an improved barrier. Furthermore, the level of oleic acid was too high, while the level of linoleic acid was too low. In a follow up study the intercellular lipids in SC of air-exposed DED cultures were visualized using freeze fracture electron microscopy. [85] The cultures clearly showed lamellar bodies in SG, terminal differentiation of keratinocytes and lipid lamellae filling the intercellular spaces. However, deviations in the appearance of lipid lamellae and corneodesmosomes were also noticed and when comparing to native human skin, higher amounts of triglycerides and oleic acid and lower amounts of CERs and linoleic acid were detected. Mak and co-workers showed that supplementation of 1,25-dihydroxy vitamin D3 and the use of DED instead of collagen resulted in an improved barrier, but deviating from that of native human skin. [68] In a subsequent study the underlying factors for this impairment were reported: an improper extrusion of the lipid lamellae into the intercellular regions and covalently bound lipids were virtually absent. [86] The FTM developed by organogenesis was also characterized. [69,87] Air-exposure created a SC with lipid lamellae in the intercorneocyte spaces together with disorganized lipid regions. The characteristic broad-broad narrow sequence (related to the LPP) as observed in RuO_4 stained native human SC (visualized by electron microscopy) was not observed. Levels of CERs were similar to that in native

Table 2
Changes in culture conditions that resulted in an improved lipid composition and/or organisation.

Induced culture changes	Improvement of the barrier lipids	Reference
Air exposure	Increased lamellar body extrusion Increase in CER synthesis (CER EO) Reduction in triglycerides	Mak [68], Ponec [44], Nolte [87]
Supplementation of fatty acids to culture medium	Increase in lipid synthesis	Boyce[91]
Reduction EGF in medium, less nutrition	Reduced cell activation Improved lipid organization	Gibbs [93], Pouliot [83]
Supplementation of Vitamin C to culture medium	Improved lipid lamellae: broad-broad-narrow appearance (EM), Formation of LPP, Improved CER synthesis Improved lamellar body extrusion Less triglycerides synthesed	Ponec [45], Pasonen-Seppanen [149]
Supplementation of Vitamin C, fatty acids, PPARα to culture medium	Improved CER composition	Michniak [74]
PPARα	Improved CER synthesis	Rivier [97]
Supplementation of Chitosan to the dermal compartment	Less cell activation, Improved CER composition	Mieremet [78]
Supplementation of LXR antagonist to culture medium	Reduction concentration unsaturated chains Improved bound lipid composition Increase in lipid chain length	Helder [32]
Supplementation of SCD-1 antagonist to culture medium	Reduction in concentration unsaturated chains	Helder [112]
Glycation	Increased in FFA, improved CER composition	Yokota [111]

human skin, but a strong reduction in the FFA level were reported confirming the findings by Ponec. [44] No information on CER subclasses was provided. The differences in lipid properties between native human skin and the organogenesis FTM probably contributed to the impaired skin barrier function reported by Roy. [69] They also showed that culturing at the air–liquid interface under reduce humidity resulted in an improved barrier, but that supplementation of lipids to the culture medium did not further improve the barrier properties. In 1994 Fartash and Ponec reported the lipid organization, morphology and lipid composition of their in-house air-exposed 3D-DED cultures and compared their model with the FTM Organogenesis. [88] Using transmission electron microscopy combined with RuO_4 staining, results of earlier studies were confirmed: an abnormal lamellar body extrusion process, disturbed formation of the lipid lamellae and an improper lamellar lipid organization. The lateral packing of the lipids in the in-house 3D-DED cultures was also examined and revealed a predominant (less dense) hexagonal packing contrasting the dense orthorhombic packing observed in native human skin. [89] In addition Michel et al. also analysed their FTM models and showed that the lipid composition was different from native human skin, irrespective of age of the donor. [71,90]

After it was shown that by air-exposure a SC was generated with intercellular lipid lamellae, the mainfocus was to improve the skin barrier further. Boyce and Williams reported that supplementation of a cocktail of various compounds including antioxidants, essential fatty acids and palmitic acid improved the morphology, increased the lipid synthesis and improved the lamellar body extrusion process. [91] Many years later, it was shown that deuterated palmitic acid supplemented to the culture medium could be traced back in the SC of the HSE. [92] Culturing at lower temperature (33 °C), omitting serum and reducing epidermal growth factor (EGF) concentration improved the lamellar organisation. [93] A similar approach was followed by Pouliot and co-workers and similar results were obtained: without EGF and serum a reduced keratinocyte activation was established resulting in improvement of barrier properties. Thus overactivation and/or excessive nutrition supplementation negatively affects the skin barrier. [94] The lipids in that model were more disordered than in human skin as revealed by the CH_2 stretching vibrations in the Fourier transform infrared spectroscopy (FTIR) spectrum. This is in agreement with the presence of a predominantly hexagonal lateral lipid packing instead of an orthorhombic one in native human skin. [89] It was not until 1997 that a major improvement in the barrier properties was achieved. Ponec et al. showed that adding vitamin C to the culture medium of an in-house air-exposed DED cultures and an in-house epidermal model resulted in a drastic improvement of the lipid composition, lipid organization and lamellar body extrusion process. [45] The amount of triglycerides reduced and the level of the most hydrophilic CER subclasses increased drastically. For the first time it was shown that lipids in the intercellular spaces formed primary the LPP. Permeability studies, however, were not published, but after a few years it was reported using corticosterone as test compound that vitamin C supplementation indeed improved the skin barrier function. [75,95] Later this improved barrier was confirmed by Batheja et al.. [74] In a subsequent study Ponec and co-workers analyzed the bound lipids. Two CER fractions referred to as CER A and CER B were identified. [96] Although not quantified, the spots after charring suggested that the quantity of bound lipids in HSE were similar to that in native human skin.

In 2001 the group of Urtti also showed, using an epidermal rat model, that supplementation of vitamin C improved the lipid and protein properties of the skin barrier of HSEs. [75,95] Keratohyalin granulae and the lipid lamellae were more abundantly present confirming the results of Ponec et al.. [45] The group of Michniak also showed that vitamin C supplementation normalized the CER composition in their in-house FTM. The group also observed that a combination of PPAR-α agonist, fatty acid and vitamin C supplemented to the culture medium improved the barrier, the lipid properties and histology of the HSE. [74] Riever et al. and Wallmeyer et al. supplemented PPARα agonist W14643 in their cultures to generate reconstructed epidermis. [97,98] Wallmeyer did not obtain improvements in the lipid properties, while Riever et al. noticed an increase in the CER synthesis. However, both studies used very high agonist concentrations in their medium.

3.3.2. Lipid properties of commercial HSEs

As described above, the available commercial HSEs showed an increased permeability compared to native human skin or pig skin. [59,60,64–66] In 2002 the morphology, lipid composition and lipid organization and the composition of bound lipids of these commercial models were reported. [96,99,100] The EpiDerm™ cultures showed a normal appearance of the SC, while the SC structure in EpiSkin™ and SkinEthic™ was very dense. Electron microscopy with RuO_4 staining revealed that all models exhibited lamellar stacks in the intercellular spaces in SC. However, some EpiDerm™ models showed an irregular lipid organization. Most probably in none of the commercial cultures vitamin C or derivatives thereof were supplemented to the culture medium as the most hydrophilic CER subclasses were almost absent. The FFA levels were very low and X-ray diffraction showed a weak presence of the LPP in EpiSkin™, while this lamellar phase was not observed in EpiDerm™. In 2014 the group of Baillet-Guffroy analyzed the EpiDerm™ model. CER subclass analysis revealed still a low concentration of the most hydrophilic CERs. [101] This strongly indicates that vitamin C or a derivative thereof was not yet added to the culture medium resulting in a highly permeable model. In addition, Raman spectroscopy showed an inhomogeneous distribution of the main lipid barrier classes, CERs, FFAs and CHOL. With respect to the Phenion model we observed an almost absence of all hydrophilic CER subclasses in 2012 (unpublished results). In 2015 Fleischli studied the CER quantity with confocal Raman and reported a higher level of CERs in SkinEthic™ than in native human skin. [67] Although confocal Raman spectroscopy is less accurate in quantifying the CERs than e.g. LC/MS this might indicate that nowadays vitamin C or one of its derivatives is used in some of these commercial cultures, but unfortunately no recent analysis of the detailed lipid profiles have been reported.

3.3.3. Current state of SC lipid properties of in-house HSEs

So far 7 CER subfractions could be separated using high performance thin layer chromatography (HPTLC) and the CER subfractions were separated by their hydrophilicity, referred to CER 1 to CER 7 in accordance to the sequence of appearance on a thin layer plate. From 2000 on, methods to analyse the lipids in SC improved and more details on the lipid properties in HSEs became available. In 2003 the various CER subfractions were further identified using NMR and gas chromatography. [102] In an epidermal model and native human skin at least 9 CER subclasses were identified composed of one of a sphingoid base (sphingosine, phytosphingosine and 6-OH-sphingosine base) linked with an acyl chains (non-hydroxy, α-hydroxy and ω-hydroxy acyl chain, see Fig. 2). Furthermore, while in native human skin, mainly linoleic acid is esterfied to the ω- hydroxyl chain of CER EO, in the epidermal model a variety of chain lengths with various degree of unsaturation were observed. [102] In 2008 the group of Uttri also focused on lipid analysis using their epidermal rat model. [103] They observed a similar ratio between CER, CHOL and FFA lipid classes as observed in native human skin. The similarity in lipid class ratio is remarkable as often the FFA levels were reported to be very low in HSEs. [44,85–87] No important differences in culture condition could be

noticed from their publication compared to conditions used in other groups, except that in their cultures keratinocytes from rat origin were used. In the rat models 7 CER subclasses were detected with the absence of CER NP and CER AP. The absence of NP and AP might be due to the rat keratinocyte origin, as e.g. in mice skin, pig skin and dog skin these CER subclasses are also less abundantly present than in native human skin. [104–106] Besides the linoleate in CER EO, also the oleate was abundantly detected similarly as observed by Ponec. [102] Covalently bound CER subclasses were also reported. The bound CERs were less abundantly present in the epidermal rat model compared to native human skin. For the first time liquid chromatography in combination with mass spectrometry (LC/MS) was employed providing information on chain length distribution. In some CER subclasses the chain length distribution was towards a longer chain length than in native human skin (CER NS and CER AS) and in other CER subclasses (CER AH an NH) it was the other way around. The epidermal rat model mimicked the native human skin barrier lipids more closely compared to all other models and it is the same model that exhibits reduced permeability compared to the commercial models. [77] The lipid organization was also assessed by small angle X-ray diffraction and revealed the presence of mainly the LPP. [107] Unfortunately, no information was provided about the lateral packing.

In 2012 Thakoersing examined the barrier properties of three in-house models (epidermal model, FTM and a fibroblast derived model). [50] A major difference between these models and native human skin is the hexagonal lateral lipid packing instead of the orthorhombic packing in native human SC. This confirmed studies dated back in 1995 using a DED-model and showed that vitamin C in the culture medium did not improve the lipid packing. [89] However, the lipid lamellae were excellently stacked and mainly a LPP was present. All CER subclasses that can be identified by HPTLC were present. A follow up study reported a more detailed lipid analysis. [108] The CERs were quantified with HPTLC and showed that the CER EO levels were increased compared to native human skin. The highest levels were found in the epidermal model. Differences between the models were also observed concerning FFA/CER ratios, but the ratios in the three in-house HSE models were much lower than in native human skin. With LC/MS the CERs and FFAs were analysed and 12 CER subclasses were present in all three HSEs similar as identified in native human skin at that time. [109] Furthermore, the chain lengths of CERs and FFAs appeared to be shorter than in native human skin. Unexpectedly, not only linoleic acid and oleic acid were present, but also unsaturated FFAs with a chain length between 24 and 34 carbon atoms were detected. The enzyme Stearoyl-CoA desaturase (SCD-1) that catalyses the synthesis of unsaturated fatty acids was abundantly present in all suprabasal layers of the epidermis, while in native human skin this enzyme was only present in the SB. This explains the high level of unsaturated lipids in HSEs. As CERs and FFAs have a common synthetic pathway, unsaturated levels of CERs were also expected to be present. This was indeed observed for CER NS and CER AS subclass. The LC/MS method to analyse CERs was further optimized. [110] With this new more sensitive method it became apparent that not only CER NS and CER AS were partly monounsaturated, but in all CER subclasses a substantial fraction of CERs were unsaturated. [32] Furthermore, the CER EO not only contained linoleate as unsaturated fatty acid chain, but a large fraction of this group of CER EO displayed oleate as the unsaturated fatty acid chain. This has consequences for the bound CERs. [102] Very recently the bound CER subclasses were analysed in details and 4 bound CER subclasses could be identified. The amount of bound CERs in the FTM was drastically reduced and the fraction of unsaturated CERs was substantially higher than in native human skin. [34] This is surprising and was not reported by Ponec, but is in agreement with studies performed by the groups of Urtti and Mak. [86,102,103] Ponec used HPTLC analysis; a high degree of unsaturation results in a more intense charring of the spots on the HPTLC plate resulting in an overestimation of the amount of bound CERs. Yokota et al. studied the effect of glycation of a 6 days old EpiSkin™ model. [111] Very interestingly glycation increased the FFA level, while CER NS/CER NP ratio decreased. Both changes are expected to improve the skin barrier. The increased level of FFA might be due to a reduced level of CER synthase 3 (CerS3), being an important enzyme to link the fatty acid with the sphingoid base. Very recently a series of papers were published aiming to improve the barrier function of FTM. This was done by either supplementation of compounds to the culture medium, (vitamin D3, palmitic acid and liver X-receptor agonist/antagonist), by changing the environmental conditions (humidity, temperature and oxygen levels) to mimic more closely the *in vivo* conditions or by stabilizing the dermis by adding chitosan to the dermal compartment. [32,78–82] Although reduction in humidity, oxygen level and temperature during culture affected the generated FTM, it did not improve the skin barrier. Modifying the dermal matrix by chitosan supplementation reduced the proliferation and activation of keratinocytes and improved the CER profile by reducing the CER NS and CER AS levels, and increasing the average chain length of the CERs. [78] This resulted in a significantly improved skin barrier as monitored by trans epidermal water loss. The liver X-receptor (LXR) stimulates the lipid synthesis and activates genes such as stearoyl-CoA desaturase-1 (SCD-1) which is upregulated in HSEs. By supplementing the LXR antagonist GSK2033 to the culture medium, the expression of SCD-1 (gene and protein level) and the fraction of unsaturation in FFAs and CERs reduced significantly, while CER chain length was increased, thereby improving the lipid organization slightly. [32] Based on these results, another attempt was to supplement the culture medium with an SCD-1 antagonist. [112] Although the concentration of unsaturated CERs and FFAs decreased, there was no effect on chain length of these lipids, which makes this supplementation less effective than LXR antagonist GSK2033 supplementation. In a subsequent study supplementation of the LXR antagonist was combined with the chitosan modified dermal matrix. The studies revealed an improved lipid composition and an increased fraction of lipids forming the orthorhombic lateral packing. (Helder et al, unpublished results) Simultaneously the barrier function was improved, but was not at the level of that in native human skin. Interestingly, an increase in the fraction of lipids forming the orthorhombic packing was associated with an increase in the functional skin barrier, suggesting that one way to go to increase the skin barrier is to improve the lateral packing of the lipids. Recently Simard showed that supplemented essential fatty acids in culture medium are taken up by the epidermis and stored in the phospholipids improving the skin barrier of this model for testosterone. [83] The phospholipids are precursors of the fatty acids in the epidermis. Therefore, it would be of interest to analyse the FFAs, but also the CERs in these HSEs.

Despite the efforts to improve the barrier function of HSEs and the progress that has been made, the barrier function is not at the level encountered in native human skin. When combining the results, in most of the models as far as the lipids are concerned the CER profile is different from that in native human skin: the CER fraction of CER EO, CER NS and of CER AS are increased, while the fraction of CER NP is strongly reduced. Furthermore, the mean chain length of FFAs and CERs is shorter and the FFAs levels are too low, an exception is the model developed by the group of Urtti with a balance between the amounts of FFA and CERs. [103] Based on studies using skin lipid membranes mimicking several aspects of the lipid phase behaviour in human SC, the

increased CER EO, CER NS and CER AS, and the low level of CER NP encountered in the models are expected not to be the main factor to improve the skin barrier. [113,114] The low level of FFAs and the shorter chain lengths of CERs and FFAs are probably key factors contributing to an increased permeability in these HSEs as this prevents the formation of an orthorhombic lateral packing. [106,115] With respect to the bound lipids the low levels of bound CERs with a higher degree of unsaturation are observed in the FTM. As the bound lipids are an essential factor in the skin barrier, this may also substantially contribute to the impaired skin barrier facilitating compounds to partition into the corneocytes making the *trans*-cellular pathway more prominent. This should affect the hydrophilic compounds more effectively than the lipophilic ones and this is indeed observed in HSEs. However, it seems that the lipid lamellar stacks are oriented similarly as in native human skin indicating that the reduced amount of bound lipids can still serve as a template to orient the lipid lamellae in the intercellular space. [50]

3.4. Role of cornified envelope in skin barrier

The cornified envelope is the other key player for a proper skin barrier function. The cornified envelope consists of a large number of structural proteins and is assembled during terminal differentiation at the interface between SC and SG. Key structural proteins are involucrin, loricrin, small proline rich proteins (SPRR), envoplakin, pariplakin and profilaggrin. [116,117] Loricrin makes up around 80% of the cornified envelope [117]. The assembly of these proteins is a complex process. In a first step in the formation of the cornified envelope involucrin, evoplakin and periplakin form heterotetramers catalyzed most probably by the enzyme transglutaminase 1. This complex is formed close to the plasma membrane. During this process and after the lamellar body extrusion process, the CERs are linked to these three proteins forming the bound lipid envelope. [118] Finally loricrin is translocated at the periphery and crosslinked with the scaffold by the enzyme transglutaminase 3. [119] Other structural proteins, such as profilaggrin, are also incorporated in this complex. In the early studies the group of Steinert used an *in vitro* model of submerged keratinocytes differentiated for one week. [120–122] Although several key structural proteins were synthesized and crosslinked in this model, which made this an attractive system to study the assembling process, in these models no terminal differentiation was obtained. To our knowledge there are no studies on the composition of the cornified envelope in terminal differentiated models generated air-exposed. However, several papers indicate that the cornified envelope in epidermal and full thickness models might not fully mimic the situation in native human skin. For example, the level of natural moisturizing factors, in which the degradation product of filaggrin (pyrrolidone carboxylic acid is a major component) is significantly reduced in HSEs compared to native skin, even when it is cultured at reduced humidity. [67,123–125] This is not due to a reduced synthesis of filaggrin as this structural protein is expressed in the epidermis similarly as in native human skin. Another difference is the protein expression of involucrin, in native human skin it is only expressed in SG, but in HSE involucrin is also expressed in SS. However, only mild changes are observed in loricrin knock-out mice showing that down regulation of one component might induce compensatory mechanisms. This indicates that several aspects in the process of forming the cornified envelope should be impaired in order to affect the skin barrier. [126] For example a triple knock-out of involucrin, envoplakin and pariplakin leads to an abnormal cornified envelope. [127] Finally the bound lipids are present in much lower quantities in the HSEs than observed in native human skin as already reported. [34,86,103]

4. Discussion

In the last decades a tremendous amount of work has been put in the engineering and optimization of human skin *in vitro*, which resulted in many in-house and commercially available human skin models mimicking native human skin to a high degree. When examining morphology, most of these HSEs harbour a well-developed epidermis consisting of a SB, SS, SG and a SC. However, a closer look reveals large differences in epidermal homeostasis and as a consequence an altered skin barrier function. In this review, we have discussed 3 main topics, including; i) homeostasis of in-house and commercial available HSEs, ii) current status of HSEs and their barrier function in penetration studies, and 3) underlying crucial factors that may influence skin barrier. Unfortunately currently no HSEs exist that fully recapitulate the *in vivo* human skin barrier, and thus improvements at different levels are still needed. The studies presented in this review also demonstrate that skin barrier biology is very complex which cannot be ignored during HSE development. In addition, homeostasis of the skin and lipid properties, including lipid organization, lipid composition and cornified envelope composition need to be considered as one system rather than as individual entities. Consequently, small changes (some examples discussed below) in one of these entities can lead to HSEs with larger changes in the other entity. Although differences exist in the barrier properties of these HSEs and one model might be better mimicking the native human skin than another model, all models seems to suffer from one or more unknown factors in the culture conditions that need to be adjusted. Nevertheless in recent years these HSE have been used as models for diseased skin. An important advantage of the use of HSEs is the possibility to study the effect of isolated factors on the barrier function. This can be done by using keratinocytes that either overexpress genes or in which the genes are knocked-down. Another possibility is to supplement compounds (e.g. cytokines) to the culture medium. [128–133] In addition, a HSE is an excellent tool to examine the effect of the human microbiome on the barrier properties.[134,135] However, in performing these studies one has to take into account that differences still exist between native human skin and HSEs. For example generated HSEs prepared from keratinocytes isolated from diseased skin will have different (lipid) barrier properties due to the selected culture conditions than the original donor skin from which the keratinocytes were isolated. Below we will summarize a few possible paths of which we believe may contribute to an improved barrier function in HSEs.

The SC lipid composition undoubtedly plays an important role in the skin barrier function. Both a reduction in chain length and an increase in the fraction of unsaturated FFAs and CERs and in addition changes in CER subclass composition were all noticed in HSEs and may contribute to an impaired skin barrier function. [32,102,103] However, when supplementing a LXR antagonist to the culture medium during generation of the HSEs, a reduced level of unsaturation in CERs and FFAs was achieved and an increase in the chain length of the CERs was noticed (except for CER EO subclasses). However, these changes did not drastically improve the SC lipid organisation and barrier function of the HSEs. Therefore a further reduction in the level of unsaturated lipids or a further increase in lipid chain length is not expected to be the key factor to normalize the skin barrier function. Other routes to improve the skin barrier should be explored.

One concern is the low level of FFAs in SC. In studies so far, the HSEs with the most optimal barrier function displayed a relatively high level of FFAs in their SC, but also harbour a thick SC. [76,103,136] Unfortunately the lateral packing in these cultures was not monitored and therefore it remains unclear whether the lipids in these cultures form a dense orthorhombic packing. However when taking the results from model lipid systems into account, an orthorhombic lateral packing is expected in these HSEs. [137] A predominant presence of an orthorhombic packing will contribute to normalizing the skin barrier function as illustrated in several lipid model systems. [106,137,138] Often a cocktail of fatty acids (including essential fatty acids) are supplemented to the culture medium, which improved the quality of the HSEs, but did not increase the fatty acid levels toward those found in native human skin. [83,91,139,140] The underlying factors that contribute to a reduced level of FFAs is still unknown. One possibility is the synthesis of a high fraction of monounsaturated FFAs that are incorporated in triglycerides via storage enzymes. However, a variation in the amount of palmitic acid in the culture medium during generation of HSE did not result in an alteration in gene expression of storage enzymes involved in the synthesis of triglycerides. [141] In addition, supplementation of the LXR antagonist reducing the synthesis of monounsaturated lipids would increase the level of FFAs, but this was not observed. [32] Another possibility is a reduction in de novo fatty acid synthesis in keratinocytes. However, as the source of fatty acids is also the basis for the CER synthesis, a reduction in CER levels would also be expected, but this has not been reported. [32,44,87]. Furthermore on gene level the fatty acid synthesis enzymes do not exhibit reduced levels. [32] Another possibility is an increased activity of CerS3, that catalyses the linkage of the fatty acid to the sphingoid base. [142] A comparison of the gene level of CerS3 between HSEs and native human skin has not been documented yet. However the ELOVL4 gene levels, the enzyme involved in the synthesis of very long acyl chains (>C28) was increased in HSEs. [32,143]. As there is a feedback mechanism between the activity of CerS3 and the activity of ELOVL4, an increased gene level and possibly activity of CerS3 remains a possible factor and has been suggested by Yokota et al. to explain the increase in FFA level after glycation. [111,142] However, it does not explain the reduced level of long chain fatty acids in HSEs. Possibly most of the very long chain FFAs are primarily used for the synthesis of CER EO, that are more abundantly present in HSEs than in native human skin. To explore this in more details might open promising paths to go.

Mieremet et al. demonstrated that supplementation of chitosan to the dermal matrix reduces the activation of epidermal keratinocytes and lowers the proliferation rate of keratinocytes in the basal cell layer resulting in an epidermis being closer to homeostasis. [78] The chitosan containing FTMs showed an improvement in CER composition and barrier function as monitored by trans epidermal water loss. [78] Whether this is also in favour of normalizing the FFAs/CERs ratio has not been reported yet as no FFA analysis was performed. However, in combination with the LXR antagonist supplementation to the culture medium, chitosan in the dermal compartment resulted in an increased fraction of the lipids forming an orthorhombic packing. As LXR antagonist supplementation only did not induce the formation of the orthorhombic packing, the chitosan containing dermis may be responsible for this improvement (Helder et al., unpublished results). Therefore creating HSEs with an epidermis close to homeostasis might be a promising route to normalize the skin barrier.

In several studies an increased amount of oleic acid was reported compared to the levels in native human skin, while the amount of linoleic acid was reduced. [44,85] The lack of linoleic acid has consequences for the unsaturated moiety of CER EO subclasses. The excess of unsaturated fatty acids are stored in triglycerides acting as source for the unsaturated esterified acyl chain of the CER EO subclasses. Consequently as already reported in 2003, and recently in more details, while in native human skin the unsaturated chain of the CER EO is predominantly linoleate, in HSEs a large fraction of these subclasses contain oleate or even acyl chains with a shorter chain lengths. [32,102] The consequences for the lamellar phase is limited as CER EOS with either an ester linked oleate or ester linked linoleate results in a similar lipid organisation. [144] However, it has huge consequences for the bound lipids. The precursors of the bound lipids and the CER EO subclasses are the glucosylceramide EO subclasses [143,145]. After releasing the lipid content of the lamellar bodies into the intercellular space, esterification of the glucosylceramide EO to the cornified envelope requires a multistep process: 12R lipoxygenase enzyme ALOX12B and lipoxigenase 3 enzyme ALOXE3 convert the linoleate into a hydroxyl-epoxide ester. [146–148] These degradation steps do not take place when an oleate moiety is ester linked to the ω-hydroxy acyl chain of CER EO, and explains the low level of bound lipids attached to the corneocyte envelope in HSEs. Although often during generation of HSEs linoleic acid has been supplemented to the culture medium and is incorporated in the phospholipids, triglycerides and ceramides, the incorporation of linoleic acid in the CER EO seems to be *in vitro* less efficient than in the *in vivo* situation. [83,139] To improve the skin barrier function, this may be another crucial factor.

Next to the possible modifications proposed above, there are several future challenges and opportunities to improve the barrier function of the HSEs. One approach is to convert current static HSE culturing towards a fluid-flow system in which culture media is pumped around between the inserts. One may argue that continuous flow of culture medium may avoid excessive accumulation of waste possibly affecting skin development and as consequence barrier properties. In addition, all commercial available HSEs harbour keratinocytes, melanocytes and fibroblast, while native skin consists of many other cell types. Therefore the epidermis could be enriched with dendritic cells, and the dermal compartment with immune cells (T, B cells, macrophages), endothelial cells, different fibroblast subpopulations (e.g. papillary and reticular) and by incorporation of hair follicles (e.g. dermal papillae, sebaceous glands). In concert with these culture adaptations, environmental factors, such as temperature, humidity, barrier function may be improved in these new generation of HSEs. Lastly genetic engineering of genes involved in epidermal cell differentiation using the CRISPR/Cas9 editing tool could open new avenues for the design of novel robust and representative HSEs, which eventually may replace animal skin permeation studies fully.

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