The importance of being three-dimensional in biology

3D Skin Models for Toxicology and Efficacy Testing

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Introduction

Any new cosmetic product must be both safe for use and effective. Consequently, every cosmetic ingredient must undergo a series of toxicological tests before being assessed as safe for the use in the respective cosmetic formulation. Since 2013, the EU Cosmetics Regulation bans the marketing of cosmetic products containing ingredients that have been tested on animals after the respective deadline. Thus, the safety assessment of cosmetic ingredients exclusively relies on the use of *in vitro* tests, in silico predictions, historic *in vivo* data and read across approaches. For some toxicological endpoints, related to single exposure scenarios, mandatory OECD test guidelines are already in place (e.g. OECD TG431, 439), whereas for the more complex endpoints with repeated exposure scenarios reliable *in vitro* tests are still under development.

Human skin is the first site of contact for many cosmetic products, be it shampoos, skin cleansing or skin care products. Thus, the skin surface can be considered as the gate for cosmetic ingredients to cross the outer barrier and to enter the inner living cell layers. This mechanism can be intentionally, e.g. in the case of anti-ageing formulations, developed to stimulate dermal collagen synthesis. However, for reasons of safety assessment, the skin absorption and penetration behavior, respectively, of formulations, intended to target e.g. only the human hair, needs to be carefully analyzed, too. For this reason, developing reliable and scientifically valid *in vitro* test methods, based on human skin cells and mimicking structure and physiology of healthy human skin, is of utmost importance for the cosmetic industry.

This review will provide a brief overview of major achievements in recent skin cell culture approaches and will then focus on properties and applications of an innovative human 3-dimensional skin equivalent, exemplified for the Phenion® Full-Thickness Skin Model.

From single cells to 3D skin models

Since more than 100 years scientific reports are published which describe approaches to culture human skin cells, especially the keratinocytes, under standardized conditions. The driving force for the early pioneers of skin culture technology was the ever-growing need for solutions to cover or treat wounds caused e.g. by burns, accidents or by health impairments like diabetes. However, for decades the technical challenges to grow keratinocytes in a sufficiently high number and to maintain them in an undifferentiated state could not be overcome properly (Horch et al., 2001). The situation changed dramatically in the mid-70s, when, for the first time, a cell culture medium was developed which sustained keratinocyte proliferation over an extended period of time (Rheinwald & Green, 1975). Based on the special medium composition which included cholera toxin, an activator of the intracellular adenylate cyclase, in low concentrations, keratinocytes isolated from small human skin samples could be multiplied under 2-dimensional culture conditions to form epithelial-like sheets. Their efficiency in dermatological applications like wound care was however limited by its delicacy and fragility when removed from the culture vessels (Horch et al., 2001). The breakthrough in favor of a mechanically more robust and physiologically competent keratinocyte culture was achieved with introducing collagen as a potent cell carrier (Bell et al., 1979, 1981, 1983). Polymerized collagen gels not only supported the development of a 3-dimensional epithelium with high similarity to the healthy human epidermis, but also served as a matrix to culture fibroblasts, the cells which build up the dermal connective tissue. Comprising an epidermis and a dermis, the full-thickness skin models not only revealed a more *in vivo*-like tissue differentiation due to intercellular cross-talks between keratinocytes and fibroblasts, but also demonstrated increased mechanical strength compared to the 2-dimensional sheets. The underlying principles of this milestone in skin cell culture technology are the basis for all 3D culture approaches until today. Supported by a plethora of publications, it is now generally accepted that only with 3D culture techniques cells of multiple tissues and organs will retain or regain pivotal physiological and structural functions which resemble the respective *in vivo*-situation (e.g. Alépée et al., 2014).

Beside several advantages the use of collagen gels has one serious drawback. They start to contract through the tensile forces of embedded fibroblasts, leading to a skin model with 1/10th or less of its original size within a few days of culture. The contraction of the collagen gels eventually results in dense structures that resemble the dermal *in vivo* situation in only limited dimensions.

This situation can be ameliorated with crosslinked and thus stabilized matrices with collagen as the main component which do not shrink even over a longer culture period (e.g. Shahabeddin et al., Mewes et al., 2007). Within the stabilized collagen matrix,

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embedded fibroblasts can adhere properly, allowing the *de novo* synthesis of native extracellular matrix components without shrinkage.

The Phenion® Full-Thickness skin model: properties

In 2007, basic morphological and physiological properties of a new full-thickness skin model, based on a proprietary lyophilized porous sponge made exclusively of bovine collagen, were published (Mewes et al., 2007). Developed by Henkel AG & Co. KGaA, Germany, and introduced into the market under the Phenion® brand, this human full-thickness skin equivalent comprises a fully stratified epidermis, including a multilayered stratum corneum, and a mechanically stable dermis. Keratinocytes and fibroblasts are isolated from human skin samples, grown in a 2D culture into the preferred passage, and then subsequently seeded into and onto the collagen sponge. After an interval under submersed conditions, the developing tissue equivalents are lifted to the air-liquid interface where they finally grow and differentiate into the 3rd dimension (Figure 1). The Phenion® Full-Thickness skin model fosters the replacement of animal experiments in the toxicological assessment of chemicals, with the development and validation of the 3D Skin Comet Assay as a milestone in this field (Reisinger et al., 2018). Furthermore, the skin model is deployed in dermatological basic research and in efficacy studies for cosmetic ingredients and formulations.

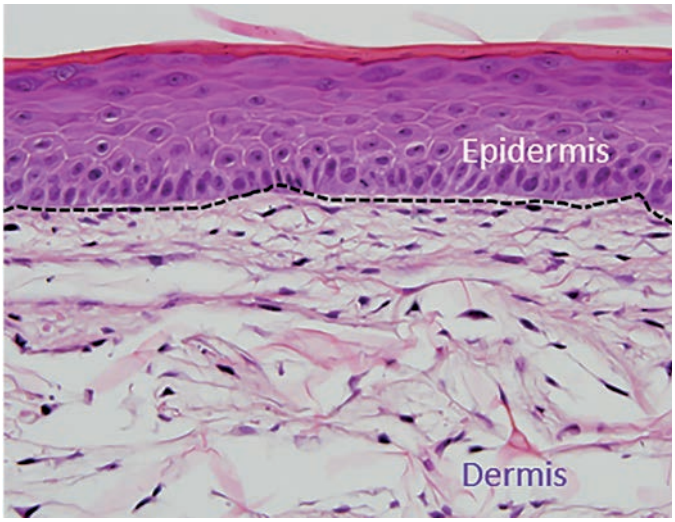

Figure 1: Histological section through the fully differentiated Phenion® Full-Thickness Skin Model. The section was stained with hematoxylin & eosin (H&E). Both compartments, the epidermis and the dermis, have developed similar to native human skin.

The commercially available Phenion FT Skin model is based on a solid matrix. Its rigid porous structure offers a favorable environment for the fibroblasts. The cells can tightly adhere to the collagen and thus enter a state of tensile stress, a prerequisite for survival and for regaining typical *in vivo*-like physiological functions. As one consequence, the fibroblasts start synthesizing and secreting extracellular matrix components, e.g. elastin and fibrillin-1, the core components of elastic fibers (Mewes et al., 2007).

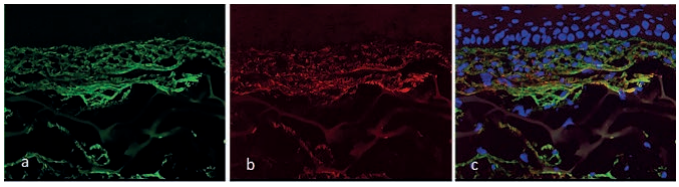

Figure 2: Detection of elastin (green, a) and fibrillin-1 (red, b) in the dermal compartment of a fully developed Phenion® Full-Thickness Skin Model. The proteins were visualized by immunofluorescence in histological sections. In fig. 2c the fluorescence labels are merged, indicating towards the co-localization of elastin and fibrillin-1 in individual elastic fibers (yellow/orange stain). The cell nuclei are counterstained with DAPI (blue).

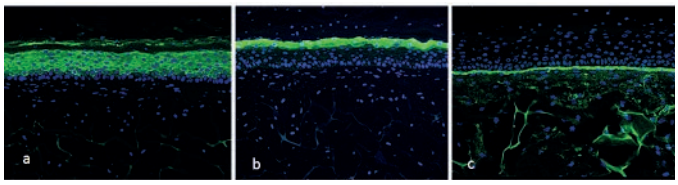

Figure 3: Detection of epidermal and basement membrane proteins by immunofluorescence (green) in histological sections through a fully developed Phenion® Full-Thickness Skin Model. 3a: Cytokeratin 10, a marker for early epidermal differentiation; 3b: loricrin, a marker for late epidermal differentiation; 3c: laminin-5, a basement membrane component. The cell nuclei are counterstained with DAPI (blue).

In a process of self-organization, with increasing culture time an elastic network is generated, mimicking the special structural and spatial features seen also in native human skin (Figure 2).

The spatially and timely correct differentiation of the epidermis is routinely assessed by immunofluorescence microscopy. With specific antibodies, the expression of proteins representing developmental key events in the epidermal layers are visualized (Figure 3 a, b). At the interface between epidermis and dermis characteristic basement membrane proteins are deposited, indicating a tight and physiological connection between both tissues comparable to native human skin (Figure 3 c). For analytical reasons, both compartments can be separated after enzymatic treatment.

By means of laser scanning microscopy (LSM) the differentiation status and structure of the full-thickness skin models can be studied non-invasively. Figure 4 demonstrates the typical structural changes which appear when moving the optical plane from

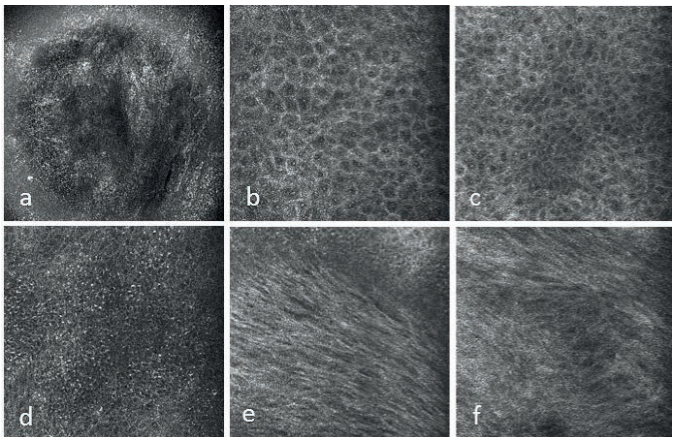

Figure 4: Laser Scan Microscopy (LSC) pictures of the the different layers of the Phenion® Full-Thickness Skin Model. a) S. corneum; b) S. granulosum; c) S. spinosum; d) S. basale; e) papillary dermis; f) reticular dermis. Within the epidermis, the keratinocyte diameter increases from the basal layer to the granular layer in the course of terminal differentiation.

the tissue surface through the epidermis to the upper dermal compartment. A striking feature, and an indicator for optimum epithelial growth, is the cobblestone-like pattern of the basal keratinocytes, which also represent the stem-cell niche of the epidermis (4d). Underneath the basement membrane, a dense mesh comprising extracellular matrix (ECM) fibers, typically composed of collagens, elastin, and others, are visible. In the upper dermal region the ECM fibres are mainly oriented in parallel to each other (4e), whereas in the deeper regions the fiber structure changes to an undirected pattern (4f). These patterns mimic the architecture of the papillary and reticular dermis, respectively, of healthy human skin. The high resolution, paired with an excellent optical penetration depth, renders the LSM a suitable device to study tissue integrity and protein deposition in the skin equivalents under different experimental conditions.

The Phenion® Full-Thickness skin model: applications

Wound healing

The detection of differentiation-specific epidermal proteins with immunofluorescence microscopy, as well as non-invasive optical techniques are well-suited tools to study wound healing processes in the full-thickness skin models. Wounds can be created in the tissues with different techniques under standardized conditions. Pure epidermal wounds are e.g. induced by mechanically removing a small strip of epithelial tissue from the center of the skin model, leaving the underlying dermal tissue exposed to the ambient air. Already within 24 hours after wounding, keratinocytes are starting to grow out of the wound margins. Throughout the following days, this process continues, thus gradually covering the naked dermis and thus closing the wound. Whereas at the leading edge the regeneration tissue mainly consists of one keratinocyte layer only, in the lagging section it successively differentiates into all layers characteristic for healthy human skin. This process of continuing epidermal regeneration can be monitored non-invasively with Optical Coherence Tomography (OCT), a technique which uses the differential scattering of coherent light by internal tissue structures to generate an image of the upper compartments of the skin model (Figure 5). With OCT an outstanding opportunity is provided as individual tissues can be monitored repetitively over a long period of time without any interference or even damage.

Using specific fluorochrome-labelled anti-filaggrin antibodies, the gradually increasing epidermal differentiation process during wound healing can be visualized with high resolution at the cellular level (Figure 6). Filaggrin is constitutively present in stratum granulosum and corneum of the intact epidermis (6a). The outgrowing keratinocytes do not express filaggrin, a clear indicator of their yet undifferentiated status. However, 48 hours after wounding, a first filaggrin signal appears in the lagging section of the regenerating tissue close to the original wound margin (6b). After 96 hours, already more than half of the newly grown tissue expresses this marker protein for terminal differentiation (6c). This observation proves that the Phenion FT Skin Model has the capacity to regenerate a structurally complete epidermis after wounding. It paves the way for studying mechanisms of wound healing *in vitro* and for developing wound care products or actives e.g. to impact impaired skin regenerating processes often associated with diabetes and other diseases.

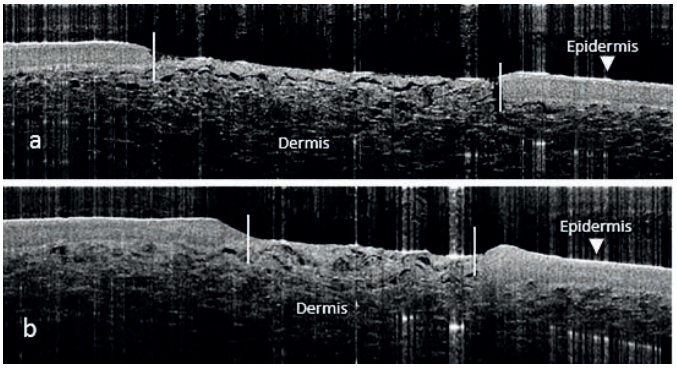

Figure 5: OCT analysis of epidermal wound healing. The epidermis was manually removed from the center of the skin model, and the wounding area analyzed daily with OCT. a) fresh wound with the dermal compartment exposed to the ambient air. b) partially wound closure 4 days after wounding; the exposed dermal surface is markedly reduced due to outgrowing keratinocytes. The vertical white bars indicate the open wound area.

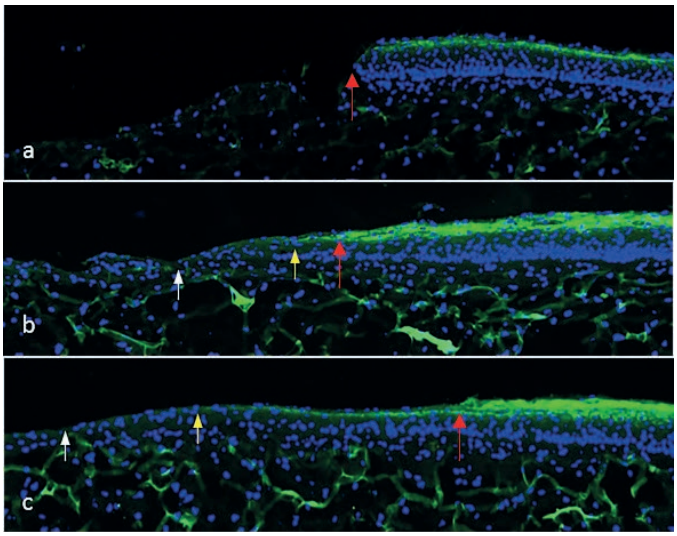

Figure 6: Filaggrin expression (green) during epidermal wound healing. The protein was visualized by immunofluorescence in histological sections through wounded skin models immediately after wounding (a), 2 days (b) and 4 days (c) after wounding. Filaggrin is present in the upper layers of the undamaged epidermis. Starting from the wound edge (red arrow), keratinocytes grow out to cover the wounded area (white arrow). Terminal differentiation of the newly grown epidermal tissue is revealed by the increasing expression of filaggrin; the yellow arrow indicates the foremost front of filaggrin expression in the regenerating tissue.

Testing of injected formulations

The unique feature of autonomous elastin expression in the Phenion FT Skin Model was exploited in a study about the biological effects of hyaluronic acid (HA) derivatives and hybrid complexes, which are used as fillers e.g. in aesthetic medicine (Stellavato et al., 2016). The authors injected small amounts of high- and low-molecular weight HA (H-HA and L-HA) and of a novel H/L HA hybrid complex (Profhilo®) in the dermal compartment of the skin equivalents (Figure 7). The tissues were then analyzed for the expression of different collagens and of elastin on the protein and the gene expression level. In contrast to the

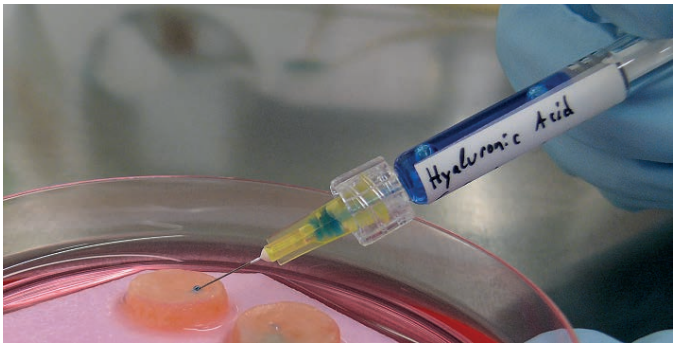

Figure 7: Injection of a hyaluronic acid (HA) based formulation into the Phenion® Full-Thickness Skin Model.

tissues treated with H-HA and L-HA, skin models which received H/L HA hybrid complex injections revealed significant increases in elastin expression after 7 days of incubation. In addition, the expression of collagens I, III, and VII was stimulated by the complex, too. Collagen IV stimulation was found only after 24 hours incubation but was decreased to background levels after 7 days. The results generated with the full-thickness skin model impressively corroborated observations already seen with keratinocyte and fibroblast monolayer cultures. In addition, due to its collagen sponge core the Phenion® skin models are simultaneously robust and flexible. These mechanical properties are ideal to allow injections of relevant amounts of filler substances like HA derivates without jeopardizing tissue integrity.

Assessement of mechanical properties

The mechanical robustness of the Phenion® FT Skin Model was just recently challenged in a series of papers published by Malhotra et al. (2019) and Pan et al. (2019). Malhotra and colleagues (2019) studied the viscoelastic properties of native human skin samples from donors of different age and compared them with the respective values measured in the 3D FT skin model and in the AGED FT version, which mimics the skin of older people in many structural and physiological features (Alili et al., 2014; Diekmann et al., 2016). The elastic and viscous moduli of the 3D skin models were on average one magnitude higher than the moduli assessed with the skin samples, however, no significant difference between the Phenion FT model and the AGED version on the one hand and between young and old skin on the other hand was observed.

Albeit mechanical differences between human skin and skin models have been elucidated, both studies clearly demonstrated that physical-mechanical analyses can be conducted reliably and with high precision on the Phenion® FT Skin Models. As Malhotra et al. (2019) stated, the mechanical properties mirror, beside others, the amount, distribution and orientation of the dermal collagen fibers on the one hand and the elastic network on the other hand. Thus, this kind of analyses can pave the way to new approaches to assess the positive influence of e.g. innovative cosmetic ingredients and actives on the biomechanical properties of the skin with the help of the 3D skin models. An overview about how the Phenion FT skin models are already employed to study the efficacy of cosmetic ingredients and actives at the gene, protein and histological level, respectively, has been published by Mewes et al., 2017.

***In vitro* genotoxicity assessment**

As already mentioned earlier, every ingredient of a cosmetic product must be thoroughly assessed regarding its toxicological profile. Genotoxicity is one of the pivotal toxicological endpoints within the safety assessment process. It must be proven that the ingredient of interest neither impacts the integrity of the DNA, the chromosome structure or the chromosome number. A collection of *in vitro* test methods has been established in the last decades, addressing one or more of the different damage classes. However, most of them are highly over-predictive, thus the chance to single out chemicals based on a false-positive result, must be considered. The 3D Skin Comet assay, developed on the basis of the Phenion® Full-Thickness Skin Model, is an innovative alternative method which overcomes some of the challenges known from other assays (Reisinger et al., 2018). It is the first test system which explicitly addresses the dermal route of exposure for genotoxicity testing, thereby taking advantage of key properties of the FT skin models, namely a functional barrier and a well-established xeno-metabolic system. In its fully differentiated state, the Phenion FT model reveals an epidermal barrier function, mainly supported by its multilayered stratum corneum. Although the barrier of this and other 3D skin models tested so far is generally weaker than in healthy human skin, it allows the permeation of chemicals through the tissue in an *in vivo*-like fashion, mainly driven by the logP value, or lipophilicity, of the topically applied chemicals (Ackermann et al., 2010).

Once a chemical has penetrated the FT skin model, it can become metabolized by phase I and II enzymes, which are either constitutively expressed or induced after chemical exposure in both the epidermal and dermal tissue (Jäckh et al., 2011; Wiegand et al., 2014). Beside other features, the ability to penetrate the skin and its subsequent metabolic fate are important parameters which determine whether a molecule acts as a genotoxin or not. This situation can be ideally simulated with the Phenion FT Skin model. In the innovative 3D Skin Comet assay, the skin models are combined with the alkaline comet assay, an approved method developed to detect DNA damages, namely mutagenic and clastogenic lesions, in cells exposed to mutagens (Figure 8). The 3D Skin Comet assay has been successfully validated in a multicentric ring trial according to EURL-ECVAM standards (Hartung, 2004) and has now entered the OECD work plan for the test guidelines programme. The first phase of the validation resulted in an accuracy of 100% in four laboratories and of 70% in the fifth facility indicating the assay's suitability to support the safety assessment

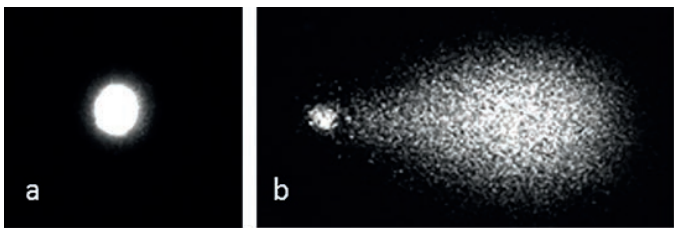

Figure 8: 3D Skin Comet Assay: Detection of fragmented DNA in keratinocytes after incubation of the Phenion® Full-Thickness Skin Model with a genotoxic chemical. DNA was stained with CYBR Gold. a) nucleus from a keratinocyte of an untreated control tissue, no DNA fragmentation visible; b) highly fragmented DNA after exposure to a genotoxic chemical resulting in a pattern which resembles a comet, thus the name of the assay.

of chemicals where classical combinations of *in vitro* genotoxicity tests give ambiguous results (Reisinger et al., 2018). Already today, 3D Skin Comet assay data for two oxidative hair dye chemicals, have been supplemented respective dossiers and supported the opinion of the European Commission Scientific Committee on Consumer Safety (SCCS) that the molecules are safe for the intended use. (Reisinger et al., 2016).

Summary

Three-dimensional human skin equivalents are state-of-the-art tools to study efficacy and toxicological effects of chemicals and actives in an *in vivo*-like environment. The Phenion® Full-Thickness Skin Model, comprising an epidermis and a dermis, reveals substantial similarities to native human skin in respect of tissue architecture, physiological features and metabolic competence. These properties allow the models to be employed in many different applications and to be investigated with different analytical methods. The mode of action of innovative cosmetic ingredients on the human skin as well as mechanistic investigations such as wound healing studies can be conducted with the FT model *in vitro* at different levels of complexity like immunofluorescence and enhanced microscopical techniques, mechanical measurements, and gene and protein expression analyses. The 3D Skin Comet assay, an innovative method to study genotoxic effects, has been included in the OECD work plan for the test guidelines programme, a pivotal step towards regulatory acceptance.

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