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REVIEW

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3D skin models for 3R research: The potential of 3D reconstructed skin models to study skin barrier function

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Abstract

The skin barrier is an important shield regulating the outside-in as well as inside-out penetration of water, nutrients, ions and environmental stimuli. We can distinguish four different barrier compartments: the physical, chemical, immunological and microbial skin barrier. Well-functioning of those is needed to protect our body from the environment. To better understand the function and the contribution of barrier dysfunction in skin diseases, 3D skin or epidermal models are a valuable tool for in vitro studies. In this review, we summarize the development and application of different skin models in skin barrier research. During the last years, enormous effort was made on optimizing these models to better mimic the in vivo composition of the skin, by fine-tuning cell culture media, culture conditions and including additional cells and tissue components. Thereby, in vitro barrier formation and function has been improved significantly. Moreover, in this review we point towards changes and chances for in vitro 3D skin models to be used for skin barrier research in the nearby future.

KEYWORDS
3D skin, microbiome, skin barrier, stratum corneum, tight junctions

1 | HUMAN SKIN: MORE THAN JUST A PHYSICAL BARRIER

To understand the need and requirements of models to study the skin barrier function, it is important to first address the different barrier functions of human skin and their role in maintaining tissue homeostasis. From the outside-in, the skin protects against diffusion of molecules, chemical exposure, ultraviolet (UV) radiation and penetration of pathogens. From the inside-out, passage of water and electrolytes is prevented thereby protecting the body from dehydration. The stratum corneum (SC) and tight junctions (TJs) form the physical barrier.[1,2] While the SC exerts most of the defensive functions of the epidermis from outside-in, TJs are the first physical barrier from inside-out. In case of a barrier defect, the Langerhans cells, keratinocytes and skin resident immune cells join forces to provide a secondary immunological barrier. The release of defense molecules by epidermal keratinocytes contributes to the chemical barrier function of the skin. Last but not least, the skin microbiome can be considered as a microbial barrier. We will briefly outline these four barrier functions which are illustrated in Figure 1.

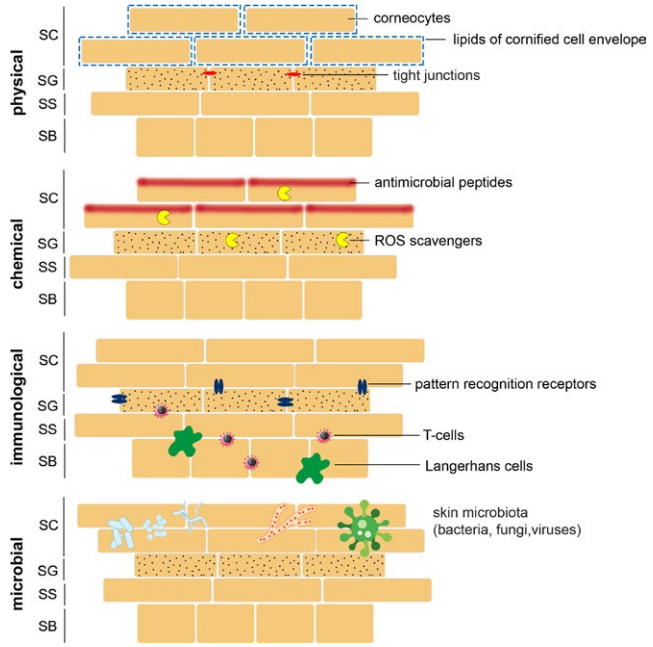


FIGURE 1 Skin barrier compartments. The human epidermal barrier can be divided into four compartments: the physical, chemical, immunological and microbial barrier. The schemes show the most important cells, molecules or micro-organisms that contribute to each particular barrier function and where those are located within the different epidermal layers (SC = stratum corneum, SG = stratum granulosum (black dots represent keratohyalin granules), SS = stratum spinosum, SB = stratum basale)

1.1 | Physical barrier

The SC consists of about 15-20 stacked layers of corneocytes with intermediate lipid layers. During the terminal differentiation process, viable keratinocytes transform into dead and dying cells that have lost their nuclei and cytoplasmic organelles, which are now called corneocytes. Terminal differentiation of keratinocytes is tightly controlled and enzymes like transglutaminases (TGases)[3] and protease inhibitors like cystatin M/E control the cornification and desquamation process.[4] The lipids form an integral part of the physical barrier, and the final steps in synthesizing the lipids occur at the interface between the viable epidermis and SC. During the differentiation process, keratinocytes express distinct epidermal proteins in the different layers of the epidermis. This feature is very useful for the distinction of the layers within the epidermis of human skin models and to check whether the in vitro generated epidermis faithfully mimics native skin. Additionally, the viable epidermis also contributes to the skin barrier function by adherens junctions, TJs and desmosomes. Adherens junctions and desmosomes are not directly linked to skin barrier function but are important for keratinocyte adhesion and differentiation that is needed for epidermal integrity. TJs are crucial for skin barrier function as they are crucial for the inside-out barrier function.[5,6] TJs are formed in the granular cell layer by several TJ proteins that are all expressed at specific layers of the epidermis, indicating the complexity of the skin barrier formation.[7]

1.2 | Chemical barrier

The skin is equipped with antimicrobial peptides (AMPs), produced by the keratinocytes, but also immune cells, that protect against bacterial infections. The most well-known keratinocyte-derived AMPs are defensins, S100 proteins, human cathelicidin LL-37 and late cornified envelope proteins. Other skin AMPs are dermcidin, SKALP/elafin and secretory leukoprotease inhibitor (SLPI). Most of these AMPs are absent or expressed at low levels during skin homeostasis. In more vulnerable areas like the hair follicles (port d'entrée) or internal epithelia (oral cavity, vagina), these AMPs are expressed at higher levels to contribute to host defense.[8]

The antimicrobial activity of reactive oxygen species (ROS) released by keratinocytes contributes to the chemical skin barrier but may also harm the host, for example when produced by excessive UV radiation. For protection, the skin produces antioxidants like vitamins C and E, and glutathione[9-11] and produces ROS scavengers (eg superoxide dismutase, catalase, peroxidase). Additionally, it was shown that small proline rich (SPRR) proteins are highly effective in quenching ROS in the epidermal cornified cell envelope, thereby contributing to epidermal protection.[12]

1.3 | Immunological barrier

In the dermis but also in the epidermis, cells and molecules from either the innate or adaptive immune system are present to protect against a large variety of pathogens, for example via the expression of pattern recognition receptors that can bind microbial-derived ligands to induce an immune response. Not only immune cells, like Langerhans cells or patrolling T cells in the epidermis, contribute to epidermal host defense, but also keratinocytes are potent producers of soluble immunomodulatory factors, like cytokines and chemokines that lead to or dampen inflammation, or can modulate T cells and dendritic cells.[13] The interplay between keratinocytes and immune cells is vital for tissue homeostasis and inflammatory processes, and has been the subject of research for decades. In the skin, there are twice as much T cells present than in the bloodstream,[14] indicating the importance of the skin as an immunological reservoir.

1.4 | Microbial barrier

The microbial barrier is formed by the commensal skin microbiome. The skin microbiome is defined as all microbiota that are present on our skin, mainly consisting of bacteria but also including fungi and viruses. Our skin commensals serve as a barrier to prevent infection by pathogenic microbes. A well-balanced microbiome contributes to our health, and it is shown that imbalance, with a shift towards pathogenic microbiota, is related to disease.[15,16] The complex host-microbe and microbe-microbe interactions on the surface of human skin, in health and disease, are poorly understood and subject of current research.

2 | REVIEW OUTLOOK AND AIMS

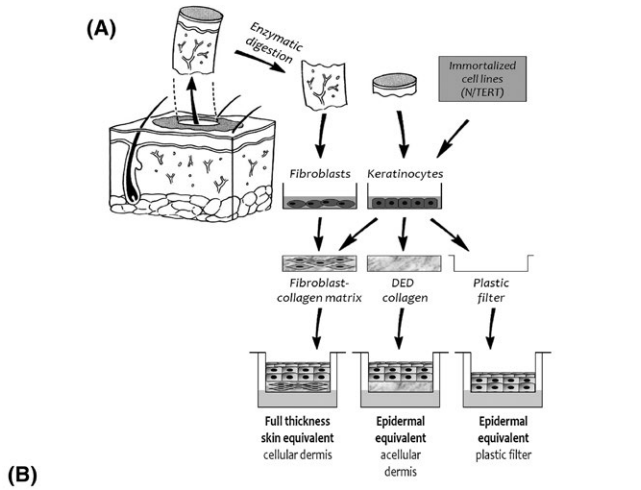
To study skin barrier function in health and disease, we need model systems in which normal skin biology and the effect of disease-related factors can be studied. The search for appropriate in vitro skin models has started decades ago and is ever rising due to limited availability of human skin, and ethical issues with regard to animal experimentation. While in vitro 3D skin models have been extensively used to study the physical barrier of the skin, the chemical barrier function of the epidermis with regard to AMPs or ROS-neutralizing molecules is less well studied. Although it is common knowledge that antimicrobial peptides are produced and secreted by keratinocytes, functional studies are lacking mainly due to the very few studies describing the incorporation of microbiota in 3D skin models. This is, however, a crucial step to take in the coming years. The same holds true for the immunological skin barrier. Three-dimensional skin models including immune cells like T cells or Langerhans cells have been reported[17-20]; however, it is challenging to mimic the dynamic patrolling of immune cells between the skin and the lymphatic system in a static in vitro system. Current developments to generate skin-on-a-chip platforms by microfluidics technology could enable the analysis of the immunological barrier function by immune cells that recognize and eliminate pathogens in the skin.[21]

In this review, we will discuss the development of 3D skin models over the years and highlight the potential of these models to study the physical and microbial barrier function of the skin. With this overview, we aim to provide a direction for future research in these fields and to enable studies on the chemical and immunological barrier as well.

3 | THE DEVELOPMENT OF IN VITRO 3D MODELS FOR HEALTHY SKIN: AN HISTORICAL OVERVIEW

The first methods that describe the separation of human skin (epidermis from the dermis) and the isolation and culturing of human keratinocytes were developed in the early fifties and lie at the heart of the knowledge we have today to generate reconstructed skin models.[22] Cultivation of adult mammalian skin epithelium in vitro was described for the first time in 1948[23] and was subsequently adapted by other groups.[24] It was demonstrated that cell suspensions obtained from slices of human epidermis after trypsinization can undergo long-term culture; however, the epithelial like cells showed more resemblance with HeLa cells than keratinocytes.[25] In 1960, it was shown that isolated keratinocytes from adult guinea pig skin were able to form colonies in culture, even in the absence of a dermal support, when seeded at high a density, while under submerged conditions cells seeded at lower densities had a tendency to differentiate.[24] A huge milestone was achieved by Rheinwald and Green in 1975 who used lethally irradiated 3T3 fibroblasts as feeder layers to generate cultures of human keratinocyte colonies that originated from a single keratinocyte. This discovery allowed scientists in the field of dermatology to generate large quantities of keratinocytes for in vitro cell culture studies and paved the way for the treatment of burn wound patients.[26-29] Initiated by the seminal work of the Rheinwald and Green laboratory, the monolayer culture of human keratinocytes on plastic culture plates has been the main technique to study skin biology and pathophysiology in vitro. Rheinwald and Green were also the first to describe that such monolayer cultures can differentiate and form multilayered structures.[30] Simplicity, high-throughput and reproducibility are major advantages of monolayer keratinocyte cultures. However, many features of a fully stratified epidermis are lacking in this model and keratinocytes are forced to adapt to artificial circumstances, like a flat surface and submerged culture, which may alter gene expression and cell function.[31] To study cell-cell interactions, regulation of proliferation and differentiation, wound healing, skin barrier function and skin-microbiome interactions, 3D skin models better resemble the natural architecture and functions of the skin and should be considered as the gold standard when performing in vitro studies on human skin.

One of the first explant 3D model was described in 1976 when inverted dead pig skin was used to establish outgrowth of keratinocytes.[32] This method was improved by the use of collagen matrices to culture keratinocytes at the air-liquid interface.[33,34] This study set the stage for generating a better differentiated epidermis on human de-epidermized dermis (DED) while preserving the basement membrane proteins.[35] Ponec et al further improved this DED to culture keratinocytes that attach to the existing basement membrane.[36] From the eighties on, culture protocols were optimized and improvements were made resulting in many different types of human skin equivalents (HSEs) and human epidermal equivalents (HEEs), also designated as organotypic cultures, cultured skin substitutes or living skin equivalent, with different types of dermal substrates (eg inert filters, DED, collagen matrices, lyophilized collagen-glycosaminoglycan GAG membranes and fibroblast derived matrices)[37-42] (Figure 2). HSEs serve as an excellent alternative to animal experimentation, which resulted (due to public pressure) in 2013 in the ban of testing cosmetic ingredients in animals. Nowadays, different types of HSEs exist harbouring other cell types like melanocytes, endothelial cells, Langerhans cells and immune cells.[20,43-45] These enriched HSEs are mostly used for research purposes to study interactions between cells and their microenvironment and open new opportunities in the field of tissue engineering and wound healing.[46-48] Next to the "in-house" HSEs, several commercial skin equivalents are available such as EpiCS® (CellSystems, Germany), Epiderm™ (MatTek, USA) and SkinEthic™ RHE (L'Oreal, France), that are used for basic research and toxicological screenings.[49-52] These 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.[49,53-58] Currently, the Organisation for Economic Co-operation and Development (ECVAM) is also putting effort in replacing the local lymph node assay (LLNA) by HSEs to predict and discriminate between skin sensitizers and irritants.[59-62] At present, the development of HSEs is accelerated due to novel technologies such as 3D printing and skin (organ) on a chip.[63-65] These technological advances enable the combination of multiple



(B)

Morphology	Barrier genes	Barrier proteins	Barrier function
Histology Imaging Stratification SC thickness	Knock-down siRNA/shRNA Transgene mRNA/qPCR	Tight junctions Cornified envelope IHC Western blot	SC dye penetration Lucifer yellow Biotin preparations SC permeation Diffusion cell Transepithelial electrical resistance Lipid classes Thin layer chromatography Mass spectrometry Lamellar phases X-ray diffraction Lateral packaging Fourier transform infrared spectroscopy

FIGURE 2 Skin barrier analyses in 3D skin models.(A) After skin biopsy, the epidermis can be separated from the dermis to isolate both keratinocytes and fibroblasts. To generate a 3D skin model keratinocyte can be grown on a cellular matrix (fibroblast-collagen matrix), acellular matrix (de-epidermized dermis, DED) or inert plastic filter. For several days, the keratinocytes are grown in submerged cell culture after which the culture medium level is lowered for culture at the air-liquid interface. About two weeks later, a multilayered stratified epithelium is formed which is similar to in vivo skin/epidermis. (B) List of diverse read out parameters that can be assessed to determine the skin barrier function of in vitro skin models

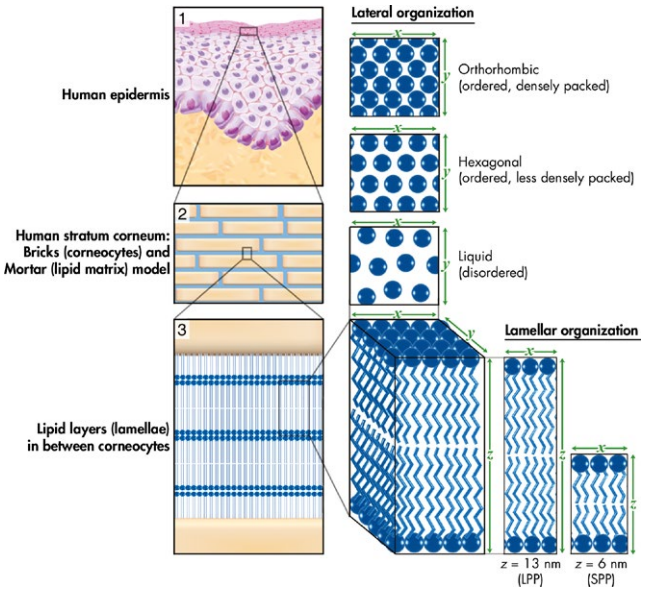


FIGURE 3 Stratum corneum lipid organization. In the intercellular spaces between the corneocytes, the lipids are arranged in stacked layers (lamellae), with two coexisting lamellar phases, either 6 nm (SPP) or 13 nm (LPP). Within the lipid lamellae, the lipids are arranged in a very dense ordered orthorhombic organization, a less dense ordered hexagonal organization or a disordered liquid organization. The former is predominantly present in healthy human SC. The figure has been adopted from J. van Smeden, thesis June 2013 entitled: A breached barrier:analysis of stratum corneum lipids and their role in eczematous patients

cell types and a dynamic fluid flow, but it remains to be seen whether optimal skin morphology and function can be achieved and monitored in these technical platforms. It will be of utmost importance to validate these novel skin models and determine their suitability to study skin barrier function like it has been carried out for the past decades for the HSEs and HEEs, which we will discuss below.

4 | 3D SKIN MODELS TO STUDY THE PHYSICAL SKIN BARRIER: FROM STRATUM CORNEUM LIPIDS TO TIGHT JUNCTIONS

4.1 | Stratum corneum lipid composition of native skin and differences with 3D skin models

A major part of the physical barrier function of the skin resides in the SC consisting of corneocytes embedded in lamellar regions. As the intercellular SC lipids are crucial for a proper skin barrier function, this section focuses on the lipid barrier as an important part of the physical skin barrier. The major lipid classes are ceramides, free fatty acids (FFAs) and cholesterol.[66] The lipid assembly of native human skin is mainly orthorhombic, which is further explained in Figure 3.[67-69] In a first attempt to create a skin barrier, keratinocytes were seeded on collagen gels or collagen-coated filters and cultured at the air-liquid interface, but the SC was not properly formed.[35] Next, keratinocytes were combined with human de-epidermized dermis[35,70-73] or collagen gels populated with fibroblasts[72,74] which improved tissue architecture, lamellar body extrusion and the SC formation.[75,76] However, lipid assembly was yet not optimal.[67,75] Further optimization by vitamin C supplementation substantially improved the lipid processing and resulted for the first time in the synthesis of also the most hydrophilic ceramide subclasses although in slightly different composition than in native human skin.[77-81] In the same period, the lipid composition and organization of various commercial was reported (eg EpiDerm, SkinEthic, EpiSkin).[82] Substantial differences were observed between the models, but at that time, none of them contained the hydrophilic ceramide subclasses in amounts similar to native skin, indicative for a lack of vitamin C in the culture medium. Importantly, the permeability of each of these models was much higher than in native human skin.[83]

In the stratum corneum of HSEs, the chain length of FFAs is shorter when compared to native human skin,[84] but improvement of the lipid profile has been achieved by the supplementation of

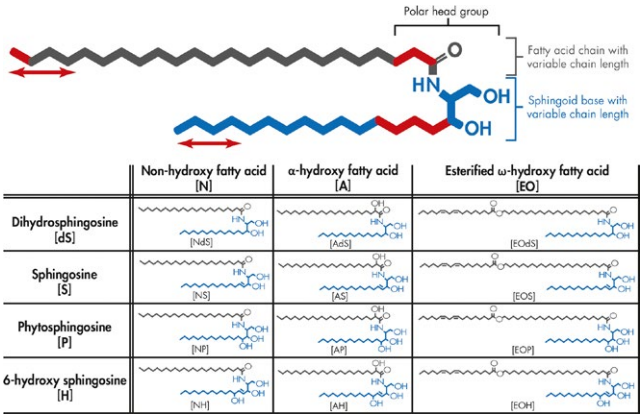


FIGURE 4 Overview of ceramide classes. Ceramides consist of a polar head group and two apolar tails, a sphingoid base linked to an acyl chain (grey). Both chains can vary in their structure by the number of carbon atoms and the head group architecture as shown in the figure. In the most abundant ceramides in human SC, 4 different sphingoid bases (dihydrosphingosine [dS], sphingosine [S], phytosphingosine [P] and 6-hydroxy sphingosine [H]) and 3 different acyl chains (non-hydroxy fatty acid [N], α-hydroxy fatty acid [A] and esterified ω-hydroxy fatty acid [EO]) are present. The figure has been adopted from J. van Smeden, thesis June 2013 entitled: A breached barrier:analysis of stratum corneum lipids and their role in eczematous patients

fatty acids in the medium.[84] More recently, two different HSE and one HEE model and their ceramide profiles were examined.[81,85] These models showed all a similar ceramide profile: all ceramide subclasses identified in native human SC were present, but the relative amounts differed from that in native human skin. The permeability of these models was 3-5 times higher than in native human skin.[86] Another HSE model shows the presence of ceramide [NS], [NdS] and [NP], but the most hydrophilic ceramides were hardly not detected suggesting absence of vitamin C in the medium.[86] For a representation of different ceramide classes, see Figure 4.

With the introduction of liquid chromatography-mass spectrometry (LC/MS), the lipid composition could be evaluated in more detail.[87] This revealed that HSEs have an increased abundance of short chain ceramides and an increased level of unsaturated chains.[87,88] This leads to organization alterations including a less dense hexagonal packing and shorter repeat distance of the lamellar phases compared to native human skin. The SkinEthic and Phenion skin models were also examined. The SkinEthic model had a similar lipid profile as the Leiden models with improved ceramide profile compared to previous studies,[82] while the Phenion model showed very low ceramide content lacking the most hydrophilic ceramides and high levels of unsaturated FFAs (unpublished results).

The presence of shorter chains and unsaturated lipids is very important observations as this provides important information to improve the lipid composition and thus the lipid barrier in culture models. Furthermore, modifications of the environmental conditions including the optimization of culture temperature both in 2D and 3D can be of influence. Recent evidence revealed the influence of reducing the relative humidity.[89-91] Recently, chitosan was introduced in the collagen matrix, which resulted in an improved skin barrier as evaluated by measurement of transepidermal water loss.[92] Optimization of culture conditions will be topic of future studies. Medium composition is also prone to optimization, as multiple studies revealed improved barrier formation after topical application of metabolic active molecules improving the barrier formation in vivo.[93-95] These molecules could potentially be applied on or dissolved in the medium of HSEs to enhance the in vitro barrier functionality. At first, the actual barrier function of the HEEs and HSEs is of importance. Nowadays, many commercial and in-house skin models show a well-developed epidermis; however, their use in screening constituents and formulations on their permeation across the skin is yet limited.[52,96] The main reason for this limitation is the difference in lipid composition and organization when compared to native skin. To conclude, improvement of the skin barrier function, can be achieved by (i) normalization of ceramide subclass composition, (ii) increase in CER and FFA chain length and (iii) reduction in the level of monounsaturated fatty acids and unsaturated CERs. The optimization of the culture medium and a more in vivo like culture environment concerning humidity, temperature and oxygen levels are important factors to take into account. Furthermore, it could be of interest to add sebocytes in the 3D models to initiate the production of sebum to modify the surface properties and more closely mimic the interactions of formulations with the skin surface in the in vivo situation.

4.2 | Human skin models to study the role of tight junctions in skin barrier function

The next physical barrier in the skin, just beneath the stratum corneum, is formed by the tight junctions (TJs) in the epidermis. In recent years, it became evident that TJs are part of the physical skin barrier.[5,7,97] In addition, they influence SC formation and function.[6,7] Furthermore, TJ proteins have been shown to be involved in keratinocyte proliferation, differentiation, migration, apoptosis and cell-cell adhesion.[98-101] Thus, TJs and distinct TJ proteins are interesting to address for investigation of epidermal barrier function, epidermal differentiation, epidermal ion gradients and wound healing.

In well-formed reconstructed HEEs and HSEs, localization of TJ proteins is similar to normal skin; that is, claudin-1 (Cldn-1) is found in all epidermal layers with a lower expression in the basal cell layer, Cldn-4 and ZO-1 are localized in the upper stratum spinosum (SS) and stratum granulosum (SG), and occludin is restricted to the granular cell layer.[102,103] In addition, TJ barrier function to molecular tracers can nicely be seen in the granular cell layer by Biotin-SH assays in well-structured models, again similar to human skin.[102-108] However, when the model is less developed, atypical localizations can be seen. TJs also contribute to transepithelial electrical resistance (TEER) often measured in 3D models, but TEER also reflects ion barrier of the SC and is therefore a measure for overall barrier function.

In general, HEEs and HSEs are elegant models to investigate TJ formation and function in the epidermis. For example, HEEs were

used already very early during epidermal TJ research to investigate the formation of TJs during epidermal maturation. In addition, HEEs and HSEs were instrumental to investigate the effect of (i) *Clostridium perfringens* enterotoxin, a toxin which is known to open TJs in the intestine by removal of—among others—Cldn-3, Cldn-4 and Cldn-7,[109] (ii) sodium caprate (C10), a molecule well known as an absorption enhancer in the intestine which influences signalling pathways and thereby opens TJs,[106,110] and (iii) knock-down of the TJ protein occludin.[98] On the other hand, 3D skin models have been used to investigate the influence of external stimuli or non-TJ-protein mutations on TJs. The models were used to test the effect of (i) various cytokines and combinations of cytokines,[103,107,111-113] (ii) histamine,[105] (iii) staphylococcal infection,[102] (iv) TLR agonists,[108] (v) cells derived from squamous cell carcinoma and actinic keratosis[114] and (vi) filaggrin mutations/knock-down.[103,112,115]

4.3 | Technical and biological variances in 3D skin models

Notwithstanding the great importance of the implementation of 3D skin models in skin barrier research, we should be aware that experimental heterogeneity and the wide variety in types of skin models based on cell sources, cell types, dermal substrates, culture medium, etc. hamper the replication of data. Raising awareness about biological variances is of great importance, and the use of multiple different biological donors (albeit resulting in larger standard deviations) within experiments is highly recommended. Furthermore, a thorough characterization of the basic model is required to evaluate the effects of interventions. The use of patient-derived, genetically defined keratinocytes or the overexpression or knock-down of genes is considered a valuable tool for the functional analysis of individual genes in an organ-like environment. However, differences in experimental procedures may impact the study outcome, which is exemplified in the next paragraph by the variety of 3D skin models to study the role of filaggrin (FLG) in skin barrier function.

4.4 | Studying skin barrier function in human skin diseases: the filaggrin story as an example

Skin barrier dysfunction is associated with several skin disorders caused by mutations encoding the main components of corneocytes, the lipid layers, or cell-to-cell contacts like desmosomes or TJs.[116] Besides these monogenic diseases, impaired skin barrier function can be a secondary event in chronic skin inflammation (eg psoriasis, atopic dermatitis). One key example of studies on the role of skin barrier function in skin disease pathogenesis is that of FLG in atopic dermatitis (AD). *FLG*-null alleles are by far the strongest and most widely replicated genetic risk factor for AD. As sensitization against common environmental antigens is a hallmark of AD, it has been tempting to postulate a leaky skin barrier as the most plausible mechanism that links genetic alterations to the disease phenotype. Murine models of FLG haplo-insufficiency, showing barrier impairment and enhanced percutaneous allergen sensitization, exemplify the detrimental effect of having less FLG expression on skin barrier function.[117] The impaired barrier integrity phenotype associated with *FLG*-null mutations in human skin is emerging, with evidence of reduced natural moisturizing factor (NMF) in the SC,[118-120] and SC integrity and cohesion impairment.[121,122] As mouse models may not faithfully recapitulate the human pathophysiology, in vitro models seem a promising tool to dissect the effect of FLG loss on skin barrier function in a controlled laboratory environment.

Over the years, several studies have appeared using knock-down strategies in 3D skin models to study the effect of FLG on skin barrier function. The majority of these models are based on the model first described by Mildner et al who used neonatal human foreskin keratinocytes and siRNA to knock-down *FLG* gene expression and observed an increased penetration of the Lucifer Yellow (LY) dye through the SC of FLG knock-down HEEs.[123] Thereafter, studies from other groups appeared using similar approaches; however, results are conflicting. Experimental models have used foreskin keratinocytes,[87,122-124] adult primary keratinocytes[125] or an immortalized keratinocyte cell line[126] to generate HSEs. Knock-down strategies varied from transient knock-down using siRNA[87,122-124] to stable transduction using lentiviral delivery of FLG-targeting shRNA.[125,127] Most of these studies obtained a significant reduction in FLG mRNA or protein expression (70%-90%), but none of them reached complete absence of expression. More recently, we and others have used patient-derived keratinocytes obtained from ichthyosis vulgaris (IV) patients carrying homozygous FLG loss-of-function mutations to generate HEEs and study in vitro skin barrier function.[103,127] This provides a unique possibility to study the role of FLG in skin barrier defects, reported for both IV and AD. Thereby, knock-down–derived off-target effects were excluded, while potential compensatory mechanisms by naturally occurring FLG deficiency were taken into account. Strikingly, no differences were found in the barrier function of the naturally FLG-deficient 3D skin models. *FLG* mutations only explain a subgroup of all AD patients, whereas all patients suffer from barrier impairment; therefore, there must be one or more additional factors than FLG mutations alone. Probably, other mutations or exogenous triggers might be necessary to induce AD, either accompanied by or resulting in an impaired skin barrier function. The advantage of the use of a 3D model was to specifically modify one parameter, in this case the use of FLG-deficient keratinocytes, to analyse the consequences thereof on the development and function of the epidermal barrier in vitro. Similarly, no changes were observed in the barrier permeability and lipid composition of FLG knock-down keratinocytes in a 3D model.[126] In our study, barrier function was tested by the polar solutes LY and biotin as these are commonly used in comparable studies.[125,128] Although alteration of the permeability for these low molecular weight tracers was not observed, this does not completely rule out alterations of FLG-deficient epidermis with respect to permeability for environmental molecules with other biophysical properties such as microbial or airborne antigens, or fragments thereof. This raises an important question: can we draw definite conclusions with the 3D skin models we are using when complex disease pathogenesis lies at the heart of the disease?

This concern also applies to in vivo animal models, and we should therefore strive to combine insights gathered from different experimental models for a better validation and extrapolation of results towards complex human diseases.

5 | THE SKIN MICROBIOME: A NOVEL PLAYER IN SKIN BARRIER RESEARCH

Besides the above-mentioned barrier compartments of the skin, the microbial barrier is of great importance.[129] Disturbance of any of these barriers can lead to a persistent inflammatory state or an insufficient host response to pathogens. Over the recent years, we have witnessed a scientific breakthrough with respect to our knowledge and understanding of the human skin microbiome.[130-137] In normal circumstances, our skin peacefully coexists with commensal bacteria; however, changes in the composition of cutaneous microbial communities (which is called dysbiosis) and an altered host immune response to these microbiota can affect the homeostatic relations. This disturbance might drive inflammatory skin diseases like psoriasis and AD, which are characterized by an impaired skin barrier function.[129,138-140] Mutations in *the FLG* gene are the major genetic risk factor for developing AD,[141] and recently, it was demonstrated that *FLG* mutations also have a strong impact on the human skin microbiome.[16] Cutaneous micro-organisms are linked to the pathogenesis of AD as skin of these patients is frequently colonized with *Staphylococcus* (*S.*) *aureus* leading to recurrent skin infections and subsequent antibiotic treatment.[142,143] A study using HSE models has shown that reduced expression of filaggrin protein resulted in increased epidermal *S. aureus* colonization.[144] *S. aureus* is also a major pathogen in skin infections in burn wound patients, and large efforts are made to prevent and treat wound infections.[145,146] The expectation is that many studies will investigate the role of micro-organisms in skin diseases and therapeutic strategies to treat these conditions. As an alternative for research animals in vitro human skin models will be the main tool to investigate the interaction between micro-organisms and human epidermis.

Since the turn of the century, several in vitro studies have shown direct interactions of skin-specific micro-organisms with keratinocytes. These studies revealed that commensal and pathogenic bacteria can activate different signalling pathways and are able to induce the expression of AMPs and proinflammatory cytokines and chemokines in these submerged cultures[13,16,147-152] and that they can biphasically influence TJ barrier function.[102] As there is no SC in conventional monolayer cultures, the bacteria are in direct contact with the keratinocytes, something that usually does not happen. Therefore, 3D models mimicking human skin to study in vitro infection and host-microbiome interactions are preferable. Until now, a few of such studies are performed. One of the first studies reported that virulent, hyphae forming, *Candida albicans* strains penetrate the protective layer of keratinocytes in in vitro reconstructed human skin and invade through the epithelial cell layers.[153] Others generated a living skin model that supported topical application and colonization of skin commensals (eg *S. epidermidis*) and a transient bacterial pathogen (*S. aureus*) for up to 72 hours of incubation and with an intact and undamaged surface showing differential host-defense gene expression or TJ protein localization and barrier function in response to *S. epidermidis* or *S. aureus*.[102,154,155] Furthermore, 3D models were used to study the fundamental effects of biofilms in wound healing[156] and to mimic thermal wound infection.[157]

However, in all above-mentioned studies just single bacterial strains are used not really reflecting our complex human skin microbiome. Future studies should focus on small composite microbiomes and patient-derived (whole) skin microbiomes. To eventually perform whole microbiome modulation and analysis in vitro the optimal collecting method of micro-organisms and time of coculture should be established. Thereafter, the interaction of HEEs with defined bacterial strains, small composite artificial and whole (patient-derived) microbiomes should be characterized to determine the stability of the in vitro microbiomes in time and epidermal host response. The viability of relevant bacterial strains upon coculture with the HEEs should be determined, as the molecular approaches that are currently in use are unable to distinguish between microbial genomic DNA derived from living vs dead organisms. We recently successfully used the propidium monoazide (PMA) treatment of micro-organisms, resulting in isolation and amplification of viable cell genomic DNA only.[158] Finally, the window of opportunity for evaluating the effect of pre-, pro- or antibiotic strategies in vitro skin microbiome models could be examined.

6 | CONCLUSIONS AND PERSPECTIVE

Three-dimensional skin models enable us to control and change different parameters of the skin, for example culture stimuli, cell genotype or application of therapeutics and measure the impact of those on skin barrier function. The 3D skin models available to date will certainly advance over the coming years by the optimization of culture conditions, but already now in vitro cultured 3D skin models have been proven to be an excellent alternative for, or addition to, experimental animal models to study skin biology, wound healing, skin ageing and disease pathology.[96,159-163] As mentioned before, improving the actual skin barrier function of 3D skin models needs serious attention. One could speculate that the sterile environment of cultured 3D skin models lacks important stimuli for a normal skin barrier function to be established. The host-microbe interaction between keratinocytes and microbiota could be a key factor for achieving the skin barrier function we observe in native skin. The sensing of microbial-derived molecules may be an essential trigger for the correct formation of the skin barrier in 3D skin models. Thus far, studies that include microbial components onto 3D skin models are scarce and have only investigated the effects of individual microbes on TEER, TJ expression and function and host-defense gene expression by keratinocytes. Studies on the effects of complete microbiomes on SC formation, composition and permeability or the keratinocyte interaction with immune cells are pivotal but still missing. Future research should be directed towards the standardization of application

methods, controlling bacterial growth and survival on the models, and optimizing techniques for the analysis of the microbes and 3D skin models.

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CONFLICT OF INTERESTS

The authors have declared no conflicting interests.

AUTHOR CONTRIBUTIONS

EB initiated and supervised the study. HN, JBo, AEG, JBr, PZ and EB wrote and edited the manuscript. All authors have approved of the content of the manuscript.

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