The Phenion Full-Thickness Skin Models: variants, barrier function, and *in vitro* testing

Easy handling of the Phenion FT Skin Models

A conversation with Dr. Karsten Mewes, Senior Manager Alternative Methods & Phenion Business Development

After studying biology at the Universities of Cologne and Mainz, Germany, Dr. Karsten Mewes obtained his PhD in Zoology in 1998. He then worked as a research scientist and lecturer at the Institute of Zoology before entering the Phenion GmbH & Co. KG in 2001, where he worked as a scientist on the development of innovative three-dimensional tissue models of the skin. After the integration of Phenion into the Henkel company in 2006, Dr. Karsten Mewes continued his work on 3D tissue models and their implementation as animal-free alternative test systems for safety assessment and dermatological research. In his current position as Senior Manager for tissue engineering and alternative methods at Henkel, Karsten Mewes is also an active member of Cosmetics Europe. Since 2017 Dr. Mewes also works as business developer for the Phenion skin model business.

EURO COSMETICS: *With more than 15 years' experience in in vitro research and tissue construction, your team of scientists make a significant contribution to reducing and avoiding animal testing. The in-vitro test methods are based on three-dimensional human skin tissue models. What is so special about your skin models?*

Dr. Karsten Mewes: The development of the full-thickness skin model, representing native human skin in many respects, started already in 2002 and is today a broadly appreciated and used test system. This human full-thickness skin equivalent comprises a fully stratified epidermis, including a multilayered stratum corneum, and – very important – a mechanically stable dermis. The keratinocytes and fibroblasts are isolated from human skin samples, amplified in 2D cell culture systems, and then subsequently seeded onto a collagen sponge. I find it noteworthy, that the cells in our skin models originate from individual donors and cell mixing or pooling is not conducted. Further our proprietary porous scaffold is made of bovine collagen, and due its structure it does not shrink during culturing and finally provides excellent mechanical robustness. After a short 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. With the air-liquid interface culture we are simulating the condition our skin experiences all day long: exposed to the air, the epidermis gets its supply of oxygen and nutrients by diffusion from the underlying connective tissue. These special conditions are responsible for the correct epidermal differentiation, including the generation of a tight, but selective barrier.

In 2007, basic morphological and physiological properties of the full-thickness skin model, were published, with the focus on the elastic network which is realized in the full-thickness skin model. The proteins which contribute to the elastic fibers, mainly elastin and fibrillin-1, are newly synthesized by the dermal fibroblasts and secreted into the extracellular space. Only in this environment, the elastic fibers assemble in a process of self-organization, a unique feature of the Phenion Full-Thickness Skin Model.

EURO COSMETICS: *This is an interesting insight into the history of the full-thickness skin model development and its production processes. What is the latest news from your product development teams?*

Dr. Karsten Mewes: It is noteworthy to

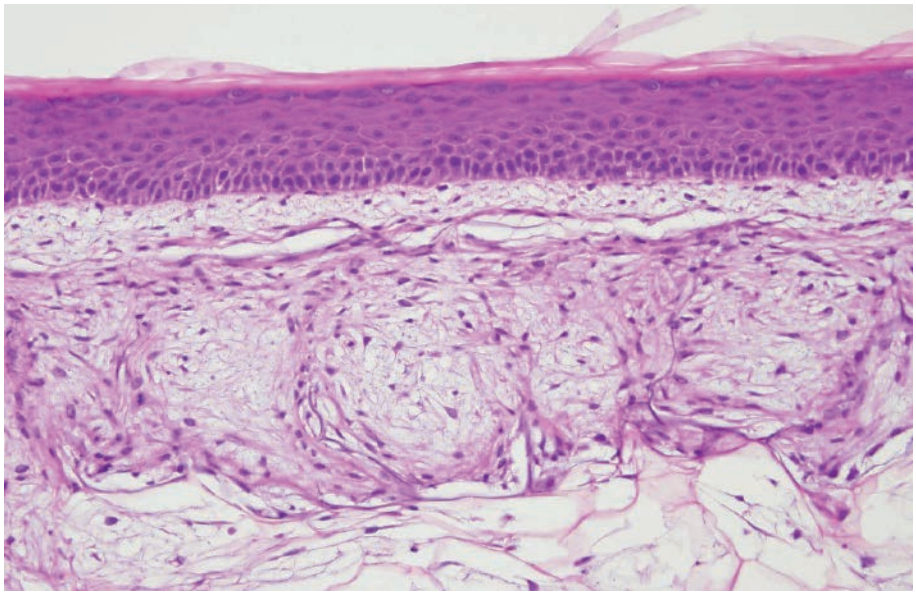
clearly state that we put the customers into the center of our ambitions. Thus, we listen very carefully what is needed to fulfill our customers needs. With this driving force we have developed during the last years new variants of the full-thickness skin model and hence markedly increased the number of products in the Phenion portfolio.

We learned e.g. that there is a need for a specific format for our standard model, that we successfully have addressed: The FT skin model is now also available in an insert-based variant. Whereas the standard FT skin model is cultured on sterile filter papers at the air-liquid interface, the new variant is confined to a cell-culture insert. It is identical with the standard FT Skin Model in terms of tissue architecture, physiological reactions, metabolic competence, and size. The new insert variant allows, beside others, the topical application of bacteria suspensions without risking that the microbes might escape into the underlying cell culture medium and consequently jeopardize a test.

With the FT LARGE model, we are offering the skin model in a bigger size for very special applications. With a diameter of 3.1 cm and a surface area of roughly 7.7 cm^2 this model variant is destined e.g. for the application of larger volumes of test items or for multiple different analyses after experimental treatment.

With the Phenion FT LONG-LIFE model, we have entered a new dimension in respect of tissue longevity. This full-thickness skin equivalent can be kept in culture for up to 50 days after arrival at your lab. Based on innovative protocols, fibroblasts and keratinocytes have achieved an advanced state of homeostasis at a very early stage during tissue production, which is maintained throughout the whole culture period. The extraordinary extended lifespanopens the gateway to completely new application fields, which finds great attention throughout the scientific community.

This variant allows to monitor physiological, biochemical, and genetic processes, e.g. after tissue exposure with chemicals, formulations, or environmental impacts, over a very long period of time. Late-onset or long-term effects can be studied as well as recovery or regeneration after tissue treatment or accumulating more delicate effects. It is easy to imagine that the LONG-LIFE model could serve as an *in vitro* platform for research into development and treatment of skin tumors or wound healing, beside other applications.



Tissue architecture of the Phenion FT Skin Model (histological section, H&E staining)

Developing formulae which can fight signs of skin ageing, is of overarching importance in cosmetic sciences. To enable research in skin ageing and work on anti-ageing mechanisms, we have invested much energy to develop the FT AGED model. This full-thickness skin model mimics the skin of older people, both in structure and physiology. It is characterized by a dermal compartment containing fibroblasts in a state of senescence. Consequently, the synthesis of extracellular matrix components like collagens and elastic fibers is markedly reduced, whereas the secretion of matrix metalloproteinases and the generation of reactive oxygen species (ROS) is enhanced, which is typical for aged skin.

To produce our IP-protected AGED skin model under very standardized and reproducible conditions, dermal fibroblasts are treated in a fine-tuned and delicate process with a cytostatic drug, leading to cell cycle arrest and ROS generation. Both effects contribute to the development of an aged phenotype. Interestingly, even after removing the cytostatic drug, the epidermal keratinocytes also reveal a wide range of signs of ageing.

For all clients who are looking for a pure epidermal model only we are offering the so-called Open Source Reconstructed Epidermis (OS-REp), grown from primary human keratinocytes in a co-culture insert. All protocols to produce the epidermal model have been published in 2016 – this is what we call "open source".

These are the latest species we are currently cultivating in our zoo of skin models. However, we are continuously working on new modifications or new types of 3D tissue models, which we will communicate in due time.

EURO COSMETICS: *And for which areas of application are the skin models suited?*

Dr. Karsten Mewes: As diverse the skin models are, as diverse are the fields of applications. Generally, the applications can be clustered in different groups. One group includes the toxicological assessment of chemicals and formulations. One prominent example is the 3D Skin Comet assay, an innovative *in vitro* test system to analyze DNA damages in the human skin exposed to chemicals. This test system is based on the Phenion FT Skin Model, thus addressing the dermal route of exposure for the first time in a lab-grown skin tissue. The advantages of the Phenion FT Skin model are, beside others, its barrier function, its well-expressed xenobiotic metabolism, and its composition of a dermis and an epidermis, which can be analyzed separately. This assay is the product of a cooperation project of several cosmetic companies under the umbrella of Cosmetics Europe, the Association of the European Cosmetics Industry and the excellent validation results were convincing enough

for the OECD expert group to include the 3D Skin Comet assay in their "Work Plan for the Test Guidelines Programme" in 2019. Thus, this method will be part of an OECD Test Guideline – a great success for the consortium and our company, and a demonstration of the capacities of the Phenion FT Skin Model.

Another group subsumes applications related to claim substantiation, e. g. for new cosmetic ingredients and formulations. Comprising an epidermis and a dermis is the key to many tests and studies which look at the connective tissue as the main target. For example, because of the well-organized elastic network in the dermis, questions regarding the increasing loss of skin elasticity during ageing can be addressed as well as screening for ingredients which might ameliorate this effect. The experimental set-up for most tests is straight-forward: you expose the full-thickness skin model to the ingredients of choice or a physical treatment like UV irradiation, and after a defined period of time you can analyze the skin tissue at every level of complexity.

Basic dermatological and pharmaceutical research defines the third group of applications with the full-thickness skin models. This application field extends, from understanding the mechanisms of skin function and integrity, over innovative transdermal drug delivery systems, to wound healing and tumor progression, etc. Let us focus on the aspect of wound healing. The full-thickness skin model can be wounded by different means, depending on the question to be addressed. We can remove the central part of the epidermis either with forceps or by applying a small metal device previously cooled in liquid nitrogen. Deep cuts can be simulated by cutting the tissue with a scalpel unto reaching the deeper layers of the dermis. Irrespective of the kind of wounding, after only one or two days the keratinocytes start proliferating and migrating out the wound margins to cover the wound bed. The wound healing progress in a single tissue can be continuously monitored non-invasively by means of Optical Coherence Tomography. Or by e. g. immunofluorescence analyses with specific antibodies directed against key proteins.

The development and progression of skin-based tumors can be addressed with the full-thickness skin model, too. For this kind of application, the LONG-LIFE model is well suited. It allows to monitor long-term effects, which is key to dissect the mechanisms leading to tumor growth and invasion, but also to monitor the effects of drugs on the tumor cells.

Franz cell adapter for skin penetration studies

EURO COSMETICS: *To understand the effects of molecules that come into contact with human skin is key for researchers across industry. Which tests do you perform in order to get a reliable statement about the portion of a cosmetic ingredient that penetrates the skin after application?*

Dr. Karsten Mewes: I agree, investigating the effects of molecules, be it a raw material or originating from formulations, or mixtures, they all can penetrate the skin barrier. A chemical which does not penetrate cannot evoke any effects in the regions of living skin cells, neither beneficial nor harmful ones. Hence, it is important to thoroughly pursue the pathway amolecule takes through the skin, and to collect information about its distribution in the different skin tissues and layers.

Many skin penetration analyses are still conducted with pig skin or human cadaver skin, following accepted OECD Test Guidelines. However, our Phenion Full-Thickness Skin Models have proven their value in this kind of analyses, too. In an *in vitro* skin penetration study, conducted at the Freie Universität Berlin, the Phenion FT models were exposed to 4 model chemicals with quite similar molecular weights, but logP values, the measure for lipophilicity, covering a broader range. Although the penetration rate through the tissue was higher for all chemicals compared with pig skin, the penetration behavior based on the respective logP value, was comparable. Thus, the capacity of the Phenion FT Skin Model to discriminate between chemicals of different lipophilicities, is *in vivo*-like. Hence, the authors of this study concluded that the skin models are suited to run penetration and dermal absorption studies when taking the somewhat higher penetration rate into account. Additionally, the Phenon FT Skin Model reflects the xenometabolism of human skin, which is e.g. for safety assessments of utmost importance. In order to facilitate penetration studies with the skin models, we have developed an adapter set to place the tissues precisely in the center of a Franz cell and to tightly seal the compartments. This so-called Franz cell adapter is an optimized version of the adapter used in the Berlin study. Constructed of Teflon, it is resistant against many chemicals and can be cleaned and autoclaved to be used several times.

EURO COSMETICS: *With which method can the extent and speed of dermal take-up of active ingredients be determined?*

Dr. Karsten Mewes: The classical test design uses radioisotopes to follow the way of a chemical through the skin and to assess the distribution in the different layers

of epidermis and dermis. The radioactive nuclides, e.g. C^{14}, H^3, or I^{125}, must be stably introduced into the molecules, and care must be taken that the nuclide is inserted in the molecular core structure. Otherwise, due to the active xenometabolism, the respective labelled group might get cleaved and eventually gets lost undetected. Producing the radiolabeled chemicals is cost-intensive and needs sound expertise of the synthetic pathways leading to the desired structure. In addition, all further work must be conducted in special labs under special safety measures. The advantage of using radioisotope, on the other hand, is its quite simple traceability and quantification.

To avoid the disadvantages of radio-labelling, we have developed a non-radioactive method for skin penetration studies. After exposure of the full-thickness skin models with the chemical of interest, the tissues can be stepwise dissected. In a first tier, epidermis and dermis can be separated enzymatically, and in a second tier, single epidermal layers can be collected in a process resembling the tape stripping procedure in dermatological studies. All tissues and epidermal layers, but also the receptor fluid, can then be subjected to chemical analyses, e. g. with HPLC or mass spectroscopy, to determine the content of the previously applied chemical. With this method we receive information about the penetration rate of a given chemical, but also generate a distribution profile within the skin model.

EURO COSMETICS: *What role does the horny layer play in skin penetration?*

Dr. Karsten Mewes: More than 95% of the epidermal barrier is defined by its uppermost layer, the Stratum corneum, or horny layer. Embedded in lipid layers of quasi-crystalline structure lie the corneocytes, the remnants of the keratinocytes at the end of their life cycle. This special structure is sometimes compared with a mortar-brick construction, with the corneocytes as solid "bricks" and the lipids as the connecting and stabilizing "mortar". During an approximately 4-week-lasting process called terminal differentiation, the epidermal keratinocytes move upwards, thereby consecutively reducing their metabolism, while simultaneously generating an intracellular cornified envelope. This envelope consists of tightly crosslinked proteins and renders the cell in a mechanically stable, but dead structure. Finally, these corneocytes are shed from the skin's surface through e.g. our daily body care, our movements, and the friction generated with our clothing. It was demonstrated that the isolated stratum corneum alone is sufficient to build up a robust barrier between our body and the environment, and to decide which substances can penetrate the skin and subsequently enter our organism or not. The underlying layers of viable keratinocytes only play a minor role in this first line of defense.

Against this background, we started intensive research into the lipid composition and the underlying biochemical mechanisms of the horny layer, and hence the barrier function, of the full-thickness skin models.

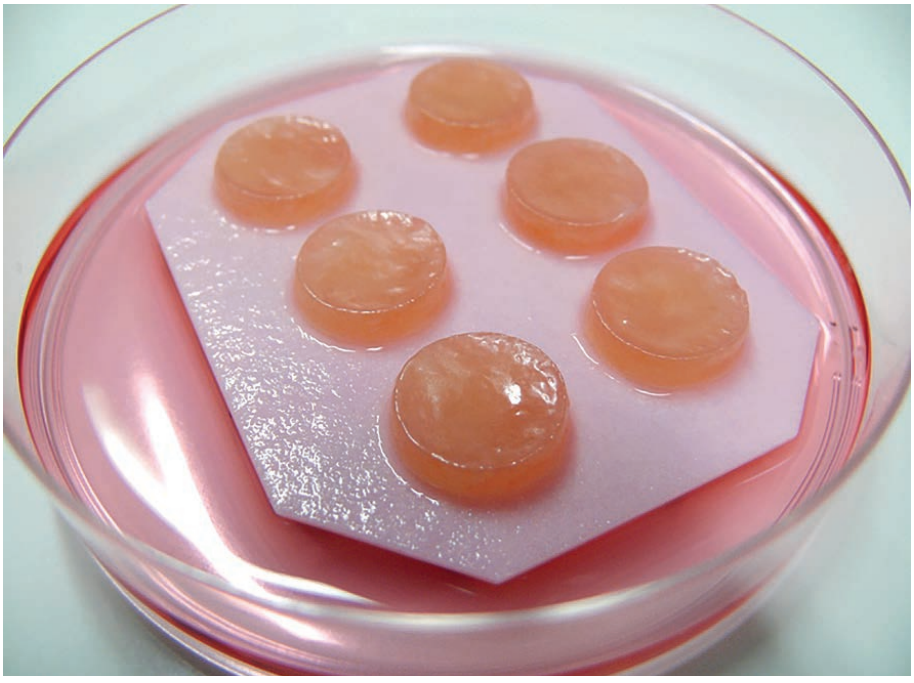
EURO COSMETICS: *Can you explain your analyses of the barrier function in more detail? Which lipids are essential for the skin barrier and how are they generated during the terminal epidermal differentiation?*

Dr. Karsten Mewes: Terminally differentiated corneocytes, together with complex lipid layers, define the epidermal barrier function. The proteins which contribute to the final structure of the corneocytes have been investigated in the skin models as part of our quality control process. Based on immunofluorescence analyses we know that structural proteins like e.g. involucrin, filaggrin, and loricrin, but also transglutaminase, an enzyme which catalyzes the crosslinking of these proteins, are expressed in a distribution pattern like in native healthy human skin.

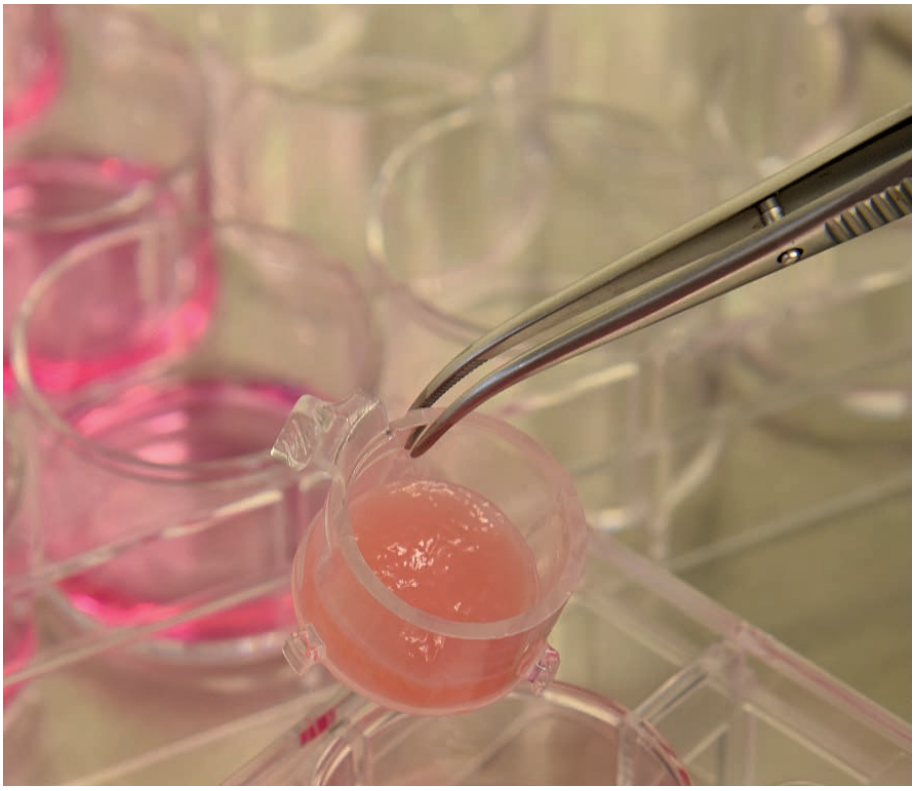
For the analysis of the lipid composition, we chose thin-layer chromatography as a well-established test system. Therefore, we prepared total lipid extracts form the epidermal tissues of the skin models, but also from native human skin biopsies as control.

It became evident, that all major lipid classes were present in the epidermal compartment of the skin models as well as of native human skin, with cholesterol, triglycerides, cholesterol esters, phosphatidyl choline, and ceramides as some prominent examples. The lipids typically found in the human stratum corneum are mostly derived from few precursors which are then processed into their final molecular structure by several enzymes located in the upper epidermal layers. In order to assess whether these enzymes are also expressed in the skin models, we conducted immunofluorescence and gene expression analyses, thereby focusing on serine palmitoyl transferase (SPT) and β-glucocerebrosidase (GBA), two key enzymes of dermal lipid metabolism.

The genes coding for both enzymes were expressed in the skin models at compara-



Phenion FT Skin Models in air-liquid interface culture



Phenion FT Insert Model

ble levels as in human skin. With means of immunofluorescence we have also demonstrated the existence of SPT and GBA on the protein level, thus again proving the high metabolic competence of the skin models. With the pivotal elements in place- the structural proteins, the lipogenic enzymes and the respective lipid species- the barrier structure of the skin models is comparable to that of human skin. These results impressively contribute to the understanding of the skin models' good performance in testing skin penetration, or dermal absorption.

EURO COSMETICS: *For studies intended to analyze the access of chemicals to the skin, knowledge of the skin barrier is essential. How can your skin models be used to address these questions?*

Dr. Karsten Mewes: Indeed, knowing if and how a substance, enters the skin and how its distribution pattern looks like is pivotal in many respects.

Being interested in substances with a beneficial effect to living skin, they must first penetrate the barrier in order to reach the target cells. Tests with the full-thickness skin models can elucidate whether a given substance reaches relevant concentrations and hence has a good chance to exert its desired positive effects. When addressing physiological reactions in the dermis, to e.g. strengthen the connective tissue, the active ingredient must reach the dermal layers without being deposited or metabolized in the epidermis.

In certain cases, it is preferable to prevent that an ingredient penetrates the skin where it might become systemically available. Again, penetration studies based on the full-thickness skin models can markedly contribute to the knowledge about tissue distribution, while at the same time respecting skin metabolism and hence can contribute to toxicological safety assessments.

Having a well-developed skin barrier in place is also crucial for assessing product claims which can aim e.g. at improving or restoring a barrier after skin damages. In general, two different study designs can be pursued. In the first scenario, the surface of the skin model is damaged under standardized conditions to evoke a phenotype similar to the stressed conditions seen *in vivo*. Then the ingredient of choice is applied, and the effects are analyzed. Here we look at the restoration of an intact barrier. The second scenario starts with the ingredient application, followed by the exposure with a damaging factor. This approach aims at preventing the skin barrier from being stressed.

Besides analyzing and comparing the lipid profiles of the treated skin models or monitoring the release of pro-inflammatory molecules like interleukins, we can now investigate the integrity of the barrier non-invasively, too. Together with Courage + Khazaka, a Germany-based company specialized in the development of skin testing equipment, we have advanced a device to monitor the transepithelial water loss (TEWL) above the skin model surface as a function of barrier integrity. Increasing TEWL's point to stressed, or damaged epidermal surface, whereas a drop in TEWL is an indicator for barrier recovery. Due to its non-invasive application, the TEWL can be recorded and analyzed several times for the same tissue model, which provides pivotal insight into the dynamics of barrier reactivity.

EURO COSMETICS: *Which are your future plans in respect of the 3D skin models/the Phenion business?*

Dr. Karsten Mewes: It is our ambition to develop and provide our expertise to replace animal testing worldwide. Knowing that we are not yet there, we nevertheless continuously strive to approach this vision step by step. Thus, we optimize the already existing tissue models and methods on the one hand and develop new tissue variants and analysis tools on the other hand. With our engagement we want to support other scientists in academia and industry to tackle urgent questions of basic dermatological and pharmaceutical research, but also of toxicological and regulatory concern, in a yet unprecedented manner.

Key for our strategic decisions is the communication with our customers. Listening to their current and prospective needs inspires us, and it is not unusual that we join forces.

Taken together, our driving force for the Phenion vision is customer centricity and sharing the science towards alternatives to animal testing.

EURO COSMETICS: *Thank you for the conversation.* ■