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Reconstructed 3D Tissues for Efficacy and **Safety Testing of Cosmetic Ingredients**

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Educational Review

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Reconstructed 3D Tissues for Efficacy and Safety Testing of Cosmetic Ingredients

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SCIENTIFIC PAPER

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Abstract

Currently, the most innovative methods for toxicology assessment and skin research are based on 3-dimensional reconstructed tissues of the human skin. Decades of research revealed that skin cells when cultured in a 3D environment can build complex tissues which closely mimic native human skin in its architecture and physiological properties. This is a pivotal prerequisite for the successful replacement of animals in toxicological tests and for assessing the efficacy, for example, of product ingredients. The present review illuminates the demands of the cosmetic industry for tissue models of the human skin. It distinguishes between their use for toxicological safety assessment as part of validated and/or scientifically accepted in vitro methods on the one hand and their use in early development of cosmetic products on the other hand. The 3D Skin Comet assay for genotoxicity testing and the in vitro skin irritation test based on reconstructed skin tissues (Phenion™ FT-Skin model, Open source Reconstructed Epidermis – OS-REp) are examples for the first aspect. The second aspect is illustrated by the use of a full-thickness skin equivalent to study the anti-aging effects of Quassia amara wood extract on human skin and of a 3D hair follicle model, derived from human skin, to analyze metabolic changes after coenzyme 10 treatment.

Introduction

Each step on the way from promising active ingredients to a high-quality consumer product requires specific tests in order to identify the best suited substance for a desired claim and for toxicological safety assessment. While in former times some safety assessment tests were conducted on animals of different species as surrogates for the human organism, *in vitro* alternatives to animal testing have been developed over the last decades to reduce, refine and replace animal tests, a principle already defined in 1959 [1].

The demand for animal-free alternative methods to describe the toxicological profile of chemicals is increasingly reflected in the national regulations of individual countries or in the legislation of economically and politically linked international unions like the European Union (EU). A milestone in the implementation of *in vitro* alternative methods in the framework of consumer protection in nationwide legislation was the 7th Amendment of the EU Cosmetics Directive 76/768/EWG defined in 2003 (replaced in 2009 by the EU Cosmetics Regulation (EC) No. 1223/2009 EU) [2]. It implemented a complete marketing ban for cosmetic products containing ingredients which have been tested on animals, starting in March 2009 for acute toxicity endpoints and in March 2013 for repeated dose and systemic toxicity endpoints [2]. Meanwhile other regions outside of the European Union like several states in Brazil, Norway, Israel, India and New Zealand have adapted their regulations, and more countries are expected to change regulations accordingly.

The aim to reduce animal tests to a maximum is also implemented in the EU chemical legislation REACh (Registration, Evaluation, Authorisation and Restriction of Chemicals), which entered into force in the European Union on June 1, 2007: "REACH is a regulation of the European Union, adopted to improve the protection of human health and the environment from the risks that can be posed by chemicals, while enhancing the competitiveness of the EU chemicals industry. It also promotes alternative methods for the hazard assessment of substances in order to reduce the number of tests on animals." (http://eur-lex.europa.eu/legal-content/en/TXT/PDF/?uri=CELEX:02006R1907-20160203) [3].

Driven by legislation as well as ethics, the development of suitable *in vitro* methods has gained momentum. Innovative test methods based on culturing animal cells

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or, more preferably, human cells have been published. Scientists as well as regulators agree that three-dimensional (3D) cell cultures, or organotypic tissue models, already play a pivotal role, with increasing importance in this context in the future. This special kind of culture promises to mimic essential aspects of human physiology and thus be the ideal tool for assessing both the beneficial and the toxic modes of action of chemicals.

Although the development of alternative methods has made enormous progress, today only in a few cases can one animal test be completely replaced by one single *in vitro* assay, bearing in mind the complexity of a living organism and its multiple interactions with a certain chemical. Experts working in this field anticipate that this limiting situation can be overcome by combining different test methods within a so-called integrated testing strategy (ITS). Ideally, the individual assays simulate different physiological traits of tissues or organs, so that a gap of knowledge which remains after running a first assay can be filled by the results of subsequent assays. In addition, such tiered approaches also allow the investigation of different mechanistic aspects of the toxicological endpoint under investigation. With a well-designed ITS in place a sufficiently precise statement can be made about the requested properties of the test item, e.g. about the toxicological potential and the efficacy of the tested substance, respectively.

The Paradigm Shift From 2D to 3D

For decades, culturing mammalian cells, including cells from human tissues and organs, in a 2-dimensional environment was state of the art. However there is increasing evidence that cells cultured under 2D conditions exhibit limited physiological properties compared to native cells and tissues in the living organism. The cells lack proper interactions with neighboring cells of the same kind and with other cell types normally present in an organ. The surface of the cell culture vessel cannot provide the adequate microenvironment that the cells prefer within a tissue to flourish and consists of a variety of proteins and carbohydrate polymers. Under these non-physiological and hence suboptimal conditions the cells cannot retain their original three-dimensional cell shape and the cell polarity breaks down. This has serious consequences at all levels of cell activity, from gene expression and signal transduction over cell division to special metabolic tasks the cells normally fulfill in an organism [e.g. 4, 11].

Many of the limitations described above can be overcome by introducing the cells of interest into a 3-dimensional environment. Starting in the late 60s with collagen-coated cellulose sponges [5, 6], many different matrix materials consisting of either natural and/or synthetic components have been developed which aim at providing a more natural 3-dimensional microenvironment to the cultured cells. The efforts of both biologists and engineers were rewarded with cultures which retained or regained their native physiological properties to a large extent. Hepatocytes, for example, maintained normal cell morphology and revealed an *in vivo*-like mRNA expression pattern when cultured in a collagen sandwich, whereas the pattern was altered significantly in monolayer culture [7-9]. Gene expression and metabolic competence of keratinocytes cultured in 3D tissue equivalents more closely resembled the situation seen in native human skin compared to keratinocytes cultured in monolayers only [10, 11]. A series of review articles have since been published dealing more generally with the advantages of the 2D to 3D transition in cell culture technique [e.g. 4, 12-15].

A prominent example for a successful transition from 2D to 3D are three-dimensional tissue equivalents of the human skin. First attempts to cover wounds and eventually restore skin functions after severe wounding were made more than a century ago with skin fragments which had been removed from a healthy site of the body [reviewed 16]. However, only after development of innovative culture media and materials to support a 3D architecture can high-quality skin models be reproducibly produced in large amounts [e.g. 17-22].

The Industry Perspective

Besides their value as transplants reconstructed skin equivalents have also been recognized as valuable tools in the framework of toxicological safety assessment of dermally applied chemicals, pharmaceuticals, and finished products [23] and are now indispensable in any kind of *in vitro* testing related to human skin. In addition, 3D skin models also display practical benefits in any test set-up, in particular when testing cosmetic ingredients and formulations. Compared to 2D cell cultures of skin keratinocytes or fibroblasts, the topical application of substances to 3D skin models reflects a more realistic exposure scenario. Researchers can easily apply formulations (e.g. emulsions, gels, etc.), solids and hydrophilic as well as hydrophobic substances taking the barrier function of the skin into account, which enables the application of doses similar to the situation of use. In addition, a broad range of solvents can be used.

The first skin models applied in such studies were tissue equivalents mirroring the outermost layer of the skin, the epidermis. Meanwhile full-thickness skin models, covering both epidermis and dermis, are available in addition to epidermal models in which melanocytes or hair follicles are embedded. Finally, mucosal tissues are commercially available to investigate effects of test substance on epithelia of the mouth or vagina characterized by a lower penetration barrier than the skin. In the course of efficacy testing for cosmetic products or ingredients, the use of these tissue models opens a vast variety of possible product claims in a similar manner as biological endpoints that can be assessed with modern analytical tools. With the help of engineered models of the target tissue, screening for potent bioactive ingredients can be conducted by analyzing, for example, modulators of skin irritation reactions, barrier function and cellular differentiation processes as well as the amount, composition and turnover of extracellular matrix molecules, scavenger systems for oxidative stress or the energy charge of the tissue.

Independently from regulatory challenges, the cosmetics industry has been making

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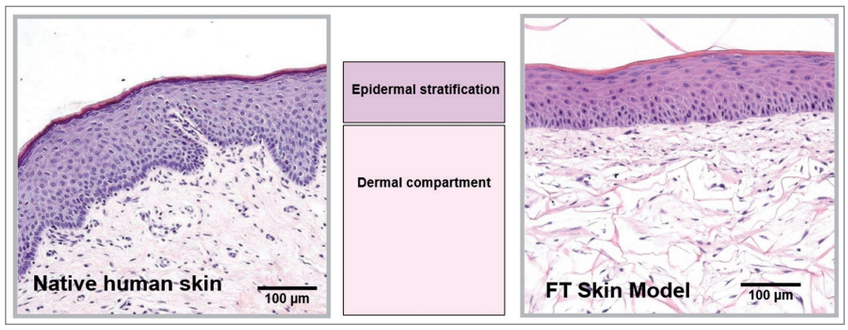

Figure 1a Comparative histological sections through native human skin and the Phenion® Full-Thickness skin model. The tissues were fixed with formaldehyde and embedded in paraffin, and the sections were stained with hematoxylin & eosin.

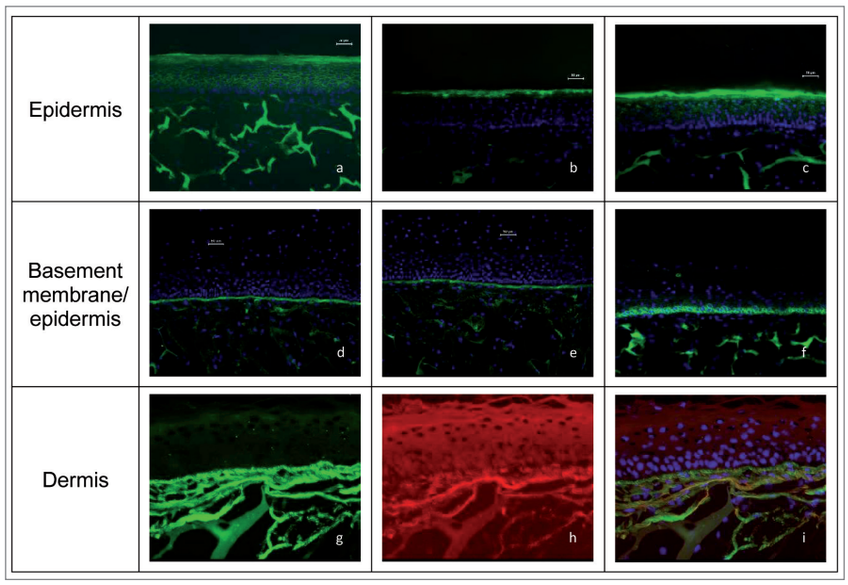

Figure 1b Immunohistochemical characterization of the Phenion® FT Skin Model. Fully differentiated FT models were fixed, cut and incubated with antibodies directed against proteins which are pivotal for the correct differentiation of native human skin. Except for the anti-fibrillin antibody, specific antibody bonding was visualized with an Alexa Fluor® 488-coupled secondary antibody (green fluorescence). Anti-filaggrin antibody binding was visualized with an Alexa Fluor® 568-coupled antibody (red fluorescence). Cell nuclei were DAPI-stained (blue fluorescence). In the merger picture orange-stained structures indicate expression of elastin and fibrillin-1 at the same place. a) transglutaminase, b) involucrin, c) filaggrin, d) laminin-5, e) collagen IV, f) integrin α3, g) elastin, h) fibrillin-1, i) merger elastin/fibrillin-1. g-i) from: [55].

fundamental investments already since the mid-1980s in close collaboration with academic institutes and regulatory authorities in order to develop and validate animal-free test systems. Since then, substantial improvement has been made also in developing three dimensional organotypic tissue models for safety assessment purposes and efficacy testing. In the following sections two different 3D tissue models will be presented which have proven their value in the development of new efficacy concepts for cosmetic actives and formulations. The commercially available Phenion® FT Skin Model, which was developed and characterized during the last years, shows high similarity with native human skin both morphologically and physiologically ***(Figure 1)***. Its *in vivo*-like physiological competence was demonstrated for the de novo synthesis of elastic fibers and the expression of phase I and II enzymes of the xenobiotic metabolism [22, 11]. The other organotypic tissue model that will be discussed, a 3D hair follicle model, is used to study diverse hair physiological processes. Since its introduction at the 5th International Congress of Hair Research in Vancouver [24], this tissue model has been used for research activities that have resulted in multiple publications [25, 26].

Use of 3D Models for Efficacy Testing of Ingredients and Formulations

Quassia amara wood with retinoid-like anti-aging effects: use of 3D human skin equivalents

Skin aging manifests itself by characteristic signs like wrinkle formation, pore enlargement, epidermal thinning and loss of elasticity in all relevant skin layers as a result of lifelong intrinsic and extrinsic stimuli. In order to develop high-performing anti-aging products, the respective cosmetic formulations need to provide efficacy against many signs of aging on the molecular level. Retinoids are widely accepted as the "gold-standard" of anti-aging treatments [see e.g. 27]. Despite the undisputed effects of retinoids in improving the appearance of wrinkles, adverse effects of topical application, such as a burning sensation, have been reported as well. Guided by the advantages of retinoids in terms of fighting signs of skin aging, a study was conducted to profile ingredients for effective anti-aging skin care formulations combined with excellent skin compatibility and reduced side effects [28]. Using human 3D skin models mimicking human epidermis and full-thickness skin, respectively, an extract from the wood of the South American plant *Quassia amara* (Fam. Simaroubaceae) turned out to provide excellent retinoid-like effects without compromising skin compatibility. Topical application of *Quassia amara* extract markedly stimulated the expression of genes coding for proteins which are crucial for proper epithelial differentiation and intact skin barrier function in a pure epidermal equivalent (OS-REp model; ***Figure 2***). The expression level even exceeded the one observed after treatment with retinoic acid. The influence of *Quassia amara* extract on dermal proteins was analyzed in the Phenion® FT Skin Model, comprising both a dermal and an epidermal compartment. After topical application of extract-con-

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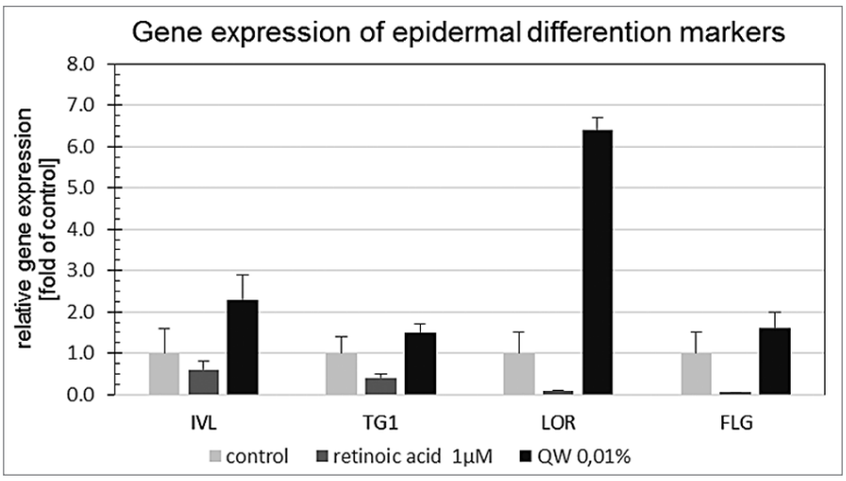

Figure 2 Induction of gene expression of essential epidermal proteins by retinoic acid (1 µM) or Quassia amara (QW) wood extract (0.01 %) in a reconstructed epidermal equivalent (open source reconstructed epidermis, OS-REp). Gene expression was analyzed for the following genes: involucrin (IVL), transglutaminase 1 (TG1), loricrin (LOR), filaggrin (FLG), (modified from [23]). The columns represent the mean values +/- SD of 3 models each, calculated as relative expression compared to the placebo control (= 1.0).

taining formulations elastin gene expression markedly increased within 48 hours of exposure ***(Figure 3a)***, an effect which could also be measured on the protein level ***(Figure 3b)***. In a former study it was already demonstrated that an increase in elastin gene expression was immediately followed by elevated elastin protein levels and an increase in the complexity of the elastic network within the Phenion® FT Skin Model [22]. Thus it can be assumed that increased elastin gene expression precedes increased protein synthesis in the *Quassia amara* study, too. As elastin comprises one of the main constituents of dermal elastic fibers, an increase can be correlated with improved skin elasticity and thus with a possible amelioration of typical signs of skin aging.

The results achieved with the *in vitro* test systems based on 3D tissue models of the skin were subsequently confirmed in clinical studies. Treating skin of volunteers with *Quassia amara* extract significantly improved parameters with regard to skin surface profile and skin firmness. Further it was shown that a face care formulation containing *Quassia amara* wood extract as well as cream containing tretinoin, a derivative of retinoic acid, increased the collagen neo-synthesis in skin, as demonstrated by measurement of procollagen peptides in the fluids of suction blisters ***(Figure 4)***. This study exemplifies a straightforward testing strategy using reconstructed 3D skin models that ultimately led to the development of innovative skin care formulations.

Q10 against aging: use of tissue-engineered hair follicle models

Recently it was described that human hair follicles change their physiology with the onset of aging [26]. In this publication the authors detailed that hair follicles are highly specialized skin appendages which undergo biological alterations as a consequence of intrinsic and extrinsic age-dependent stimuli. Although phenomena like hair graying were well recognized, molecular alterations in terms of hair structure remained obscure and were described by these authors for the first time. A comparative study revealed

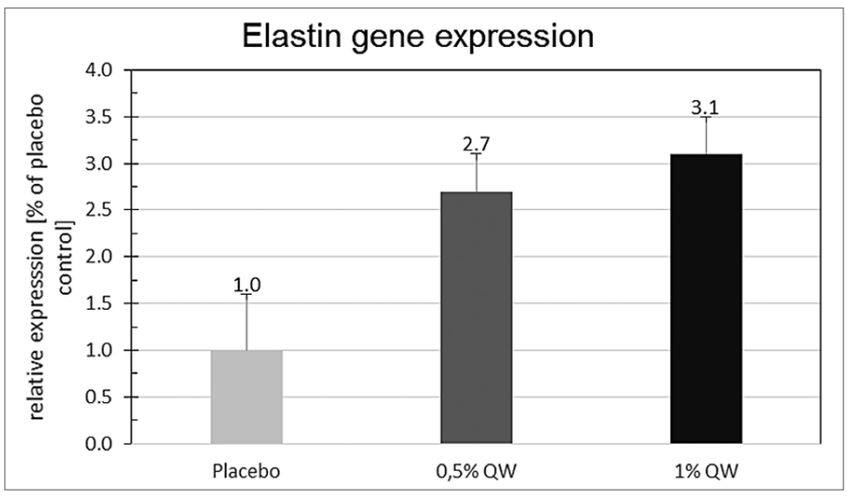

Figure 3a Induction of elastin gene expression in Phenion® Full-Thickness skin models after 48 hours of topical treatment with 0.5 % and 1.0 % Quassia amara (QW) extract, respectively. The columns represent the mean values +/- SD of 4 models each, calculated as relative expression compared to the placebo control (= 1.0).

Treatment	Intensity	SD	Rel. expression	SD
Control	64142	7395	100	11
Placebo	68461	1935	102	3
QW 0.5%	69116	3119	103	5
QW 1.0%	76236	2874	114	4

Figure 3b Induction of elastin secretion in Phenion® Full-Thickness skin models after 48 hours of topical treatment with 0.5 % and 1.0 % Quassia amara (QW) extract, respectively. After treatment the skin models were fixed, cut and incubated with an anti-elastin antibody followed by a secondary HRP-coupled antibody. Elastin was visualized with the HRP substrate DAB and quantified with the ImageJ analysis software (mean intensity ± standard deviation). For each treatment 4 tissue sections were analyzed. Elastin content is indicated as the relative expression compared to the untreated control (mean ± standard deviation). Placebo: basis cream formulation without active.

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that the expression of certain keratins as well as keratin-associated proteins (KAPs) differ significantly between volunteers under 25 and volunteers over 50 years of age ***(Figure 5)***. With the help of tissue-engineered 3D hair follicle models the researchers conducted a screening of bio-active ingredients which finally led to the identification of coenzyme Q10 [25].

In the 3D hair follicle model key players in the human hair follicle are integrated in a way which allows them to interact with each other almost the same as in the *in vivo* situation ***(Figure 6)***. Briefly, dermal papilla cells are grown on porous Cultisphere® carrier beads and subsequently embedded in a collagen gel populated with human dermal fibroblasts. Finally, outer root sheath keratinocytes are added on top of the collagen gel to form an epithelial-like cell layer. The cells used to construct the hair follicle model had been isolated from human plucked hair follicles and human skin, respectively, according to well-established protocols [29, 30; proprietary standard operation procedures].

As shown in ***Figure 7***, application of Q10 on top of the hair follicle model stimulated the expression of genes coding for the hair keratins KRT33A, KRT34 and, to a minor extent, KRT86. These keratins are known to be involved in the age-related changes in the native human hair follicle. It had been demonstrated previously that their expression is reduced on the gene expression level in the hair follicles of mature volunteers (≥ 50 years) compared to younger individuals (≤ 25 years; [31]). The effectiveness of coenzyme Q10 according to results of the *in vitro* approach was confirmed in a placebo-controlled volunteer study looking at keratin gene expression in plucked hair follicles with and without prior Q10 treatment. After Q10 treatment the age-related keratins were upregulated, which corroborated the results achieved with the reconstructed hair follicle model [32]. These findings clearly underline the biological relevance and predictivity of the bioengineered 3D hair follicle model.

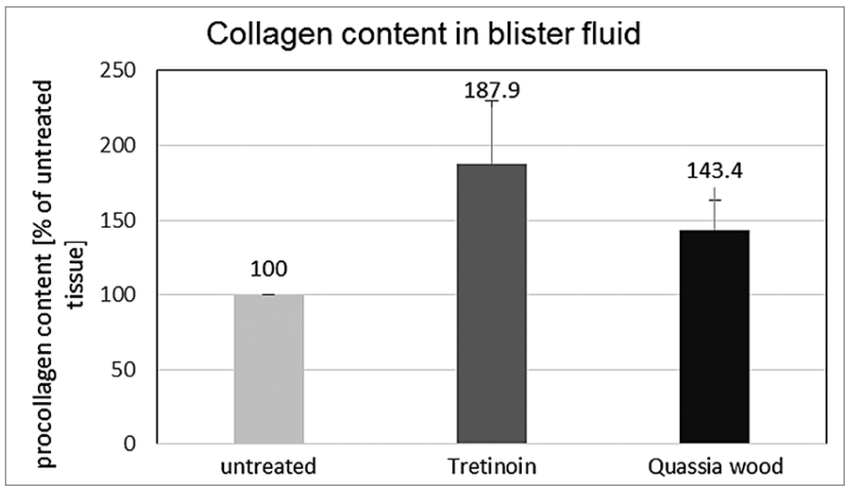

Figure 4 Induction of collagen by tretinoin, a derivate of retinoic acid (0.05 %), and *Quassia amara* wood extract (1 %). After eight weeks of exposure with respective products on four randomized test sites on the volar forearm suction blisters were generated and the content of collagen propeptides measured in the suction blister fluid. Samples were collected from 25 female volunteers (40 and 60 years of age) with three blisters from each of the four test areas. Values are given as percent increase relative to untreated controls after normalization to the total protein content in the respective blister fluid (mean ± SD).

Use of 3D Skin Model for Regulatory Toxicological Purposes

Three-dimensional models of the skin and other organs are not only valuable tools for basic research and efficacy studies, but also for the development of *in vitro* methods used within the framework of toxicological safety assessment. The increasing

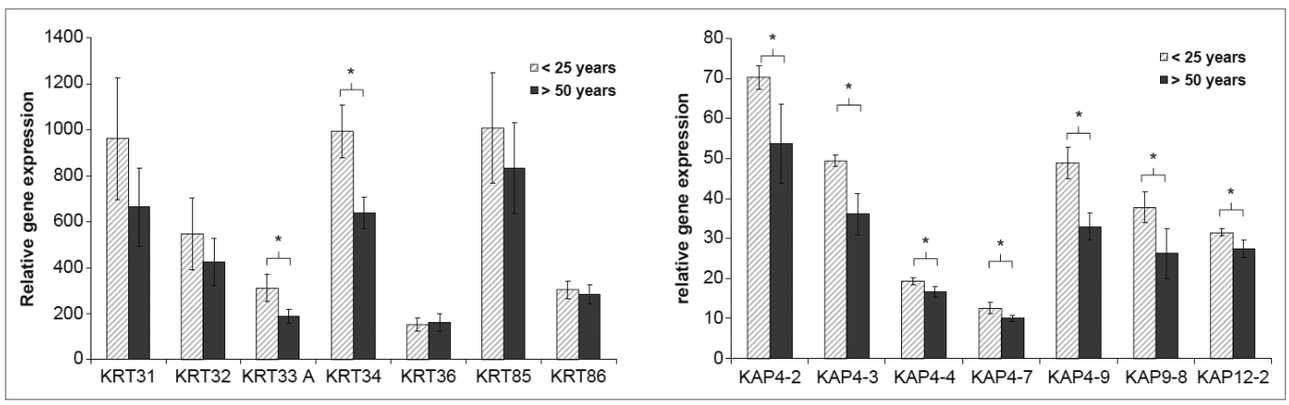

Figure 5 Differences in the gene expression profile in hair follicles of young (< 25 years) and middle-aged (>50 years) humans. a) Gene expression analysis of hair keratins (KRT) using a quantitative PCR method. Data show significant downregulation of KRT33A and KRT34 in the older hair follicles compared to younger ones. b) Gene expression analysis of keratin-associated proteins (KAP) using a cDNA microarray. Data show significant downregulation of different KAPs in the older follicles compared to the younger ones. Ten scalp hair follicles were plucked from 20 healthy volunteers each (10 women and 10 men) under 25 years and over 50 years of age, and total RNA was isolated. All statistics are given as mean +/- SD, *P < 0.05. (modified from [26]).

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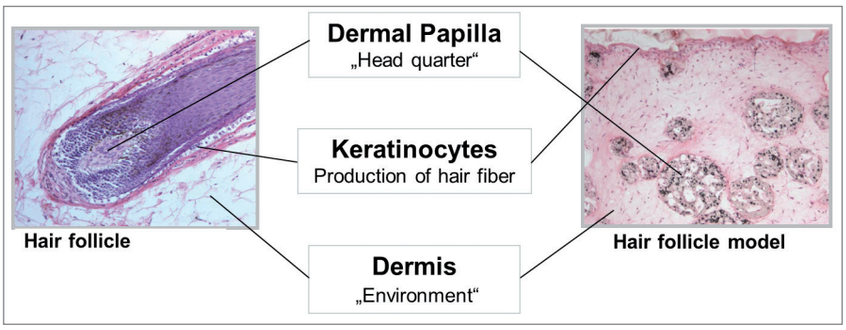

Figure 6 Tissue architecture of the hair follicle model compared to the native human hair follicle. The major components of the native human hair follicle and its environment – namely the dermal papilla cells, the outer root sheath keratinocytes and the fibroblasts of the surrounding connective tissue- are integrated in the 3D model. The tissues were fixed, paraffin-embeded and cut, and the sections were stained with hematoxylin & eosin.

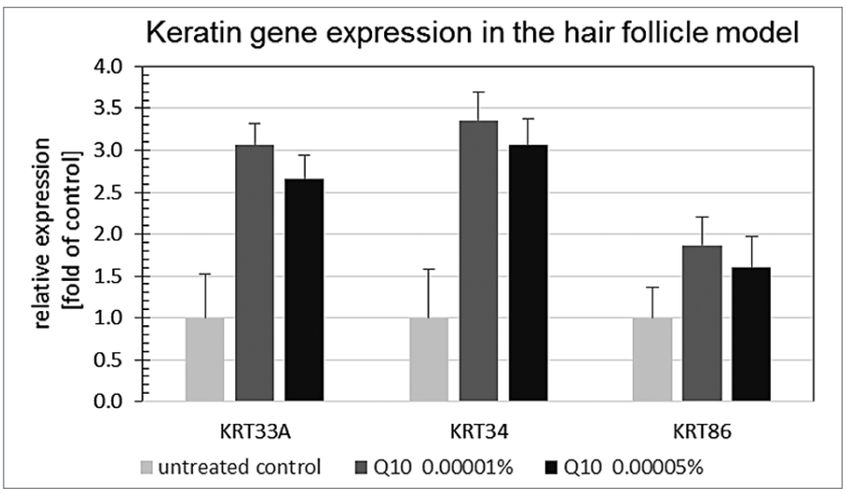

Figure 7 Induction of hair keratin gene expression after a six-hour treatment of hair follicle models with coenzyme Q10 at $1x10^{-4}$ % and $5x10^{-4}$ %. The expression is given as the fold induction compared to the expression in untreated control follicle models. Each column represents the mean value +/- SD of the results from three follicle models. After sample lysis total RNA was isolated using the Qiagen RNeasy MinElute Spin Column system according to the manufacturer's instructions. Quantitative PCR analysis was performed using gene-specific primer sets for the hair keratins KRT33A, KRT34 and KRT86.

importance of tissue model-based assays is illustrated in the following representative examples [for more information, see e.g. 33].

Only recently the alkaline COMET assay, an *in vitro* method for the detection of genotoxic effects, was transferred successfully to the Phenion® Full-Thickness Skin Model. This so-called 3D Skin Comet assay is currently being validated in a 2-tiered ring trial funded by Cosmetics Europe and the German Federal Ministry for Education and Research in which 30 chemicals are tested blinded. The outcome of the first tier in which eight chemicals were investigated independently in five laboratories was promising in terms of reproducibility and predictivity (four laboratories obtained a 100% predictivity, the fifth an 80% predictivity; [34]). The second tier in which 22 additional chemicals will be tested blinded will be finished in 2017.

This good predictivity may be also based on the clear intrinsic metabolic capacity of the tissue model, making it unnecessary to add an external metabolism system such as rat liver S9 mix to convert so-called promutagens into the DNA-reactive metabolite [36]. In preliminary studies it was shown that gene expression and activity of key enzymes of the xenobiotic metabolism in cells of the Phenion® FT were found to be similar to native human skin, in contrast to cells from the same donor grown in 2D culture [11]. In addition, differences in the enzyme pattern were more prominent between epidermis and epidermis than among the three donors investigated.

Meanwhile, the intended regulatory use of the 3D Skin Comet assay has already been demonstrated in an example supporting the safety assessment of a hair dye ingredient, namely Basic Brown 17 [35]. Initial positive findings of the standard *in vitro* test battery were addressed in follow up *in vitro* tests including the 3D Skin Comet assay. This weight-of-evidence approach was accepted by the Scientific Committee on Consumer Safety (SCCS) and the hair dye ingredient declared as 'safe for use' in terms of genotoxicity.

To assess the skin-irritating potential of new cosmetic ingredients *in vitro*, viability tests based on 3D human epidermal equivalents are currently the method of choice. The prediction model is quite simple: tissue viability after substance exposure >50 %, compared to an untreated negative control, indicates a non-irritant and viability ≤50 % a skin irritating substance. Currently, four commercially produced epidermal models are listed in the respective OECD Testing Guideline 439 [38]. The skin-corrosive effects of chemicals can be assessed *in vitro* according to OECD TG 431 by using one of four commercially available epidermal equivalents with a different experimental setup and a different prediction model compared to skin irritation [39].

However, to circumvent trade hurdles for living tissues in several countries, become independent from commercial suppliers and foster worldwide dissemination of non animal test methods, an *in vitro* skin irritation assay based on an open source reconstructed epidermis (OS-REp) model was developed. Its high predictive capacity and reproducibility was proven in a 2-tiered catch-up validation study, which was conducted according to the OECD

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Performance Standards for *in vitro* skin irritation testing [40-42]. The open source concept includes freely available and accessible standard operating procedures for both epidermal model construction and assay performance. It enables users worldwide to produce their own tissue models and run the skin irritation test independently from any legal or intellectual property issues. With this concept the OSREp-based skin irritation test sets a precedent within the framework of alternative methods to animal testing.

Future Developments / Outlook

All examples presented in the previous sections are representative of many other studies conducted in and for the cosmetic industry to screen for new ingredients, conduct safety assessment, develop new concepts for active ingredients and provide further evidence for the efficacy of the finished product. 3D equivalents of the target tissues have not only facilitated the testing of substances with the most diverse physical and chemical properties. They also contributed to the development of test methods which mimic human physiology in a yet unprecedented manner.

Therefore it is not surprising that tissue engineering is a thriving business which is reflected, for example, by the steadily increasing number of scientific publications (***Figure 8***; cit. from PubMed; [43]).

At least three trends can be identified for the future of tissue engineering: the tendencies towards standardization and miniaturization of 3D tissues cultures, respectively, and the tendency towards more complex cultures. One possible way to standardize the production process for 3D tissue equivalents and consequently enhance tissue quality is pursued by the German-based Fraunhofer Gesellschaft. At their Institute for Manufacturing, Engineering and Automation (IPA) scientists developed a fully automated production facility for 3D tissues models, the so-called "Tissue Factory", a high-tech device which ideally combines biological and technical engineering knowledge [44]. One of the first 3D models run on the tissue factory is the open source reconstructed epidermis, which, in its manually produced version, has been successfully validated as an *in vitro* alternative method for skin irritation testing of chemicals (see above).

To enable high-throughput testing and thus save costs and time, smaller tissue equivalents are needed. Tissue miniaturization started by transferring the once-established culture conditions from e.g. the 12-well or 24-well size to the much smaller 96-well format (e.g. EpiDerm™; www.mattek.com). However, decreasing tissue size must not be limited by the size of the currently available wells or inserts. Innovative small-scale culture systems which are, for example, connected to a perfusion system will pave the way to even smaller skin tissue entities [45-47]. Once established, a large number of substances can be tested in parallel, requiring only small volumes of the test substances.

Increasing complexity of tissue equivalents can be achieved by incorporating different cell types in a precisely defined spatial pattern. Different concepts of so-called 3D-bioprinting are currently pursued in order to achieve this goal [e.g. 48]. 3D tissue equivalents can be assembled in such a way that the different cells types are precisely arranged in their *in vivo*-like 3D matrix before starting the culture. The different cells come into close contact with its neighbors, thus mirroring the spatial organization in an intact organ. Rapid prototyping technologies paved the way and have widely opened the door for standardized organ construction.

On the other hand so-called "organ-on-a-chip-technologies" make use of small aggregates of cells from one specific tissue that can be placed downstream of the respective aggregates from another tissue within an interconnected network of channels. This technology paves the way towards miniaturized organ-like constructs which are cultured using principles of microfluidics. Although the specific microtissues are spatially separated from each other, they are able to communicate *via* the medium-perfused channel network, intended to mimic a functional organ or even organism in the future. Although the technology is still in an early research phase, first biological applications have been announced [e.g. 23, 49-53]. By using laser technology it is now possible to carve 3D microfluidic networks inside cell-populated hydrogel scaffolds in order to construct, for example, a vascular system or other complex or branched tissues [54]. All the aforementioned technologies will play a pivotal role in the cosmetics industry in the future. They will pave the way to screening for new and promising raw materials, conducting the respective toxi-

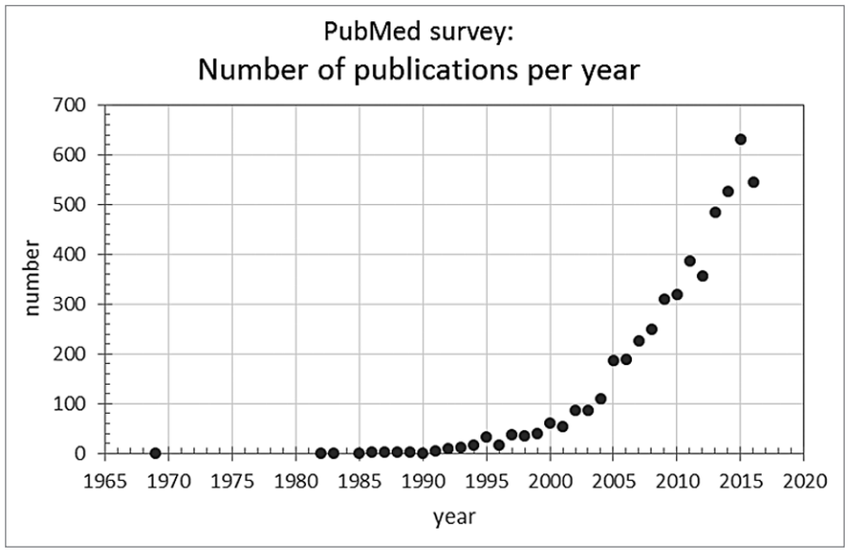

Figure 8 Number of manuscripts published per year dealing with three-dimensional tissue models when searched for "3D AND tissue AND models" in PubMed. In total, 5029 citations were recorded until the end of 2016 with a marked increase in number starting around 1995.

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cological assessment and proving the efficacy of end products *in vitro* with a previously unprecedented precision and wealth of information. The switch from less complex animal cells to complex human-based cell cultures will add to the predictivity of the different areas of application.

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