3D skin models for inflammation & pigmentation

Dr Michel Salmon - StratiCELL, Belgium

Monolayer skin cell cultures (keratinocytes, fibroblasts, melanocytes, etc.) provide us with valuable tools for the evaluation of therapeutic or dermo-cosmetic compounds, particularly in large-scale screening studies. The cost of their use is low, especially if one can work on immortalised lines, in simple and inexpensive culture media. Depending on the nature of the studies, they have limitations and do not always accurately represent the situation encountered *in vivo*. A culture of differentiated keratinocytes in a monolayer, for example, is not suitable for evaluating the effect of active compounds on the barrier function of the epidermis, which needs a stratum corneum. Furthermore, they do not allow topical contact with finished products or liposoluble compounds. Their environment is also very simple and different from the skin, in terms of mechanical forces, spatial orientation, pH and oxygen gradient, and interactions with the extracellular matrix.

There is therefore a real need for models that are closer to skin physiology, representative of epidermal differentiation and stratification, and that are more predictive for active ingredients in the preclinical phase, for the purposes of cosmetic objectification or toxicological evaluation.

The beginnings of 3D reconstructed skin models as we know them today originate from the work of James Rheinwald and Howard Green in the late 1970s, who first succeeded in growing a sheet of keratinocytes on a 3T3 murine fibroblast support. However, the model was devoid of a horny layer and not very representative of the skin.[1]

The first epidermal model with a functional stratum corneum was published by Michel Prunieras and his team in 1979 on the basis of an emersion keratinocyte culture on an acellular dermal matrix.[2]

During the 1990s, models that were easier to manipulate and standardised were developed, notably through the work of Martin Rosdy and LC Clauss, which were cultured at the air-liquid interface on a porous polycarbonate membrane, in a hyper-calcic medium and in the presence of Epidermal Growth Factor (EGF) and vitamin C, among other factors, to promote the formation of a functional epidermal barrier.[3]

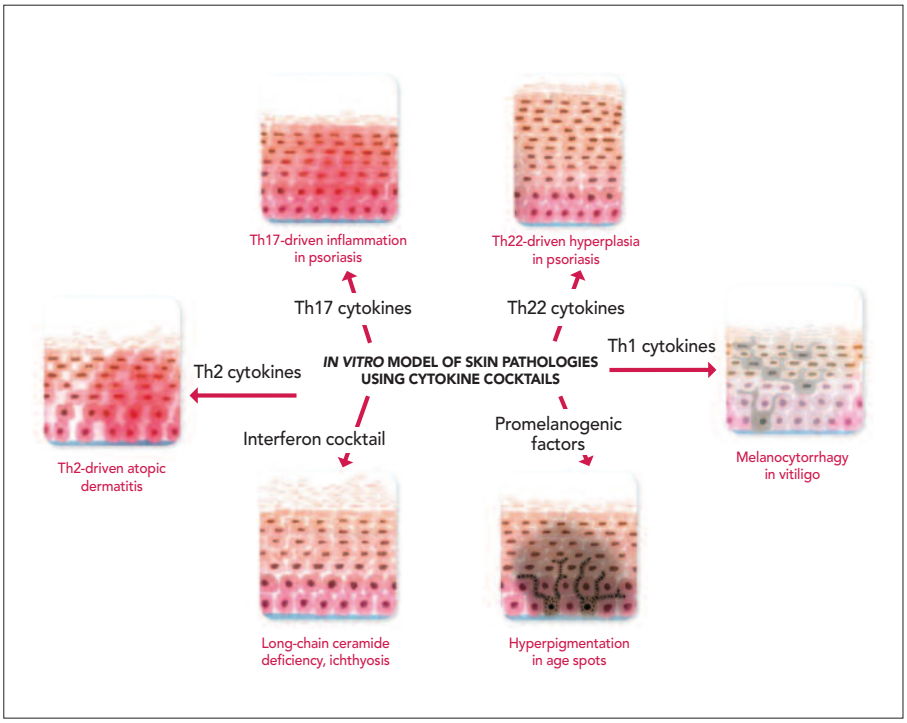

Figure 1: StratiCELL's 3D *in vitro* models of skin pathologies.

The different types of 3D skin models

To date, there are essentially three types of 3D skin models that are compatible with industrial-scale testing, namely reconstituted human epidermis (RHE), human skin equivalent (HSE) and human skin explant, which is taken from human skin during surgery.[4]

RHEs are most often produced using highly standardised protocols, in culture inserts of varying sizes on polycarbonate membranes, based on Rosdy's work mentioned above. They have the advantages of a reasonable unit cost and are commercially available from multiple suppliers (EpiSkin/SkinEthic, EpiDerm/MatTek, epiCS/CellSystems, EPI-001/StratiCELL, EPI-model/LabCyte, etc.). The reconstruction protocol is mainly based on primary keratinocytes derived from neonatal foreskin or abdominal or mammary plasties, but it can easily be applied to keratinocytes derived from iPSC cells, cells from older donors or patients with various pathologies, immortalised keratinocytes (e.g. N-TERT) or genetically modified cells (knock-down, recombinants).

Skin equivalents combining a dermal and an epidermal compartment, are also commercially available (Phenion FT, LabSkin, T-Skin/EpiSkin, EpiDerm FT-200/MatTek), and differ in the nature of the dermal matrix, which may be produced on the basis of collagen, fibrin, other hydrogel-forming proteins, and possibly contain other components such as chitosan and/or hyaluronic acid. The primary advantage of this type of model is that it allows paracrine communication between the cells of the dermis and the epidermis.

However, these models are more expensive and time-consuming to produce than reconstructed epidermis models.

Finally, *ex vivo* skin explants from abdominal or breast, surgeries may be acquired from specialized providers or hospital departments, with delivery delays depending on surgical programmes, required specificities, quality criteria and amount of required sample. Even though they are good representative of *in vivo* skin physiology, they display a high variability of tissue response to treatment or stress, depending on the identity of the donor or the collection site. They are also rapidly found in a pro-inflammatory state linked to survival conditions, and present certain limitations due to preoperative treatment. For example, residual surgical antiseptic compounds exclude *ex vivo* explants from studies on the skin microbiota.

It is also worth emphasising the spectacular advances in cutaneous tissue bio-imprinting techniques, opening up new perspectives towards the complexification of models in particular for applications in toxicology, pharmacology or regenerative medicine.[5]

While these basal state models are perfectly adapted to evaluate the effects of compounds, plant or marine extracts, or even finished products on skin physiology and epidermal homeostasis, they can also be subjected beforehand to stressful conditions to which we are exposed daily, such as UV, infrared or blue light, urban pollution, organic solvents, temperature, or microbial challenges. The disturbances caused by these environmental aggressions and the skin defence mechanisms are increasingly well characterised and can easily be monitored by cellular and molecular biology assays.

In addition, many publications report the integration of other cell types for specific applications, such as primary melanocytes of different phototypes, immune T cells, iPSC-derived human sensory neurons, or components of the skin microbiota (commensal or opportunistic bacteria, Malassezia yeasts, dermatophytes, etc.).

In a concern for quality and standardisation, a discussion that followed the keynote session New Developments in Skin and Epidermal Equivalent Models at the 2019 Barrier Function of Mammalian Skin Gordon Research Conference, pointed out that there is a clear need for a consensus on the quality standard and validation of which organotypic 3D skin models are suitable for skin barrier research and a recommendation paper has been published.[6]

Reconstructed epidermis for the modelling of skin pathologies and pigmentation disorders

Multiple skin pathologies or disorders originate from a deregulation of the immune system, leading to the secretion of pro-inflammatory cytokines by effector cells such as CD4 or CD8 lymphocytes, to a cascade of inflammatory responses from keratinocytes, and to a loss of efficiency of the epidermal barrier.[7]

Additionally, deregulation of paracrine communication between fibroblasts, keratinocytes and melanocytes, may also lead to the development of pigmentary disorders such as actinic or solar lentigo. Prevalence of those pigmentary spots, increases significantly with age, in areas exposed daily to solar radiation and atmospheric pollutants. Even though little is known about the origin of hyper-pigmented spots, it seems to involve fibroblastic growth factors with melanogenic properties.[8]

RHE is a simple yet relevant approach for modelling certain inflammatory pathologies such as psoriasis or atopic eczema, or pigmentary disorders such as lentigo actinic or vitiligo. The role played by lymphocytes or fibroblasts can in fact be substituted by the addition of a cocktail of cytokines or growth factors in the culture medium,

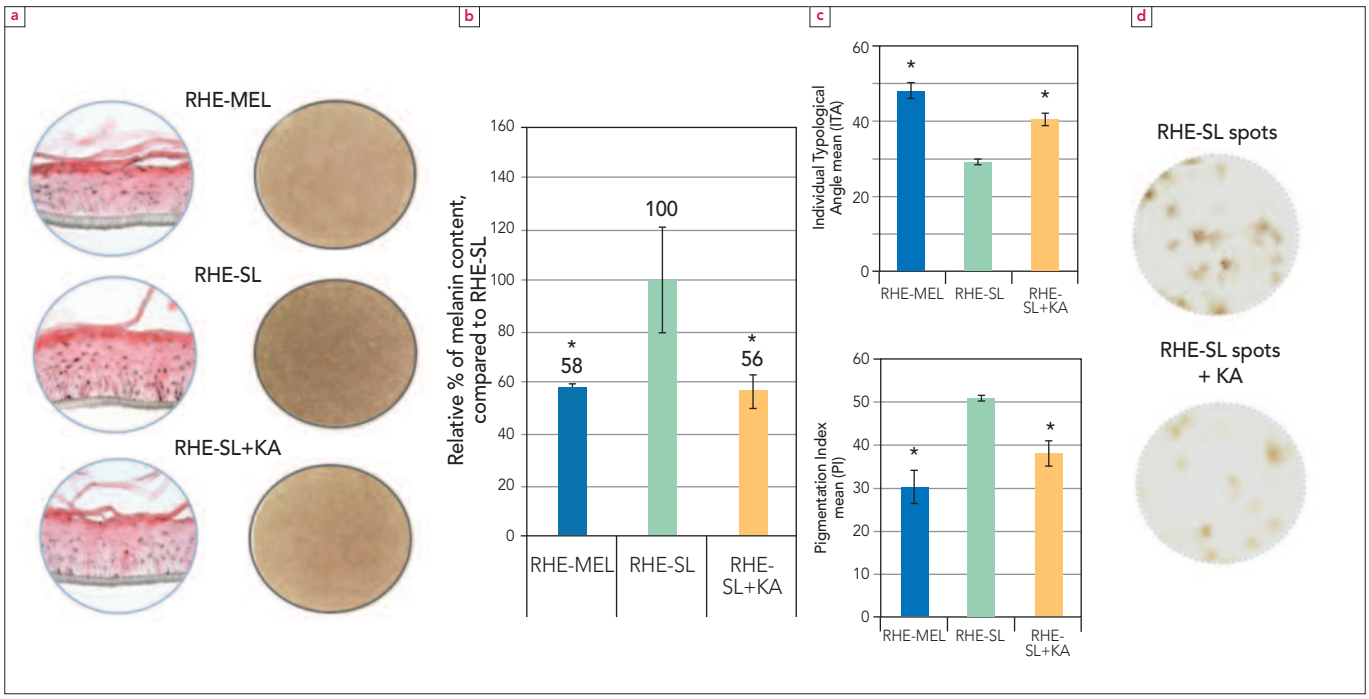

Figure 2: StratiCELL's*in vitro* model of hyperpigmented reconstructed human epidermis, with or without treatment with kojic acid. (a) Fontana-Masson staining (left) and high-resolution images taken by dermoscopy (right). (b) Quantification of melanin by image analysis after Fontana-Masson staining. (c) Measurement of pigmentation parameters by dermoscopy image analysis. (d) High-resolution images of hyperpigmented spots, taken by dermoscopy. RHE-MEL: reconstructed human epidermis with melanocytes; RHE-SL: hyperpigmented reconstructed human epidermis; KA: kojic acid.

leading to a response of the epidermis close to the physiological response (Fig 1).

Modelling of inflammatory pathologies (atopic eczema, psoriasis) and disturbance of the epidermal barrier

Epidermal disturbances in the context of atopic eczema can be induced in RHE by the application in the culture medium of a cocktail of Th-2 cytokines (interleukins IL-4 and IL-13), produced in a physiological context by Th-2 polarised CD4 lymphocytes.[9] Stimulation of RHE by these two cytokines disturbs tissue morphology (observation of spongiosis by histological analysis), reduces the efficiency of the cutaneous barrier, and induces deregulation of the synthesis and transport of lipids and components of the cornified envelope. An increase in cytokines produced by keratinocytes and possessing chemotactic properties is also observed. These perturbations can be attenuated
by different classes of molecules, such as Liver X receptor (LXR) agonists,[10] Janus kinase inhibitors (JAK), or antibodies directed against the IL-4 receptor, blocking the stimulation of the pathway.

The same approach can be applied to psoriasis.[11] For example, the addition of Th-17 cytokines has the effect of inducing the pro-inflammatory cascade dependent on IκB-ζ and NF- κB, leading to overexpression of psoriasis genes IL-19, IL-23, β-defensin-2 and psoriasin (S100A7). Here too, inhibitors of the p38 protein kinase or the NF-κB pathway can be used as reference therapy.

A Th-22-type stimulation can reproduce the phenomenon of hyperplasia characteristic of psoriasis lesions, as well as a loss of barrier components such as filaggrin and loricrin, and a disruption of the involucrin expression profile.[12] Hyperplasia can be limited, for example, by a treatment in parallel with cyclosporine.

Furthermore, in both atopic eczema and psoriasis, a disturbance in the ceramide profile is associated with excessive permeability of the stratum corneum. In addition, a correlation between the average length of the fatty acid chains from ceramides and the effectiveness of the barrier function has been demonstrated.[13,14] Deregulation of Th-1 cytokine release, in particular interferon(IFN)-γ, leads to a decrease in the expression of elongases (ELOVLs) and ceramide synthases (CerS), with an impact on the number of carbons in the fatty acid chains of ceramides. This has been demonstrated upon a 7-days IFN-γ challenge of RHE. Downregulation of ELOVLs and CerS by IFN-γ is mediated through a STAT1-independent pathway, and is prevented by pyridone-6, a pan-JAK inhibitor. [15,16]

Modelling of skin pigmentary disorders (lentigo, vitiligo)

Solar lentigines, commonly known as 'age spots', appear on areas exposed to the sun (back of hands, shoulders, face, forearms). They are very common in elderly people of Caucasian or Asian origin. A link with excessive exposure to air pollutants has recently been established on the basis of epidemiological studies, in particular on populations living in extremely polluted areas in Beijing province in China.[17]

Several studies suggest an involvement of dermal fibroblasts in the appearance of pigmentary spots, through an increased secretion of cytokines having an influence on the growth of melanocytes and the intensity of pigmentation, such as growth factors like HGF, KGF/FGF-7 or SCF.[8,18]

Based on the publication of Chen *et al.*,[19] StratiCELL has developed a model of hyper-pigmented RHE or individualised spots by applying a cocktail of melanogenic factors (Fig 2). In addition to overproduction of melanin, which can be demonstrated by dermoscopy, spectrophotometry or histology (Fontana-Masson staining), the epidermis also presents a hyperplasia characteristic of pigment spots *in vivo*. These can be attenuated by depigmenting factors such as kojic acid. A model of pigmentary spots induced or accentuated by components of urban pollution is currently under development.

Vitiligo is a chronic autoimmune disease of the epidermis. It is characterised by a loss of melanocytes resulting in white spots or area on the skin, of variable size, appearance and location, which tend to enlarge. The loss of pigmentation is thought to be due to detachment of

melanocytes from the basal layer and their apoptosis, induced by an excess of type-I cytokines such as IFN-γ and TNF-α produced by an exacerbated response of self-reactive CD8 lymphocytes. These cytokines induce an overproduction of matrix metalloproteinase 9 (MMP-9) by the keratinocytes which causes the cleavage of E-cadherin, a melanocyte anchoring molecule in the basal layer of the epidermis.[20]

In vitro, the stimulation of RHE by these two cytokines results in the characteristic detachment of melanocytes that can be observed by immunostaining in the supra-basal layers, an increase in MMP-9 and in the soluble form of E-cadherin.

Simple and relevant models

In conclusion, since the 1970s, modelling skin in 3D has remarkably evolved to complement information provided from monolayer cell cultures. As close representative of the *in vivo* physiology, 3D skin models are now considered as essential tools to fully understand the skin biology in all its states since the combination of reconstructed epidermis and selected cellular mediators such as cytokine cocktails or growth factors, allows the modelling of various inflammatory pathologies or pigmentary disorders. This very simple conceptual approach, in which the experimenter plays the role of the immune system or paracrine communication, makes it possible to demonstrate *in vitro* the effects of therapeutic or preventive compounds in a low or medium-throughput format, to identify their targets and to study the mechanisms of action involved using cellular and molecular biology techniques.

References

1 Rheinwald JG, Green H. Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. *Cell.* 1975;**6**(3):317–30.

2 Prunieras M. Epidermal cell cultures as models for living epidermis. *J Invest Dermatol.* 1979;**73**(2):135-7.

3. Rosdy M, Clauss LC. Terminal epidermal differentiation of human keratinocytes grown in chemically defined medium on inert filter substrates at the air-liquid interface. *J Invest Dermatol.* 1990;**95**(4):409-14.

4 Mathes SH, Ruffner H, Graf-Hausner U. The use of skin models in drug development. *Adv Drug Deliv Rev.* 2014;69 70:81-102.

5 Derr K, Zou J, Luo K, et al. Fully Three-Dimensional Bioprinted Skin Equivalent Constructs with Validated Morphology and Barrier Function. *Tissue Eng Part C Methods.* 2019;**25**(6):334-43.

6 van den Bogaard E, Ilic D, Dubrac S, et al. Perspective and Consensus Opinion: Good Practices for Using Organotypic Skin and Epidermal Equivalents in Experimental Dermatology Research. *J Invest Dermatol.* 2020;

7 Guttman-Yassky E, Krueger JG. Atopic dermatitis and psoriasis: two different immune diseases or one spectrum? *Curr Opin Immunol.* 2017;48:68-73.

8 Bastonini E, Kovacs D, Picardo M. Skin Pigmentation and Pigmentary Disorders: Focus on Epidermal/Dermal Cross-Talk. *Ann Dermatol.* 2016;**28**(3):279-89.

9 De Vuyst E, Salmon M, Evrard C, Lambert de Rouvroit C, Poumay Y. Atopic Dermatitis Studies through In Vitro Models. *Front Med (Lausanne).* 2017;4:119.

10 Hubaux R, Bastin C, Salmon M. On the relevance of an in vitro reconstructed human epidermis model for drug screening in atopic dermatitis. *Exp Dermatol.* 19 2018;

11 Desmet E, Ramadhas A, Lambert J, Van Gele M. In vitro psoriasis models with focus on reconstructed skin models as promising tools in psoriasis research. *Exp Biol Med (Maywood).* 2017;242(11):1158-69.

12 Tjabringa G, Bergers M, van Rens D, de Boer R, Lamme E, Schalkwijk J. Development and validation of human psoriatic skin equivalents. *Am J Pathol.* 2008;173(3):815-23.

13 Ishikawa J, Narita H, Kondo N, Hotta M, Takagi Y, Masukawa Y, et al. Changes in the ceramide profile of atopic dermatitis patients. *J Invest Dermatol.* 2010;130(10):2511-4.

14 Joo K-M, Nam G-W, Park SY, et al. Relationship between cutaneous barrier function and ceramide species in human stratum corneum. *J Dermatol Sci.* 2010;**60**(1):47-50.

15 Tawada C, Kanoh H, Nakamura M, Mizutani Y, Fujisawa T, Banno Y, et al. Interferon-γ decreases ceramides with long-chain fatty acids: possible involvement in atopic dermatitis and psoriasis. *J Invest Dermatol.* 2014;**134**(3):712-8.

16 Kanoh H, Ishitsuka A, Fujine E, et al. IFN-γ Reduces Epidermal Barrier Function by Affecting Fatty Acid Composition of Ceramide in a Mouse Atopic Dermatitis Model. *J Immunol Res.* 2019;2019:3030268.

17 Peng F, Xue C-H, Hwang SK, Li W-H, Chen Z, Zhang J-Z. Exposure to fine particulate matter associated with senile lentigo in Chinese women: a cross-sectional study. J Eur Acad *Dermatol Venereol.* 2017;**31**(2):355-60.

18 Salducci M, André N, Guéré C, et al. Factors secreted by irradiated aged fibroblasts induce solar lentigo in pigmented reconstructed epidermis. *Pigment Cell Melanoma Res.* 2014;**27**(3):502-4.

19 Chen N, Hu Y, Li W-H, Eisinger M, Seiberg M, Lin CB. The role of keratinocyte growth factor in melanogenesis: a possible mechanism for the initiation of solar lentigines. *Exp Dermatol.* 2010;**19**(10):865-72.

20 Boukhedouni N, Martins C, Darrigade A-S, et al. Type-1 cytokines regulate matrix metalloprotease-9 production and E-cadherin disruption to promote melanocyte loss in vitiligo. *JCI Insight.* 5 2020;

PC