Journal of Cellular Biotechnology 3 (2017) 21–39
DOI 10.3233/JCB-179004
IOS Press

In vitro skin three-dimensional models and their applications

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Abstract. Skin fulfils a plethora of eminent physiological functions ranging from physical barrier over immunity shield to the interface mediating social interaction. Prone to several acquired and inherited diseases, skin is therefore a major target of pharmaceutical and cosmetic research. The lack of similarity between human and animal skin and rising ethical concerns in the use of animal models have driven the search for novel realistic three-dimensional skin models. This review provides a survey of contemporary skin models and compares them in terms of applicability, reliability, cost and complexity.

Keywords: Skin, phenotypic screening, 3D models, pharmaceutical research

1. Skin – composition and function

Human skin covers an area of almost 2 m^2 in the adult and consists of the three major layers, subcutis, dermis, and epidermis. The subcutis is composed of adipose and epithelial cells. It harbours blood vessels, neurites of peripheral neurons, Vater-Pacini mechanosensors, and, partially, also sweat glands and hair follicles. It connects the skin to periosteum and fascia, absorbs forces, and mediates thermal insulation. The dermis supplies the epidermis with mechanical support and nutrients. It is stratified into an inner, reticular, and an outer, papillary, zone. The dermis houses most sebaceous glands, sweat glands, hair follicles, smooth muscle cells, and capillary beds and, thus, regulates skin moisture, body temperature, and performs the secretory function of skin. The papillary layer is characterized by relatively loose connective tissue, where Meissner corpuscles sense touch. Immune cells, particularly mast cells and dendritic cells, are patrolling in the papillary layer and mediate local inflammatory reactions and immune surveillance. Finally, dermal fibroblasts secrete extracellular matrix (ECM) and basement membrane components. These are primarily collagens I and III, and a proteoglycan-rich ground substance [1]. The resulting ECM mediates tensile strength of the dermis. The dermo-epidermal junction is centered around a special basement membrane. This is composed of a laminin/collagen IV scaffold and further typical basement membrane components such as perlecans and nidogens [1].

The epidermis is a squamous epithelium of 50–100 μm thickness. It is devoid of blood vessels but contains keratinocytes, Merkel cell mechanosensors, Langerhans immune cells, and melanocytes. The

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Table 1
Marker molecules for the analysis of epidermal stratification

Protein	Epidermal layer	Physiological function	Ref.
KRT5/KRT14	Stratum basale	Resilience	[2, 95]
KRT1/KRT10	Stratum spinosum	Resilience, part of cornification	[2, 95]
KRT6/KRT16	Activated keratinocytes	Resilience	[6]
KRT17	Contractile keratinocytes	Resilience, regulation of protein synthesis and cell size	[6, 7, 15]
Involucrin	Stratum spinosum, Stratum granulosum, Stratum corneum	Cornification of plasma membrane, scaffold for other envelope proteins	[8, 96]
Loricrin	Stratum granulosum, Stratum corneum	Major cornified envelope component	[8, 97]
Profilaggrin	Stratum granulosum	Filaggrin precursor	[8, 98, 99]
Filaggrin	Stratum corneum	Keratin crosslinking	[8, 98, 99]
Ki67	Stratum basale, Stratum spinosum	Undefined role in cell division, rRNA synthesis, maintenance of mitotic spindle	[100–102]
Cleaved caspase 3	Stratum granulosum	Execution of apoptosis	[102–104]

epidermis shows two germinal layers (stratum basale and spinosum), a secretory layer (stratum granulosum), and a layer of dead cells aka corneocytes, and secreted lamellar bodies (stratum corneum). Thick skin on palms of hands and feet features an additional layer, stratum lucidum, between stratum granulosum and stratum corneum. Keratinocytes give ultimate strength to skin through intracellular intermediate filament systems made up of keratins, which connect to neighbouring cells via desmosomes containing desmoplakin and to the basement membrane via hemidesmosomes containing plectin [2]. For this reason, mutations in keratin genes (*KRT*) lead to various threatening skin diseases, such as epidermolysis bullosa simplex, which is due to mutations in either *KRT5* or *KRT14* [3].

Epidermal keratinocytes progress from the basal to the cornified layers, allowing for the regeneration of epidermis within 60 days in an average adult [4]. Accordingly, keratinocyte gene expression undergoes robust longitudinal changes, which is particularly well understood and characterized for keratins [2, 5–7]. As outlined in Table 1, KRT5 and KRT14 are typical for basal keratinocytes, while KRT1 and KRT10 are increasingly expressed during differentiation and postmitotic stages. The switch from KRT5/14 to KRT1/10 occurs in a gradual transition process [2]. Therefore, a mixed composition of keratins can be observed in intermediate differentiation stages. The terminal differentiation of the water-repellent and antibiotic stratum corneum happens in the stratum granulosum and stratum corneum [8]. It involves the release of lamellar bodies leading to an extracellular, highly-ordered lipid matrix that consists of ceramides, cholesterol, and fatty acids [9] and the intracellular aggregation of an envelope composed of keratins, involucrin, filaggrin, and loricrin [4, 8]. The lipid matrix serves as a bedding for corneocytes and the proteinaceous envelope in corneocytes adds mechanical stability. Terminal differentiation of keratinocytes occurs in a temperature- and Ca^{2+}-dependent manner [4, 10]. Indeed, envelope formation is favoured in the presence of millimolar amounts of Ca^{2+} and lowered temperature [4, 10]. Also, the secretion of lamellar bodies is preceded by Ca^{2+}-influx into keratinocytes [4] and coincides with apoptotic cell death conversion from keratinocytes to corneocytes [11]. Conversely, cell proliferation in the basal layers necessitates low, micromolar Ca^{2+}-levels. The phenotypic analysis of proliferation versus apoptosis in keratinocyte three-dimensional (3D) cultures is shown in Fig. 1. Due to the ambivalent function of Ca^{2+}, a steep Ca^{2+}-gradient needs to be maintained between inner and outer layers of the epidermis. Two principal mechanisms for its establishment are being discussed, i.e. the barrier function of the stratum corneum and the presence of tight junctions [12].

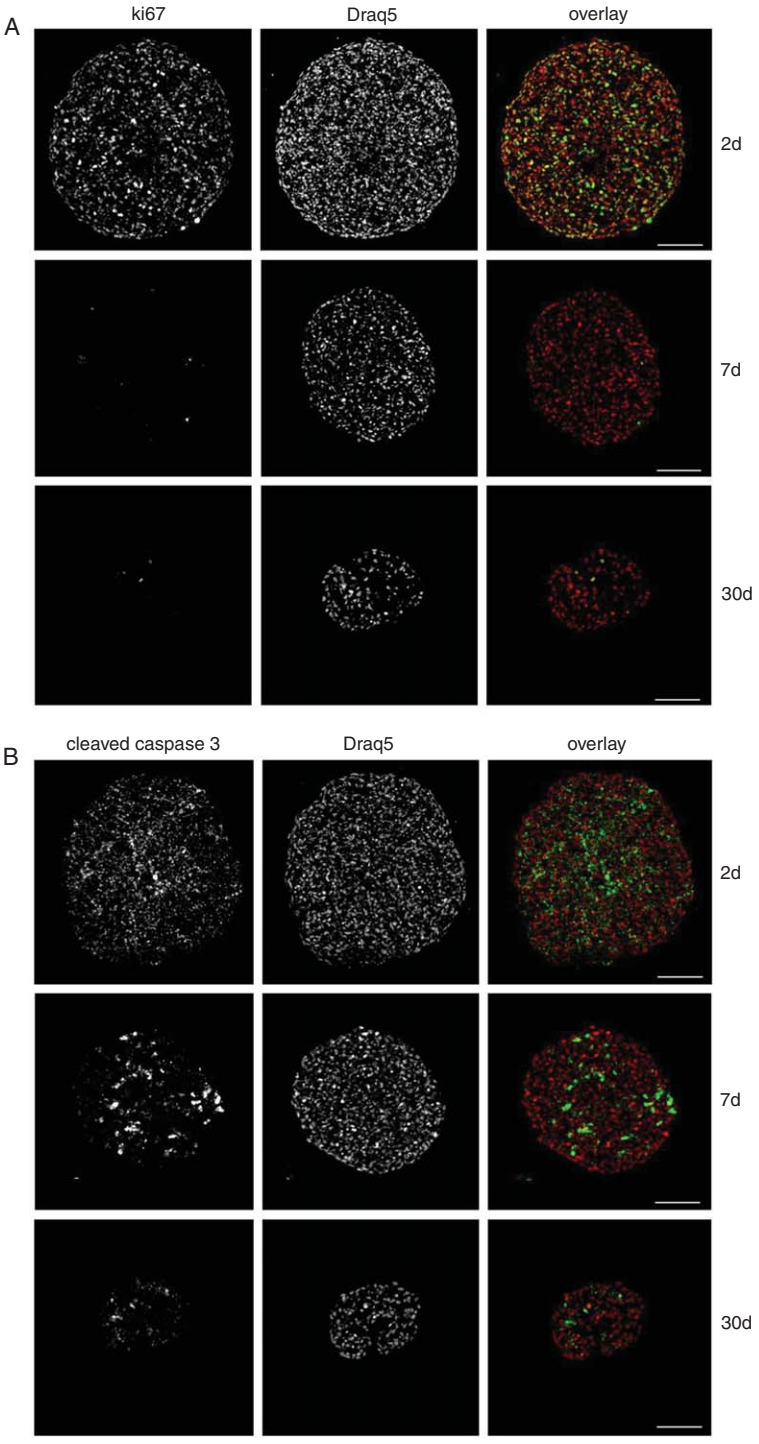

Fig. 1. Phenotypic analysis of HaCaT spheroids. HaCaT cells were grown as spheroids. After 2, 7, and 30 days, spheroids were sectioned into 10-μm thick slices and immunostained for Ki67 (A) or Cleaved caspase 3 (B), respectively. Nuclei were labeled with Draq5. Scale bars, 100 μm. Ki67 is a well-known marker to differentiate between dividing and postmitotic cells [102]. This is found within the nucleus during G_1, S and G_2, while in mitosis it is bound to dividing chromosomes. At G_0 it is not present at all [100]. Caspase 3 is a major executioner caspase in all types of apoptosis. Its prodomain is cleaved upon apoptosis induction thereby activating its function that leads to the execution of apoptotic cell death [102, 104, 105].

The latter can be found at the stratum granulosum and serve as a major barrier of skin. Tight junction proteins claudin-4 and occludin can be found in that skin layer [13].

The differentiation from basal keratinocytes to shielding corneocytes occurs in normal, uninjured skin; but disturbance of this well-orchestrated process can be associated with chronic skin disorders such as atopic dermatitis and psoriasis. In psoriasis, inflammatory cytokines (e.g. TNF-alpha and IL-17) are secreted from immune cells, which trigger drastic gene expression changes ultimately leading to hyperproliferative, incompletely differentiated epidermal keratinocytes [14]. Such psoriatic keratinocytes show increased expression of hyperproliferative markers KRT6 and KRT16, downregulation of differentiation markers KRT1 and KRT10 as well as early onset of IVL expression. As a consequence, the psoriatic skin lesion is characterized by hyperplasia of the epidermis with excessive cornification but poorly adherent stratum corneum, which results in the psoriatic characteristic flakes [14].

In the case of acute skin lesions, keratinocytes undergo a different process, termed keratinocyte activation cycle [6]. Here, injured keratinocytes release IL-1, which triggers local blood vessel formation and immune response. Also, IL-1 switches keratinocytes to become proliferative and migratory, and to change gene expression. This upregulates the activated keratinocyte markers *KRT6* and *KRT16* (Table 1) and stimulates secretion of several paracrine factors. Activation is maintained by some of these signalling molecules and results in a release of chemoattractants for immune cells. The concerted action of keratinocytes, endothelial cells, and immune cells will eventually resolve the acute local inflammatory stimuli and allow a partial closure of the wound. Further healing involves the release by fibroblasts of a new basement membrane and its contraction by contractile keratinocytes [6, 15]. These express *KRT17* (Table 1), and are transformed from activated keratinocytes by IFN-gamma. Close to definitive wound healing, fibroblasts release TGF-beta, which shifts keratinocytes to the basal phenotype, i.e. suppressing hyperproliferation, activating the standard keratinocyte differentiation program, stimulating the production of ECM components, and inducing normal keratinocyte layering [6, 16]. The critical role of enrichment of TGF-beta for sheeting behaviour was shown in a study comparing standard spheroid versus microbubble 3D cultures of keratinocytes [16]. While in the first, secreted molecules such as TGF-beta could diffuse freely, the compartmentation induced by the microbubble cultivation technique arguably led to a local increase in TGF-beta which induced a sheeting cell growth [16]. Using a similar chip-based technology featuring micro-fenestrated 300-μm wide compartments (KIT Chip), we could recently confirm these findings and show a regular layered expression of keratinocyte differentiation markers, *KRT14* and *KRT10* (Fig. 2).

2. Skin – current *in vitro* test systems and which functions can be tested with these

The important functions of skin make it a principal organ for body health and physiology. Accordingly, pharmacological and biomedical research necessitates appropriate test systems for finding novel drugs and treatments. The same is true for the development of new cosmetics, which have always been central to mankind. For both applications, though, animal models are not ideal. On the one hand, frequently used and relatively economic species such as rodents do have a skin composition that is too different to human skin to be comparable. On the other hand, animals with more human-like skin, such as pigs, are too difficult to manage and too expensive for regular use. On top, ethical reasons preclude this as well, rendering animal models for skin research less and less attractive. However, also two-dimensional (2D) skin cell cultures that have been used since decades now, come with an overt lack of physiological relevance. Most principal functions of skin, like barrier function, resilience, cell sheeting, cell layering, developmental profiles, immune function, blood perfusion, and innervation, are not recapitulated in simple 2D cultures. The following chapters introduce major 3D skin models used

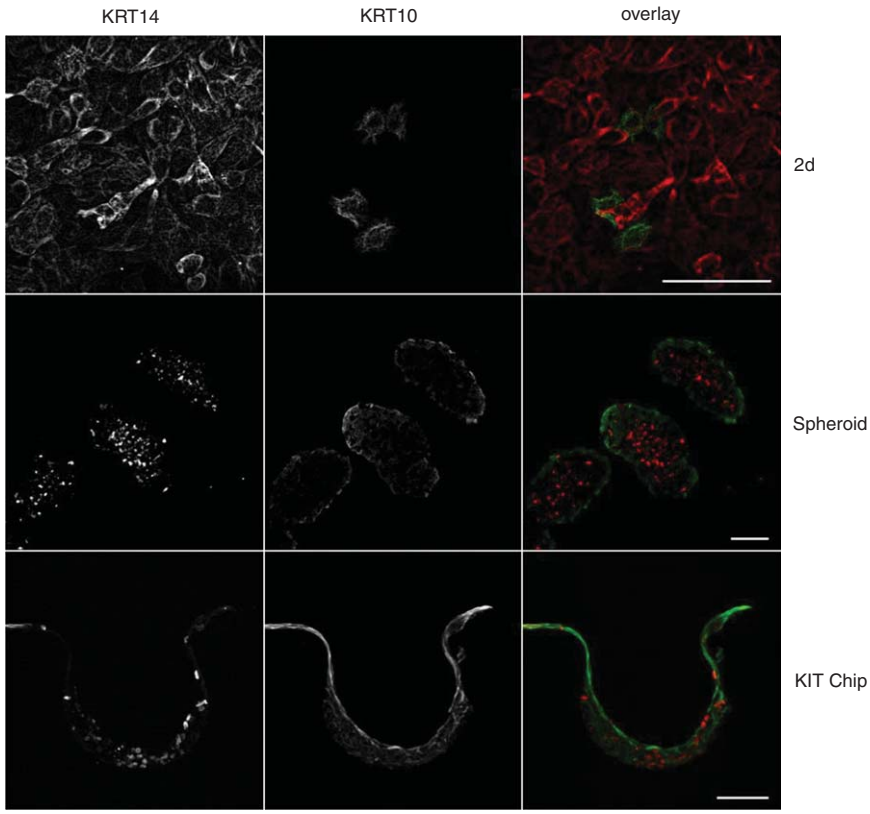

Fig. 2. Analysis of differentiation of HaCaT cells in 2D and 3D models. HaCaT cells were grown in 2D, as spheroids, and in a microbubble format (KIT Chip). KIT Chips were assembled as described previously [106]. After 7 days, spheroids were cryosectioned into 10-μm slices and the KIT Chip was cut with a microtome. Samples were stained for KRT14 and KRT10 to mark basal cells and differentiated keratinocytes, respectively. Scale bars, 100 μm.

in research and drug discovery and briefly discuss their advantages and disadvantages. An overview of these models and their main applications in basic, toxicological, and pharmaceutical research is given in Table 2.

2.1. Spheroids

The observation, that malignant cells are more likely to aggregate than normal cells when cultured in an Erlenmeyer on a shaker [17] gave rise to the spheroid technology and its use in oncology research. Sutherland and colleagues termed their Chinese hamster V79 lung cell aggregates "multicellular spheroids" and further described the three typical zones found in most of these 3D spherical cultures: an inner necrotic layer, an intermediate zone of quiescent cells as well as an outer stratum of proliferating cells [18]. However, depending on the application, small spheroids can be generated to avoid a necrotic core [19]. Spheroids are mostly made by the hanging drop method [20, 21] or by the use of non-adherent U-bottom shaped plates [22]. Furthermore, some cells like melanocytes need the addition of a coating such as chitosan to form spheroids [23]. More recently, new methods have become commercially available that allow the aggregation of magnetised cells [24, 25].

Spheroids are not amongst the classical 3D models for skin research. Indeed, until now there have been only very view papers using HaCaT cells or fibroblasts co-cultured with melanoma cells in the spheroid format to gain tumour models. Okochi and colleagues investigated the interaction of

Table 2
Types of 3D cell culture systems and their application to skin basic, pharmaceutical, and cosmetic research

System	Special adaptations	Complexity	Cell types	Reference	Applications
Spheroids	Rotating wall vessel bioreactor system	Epidermis with melanoma	HaCaT, mouse melanoma cells (B16.F10)	[26]	Research on cancer development and new potential treatment regimens
	Magnetic force-based cell patterning	Dermis with melanoma	Human melanoma cell line (M-1), mouse fibroblast cell line (NIH 3T3)	[25]	Study of fibroblast interaction to invasive capacity of melanoma
	U-bottom shaped wells were treated with low-electroendosmotic agarose to form a nonadhesive surface	Dermis and epidermis	Primary human dermal fibroblasts, primary human keratinocytes, HaCaT	[27]	Production of synergistic factors for clinical settings
	3D InSight(TM) Skin Microtissue	Dermis and epidermis	Primary human dermal fibroblasts, HaCaT	[28]	Drug screening
Hydrogel systems	Photocrosslinkable gelatin with tunable mechanical, degradation and biological properties	Epidermis	HaCaT	[48]	Skin tissue engineering
	Collagen gel			[45]	Epidermal stratification/differentiation studies
			Primary human keratinocytes	Episkin (L´Oreal; SkinEthic, Nice, France)	Skin irritation, corrosion, UV exposure, DNA damage, bacterial adhesion, omics, permeability
	Collagen gel containing fibroblasts	Dermis and epidermis	HaCaT, postmitotic fibroblasts (HDFi)	[35]	Cytotoxicity testing of hair dyes to human skin
			Primary human fibroblasts, primary human keratinocytes	[34]	Disease processes of cancer and wound re-epithelialization
				Apligraf (Organogenesis Inc., Canton, MA, USA)	Tissue engineering

			HaCaT, primary human fibroblasts	[38]	Analysis of HaCaT differentiation capacity *in vitro* for the use in pharmacotoxicity
		Dermis and epidermis containing melanocytes and melanoma cells	Primary cultures of skin cells (keratinocytes, melanocytes and fibroblasts), metastatic melanoma cell lines	[43]	Drug testing on an artificial skin model containing melanoma
	Fibrin gel	Dermis and epidermis	HaCaT and primary human fibroblasts	[37]	Epidermal stratification/differentiation studies in homeostasis and skin cancer development
			Primary human fibroblasts, primary human keratinocytes	Labskin (Innovenn, Dublin, Ireland)	Skin irritation, drug delivery, cosmetic testing, impact on skin microflora, efficacy against pathogenic bacteria
		Dermis and epidermis, subcutis	Primary human fibroblasts, primary human keratinocytes, primary human adipose-derived stem cells and adipocytes	[42]	Research and clinical applications
	Agarose gel	Epidermis	Primary human keratinocytes	EpiDerm (MatTek, Ashland, MA, USA)	Skin irritation, hydration, corrosion, phototoxicity, drug delivery, epidermal differentiation, dermal genotoxicity
3D bioprinting	Laser-assisted bioprinting is used to arrange cells in a collagen gel in layers	Dermis and epidermis	Mouse fibroblast cell line (NIH-3T3), HaCaT	[60]	Tissue engineering, study of tissue function

(*Continued*)

Table 2
(*Continued*)

System	Special adaptations	Complexity	Cell types	Reference	Applications
	Use of 3D bioprinting to engineer human skin in a layer-by-layer assembly in a collagen gel		Primary human fibroblasts, HaCaT	[49]	Transdermal and topical formulation discovery, dermal toxicity studies, designing autologous grafts for wound healing
	Bioprinting a full-thickness biomimetic skin with melanocytes at the epidermal layer		Primary human dermal fibroblasts, primary human epidermal keratinocytes, primary human epidermal melanocytes	[61]	Biomimetic skin structures, tissue engineering, regenerative medicine
	A robotic platform printing a collagen hydrogel precursor, fibroblasts and keratinocytes layer by layer		Primary adult human dermal fibroblasts and primary adult human epidermal keratinocytes	[58]	Creating skin grafts tailored for wound shape or artificial tissue assays for disease modelling and drug testing
Skin-on-a-chip	Microfluidic device using conventional soft lithography and PDMS molding techniques	Epidermis	Primary human epidermal keratinocytes (HEK), primary normal neonatal human epidermal keratinocytes (NHEK)	[68]	Skin functionality studies, tissue engineering
	Skin-on-a-chip device fabricated by assembling four polymer layers (two patterned PMMA sheets, a PS sheet and a PDMS sheet)	Epidermis, immune cells (dendritic cells)	HaCaT, human leukemic monocyte lymphoma cell line (U937)	[74]	Effect of chemical and physical (UV irradiation) stimulation on the skin barrier

Pumpless microfluidic platform consisting of two PDMS layers separated by a polycarbonate porous membrane	Dermis and epidermis	Primary human fibroblasts and primary human keratinocytes	[82]	Long-term maintenance of full thickness human skin equivalents, drug testing
Culture device composed of a main part and a bottom plate fabricated by 3D printing, anchoring structures to keep the skin-equivalent fixed to the device	Dermis and epidermis, vascular layers	Primary human dermal fibroblasts (NHDF), primary human epidermal keratinocytes (NHEK), human umbilical vein endothelial cells (HUVEC)	[81]	Drug development, cosmetic testing, and studying skin biology
Microfluidic device fabricated using conventional soft lithography with an elastomeric material, PDMS		HaCaT, primary human fibroblasts (Hs27), human umbilical vein endothelial cells (HUVEC)	[79]	Skin functionality studies, drug/cosmetic testing, skin disease models
Skin chip device was made from PDMS by soft lithography		Primary human dermal fibroblasts, primary human keratinocytes, HaCaT, human umbilical vein endothelial cells (HUVEC)	[69]	Testing skin reaction to cosmetic products and drugs
Dynamically perfused micro-channel system combining an on-chip micropump with variable tissue culture compartments	Dermis, epidermis, hair	EpiDermFT (Mattek, Ashland, MA, USA), Human juvenile prepuce, primary human hair follicles	[73]	Assess the effect of dynamic perfusion in comparison to common static culture conditions; *ex vivo* hair elongation and substance testing

(*Continued*)

Table 2
(*Continued*)

System	Special adaptations	Complexity	Cell types	Reference	Applications
	A single PDMS layer, containing the respective arrangement of channels, micropumps and openings for culture compartments bonded to a glass microscope slide	Skin (epidermis), liver	Human hepatocyte cell line (HepaRG), primary human hepatic stellate cells (HHSteC), human juvenile prepuce, skin biopsies	[76]	Substance testing
		Skin (epidermis, dermis, subcutis), liver, vasculature	Human hepatocyte cell line (HepaRG), primary human hepatic stellate cells (HHSteC), human juvenile prepuce, skin biopsies, human dermal microvascular endothelial cells (HDMEC)	[77]	Substance testing
	Device comprising two polycarbonate cover-plates and a PDMS-glass chip	Intestine, liver, skin (epidermis), kidney	Human hepatocyte cell line (HepaRG), primary human hepatic stellate cells (HHSteC), human proximal tubule cell line (RPTEC/TERT-1), human skin biopsy, EpiIntestinal™	[78]	Microfluidic ADME profiling and repeated dose systemic toxicity testing of drug candidates

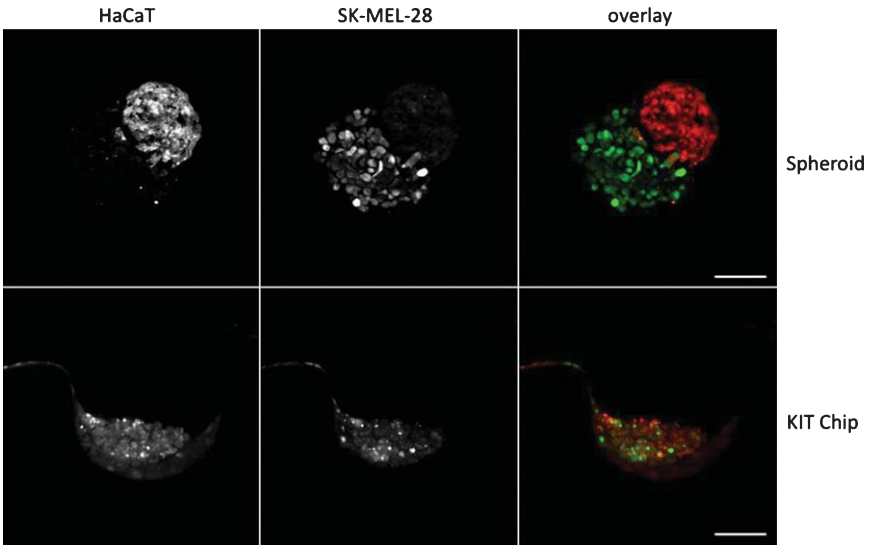

Fig. 3. Co-culture of keratinocytes and melanoma cells. HaCaT and SK-MEL-28 cells were stained with Cell Tracker Red (red in overlays) and Cell Tracker Green (green in overlays), respectively. Then, cells were seeded simultaneously to grow as spheroids and on the KIT Chip. After 7 (spheroids) or 12 days (KIT Chip) in 3D culture, spheroids and KIT Chip were sectioned into 10-μm and 30-μm thick slices, respectively. KIT Chips were assembled as described previously [106]. Scale bars, 100 μm.

fibroblasts on the invasion of melanoma using magnetically labelled fibroblasts and melanoma to generate co-culture spheroids [25]. Another melanoma system used HaCaT spheroids as a scaffold for melanoma cells in a bioreactor system [26]. Also, Peura et al. showed the importance of a spheroid network to harvest released factors from the cells to produce an active matrix [27]. However, none of these studies characterized the development of typical epidermal stratification. In our laboratory, HaCaT spheroids were generated with cell repellent U-bottom shaped plates from Greiner and cultured in growth media (DMEM + 10% fetal calf serum + 1% penicillin/streptomycin). The resulting spheroids were immunostained for the differentiation markers KRT14 and KRT10, showing an inner, basal and an outer, differentiated epidermal layer (Fig. 2). Furthermore, we established 3D skin-melanoma *in vitro* models to gain information about the interaction of keratinocytes and melanoma cells. We show the spatial arrangement of these cells when grown as spheroids or on the KIT Chip. First, cells were stained with Cell Tracker dyes (HaCaT keratinocytes were stained with Cell Tracker Red and SK-MEL-28 melanoma cells with Cell Tracker Green). Both cell types were seeded simultaneously to grow as spheroids or on the KIT Chip. Depending on the 3D culture condition, the cells arranged differently. When grown as spheroids, they formed separate entities with a central HaCaT spheroid and SK-MEL-28 aggregates around the spheroid (Fig. 3A). Conversely, when grown on the KIT Chip, the two cell types were mixing, building a common sheet of keratinocytes and melanoma cells (Fig. 3B).

It still needs to be determined, if skin cell spheroids can be grown to reach complete differentiation and full layer stratification as for standard hydrogel or 3D printing models. First promising results were reported, which describe a spherical skin microtissue (3D InSight(TM) Skin Microtissue, InSphero, Schlieren, Switzerland) composed of different keratinocyte layers and a dermal fibroblast core, which produces exctracellular matrix proteins without the need for exogenous collagen [28]. Spheroids are potentially very interesting systems also for skin research due to their simplicity, low cost and high reproducibility. Hence, they are suitable for high-throughput cell function and cytotoxicity analysis [29]. Most spheroids can be dispersed by lysis buffer or enzymes and therefore, they are also appropriate

for biochemical analysis [30]. Whole mount microscopic analysis of spheroids can be performed by confocal microscopy [31] or light-sheet microscopy [32], while for conventional microscopy one has to section spheroids prior to imaging [33].

2.2. Hydrogel systems

The most dominant technique for creating an *in vitro* skin model is the use of hydrogels that serve as a scaffold for dermal fibroblasts, which is then co-cultured with keratinocytes on the surface. Collagen I, the leading class of ECM protein, is the typically used hydrogel material, and dermal fibroblast cells are usually distributed within the collagen gel to mimic the dermal layer [34, 35]. The first hydrogel system using epidermal cells on top of a primary rat fibroblast-seeded collagen scaffold was described by Bell and colleagues [36]. Apart from collagens, also other ECM interacting proteins can be used to generate hydrogels. For example, Alameda et al. generated 3D cultures of HaCaT keratinocytes and fibroblasts in fibrin gels to obtain human skin equivalents that comprise an epidermal and a dermal compartment that resembles both the structure and stratification of normal human skin [37]. Increasing numbers of commercially available skin models, that are based on hydrogels, have reached the market, such as EpiDerm (MatTek, Ashland, MA, USA), Episkin (L´Oreal; SkinEthic, Nice, France), Apligraf (Organogenesis Inc., Canton, MA, USA), and Labskin (Innovenn, Dublin, Ireland).

Using hydrogel systems, one can assemble the dermal layer and the stratified layers of the epidermis. Special protocols of air exposure [38, 39] or combinations of high Ca^{2+} and low temperature [10] induce complete keratinocyte differentiation, including cornification. Its high level of differentiation makes this system a good approach to study effects on specific epidermal layers. Typically applied cell types are primary human dermal fibroblasts and primary keratinocytes [34, 40], but also keratinocyte cell lines like HaCaT cells are used [38]. Simple psoriasis models, which are also commercially available (MatTek Corp., Ashland, MA, USA), are composed of healthy keratinocytes and diseased fibroblasts isolated from psoriatic lesions of patients. However, the use of psoriatic epidermal keratinocytes more closely resembles psoriatic conditions [41].

Depending on the application, all three major skin layers including the subcutis with human adipose-derived stem cells and adipocytes can be assembled [42]. To generate a cancer model, melanocytes and/or melanoma cells can be added [43]. Since skin cancer, psoriasis, and other skin diseases involve the complex cross-talk between various cell types and cytokines, 3D skin models that also contain immune cells are of great value. Van den Bogaard et al. were the first, who achieved to generate 3D skin equivalents that included different T-cell populations. It allowed to study migration of immune cells and secretion of proinflammatory cytokines in the context of psoriasis [44]. However, hyperproliferation was not observed and cytokine levels were much lower compared to the *in vivo* situation, suggesting that crucial components and relevant cell types are still missing to create a more complete psoriasis model.

3D *in vitro* hydrogel systems can be used to evaluate epidermal behaviour, e.g. to address the impact of epimorphin modulation on epidermal growth factor receptor-driven epidermal differentiation in an organotypic skin model [45]. Furthermore, Zanoni and co-workers investigated the cytotoxicity of different chemicals, like hair dyes on a hydrogel-based skin system [35]. The major advantages of hydrogel based models are their defined biomimetic functions. Cell adhesion sites can be varied by the use of peptides like RGD or IKVAV [46, 47]. In addition, the cell-mediated degradability, the low stiffness of the gels as well as the gel material itself can be modified (e.g. collagen, fibrin, gelatin) [37, 45, 48]. Since the cells can be recovered from the gels by proteases hydrogel systems are also convenient for molecular analysis (Western Blot, Polymerase Chain Reaction (PCR)) [40, 43] and their transparency allows microscopy [38]. The costs are moderate. They are cheaper than microfluidic devices but more expensive than simple spheroid systems. Their handling is easy but based on the

involved biological components the gels might have a high batch-to-batch variability which makes it difficult to repeatedly achieve the same results.

2.3. 3D bioprinting

Bioprinting allows the automated generation of complex tissue architectures [49]. Different layers of a desired material, mostly in the forms of hydrogel or biodegradable scaffolds, can be printed to form such geometries. Subsequently, biomolecules or cells can be added to defined positions to form various biological structures of interest [50]. As a pioneer of 3D printing, Charles W. Hull introduced the stereolithography method by uniting thin layers of certain materials with ultraviolet light [51]. Later, the technology was improved leading to new methods, such as laser printing [52] and ink printing [53]. In addition, soft lithographic methods were developed, which utilize 'stamping' of each cell-containing layer by creating layer-specific molds and stacking of these layers to provide a 3D shape [54, 55]. These approaches, however, often include complex manufacturing processes containing the spatially accurate location of cell-containing tissue layers.

As 3D bioprinting has the ability to manufacture the 3D structures in an on-demand fashion, it has been applied to create various biomimetic structures, such as fluidic channels [56], vascular-like structures [49], growth-factor releasing matrices [57], 3D neural tissues [58], and tumor cell-bearing tissues for angiogenesis models [59]. With regard to bioprinted skin, complex skin tissues with dermal and epidermal layers containing keratinocytes, melanocytes and fibroblasts were generated [49, 58, 60, 61]. More complete models comprising also subcutis and other structures have not yet been presented.

Using the potential of 3D bioprinting, Lee and colleagues fabricated a biomimetic human skin with a similar morphology to native skin layers, in which keratinocytes and fibroblasts resembled the epidermis and dermis, and collagen the dermal matrix [49]. Using laser-assisted bioprinting to embed keratinocytes and fibroblasts in collagen, Koch and colleagues showed, that the printing process does not harm cells and that cells differentiate and form functional gap- as well as adherent junctions [60].

Modern 3D bioprinting is mostly automated and therefore provides high reproducibility and high-throughput. As the scaffolds can be varied on demand and as different cell types and active molecules can be applied, bioprinting is a highly flexible method. Consequently, this system has potentials in tissue engineering as well as in cytotoxicity testing and pathophysiology of skin diseases [49]. Furthermore, bioprinted tissue can be imaged by fluorescence microscopy and analysed by molecular biology methods like Western Blot and PCR [49, 62]. However, 3D printing is only available in a few laboratories due to its high costs and complex printing system.

2.4. Organ-on-a-chip

Organ-on-a-chip technology is the reconstruction of tissue and organs by culturing living cells in a continuously perfused microscale environment with spatiotemporal control [63, 64]. It is here not the goal to form a completely living organ but to create minimal functional units that summarize functions of tissue and organs. The easiest system is a single, perfused microfluidic chamber containing one type of cells that displays functions of one tissue. In more complex designs, two or more microfluidic chambers are connected by porous membranes with different cell types to mimic interfaces between different tissues [64]. The chips are mostly made by a modified form of photolithographic etching. Microfluidic culture systems are often made by 'soft lithography', which is the etching of replicating patterns into silicon chips in more biocompatible and flexible materials. For this, a liquid polymer, such as polydimethylsiloxane (PDMS), is poured on an etched silicon substrate and allowed to polymerize into an optically clear, rubber-like material, basically creating a rubber stamp [65]. While most of the chips are perfused with a pumping system [66], some are pumpless using a rocking platform [67].

In comparison to the above described 3D *in vitro* systems, microfluidic skin-on-a-chip provides the best physiology and functionality. Hence, all systems have a perfusion whereby shear stress is produced which in turn increases cell viability and proliferation in contrast to static cultures [68]. Additionally, almost all systems offer an air-liquid-interface culture [69]. Depending on the application, either skin biopsies, primary cells or cell lines can be used [66, 68, 69]. Also, human induced pluripotent stem cells provide a source to mimic healthy and diseased skin models as they can be differentiated into keratinocytes [70], fibroblasts [71], melanocytes [72] and endothelial cells [67]. Besides simplified models with dermis and epidermis, also more complex models with hair follicles and adipocytes [73], immune cells [74] and features resembling sweat gland pores [75] exist. In addition, skin-on-a-chip can be expanded to multiple organ chips to test drug metabolism. Accordingly, Maschmeyer et al. and Wagner et al. engineered two and four organ chips with intestine, liver, skin and kidney [76–78]. Furthermore, disease models simulating inflammation and edema exist [79].

After Black et al., who generated capillary-like structures in a co-culture of fibroblasts and keratinocytes [80], Groeber and colleagues published the first full thickness skin model with populated vessels that can be physiologically perfused [66]. Groeber's model is based on primary keratinocytes, fibroblasts as well as endothelial cells, which were cultured at the air-liquid-interface on a decellularized biological vascularized scaffold in a bioreactor. After two weeks, they reported the formation of human dermis and epidermis architecture containing repopulated functional vessels [66]. In contrast, Lee and co-workers used a human engineered chip with a chamber for skin cells and microfluidic channels for endothelial culture [69, 81] and Abaci et al. achieved vascularization with the use of induced pluripotent stem cells (iPSCs) [82].

The major advantage of the microfluidic chip is the physiological flow of medium through the vascular-like system, which permits a constant oxygen and nutrient supply. This affects cell–cell communications and local concentrations of secreted ligands of tissues [73]. The presence of flow also allows the study of circulating cells, such as circulating breast cancer cells with microvascular endothelium at potential sites of metastasis [83] or immune cells [84]. Using a multi-organ-chip, one can link different organs into a systemic arrangement imitating that of the human organism at a miniaturised scale [76]. Combining the four organ equivalents, skin, intestine, liver, and kidney, this model allows for *in vitro* microfluidic ADME – absorption, distribution, metabolism and excretion – profiling [78]. Prospectively, iPSCs have the potential to predict patient-specific response to therapies [85]. Accordingly, the chips are the best model to predict efficiency, pharmacokinetics, pharmacodynamics and for screening assays. As the chips are mostly made of PDMS, they allow for *in vitro* microscopy [77]. However, PDMS has the disadvantage that it might absorb small molecules such as drugs [86]. Molecular analysis, like PCR, is feasible [79]. The main limitations are the high costs of this system and the demanding skills due to its complex assembly. Another point is that arising bubbles can impair biological function and often viability as it increases the wall shear stress in a liquid-perfused microchannel [87, 88].

2.5. Outlook: Skin organoids and their potential use for personalized approaches

Organoids can be defined as 3D cell culture models mimicking the organ the cells originated from [89]. More accurately, organoids [i] contain more than one cell type, [ii] show physiological function of the organ they model, and [iii] are formed by self-assembly of cells and show organization of the specific organ [90, 91]. The present models arose with the introduction of the hanging drop method by Harrison in 1906 [20] and Wilson who showed self-organization of sponge cells forming a whole organism [92]. Since those progenitor models mostly required large numbers of primary cells from organs, high-throughput screening was not feasible and viability was limited [89]. Nowadays, organoids

are mostly generated from two main types of stem cells: [i] pluripotent embryonic stem (ES) cells and their synthetic iPSCs counterparts and [ii] organ-restricted adult stem cells (aSCs) [93].

To our knowledge, skin organoid models have not been described yet. Nonetheless, it was shown that human iPSC provide a source to mimic healthy skin models as they can be differentiated into keratinocytes [70], fibroblasts [71], melanocytes [72], and endothelial cells [67]. Disease models can be generated with iPSCs derived from somatic cells having a genetic mutation. To mimic keratinocytes in recessive dystrophic epidermolysis bullosa, Itoh et al. isolated cells from diseased patients, reprogrammed them into iPSCs and differentiated them into keratinocytes [94]. Prospectively, iPSCs have the potential to predict patient-specific response to therapies. This is of particular importance since current treatments for psoriasis and other skin disorders are not effective in all patients [41]. Furthermore, it is an unlimited source of cells. However, iPSCs are labor-intensive, reprogramming and differentiation are not matured yet, and there are few high-throughput approaches [85].

Declaration of interest

No competing interests are declared.

Acknowledgments

This work was funded by the German Federal Ministry of Research (BMBF) as part of the Innovation Partnership M^2Aind, project M^2OGA (03FH8I02IA) within the framework "Starke Fachhochschulen – Impuls für die Region“ (FH-Impuls). This research project is part of the Forschungscampus M^2OLIE and funded by the German Federal Ministry of Education and Research (BMBF) within the Framework “Forschungscampus: public-private partnership for Innovations”.

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