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ALTERNATIVE METHODS & *IN VITRO* SKIN 3D TISSUE MODELS: WHAT'S NEW FOR TOXICITY TESTING AND COSMETIC?

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INTRODUCTION

The safety assessment of cosmetic ingredients is a rapidly progressing field of research, including evaluation of alternative approaches suitable for regulatory implementation and development of both *in vitro*, in chemico and in silico methodologies avoiding any use of animals. The emerging need of new integrated approaches for testing and assessment originated from the marketing ban of cosmetics ingredients, as well as finished products, tested on animals within the European Union through the Cosmetics Regulation (EU No. 1223/2009) [1]. This step was taken after the Organization for Economic Co-operation and Development (OECD) approved *in vitro* methods for skin irritation, eye irritation, and skin sensitization. It was a milestone to support the ban for animal testing [2] (Table 1).

Table 1: Summary of selected in vitro assays available or under evaluation to support skin research

Category	Assay	Available models	TG	Primary endpoint
Corrosion	Transcutaneous electrical resistance (TER) test method	Excised skin	430[44]	TER
	Membrane barrier test method	Corrositex (artificial membrane)	435[45]	Color change in receiver fluid
	Reconstructed human epidermis (RHE) test method	EpiSkin™, EpiDerm™, SkinEthic™, epiCS®	431[42]	Viability *via* MTT reduction
Irritation	Skin irritation epidermis method	EpiSkin™, EpiDerm™, SkinEthic™, epiCS®, EPI-MODEL	439[43]	Viability *via* MTT reduction
Sensitization	Direct peptide reactivity assay	*In chemico*	442C[4,46]	HPLC detection of unbound peptides[5,47]
	ARE-Nrf2 luciferase test method	KeratinoSens™, (HaCaT human keratinocytes)	442D[48,49]	Luciferase activity[8,50]
	NCTC 2544 IL-18 test	NCTC 2544 cells (human keratinocyte cell line)	N/A[a]	IL-18 levels *via* ELISA[51,52]
	LuSens assay	Immortalized keratinocytes	N/A[a]	Luciferase activity[53,54]
	SENS-IS® assay	EpiSkin™	N/A[a]	qPCR analysis of selected biomarkers[5,12,55]
	SenCeeTox® assay	In chemico GSH reactivity Activation of Nrf2 pathway in HaCaT cell or EpiDerm™	N/A[a]	GSH depletion qPCR analysis of selected Nrf2 target genes[56]
	Human cell line activation test (h-CLAT)	THP-1 cells (human monocytic leukemia cell line)	442E[5,57]	Upregulation of CD86 and CD54[58]
	U-SENS™	U937 cells (human myeloid cell line)	N/A[a]	Upregulation of CD86 and CD54[59,60]
	GARDskin (genomic allergen rapid detection)	MUTZ-3 cells (dendritic cell-like human myeloid cell line)	N/A[a]	Array analysis of 196–200 genes[4,5,33,61,62]
	VITOSENS™	Human CD34+ dendritic cells	N/A[a]	Gene expression analysis of CCR2 and CREM[63,64]
	Human T cell priming assay (hTCPA)	Human monocyte-derived dendritic cell and naïve T cell co-culture	N/A[a]	Flow cytometry analysis of T cell activation markers (*e.g.*, IFN-γ and TNF-α)[65,66]
Genotoxicity	Reconstructed skin micronucleus assay	EpiDerm™	N/A[a]	% micronuclei (modification of TG 487)[34,37,67,68]
	3D skin comet assay	EpiSkin™, EpiDerm™ FT, Phenion® FT	N/A[a]	Comet tail (based on TG 489)[69–71]
Phototoxicity	3T3 NRU assay	Balb/c 3T3 fibroblasts	432[72]	Viability ± UVR
	EpiDerm™ Phototoxicity test	EpiDerm™ H3D-PT	N/A[a]	Viability ± UVR (based on TG 432)[72,73]
Absorption	Skin absorption: *in vitro* method	Excised dermatomed or full thickness skin	428[74]	Concentration in receptor fluid, skin surface (wash), and skin layers

[a] Not available and/or not yet approved.

From Hardwick et al. (2019)

OPPORTUNITIES FOR DEVELOPMENT OF NEW *IN VITRO* MODELS FOR SKIN TOXICITY

Recently, 3D cell culturing techniques have improved the relevance of the available models and demonstrated the synergistic effects that different cell types have on each other. These models can be assembled into complex structures to simulate physiologically relevant conditions. Both Reconstructed Human Epidermis (RHEm) and Full-Thickness Skin models (FTSm) have been used for many applications including basic, pharmacological and cosmetic research. Innovative techniques such as 3D printing and scaffolds are promising approaches to increase the relevance of these models [3]. In this line, recent development in microfluidic-based cell culture technology has demonstrated the feasibility of using micro-scale *in vitro* physiological models "organs-on-a-chip" models for drug screening. These Organ-on-a-Chip devices (OoC) aim to mimic the architecture and function of an organ by combining 3D bioengineered constructs such as multicellular spheroids and organoids, and bioprinted constructs (Figure 1).

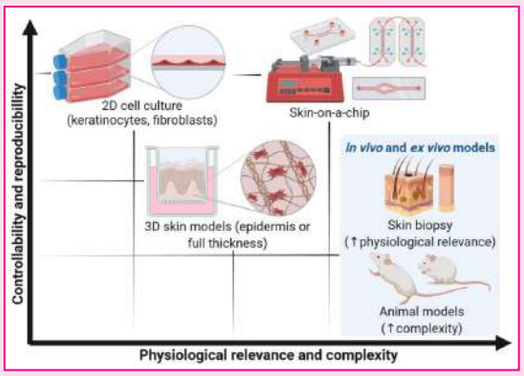

Figure 1. Highlights the potential significance of Organ-on-a-Chip (OoC) technology to provide a reproducible and physiologically relevant approach to to provide a reproducible and physiologically relevant approach to systematically evaluating skin responses (Zoio et al., 2022).

KEY REQUIREMENTS FOR THE DEVELOPMENT OF SKIN-ON-A-CHIP DEVICES

A physiologically relevant SoC model is expected to include the main layers of the human skin (dermis and epidermis) and a vascular system. The cell source and scaffold. The cell source and scaffold type are crucial to obtain a final model. The integration of mechanical stimuli such as cyclic stretching and shear stress should also be considered to reproduce the in vivo-like microenvironment. Finally, the integration of sensors in the SoC should be considered for real-time monitoring of skin function (Figure 2).

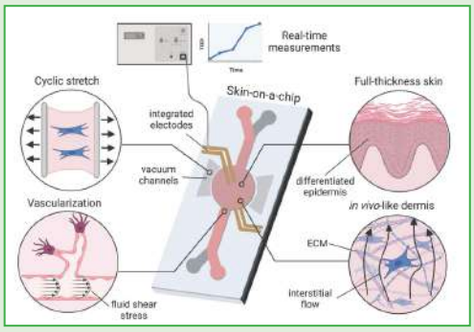

Figure 2. Development of biomimetic Skin-on-a-Chip platforms. Schematic drawing representing the main factors to be considered when developing physiologically relevant Skin-on-a-Chip (SoC) models, including technical and biological factors (cell sourcing, cell scaffold, perfusion, cyclic stretching, design and fabrication and sensor integration).

CONCLUSION

Next-generation *in vitro* skin models will reflect more closely the skin architecture and cell composition to allow for more precise toxicological profiling (Figure 3). The development of OoC technologies is promising. Inclusion of immune components for example in the 3D skin model offers a new perspective. The next step is to generate multi-OoC platforms that emulate entire biological processes: incorporating immune system, organ innervation and vascularization are the keys allowing to improve these platforms. Besides these challenges, the technology needs to be validated and accepted by the regulatory organizations as an efficient method . Collaborations between researchers, regulatory organizations and the industry would be necessary to obtain this validation (Figure 3).

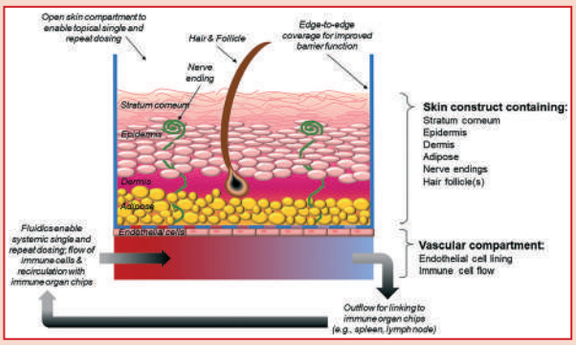

Figure 3. Desired physical features in future skin models. Several physical features desired in future skin models are shown including fluidics and drug administration considerations. It is expected that many features may work in concert to achieve an improvement such as that of improved barrier function.

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