Advanced Drug Delivery Reviews 69–70 (2014) 81–102

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/addr

The use of skin models in drug development ☆

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ARTICLE INFO

Article history:
Accepted 17 December 2013
Available online 27 December 2013

Keywords:
Skin
Drugs
Disease
Equivalents

ABSTRACT

Three dimensional (3D) tissue models of the human skin are probably the most developed and understood *in vitro* engineered constructs. The motivation to accomplish organotypic structures was driven by the clinics to enable transplantation of *in vitro* grown tissue substitutes and by the cosmetics industry as alternative test substrates in order to replace animal models. Today a huge variety of 3D human skin models exist, covering a multitude of scientific and/or technical demands. This review summarizes and discusses different approaches of skin model development and sets them into the context of drug development. Although human skin models have become indispensable for the cosmetics industry, they have not yet started their triumphal procession in pharmaceutical research and development. For drug development these tissue models may be of particular interest for a) systemically acting drugs applied on the skin, and b) drugs acting at the site of application in the case of skin diseases or disorders. Although quite a broad spectrum of models covering different aspects of the skin as a biologically acting surface exists, these are most often single stand-alone approaches. In order to enable the comprehensive application into drug development processes, the approaches have to be synchronized to allow a cross-over comparison. Besides the development of biological relevant models, other issues are not less important in the context of drug development: standardized production procedures, process automation, establishment of significant analytical methods, and data correlation. For the successful routine use of engineered human skin models in drug development, major requirements were defined. If these requirements can be accomplished in the next few years, human organotypic skin models will become indispensable for drug development, too.

Contents

☆ This review is part of the *Advanced Drug Delivery Reviews* theme issue on "Innovative tissue models for drug discovery and development".

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0169-409X/$ – see front matter
http://dx.doi.org/10.1016/j.addr.2013.12.006

1. Introduction

The human skin is a large, complex organ containing a multitude of different cell types which interact with each other in a highly concerted manner during homeostasis. The barrier function of the skin is realized by a cornified layer of protein-rich dead cells (corneocytes) embedded in a lipid matrix, namely the stratum corneum (SC). This layer is formed at the end of a balanced differentiation process, beginning at the basal layer of the epidermis, where progenitor cells divide and newly emerged keratinocytes are pushed towards the apical side of the epidermis (Fig. 1). In addition to keratinocytes, melanocytes and Langerhans cells are present in the epidermis. The epidermis is connected *via* the basal membrane (consisting of at least one member of the protein family of laminin, type IV collagen and nidogen, and the proteoglycan perlecan) to the underlying dermis. The dermis is divided into an upper papillary layer containing loosely arranged collagen fibers and a reticular layer with dense collagen fibers arranged in parallel to the surface of the skin. As well as collagen, the dermal matrix comprises a high amount of elastin, to provide the elastic properties of the skin. This matrix is produced by fibroblasts, which are the main cell type of the dermis. In contrast to the epidermis that represents an avascular structure, the dermis is pervaded by blood and lymph vessels. Nerves, sweat and sebaceous glands as well as hair follicles and shafts are embedded in the dermis. Beneath the dermis lies the subcutis, also known as the hypodermis. The subcutis functions as both an insulator, conserving the body's heat, and as a shock-absorber. Next to fibroblasts, adipocytes are the most prominent cell type in this compartment (Fig. 2).

Disorders of the skin can occur by several means, such as by an imbalance in molecular events that govern communication between the cells, infections, neoplastic transformation, immunological and non-immunological responses to foreign agents and autoimmune events, venous insufficiency and pressure or shear on the skin, as it is the case for certain ulcers, as well as by physical and chemical external insults. By being aware of the multiple factors and circumstances, which can lead to the need of a therapeutic intervention, the translation to *in vitro* human skin models simulating the different states of homeostatic imbalance for drug development is challenging. The remarkable regeneration capacity of the skin is able to maintain skin homeostasis throughout a lifetime and in response to some of the above mentioned disturbances. Epidermal regeneration is initiated by epidermal stem cells, which are present in different compartments of the skin, *i.e.* in interfollicular compartments as well as in epidermal appendages, such as sweat glands and hair follicles with their associated sebaceous glands [1–3]. Some of these stem cells can be multi-functional, *e.g.* while certain stem cells present in the isthmus mainly contribute to maintenance of the isthmus and sebaceous glands during homeostasis, they become activated by skin wounding to migrate to the interfollicular epidermis and contribute to

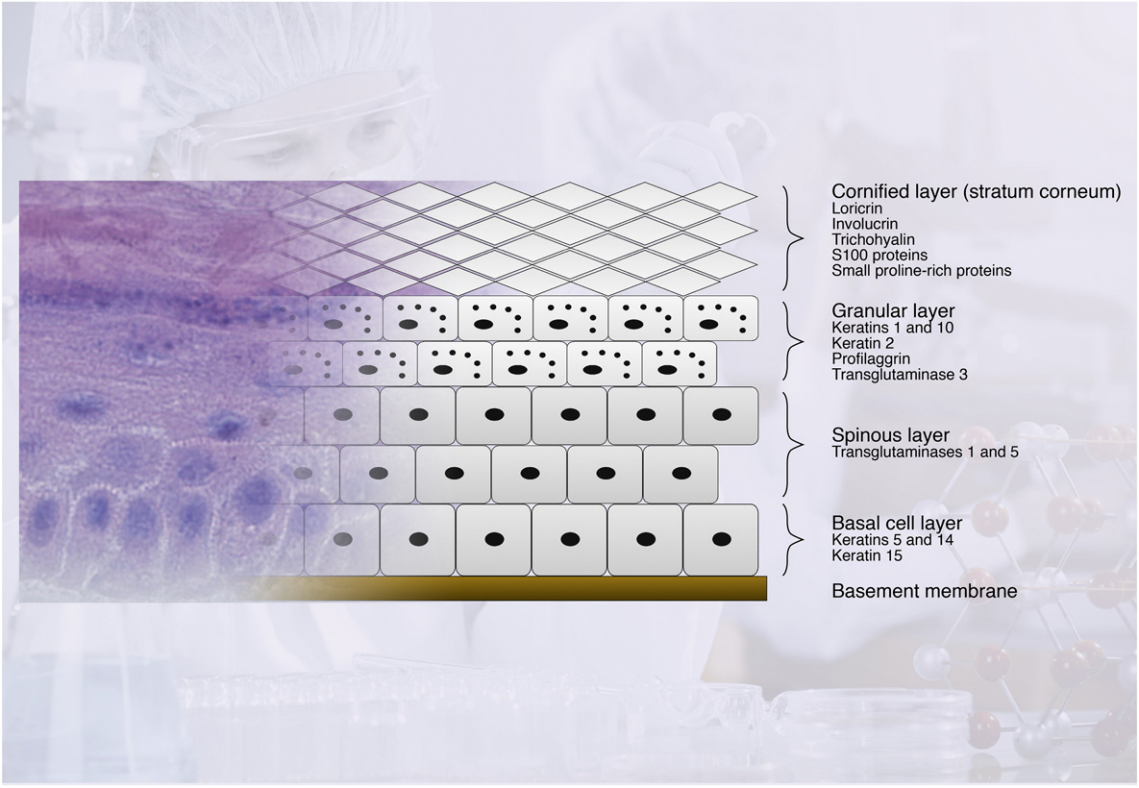

Fig. 1. Alignment of epidermal layers contributing to the barrier function of the skin with relevant markers for each zone. The cellular layers correlate to the histologic picture of a human reconstructed epidermis [4].

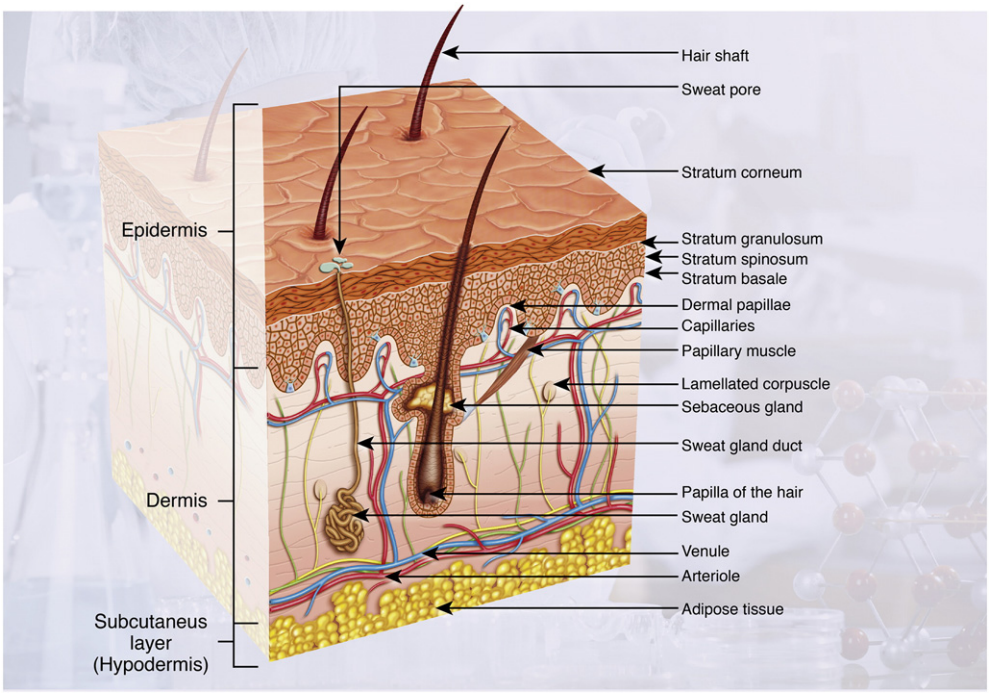

Fig. 2. Schematic picture of the native skin that is sub-classified into three main compartments: epidermis, dermis and subcutis (hypodermis). Skin appendices like hair with sebaceous glands, sweat glands as well as blood vessels are embedded in the skin.

wound repair [5]. Similarly, sweat glands and their ducts contain distinct populations of stem cells which behave differently not only during homeostasis but also in response to different skin insults [6]. Furthermore, mesenchymal stem cells (MSCs) derived from bone marrow or adipose tissue have been suggested to be recruited to wounded skin and contribute to skin repair [7]. Hence, it is not surprising that there is an increasing demand for skin models harboring stem cell populations for the identification of low molecular weight and biological agents to stimulate skin regeneration.

On the other hand *in vitro* engineering of the human skin has been a target issue for the cosmetics industry for about three decades due to legal and ethical demands. Validated reconstructed skin models have been optimized for toxicological screening and results were correlated with *in vivo* data to enable a robust predictive model. This is true for skin irritancy and corrosion, and will also apply to prediction of skin sensitizers soon. From cultivation on feeder layers to epidermal equivalents and co-culture systems a broad range of research and development has been undertaken to improve human skin model creation. Two main branches have emerged, one focusing on more sophisticated models for obtaining deeper insight into skin homeostasis and development of skin diseases for therapeutic intervention, and the other on production and validation of models for risk assessment. A great deal of knowledge has also been gathered by the use of *ex vivo* explant cultures, which feature all important cells, organelles and matrix components, but are generally not feasible for standard tests due to donor variability and limited availability.

Although considerable work has been done (and engineered skin is by far the most accepted and best established organotypic model), we want to raise the question of usability for the pharmaceutical industry. This review focuses on the demands and benefits that can emerge for the prediction of drug efficacy in 3D human skin models.

There are two main areas where *in vitro* skin models are beneficial for drug development: using the skin for application of systemically acting drugs (overcome the barrier and transfer the drug into vessels or adipose tissue), and for efficacy testing of drugs at sites of action (disease models). Nevertheless, in both cases issues of compound application, penetration, metabolism and action have to be tackled (Fig. 3). Depending on the target value, the human skin equivalents have to fulfill appropriate criteria, such as having a suitable barrier function (thickness of stratum corneum and lipid composition) for penetration studies or exhibiting the typical phenotype of a specific disease for drug efficacy testing.

2. State-of-the-art approaches

If we take a look at the existing 3D skin model landscape, we find a broad spectrum of models and creation conditions, which reveal different levels of biological complexity. This is due to different demands that the researcher or industry places on these tissue models. Models which consist of one cell type only (reconstructed human epidermis made of keratinocytes, RHE) have to exhibit high reproducibility when it

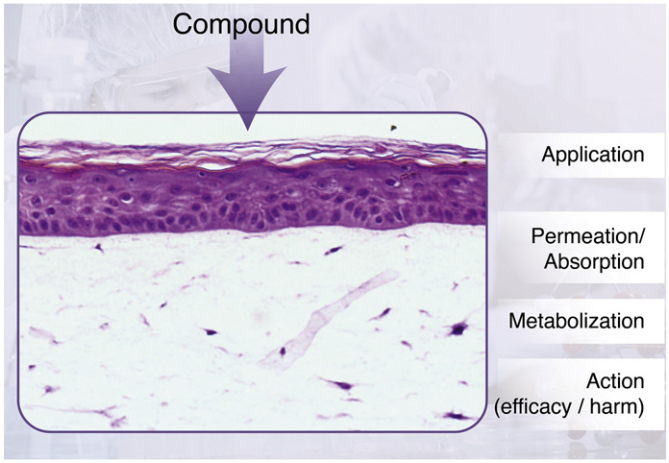

Fig. 3. Potential topics of interest of compound action on organotypic human skin models for drug development.

Table 1
Summary of state-of-the-art models and their applicability for drug development.

Type of skin model	Application for	For the purpose of drug development	
		Advantages	Disadvantages
RHE	• Skin irritation (ECVAM validated) [8,9] • Skin corrosion (ECVAM validated)[10] • Phototoxicity [11] • Epidermal genotoxicity [12] • Transdermal drug delivery [13] • Skin sensitization [14,15] • Metabolization [16]	• Validated models (EpiDerm™, EpiSkin®, epiCS and EPI-MODEL 24) are available • High degree of standardization	• Barrier function impaired • Low complexity, no cross talk with other cell types • Optimized for safety testing • Narrow test window period after arrival (2–3d)
RHE + melanocytes	• Skin Lightening [17] • Pigmentation	• Standardized models to perform studies on skin pigmentation	• Low relevance for drug development
Full thickness (FT) models	• Percutaneous absorption [18,19] • Wound healing [20] • Bacterial adhesion [21]	• Standardized, commercially available • Relevant tools to study wound healing processes	• Cost intensive (one model around $80) • No long term culture possible
FT + melanocytes	• Investigating expression of melanogenetic proteins [22–25] • Assessment of agents acting on vitiligo pathogenis [26]	• Applicable for drug development for drugs acting on vitiligo • Basic melanogenesis research	• Non-standardized model • Approved standards missing
FT + Langerhans	• Assessment of allergens [27] • Investigating maturation and migration of epidermally incorporated Langerhans cells [28]	• Immunocompetent model to asses sensitization potential	• Non-standardized model • Cell line used • Meanwhile equivalent conclusions can be gained with standardized models
FT + endothelial cells and subcutis	• Assessment of angiostatic therapies [29] • Adipose metabolism [30]	• Feasibility to assess impact of drugs on adipose tissue and angiogenesis • Long term cultivation feasible	• Non-standardized models • Approved standards missing • Technically still immature
FT + stem cells	• Assessment of epidermal development [31,32] • Wound healing studies [33,34] • Pigmentation disorders [35,36] • Enabling autologous transplantation [37]	• Enabling the development of a broad spectrum of skin equivalents and disease models with defined and standardized cell sources	• No feasibility shown • Technically demanding
FT + hair follicles	• Substance penetration [38]	• Influence of hair follicles on substance penetration • Absence of artificial components (*e.g.* matrix)	• Low throughput method • Other skin appendages not recapitulated (*e.g.* sweat glands)
Explants	• Epithelial migration [39–41] • Activation and migration of antigen-presenting cells [42–44] • Microbial impact on skin inflammation [45] • Compound screening [46–50] • Neuronal interaction and outgrowth [51–53]	• All cell types are present • Explants of diseased skin can be used for drug development	• Limited amounts of viable skin available (logistical obstacles) • Donor variability
Explants + hair follicles	• Study molecular processes of hair follicle formation [54]	• Analysis of *de novo* hair follicle formation • Close-to-physiologic skin environment initially after cell injection	• Non-uniform developmental stages of hair follicles • No influence of skin specimen epidermis in reorganization of hair follicles, small-sized hair follicles • Sub-optimal to study growth of developed hair follicles

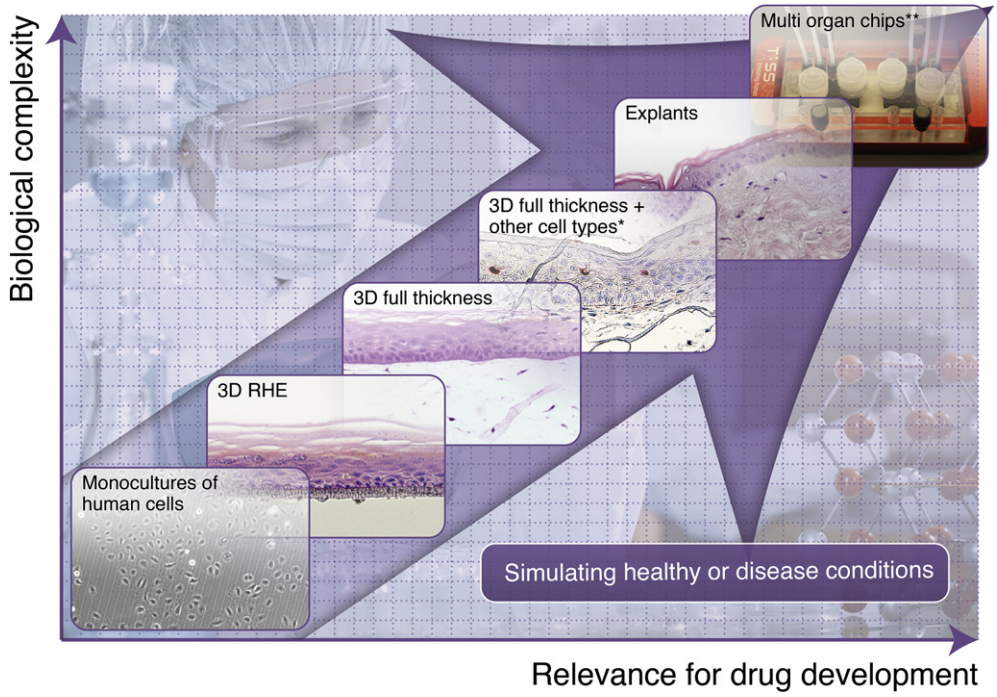

Fig. 4. General correlation of *in vitro* model complexity and relevance for drug development (RHE — reconstructed human epidermis). * Langerin staining of MUTZ-3-LCs cells integrated into a FT model; provided by Dr. V. Laubach, Goethe University Hospital, Germany. ** Multi-organ chip for dynamic cultivation of human skin explants or *in vitro* models; provided by Dr. I. Wagner, TU Berlin, Germany.

comes to the assessment of risks. More sophisticated models are typically used for proving drug or treatment efficacy, but are harder to standardize due to their complexity of creation and readout. Already well-established are full thickness (FT) models which consist of dermal compartment enabling cellular cross-talk between keratinocytes and fibroblasts. Raising the complexity of the models is realized by the integration of additional cell types (melanocytes, stem cells, Langerhans cells and others) or by enabling interaction with other organotypic models (organ-on-a-chip approaches). The different strategies that have recently been realized are discussed below in more detail. Table 1 gives an overview of the different types of *in vitro* skin models as well as the advantages and disadvantages they exhibit in the context of drug development.

In general complex tissue models reflect the *in vivo* situation more reliably than simpler 2D or 3D systems. But high complexity raises problems concerning maintenance, reproducibility, analysis and readout, costs, validation and other matters. This dichotomy needs to be bear in mind.

Fig. 4 summarizes the existing models in respect of their complexity and usability for pharmaceutical drug development. We believe that the highest complexity of *in vitro* models for the purpose of drug development can be achieved with "organ-on-a-chip" approaches, in which different organotypic models can be integrated and communication is provided by fluidic systems. Generally it is feasible to make use of any kind of *in vitro* skin model in such a system. Therefore the different state-of-the-art models are discussed in detail below, whereas the integration of the equivalents into systemic models is taken up in the discussion.

2.1. Models of reconstructed human epidermis (RHE)

In 1998 the first skin models namely EpiSkin® and EpiDerm™ were validated next to the rat TER (trans epidermal resistance) test by the ECVAM (European Centre for the Validation of Alternative Methods) as a predictive model for skin corrosion, and internationally accepted in 2004 when the OECD test guideline was published [10]. Other models that met the demands of the test guideline (TG) were subsequently accepted [55,56]. Soon after, the models were evaluated for prediction of skin irritancy. The modified EpiDerm™ SIT and the SkinEthic® Reconstructed Human Epidermis (RHE) were approved in 2008 to assess skin irritation potential [8,9]. Recently the LabCyte EPI-MODEL 24, a reconstructed human epidermis model produced in Japan, was also validated for hazard prediction. The analysis of the hazard potential is an endpoint determination of cell viability (usually by tetrazolium-based assays like 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) and in case of irritancy determination of IL-1α release. These validated and accepted models are formed by human keratinocytes (foreskin or mammary skin tissue) under optimal conditions at the air–liquid interface to create a stratified epithelium during the time course of around 14 days (Fig. 5). In order to guarantee independent reproducibility, certain functional

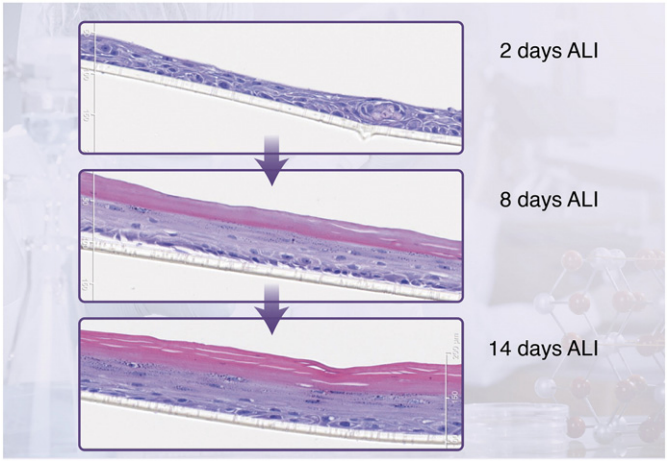

Fig. 5. Developmental process of an *in vitro* grown epidermis demonstrated after 2, 8 and 14 days following air-lift (ALI); arrows indicate the chronological sequence of this process.

conditions were defined that have to be fulfilled by the various test systems. These criteria can be found in OECD TG 439 (2010) (http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/OECD/OECD-TG439.pdf):

- Viability: Each batch of the RHE models should meet the criteria of the defined negative control (NC)
- Barrier function: The stratum corneum and its lipid composition should be sufficient to resist the rapid penetration of cytotoxic marker chemicals
- Morphology: Epidermal layer structure has to be confirmed by histological analysis
- Reproducibility: The results of the positive and negative controls of the test method should demonstrate reproducibility over time
- Quality control: The test user should be equipped with the necessary information about viability, barrier function and morphology. Acceptability ranges of IC50 and ET50 should also be provided

In general, all these criteria are also valid for the evaluation of drugs for the pharmaceutical industry. But if we take a closer look at the presupposed criteria, especially the demands in respect to barrier function do not entirely meet the requirements for drug penetration studies. Concerning transdermal drug delivery, van Gele et al. [13] summarized recent work concerning the permeation patterns of different drugs and substances using different tissue models. Although Schmook et al. [57] did not support the RHE models as alternative methods for penetration studies, van Gele et al. suggested that these models could serve as alternatives and improvements to the models should focus on the composition of the lipids of the stratum corneum. In a pre-validation study [58] the models EpiDerm™, EpiSkin® and SkinEthic® were compared concerning the permeability of caffeine and testosterone. As the permeation coefficients differed from those of human skin, it was concluded that the barrier properties of these models were less developed. The following validation study with permeation substances, which differ in their physio-chemical properties, was also performed with the commercially available epidermal models [59]. In general, permeation of the RHE models exceeded that of human epidermis and pig skin (the SkinEthic® RHE was found to be the most permeable), yet the ranking of substance permeation through the three RHE models and the pig skin tested reflected permeation through human epidermis. Interestingly, the statistical data analysis demonstrated that the significantly better reproducibility expected (compared to excised skin) was not found in the RHE models, but showed a tendency towards lower variability of the data. Differences concerning distribution and amount of water, keratin, ceramids and natural moisturizing factors (NMFs), which contribute to the barrier function as well as penetration profiles, can be assessed by Raman spectroscopy. Spatial distribution coefficients of penetrated active glycerol and octyl methoxycinnamate (OMC) were correlated to the water coefficients in RHE models [60]. The obtained data confirm the data of the prevalidation study [58], but with the advantage of a non-disruptive method.

2.2. Full thickness skin models

Full thickness (FT) skin models consist of a dermal equivalent in proximity to an epidermal one. Generally, collagen I with integrated human dermal fibroblasts is used to create the dermal compartment. After a period of typically 5–7 days, where fibroblasts spread and adhere to the collagen fibers to contract the gel, the keratinocytes are seeded on top. In order to establish a differentiated epidermal equivalent, the construct is cultivated at the air–liquid interface. In addition, nutrients and supplementary factors are adapted to promote organotypic structuring [61–64]. In contrast to the epidermal models, where the cultivation occurs under defined conditions, the FT models are mostly cultivated in the presence of serum (for fibroblast survival). Serum is a natural product, which can differ in protein composition (important for growth factors and cytokines). It is thus subject to batch-to-batch variations and ultimately to the biological outcome of the organotypic model, which is supplemented with serum. Therefore, defined media are currently under development for the co-cultivation models as well.

Concerning the epidermal barrier it was shown that the barrier properties of full thickness models are much closer to the ones of *in vivo* skin, as permeability was only 3–4 times superior to human cadaver control skin for caffeine, tamoxifen and hydrocortisone, while permeability of the epidermal model EpiDerm™ was 10 times higher. The strengthening of the barrier property can be explained by adapted culture conditions, namely by lowering the relative humidity to 75% and the omission of serum prior to the air-lift [18]. Analysis of the lipid composition revealed that all lipids of human skin were also present in the cultured FT models. Significant differences were detected concerning relative lipid abundance. While the amount of triglycerides in human stratum corneum makes up 0–3% of total lipid weight [65,66], they were present in a relative amount of $55.9 + -4.65\%$ in FT models. Indeed it seems that the relative humidity during cultivation of the organotypic models at the air–liquid interphase is a critical factor not only in terms of lipid occurrence. Bouwstra et al. [67] decreased relative humidity in their system to 40% to analyze water content and occurrence of pyrrolidone carboxylic acid as a NMF. They concluded that stratum corneum water level in human skin equivalents is regulated by other, as yet unknown, factors in addition to NMF, as the water content is still lower even at low humidity conditions compared to native skin.

The same research group established different human skin equivalents (HSEs) and studied the stratum corneum lipid organization. They established the fibroblast-derived matrix model (FDM), the Leiden epidermal model (LEM), the full-thickness collagen model (FTM), and the full thickness outgrowth (FTO) [68,69].

In general, all HSEs were more permeable than human skin. Concerning the lipid composition, the FDM and LEM had relatively lower free fatty acid content than the FTM and human skin. Further analysis revealed that the lipids in the SC of all HSEs were arranged into lipid lamellae, similar to human skin but with a higher number of lipid lamellae. Moreover, the SC lipids of all HSEs show a less densely packed lateral lipid organization compared with human SC. They concluded that the HSEs mimic many aspects of native human skin, but differ in their barrier properties. In a recently published article [70] the same group tried to elucidate the cause of the altered SC lipid organization in the HSEs. In line with the findings of Asbill et al. [18], they also detected the presence of 12 ceramide subclasses, similar to native human SC. In addition, the HSEs had an increased presence of monounsaturated free fatty acids (FFAs) compared with human SC, although levels of total FFAs were lower. Moreover, the HSEs displayed the presence of ceramide species with a monounsaturated acyl chain, which were not detectable in *in vivo* skin. All HSEs also exhibited an altered expression of stearoyl-CoA desaturase, the enzyme that converts saturated FFAs to monounsaturated FFAs. Polyunsaturated fatty acids such as arachidonic and linoleic acid are efficient activators of two PPAR (peroxisome proliferator-activated receptors) isoforms and contribute essentially to barrier homeostasis [71–73]. Thakoersing et al. assumed that the level of linoleic acid, arachidonic acid and palmitic acid in the culture media may therefore lead to a change in the activation of the various PPAR isoforms as well as subsequent alterations in epidermal homoeostasis and SC lipid properties [70]. To summarize, the barrier function of existing skin models is insufficient, especially for their application in drug development, as lipid composition and packaging differ from *in vivo* human skin. Furthermore, there are other features which are not yet implemented in the validated models. Additional points to integrate are immunological, photorelevant and systemic issues that require additional cell types or other matrix components.

2.3. Models with additional cell types

2.3.1. Pigmentation

One of the most established and physiologically proved models today is the 3D pigmentation model from Duval et al. [26]. Although melanocytes were the first cell type to be integrated into a reconstructed skin [74], it took almost three decades to construct a relevant model. The incorporation of melanocytes into a full thickness model is necessary for increased physiology — as keratinocytes regulate melanocyte adhesion, survival and morphology by E-cadherins and endocrine factors. Moreover, keratinocytes are able to modulate expression of melanogenetic proteins [22–25]. In order to achieve closer insight into the regulatory impact of the dermal compartment on melanocyte behavior, Hedley et al. [75] established a full thickness skin model on a de-epidermized acellular dermis. The study revealed that the presence of basement membrane (BM) antigens was necessary for positional orientation of the melanocytes. Addition of fibroblasts suppressed the extent of spontaneous pigmentation of melanocytes in this model, supporting the regulatory role of the mesenchymal cells, probably by the secretory factor neurogulin-1 [76]. In contrast to the model of Hedley et al. [75], which didn't respond to the pro-pigmenting agent α-MSH, the system of Duval et al. [26] showed functionality after α-MSH and forskolin stimulation. This was achieved by an adaption of cultivation parameters and addition of promelanogenic growth factors. In the context of pharmaceutical drug development, these pigmentation models may serve as valuable tools for the assessment of agents acting on vitiligo pathogenis. Patients suffering from vitiligo are faced with a proceeding loss of functional melanocytes as a result of a multifactorial process, and there is significant interplay between oxidative stress and the immune system [77].

2.3.2. Langerhans cells

As one of the essential roles of the skin is to maintain a barrier to foreign particles, it is obvious that dermal and epidermal layers are highly immunogenic compartments. Epidermal cells with proinflammatory and immune regulatory functions include keratinocytes, melanocytes, Merkel cells, and the population of epithelial dendritic cells (DCs), namely Langerhans cells (LCs) [78]. In the dermal compartment, dermal dendritic cells (DDCs) belong to the dermal immune system. In contrast to their epidermal counterparts, DDCs do not express E-cadherin [79] and can be distinguished by expression of langerin (only occurring in LCs) [80]. Because of the pivotal role of epidermal LCs in immunologic responses, much emphasis has been placed on the *in vitro* cultivation and integration of epidermal dendritic cells into organotypic models. The cultivation and incorporation of human primary Langerhans cells have only been achieved in outgrowth models to date. In 1998 Fransson et al. [81] published the results of the outgrowth model, which was meant to act as a model predicting contact dermatitis. Other reconstructed models use cell lines, such as human MUTZ-3 [27,82,83]. The system created by Ouwehand et al. [27] has already proved its feasibility as a system stimulated by skin sensitizers. Exposure of the skin equivalent to subtoxic concentrations of the allergens NiSO4 and resorcinol resulted in LC migration out of the epidermis towards the fibroblast-populated dermal compartment. A significant dose-dependent upregulation of the dendritic cell maturation was observed and related to chemokine receptor (CCR) 7 and IL-1-transcript levels and of CD83 at protein level after epidermal exposure to both allergens. Hence, these results indicate the maturation and migration of epidermally incorporated Langerhans cells in an *in vitro* skin model. In a recently published paper [28] the same group found evidence of the role of keratinocyte-derived chemokine ligand (CCL) 5 and CCL20 in the chemo-attraction of MUTZ-3 , as neutralizing antibodies against CCL5 and CCL20 blocked LC migration towards keratinocytes.

2.3.3. Endothelial cells and subcutis

When we think about important features of skin equivalents usable in drug development we need to consider the reconstruction of the subcutis and blood vessels. Especially for the assessment of angiostatic therapies, *in vitro* models can ameliorate the screening process as the essential players are available in a very physiologic environment. In the beginning, *in vitro* tissue engineered models were designed to serve as substitutes for xenografts or cadaver skin in cases where the autologous tissue is limited. Fibroblasts and HUVEC (human umbilical vein endothelial cells) seeded onto a chitosan/collagen biopolymer resulted in an endothelialized skin equivalent and promoted spontaneous formation of capillary-like structures in a highly differentiated extracellular matrix [84]. As HUVEC cells are allogenic per definition, other cell sources were investigated which could be integrated to form capillary structures. Just recently, Auxenfant et al. [85] demonstrated that human adipose derived stem cells represent a valuable source to reconstruct capillary-structures *in vitro*. Again, differentiated endothelial cells were seeded along with human fibroblasts into a porous collagen–glycosaminoglycan–chitosan scaffold to obtain an endothelialized dermal equivalent. Addition of human keratinocytes gave rise to an epithelialized skin equivalent. This study supports the assumption that adipose tissue can serve as an excellent source of autologous endothelial cells to reconstruct endothelialized tissue equivalents.

In another reconstructed soft tissue model that was composed of human endothelial cells isolated from umbilical cord or from newborn foreskin (human microvascular endothelial cells, HMVEC) and dermal fibroblasts on a collagen sponge, three inhibitors of angiogenesis were assessed. A dose–response-dependent significant inhibition of the capillary-like formation was detected when increasing concentrations of tamoxifen, ilomastat, or echistatin were added to the culture medium of the soft tissue endothelial model for 1 week [29]. This study is a good example of the usefulness of tissue models for efficacy testing and prediction of action modes.

In order to create a full thickness skin model which comprises all three compartments of the *in vivo* skin, Bellas et al. [30] developed a system combining the epidermal/dermal compartment on a collagen matrix [64,86] and the adipose/endothelial compartment on a silky sponge scaffold [87–89]. As stated by the authors, the model was easy to handle for more than 14 days and exhibited physiological morphologies of the epidermis and dermis, seen by keratin 10, collagens I and IV expression. Moreover, the skin equivalent produced glycerol and leptin, markers of adipose metabolism.

2.3.4. Tissue stem cells

Stem cells derived from a variety of tissues have been assessed for their functional relevance in 3D skin models. Adult mesenchymal stem cells (MSCs) have been highlighted in the past for repair and regeneration of a multitude of tissues and organs [90]. Human bone marrow-derived MSCs were introduced into a human 3D skin equivalent and substantially contributed to the formation of a stratified epidermis mimicking native skin [31]. Furthermore, adipose-derived MSCs incorporated into the dermal compartment of a human skin equivalent were shown to increase epidermal healing following laser injury [33]. In the context of tissue development epidermal stem cells were also investigated concerning their differentiation potential to a stratified epidermis. In the presence of dermal fibroblasts embedded in collagen, epidermal tissue stem cells located in the outer root sheath of plucked hair follicles were able to reconstitute a multi-layered cornified epidermis, and combining follicle dermal sheath and outer root sheath cells, the former assumed to harbor stem cell properties, gave rise to skin equivalents consisting of distinct epidermal and dermal compartments separated by a basement membrane [34,37]. But not only multipotent stem cells can be used for the establishment of an *in vitro* skin model. Pluripotent stem cells such as germline-derived pluripotent stem

Table 2
Collocation of three-dimensional models of diseased or damaged human skin.

Type of cells	Scaffolding	Pre-treatment of cells or models	Drugs tested	Reference
3D models of psoriatic skin				
Normal and psoriatic human primary fibroblasts	Psoriatic/normal biopsy on collagen-fibroblasts dermis equivalent	–	–	[120]
Human primary cells, explant cultures	Psoriatic/normal biopsy on epidermal surface of non-viable de-epidermized dermis	–	–	[121]
Normal and psoriatic human primary keratinocytes and fibroblasts	Collagen type I	–	–	[122]
Normal and psoriatic human primary keratinocytes and fibroblasts	Fibroblasts own ECM (sheet formation, 2 sheets per dermis model)	–	–	[123]
Human primary keratinocytes	Human de-epidermized dermis	*In vitro* skin equivalents treated with IL-1α, TNF-α, IL-6, IL-22 to simulate psoriatic skin	–	[124]
Combinations of healthy and psoriatic human keratinocytes and fibroblasts	Fibroblasts own ECM (sheet formation)			[97,98]
Phenion® organotypic skin models (FTM's) (Henkel AG)	Collagen I	FTMs treated with IL-22 to simulate psoriatic skin	Calcipotriol	[105]
Human normal fibroblasts, human normal keratinocytes, endothelial cells	Human de-epidermized acellular dermis	Models treated with putrescine	–	[125]
Normal human epidermal keratinocytes	–	RHE treated with IL-4, IL-13 and IL-17	–	[101]
Normal human epidermal keratinocytes and psoriatic fibroblasts harvested from psoriasis lesions	Collagen I	–	Claimed for anti-psoriatic drug testing (calcipotriol hydrate)	SOR-300-FT by MatTek
3D skin models of vitiligo				
Normal human primary keratinocytes and melanocytes (foreskin), human primary vitiligo keratinocytes and melanocytes	Human dead de-epidermized dermis	–	–	[126]
3D melanoma models				
Cutaneous melanoma cell line, human primary fibroblast, melanocytes and keratinocytes	Human de-epidermized acellular dermis	–	–	[127]
Human primary keratinocytes and fibroblasts, human primary and metastatic melanoma cells	Rat tail collagen type I	–		[128]
Human fibroblasts, human keratinocytes, melanocytes or melanoma cells	Bovine collagen I	–	–	[129]
Human keratinocyte cell line HaCaT, mouse melanoma B16.F10 and human melanoma SKMEL-5	–	–	–	[130]
Human primary keratinocytes and fibroblasts, human melanoma cell lines SBCL2 (RGP), WM-115 (VGP) and 451-LU (MM)	Rat tail collagen type I	–	Combinatory treatment of TRAIL and UVB or cisplatin	[112]
Human primary keratinocytes and fibroblasts, primary HUVEC, human melanoma cell lines A375 and SK MEL 28 derived from skin, RPMI 7951 and Malme 3 M from a metastatic site	Cell sheets	–	–	[131]
Full thickness melanoma skin model (MLNM-FT-A375) MatTek: Human melanoma cell line (A375), normal human epidermal keratinocytes (NHEK) and normal human dermal fibroblasts (NHDF).	Collagen type I	–	Claimed for anti-melanoma drug screening; shown for roscovitine	[132,133]
3D skin models of squamous cell carcinomas				
HaCaT-ras cell line A-5 and transfectants derived from it, containing eukaryotic expression vector with the coding sequences for either G-CSF, GM-CSF or mock	Bovine collagen type I	–	–	[134]
A-5IL-6E cells (high expression of IL-6) or A-5C3 (low expression of IL-6) and human dermal fibroblasts	Collagen I, rat	–	–	[135]

Normal human dermal fibroblasts, squamous cell carcinoma cell lines and biopsies	Rat tail collagen	–	–	[136]
Primary normal human epidermal keratinocytes (NHEKs) and primary normal human dermal fibroblasts (NHDFs), SCC-12B2 and SCC-13 cell lines	Rat tail collagen	5 ng/mL, 20 ng/mL or 50 ng/mL EGF	10 μM erlotinib	[111]
3D skin tumor models				
Human and murine dermal fibroblasts, human and murine macrophages	Rat tail collagen type I	IL-4 for M2 polarization of macrophages, LPS and IFN-γ for M1 polarization of macrophages	–	[137]
3D skin models of dermatitis				
Normal human dermal fibroblasts and normal human epidermal keratinocytes	Collagen type I	Histamine	–	[107]
Human foreskin fibroblasts, HaCaT cells, CD45RO + T cells (memory effector)	Rat tail collagen type I, fibronectin	–	Dexamethasone and FK506 (tacrolimus)	[110]
Human keratinocytes (Knock down filaggrin expression)	Bovine collagen I	–	Percutaneous absorption of testosterone and caffeine	[138]
Normal human dermal fibroblasts and normal human epidermal keratinocytes knock down of filaggrin by gene silencing	Collagen type I			[108]
Normal human epidermal keratinocytes	–	RHE treated with a combination of IL-22, TNF-a, IL-4, and IL-13	–	[101]
Human primary atopic dermatitis/normal keratinocytes	De-epidermized dermis	IL-4 and IL-13	Coal tar	[139]
3D skin models of dermatitis				
Human normal fibroblasts and keratinocytes	Pre-glycated bovine skin collagen			[140,141]
3D photodamaged skin models				
Human normal fibroblasts and keratinocytes	Collagen type I	Models exposition to UVA and UVB radiations	Sunscreens	[142]
Human normal fibroblasts and keratinocytes	Collagen type I or de-epidermized dermis	Models exposition to different types of UV radiation	Sunscreens	[143]
3D skin models of pressure ulcers (decubitus)				
EpiDerm™ models (Biodermis)		EpiDerm™ equivalents subjected to loads		[144]
Wounded 3D skin models				
Human normal keratinocytes	Collagen I	Incision wound in a stratified squamous epithelium		[116]
Normal primary fibroblasts and human primary fibroblasts isolated from wound-healing tissue	Bovine collagen type I			[145]
Human fibroblasts and keratinocytes	–	Excisional wound by 6 mm biopsy punch	20 μl of 0.9% NaCl saline solution, culture medium, platelet-rich plasma or fibrin	[115]
Human dermal fibroblasts and human normal keratinocytes	Rat-tail collagen	Full thickness wounds and incision wounds		[118]
Human foreskin fibroblasts and keratinocytes	Bovine tendon Type I collagen	Incision model (2-3 mm width)		[86,117]
Human keratinocytes	De-epidermized dermis	Full thickness wound using a 2- or 3-mm biopsy punch	Evaluation of dermal substitutes: two porous and two non-porous matrices	[119]

cells can also induce the establishment of a stratified epithelium under defined cultivation conditions [32].

Another reason for the integration of stem cells into organotypic skin *in vitro* models is the generation of cell types different from keratinocytes, *e.g.* melanocytes. The integration of spheroids of dermal stem cells into a reconstructed skin equivalent showed that the dermal stem cells were able to settle in the basal cell layer of the epidermis and to differentiate into melanocytes [35]. These findings raised the question whether early epigenetic or genetic alterations leading to transformation take place in the dermis rather than in the epidermis. Besides multipotent stem cells like the aforementioned dermal stem cells, embryonic and induced stem cells exhibiting pluripotency were differentiated to melanocytes and subsequently integrated into a reconstructed epidermal model [36].

2.3.5. Hair follicles

To generate artificial 3D cultures that can either be used *in vivo* as skin substitutes or *in vitro* as predictive models, appendages such as hair follicles with their associated sebaceous and sweat glands need to be integrated as functional units [91]. When studying substance penetration, the presence of hair follicles might significantly influence penetration characteristics. Whereas the interfollicular epidermis constitutes a tight barrier to the penetration of hydrophilic substances, hair follicles allow increased transport of substances through the skin [92]. Moreover, hair follicles can serve as reservoirs for topically applied agents, thereby acting as local depots for substance delivery. To generate skin models containing hair follicles, one can transplant either entire functional units or precursor cells which are then induced to generate hair follicles in skin models. A human fibroblast-keratinocyte 3D model harboring transplanted human complete pilosebaceous units substantiated the influence of the presence of hair follicles on the penetration rate of the test substance hydrocortisone [38]. Another study described the feasibility of generating hair follicle-containing skin models without follicle transplantation [54]. In brief, primary dermal papilla and outer root sheath cells were individually isolated from human hair follicles, expanded in culture and co-injected into human skin explants to allow *de novo* formation of hair follicles with sebaceous glands. In the future, similar approaches might enable the generation of standardized hair follicle-containing skin models which are appropriate for substance testing and screening. Recent efforts have highlighted the importance of producing robust hair follicle models at scale as experimental or screening tools for drug discovery approaches and possibly even cell therapy [93–95]. The identification of markers and potential key molecular pathway regulators of different stem cell populations present in hair follicles might enable the development of drug target opportunities aimed at hair follicle and epidermal skin regeneration [96].

2.4. Explant models

Pieces of skin from healthy donors or donors with skin diseases can be maintained or propagated in culture, either as full or partial thickness explants, the latter comprising dermal or epidermal compartments. Full thickness explants harbor all cell types present in the skin, representing a snapshot of their physiological and functional interdependences at the time of explant generation. Like other 3D cultures, explants display certain limitations, such as loss of the vascular and nervous systems, although their highly contextual nature facilitates the study of processes contributing to disease [53]. Because a multitude of these features can be studied in cell culture conditions, skin explants represent an increasingly valuable system to study biological processes, molecular consequences resulting from disease imbalances, co-cultivation of particular cell types and influences of exogenously added factors and even pharmacological modulators on cell behavior. The utility of explant models has been documented for the study of migration of keratinocytes and sub-populations of dendritic cells. Full thickness epiboly culture models enable the measurement of epithelial outgrowth, such as from mouse ear skin pieces, allowing keratinocytes to migrate over the cognate substrate surface [39]. Human epidermal skin explants facilitate the study of progression from the initial epithelial migratory, non-proliferative phase into the proliferative phase during epithelial outgrowth [40]. Partial thickness skin explants consisting of the full epidermis and a fraction of the adjacent dermis can also be cultured on de-epidermized dermis to quantitatively measure keratinocyte outgrowth [41]. However, these models reflect only part of the complexity of human skin, since certain cell types, such as endothelial cells, melanocytes and Langerhans cells, are lost in culture over time. If cultivated for short periods, *i.e.* less than 3 days, full thickness skin explants are very suitable for studying the activation and migration of dendritic and Langerhans cells upon intradermal injection of TLR agonists [42,43]. In principle, explant cultures can be applied to compound screening, as evidenced when scoring for keratinocyte outgrowth, yielding a decrease by pharmacological p38 MAP kinase pathway or antibody-based epidermal growth factor receptor signaling inhibition [40,46] or increase by Rho-kinase inhibition [47]. Furthermore, a trifunctional peptide was able to stimulate the production of matrix constituents by dermal fibroblasts in an aging skin explant model [48]. When applied within few days of human explant retrieval, these models can also be employed to assess skin inflammatory status and skin barrier function reconstruction in response to a probiotic strain using a substance P-induced inflammation model [45]. Moreover, the biology of dermal antigen capture and presentation by skin dendritic cells, *e.g.* epidermal Langerhans cells, can be investigated in skin explants over a couple of days [44]. Lastly, skin explant models can be employed as co-culture models with heterologous cell types to study their interactions, as recently shown for the influence of primary neurons on the integrity of the epidermis [51] and, reciprocally, the effect of skin explants on neurite growth of the co-cultured PC12 cell line [52]. Explant models or *ex vivo* cultures are also alternatives for the assessment of drug impact. But although they are closer to the *in vivo* situation concerning the complexity of the tissue, they are not yet regularly used in routine drug or substance testing, mainly due to inter-donor variability, availability and biological limitations. They are clearly eligible for basic research and proving principles of action, but not for medium or high throughput screening in drug development and substance testing. Nevertheless, they represent valuable tools to study relevant processes of skin homeostasis, such as the aforementioned nervous re-innervation [51,53] or the impact of chemotherapeutics on hair loss or general proliferation [49,50].

2.5. Disease models

Drugs, which are intended to act at the site of application, have to show their capacity in models which display key features of the impaired skin. In Table 2 relevant 3D *in vitro* models for selected pathological skin alterations are summarized. Dominant characteristics of different types of diseases either based on inflammatory reactions (vitiligo, psoriasis, atopic dermatitis), damage (wounding, burns, photodamage) or abnormal cell behavior (melanoma, squamous cell carcinoma) have been simulated in approximated models. Cells are either harvested from affected patients or pre-conditioned by certain cytokines [97,98] able to provoke a cellular phenotype that is typical for the simulated disease. Some examples are given below.

Although quite a few disease models have been published over the last few years, only a certain number were investigated in respect to their ability to respond to known drugs and proved their relevance by correlation to previously gained *in vivo* data. As we raised the question about the usability of *in vitro* models for drug testing, we should now focus on the studies performed to assess the effectiveness of certain compounds.

In case of psoriasis, the skin is spotted with raised plaques of red, scaly skin. Histological analysis has revealed a vast infiltration of

Table 3
Molecular analyses of skin.

Readout	Characteristics	Variables	Cell source	Method	References
Nucleic acid	mRNA (expression levels)	Cell stimulation, differentiation, RNA silencing	Cells *in situ* or following isolation	qRT-PCR, microarray using oligonucleotide primers, arrays	[153]
	mRNA (expression levels, spatial and temporal resolution)	Cell stimulation, differentiation, RNA silencing	Cells *in situ*	*In situ* hybridization using labeled (direct, indirect) RNA probes	[158]
	mRNA, miRNA, ncRNA (expression levels, isoforms, mutations, non-coding RNAs)	Cell stimulation, differentiation, RNA silencing	Cells *in situ* or following isolation	RNA-Seq by hybridization-based methods	[153–157]
	DNA (cell proliferation, cell cycle)	Cell stimulation, differentiation, RNA silencing	Cells *in situ*	Autoradiography immunohistochemistry, fluorescence microscopy using modified nucleoside (tritiated thymidine, anti-BrdU antibody, EdU click chemistry)	[116,159]
	DNA (fragmentation, apoptosis)	Cell stimulation, differentiation	Cells *in situ*	TUNEL	[184]
Protein	Protein levels, protein modifications	Effect of mRNA silencing, external stimulation	Total lysate	Western blot analysis using enzyme-conjugated antibodies	[162]
	Sub-cellular and cellular localization, abundance, proliferation	Cell differentiation, external stimulation	Cells *in situ* or following isolation	Immunocytochemistry, immunohistochemistry with protein-specific antibodies, fluorescent or color detection	[160,161,185]
	Plasma membrane expression, abundance	Cell differentiation, external stimulation	Single cells following isolation	Flow cytometry with fluorescent labeled antibodies or SOMAmers	[163,164,167]
	Signaling events, such as protein phosphorylation	Cell differentiation, external stimulation	Single cells following isolation	Mass cytometry with antibodies tagged with elemental isotopes	[165,166]
Architecture	Cellular staining	External stimuli, maturation	Cells *in situ*	Histochemistry (eosin, hematoxylin, Fontana–Masson, phycoerythrin-conjugated phalloidin, Hoechst)	[35,168,186]
	Cell tracking	Cell migration, cell differentiation	Single cells, cells *in situ*	Fluorescence microscopy (epifluorescence, laser scanning, 2-photon, multi-photon, light sheet fluorescence microscopy, Fluorescence lifetime imaging)	[169–175]
	Cell tracking	Cell migration	Cells *in situ*	β-galactosidase (genetically marked cells)	[116]
	Cell tracking	Cell migration	Cells *in situ*	Second and third harmonic generation microscopy by label-free imaging, 3D reconstruction	[176]
	Structural information	Ultrastructure, topography, morphology, composition	Cells *in situ*	Transmission electron microscopy, scanning electron microscopy	[178,179]
	Mechanical properties	Shear strength, viscosity and elasticity, indentation and friction	Cells *in situ*	Rheometer, atomic force microscopy indentation, bio-tribometer	[180–182]

immune cells including T-lymphocytes, dendritic cells (DCs), macrophages, and granulocytes in the dermis and epidermis. By addition of IL-22 in combination with other (pro)-inflammatory cytokines and growth factors (OSM, IL-17A, IL-1α, INF-γ, TNF-α *etc.*) a psoriasis-typical inflammatory milieu can be generated *in vitro* [99]. Although the first organotypic models were created by addition of IL-22 and a psoriatic phenotype was postulated [100], recent studies revealed a more differentiated picture of the action of pro-inflammatory cytokines [101]. IL-22 secreted by Th17 cells and acting *via* pSTAT 3 is the strongest activator of keratinocytes. Gene expression analysis showed that IL-22 treatment of normal human epidermal keratinocytes results in the decreased expression of keratinocyte differentiation markers such as filaggrin, loricrin and involucrin [102,103]. Typical proteins of psoriatic plaques associated with innate immunity such as S100A7 and BD-2 are upregulated by another Th17 release cytokine, IL-17 [99]. Hence, with the addition of a cytokine cocktail, it is possible to induce the loss of terminal differentiation, epidermal thickening and hypergranulation. Some companies like Dermatest GmbH, MatTek Corporation, BIOalternatives or Straticell use this knowledge and offer psoriatic organotypic models or services to test compounds [101]. Standard control is calcipotriol, a synthetic derivate of 1 alpha,25-dihydroxyvitamin D3 (1 alpha,25-(OH)2-D3), which already proved its efficacy in monolayer cultures [104]. Although histological analysis of calcipotriol-treated organotypic psoriatic models showed a restoration of barrier-specific structures [105] and extensive studies have been undertaken to define the cytokine mix for simulating a disease typical phenotype [101], standards are still rare and systematic studies on compounds lacking.

As stated earlier, IL-22 induces a decrease in differentiation markers. The absence of correct barrier function is also a hallmark of atopic dermatitis. But in contrast to psoriatic skin, IL-17A is unaffected in atopic lesions compared to normal skin [106]. Organotypic skin models of dermatitis are either afforded by the addition of cytokines [101], histamine [107] or filaggrin gene silencing [108,109]. Knock-down of filaggrin results in altered penetration profiles for lipophilic and hydrophilic drugs as also seen for atopic skin. A dose-dependent protective effect on impaired barrier function was demonstrated by the application of dexamethasone (glucocorticoid, which acts on cytokine release) and FK506 as an immunosuppressant to organotypic skin models of eczematous dermatitis [110]. Besides the models that belong to a group of inflammatory disease state models, there are organotypic models which represent abnormal cell behaviors, such as melanoma or squamous cell carcinoma.

Among these, one model which has already proved its usability for drug development is the human cutaneous squamous cell carcinoma which was developed by Commandeur et al. [111]. They investigated the effects of epidermal growth factor receptor (EGFR) activation and inhibition on normal and malignant *in vitro* human skin equivalents (HSEs). In healthy HSEs, increasing epidermal growth factor (EGF) levels resulted in a dramatic decrease in epidermal proliferation and increased epidermal stress. Moreover, higher concentrations of EGF induced remarkable epidermal disorganization with loss of proper stratification. Similar effects were observed in HSEs generated with the cutaneous squamous cell carcinoma (SCC) cell lines SCC-12B2 and SCC-13. Treatment of both healthy and SCCHSEs with 10 μM erlotinib resulted in an efficient reduction of epidermal thickness from 10 to 3 viable cell layers and counteracted EGF-induced epidermal stress. Remarkably, erlotinib treatment caused severe desquamation in healthy HSEs, reminiscent of xerosis as a known side-effect in patients treated with erlotinib. Most recently, the development of a hybrid system simulating metastatic melanoma in an organotypic full thickness skin equivalent was published [112]. This model harbors melanoma tumor spheroids of defined sizes that were seeded into dermal matrix or melanoma cells which had previously been seeded on top of the dermal layer before addition of keratinocytes. In a previous monolayer-based study, it was shown that a high percentage of melanoma cell lines were synergistically sensitized to TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis by co-application of sublethal UVB radiation [113,114]. In contrast to previously performed 2D tests, skin equivalent-embedded melanoma spheroids remain almost unaffected by the TRAIL/UVB combinatory treatment. This result clearly justifies further studies to develop 3D disease models for drug development.

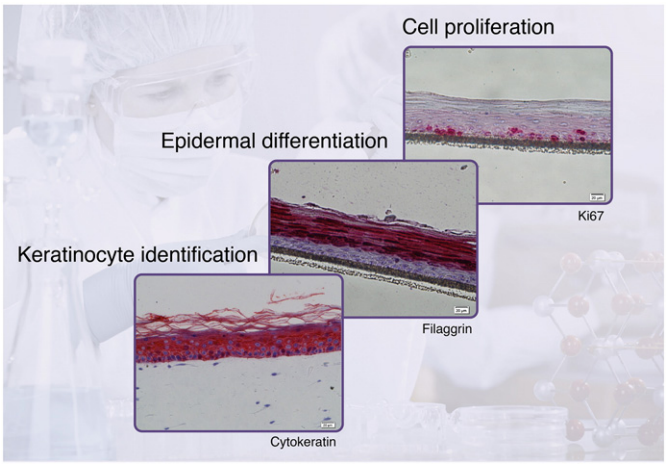

Fig. 6. Examples of immunohistochemical stainings of cytokeratin, filaggrin or Ki-67 (each in red, cell nuclei counterstained in blue) in epidermal or FT models in order to demonstrate keratinocyte presence (cytokeratin), epidermal differentiation (filaggrin) or proliferating cells (Ki-67).

Alterations of the skin can also be achieved by any kind of physical burden, leading to partial or entire disruption of the epidermis and underlying tissue. There are two commonly used damage models, incisional and excisional wounds. Both are similar, but the latter generates a larger wound area that takes longer to heal, enabling all stages of the re-epithelialization process to be investigated [115]. The generation of excisional wounds, whereby an epidermal–dermal portion is removed from a 3D model, allows keratinocyte movement over the fibroblast/collagen compartment from the surrounding wound margin towards the center to repopulate the wound bed. This re-epithelialization process is completed by epithelial stratification [116,117]. The dermal compartment plays a major role, as the fibroblasts strongly influence the re-epithelialization rate in dependency on the ECM on which they have been cultivated prior to 3D model generation [117]. Furthermore, the presence of fibroblasts in superficial incisional wounds, in which only the epidermis was removed, positively affected re-epithelialization in comparison to excisional wounds with concomitant fibroblast loss [118]. Excision models allow dissecting the biology of the re-epithelialization process, such as keratinocyte and fibroblast migration, and the reorganization of the dermo-epidermal junction (DEJ) [115]. Cellular analyses indicated two complementary and independent mechanisms by which keratinocytes are displaced towards the wound center, both contributing to the regeneration of a functional epidermal barrier. Exogenously added agents, such as fibrin, platelet-rich plasma, EGF and keratinocyte growth factor (KGF), enabled modulation of the wound closure progression, *i.e.* enhanced re-epithelialization with concomitant retarded deposition of DEJ proteins [115,118]. The physiology of wounded human 3D skin models was changed even further by the addition of fresh human blood, and inclusion of fibroblasts and endothelial cells to study the granulation process in conjunction with different matrices [119]. By adapting human 3D skin models stepwise to the characteristics of human skin, these models are suitable for screening of substances which promote individual steps of the wound healing cascade under a given experimental design at a medium throughput scale. These models can be further adapted towards disease indications by incorporating stimuli, such as chronic wound fluid in order to mimic manifestations of chronic wounds.

However, the settings are only approximations of a clinical situation, since certain attributes are not recapitulated in the models, such as a vascularized dermal matrix. Therefore, it is crucial to validate identified bioactive substances in suitable pre-clinical models.

3. Analyses of skin models

To implement 3D skin models into routine application for drug development, efficient methods for their analyses and reliable readouts are crucial.

A variety of molecular signaling pathways govern cellular processes. Extracellular stimuli, such as soluble ligands or cell membrane-associated proteins, induce intracellular signaling cascades, leading to changes in cell behavior. As perturbations of signaling pathways can result in disease, exploring the dynamics and intersection of signaling pathways helps to understand disease mechanisms and enable drug discovery research [146].

Mechanical properties of the skin play a major role as to how physical external stimuli are received and signal transduction cascades are initiated. When keratinocytes grown on a solid surface were subjected to cyclic pressure treatment, they started to differentiate into multilayered structures with concomitant changes in cytokeratin expression [147]. Moreover, mechanical stretching of cultured human keratinocytes led to phosphorylation of EGFR and subsequent activation of the extracellular signal-regulated kinases (ERK)1/2 pathway components, increased cell proliferation, and decreased differentiation with upregulated keratin K6 and downregulated keratin K10 expression [148]. These mechanisms may mediate epidermal hyperproliferation in response to mechanical stretching of skin. To assess the consequences of pressure application in a 3D epidermal model, a human epidermal equivalent (EpiDerm™) was treated with increasing degrees of pressure and analyzed by histology and for viability as well as cytokine and chemokine release [144,149]. Sustained pressure loading led to enhanced release of IL-1α, IL-1RA, TNF-α and CXCL8/IL-8, and swollen cells, vacuoles, necrosis and affected cell membranes were observed at higher pressures. Such models may prove valuable for clinical marker identification and prevention of pressure ulcers.

How are external pressure stimuli to the skin transformed into signal transduction events? A recent study performed in *Caenorhabditis elegans* may provide some clues. The authors demonstrated the existence of a mechanotransduction pathway that functions *via* hemidesmosomes and operates between the body-wall muscles and the epidermis [150]. This or similar pathways were suggested not only to contribute to the morphogenesis of epidermal and muscle tissues but also to epithelial morphogenesis or even wound healing in other organisms. Another study revealed how mechanical cues, such as extracellular matrix rigidity, and cell shape can result in alteration of transcription regulation [151]. This mechanism involves the Yorkie-homologues YAP (Yes-associated protein) and TAZ (transcriptional co-activator with PDZ-binding motif) regulators. YAP has also been implicated in balancing growth and differentiation in skin [152], potentially linking mechanosensing and epidermal cell fate regulation.

Cultured cellular skin models replicate multiple aspects of *in vivo* skin biology due to the presence of a subset of relevant cell types as well as the microenvironment and preservation of signaling pathways in these cells. Therefore, the cellular consequences of skin disease states and perturbations, be they of chemical, biological or physical nature, can be investigated by functional, molecular and biochemical methods in 3D skin models. The influence of these stimuli on cells can be assessed at various levels. Often, only a multitude of different readouts permit a biological question to be answered in a meaningful manner. Such studies can be performed on homogenous cell populations isolated from a mixture of cells based on particular purification strategies, such as fluorescence activated cell sorting (FACS), providing insights into spatio-temporal events when different cell samples are taken at different time points over the duration of an experimental procedure. In fact, even single isolated cells can be analyzed, allowing the characterization of differences between *e.g.* a tissue stem and differentiated cell. On the other hand, intact pieces of 3D models comprising different cell types can be analyzed *in toto*. These models consist of mixed cell populations, allowing the study of temporal events by embracing context-specific information due to the presence and interdependence of different cell types.

3.1. Molecular analyses

Cellular proteins, lipids, carbohydrates and nucleic acids all serve essential physiological functions. In order to understand the functional consequences of an insult on or an interaction between different cell types in a skin model, one needs to understand the relative quantitative and qualitative changes of these molecules. In the following, we focus on protein and nucleic acid analyses (Table 3).

Events which result in changes of gene expression can be visualized using methods measuring relative mRNA transcript levels, such as qRT-PCR and microarray analyses [153]. qRT-PCR studies are typically performed at lower throughput with an emphasis on particular genes only, whereas microarrays allow parallel assessment of a multitude of defined genes in a standardized manner. A relatively novel method, RNA-Seq, is based on deep-sequencing technologies and enables the analysis of transcriptomes with high accuracy and reproducibility [154]. Importantly, this "*de novo*" sequencing approach does not require sequence knowledge of individual mRNAs and is suitable for measuring mRNA isoforms, mRNA mutations (including post-transcriptional mutations), editing and gene fusions, and also non-coding RNAs [153,155–157]. Even though RNA-Seq datasets are generally more complex than microarray datasets, both require expert knowledge concerning data processing and analysis, since multiple parameters, such as experimental setup, RNA and reagent quality as well as nuances in sample handling can dramatically influence data quality. As deep-sequencing technologies will become more established, RNA-Seq might eventually replace microarrays. Furthermore, *in situ* hybridization techniques on whole 3D model sections serve to visualize RNA expression at spatial and temporal resolutions. Fluorescence RNA *in situ* hybridization (RNA FISH) studies are performed on formalin-fixed paraffin-embedded (FFPE) or frozen sections and visualized using a fluorescent microscope [158]. Narrow detection sensitivity coupled with limited multiplexing capability and throughput as well as challenges in automated data analysis might pose some issues for these methods. To assess the effect of experimental variables on cell proliferation, in particular on the basal layer keratinocytes, modified nucleosides (which are either radioactively labeled or can be specifically detected *via* fluorescently labeled antibodies) or click reaction reagents are added to cells [116,159]. The signal recorded is proportional to the rate of DNA synthesis and hence cell proliferation.

A major functional effector class in cells consists of the mRNA translation products, the proteins. Proteins rarely act alone, but they engage with other proteins in a dynamic fashion in time and space to fulfill their cellular functions. To analyze 3D skin models in response to stimulation, one needs to assess various aspects of protein biology, such as relative abundance, post-translational modification (PTM), sub-cellular localization and protein complex composition.

Immunohistochemistry (IHC) studies are often performed to monitor protein abundance, cellular protein expression and protein PTMs on sections of 3D cultures using antibodies directed against respective epitopes. For example, the proliferative status of cells is often quantified by measuring the levels of Ki-67 protein, which is strictly associated with cell proliferation, whereas the presence of keratinocytes can be identified by specific staining of cytokeratins and epidermal differentiation by antibody-based detection of filaggrin (Fig. 6). Similarly, the presence of p63 can be quantified in IHC experiments using a labeled antibody to visualize the proliferative potential of basal keratinocytes [160]. The effect of external perturbations can be measured by reagents which specifically recognize transient alterations of proteins, such as the

phosphorylation of histone H2AX (γ-H2AX) in response to DNA double-strand breaks [161]. IHC analyses are subjected to shortcomings such as semi-quantitative scoring, variability in sample preparation and pitfalls of computer-assisted image analysis. Western blot analysis is suitable for gaining semi-quantitative or qualitative information about the relative level and the extent of posttranslational modifications of proteins, *e.g.* in response to downregulating tight junction or filament-associated proteins using siRNAs in 3D skin models [108,162]. These methods are typically carried out at a low throughput and require, in contrast to PCR- and IHC-based methods, higher numbers of cells.

For comparative analyses of skin cells derived from different sources, such as normal and pathologic tissues, quantitative flow cytometry following dissociation of cells is a method of choice [163]. Multiparameter flow cytometry analyses can be instrumental in understanding epidermal cell growth control in healthy and disease conditions [164]. A recently developed method called single-cell mass cytometry enables the quantification of network signaling dynamics at single cell level in a complex cellular mixture in a comparative and quantitative mode, thereby addressing communication between different cell types in cellular models [165,166]. This method might eventually enable assessment of the effect of different drug combinations on patient-derived 3D skin models to guide therapeutic intervention. A successful application of this method will require rapid single cell isolation and analysis from 3D models without interfering with cellular signaling marks. However, a limitation of the current method is that cells need to be brought into single cell suspension before analysis, preventing *in situ* analyses and potentially introducing unwanted cellular alterations due to the single cell isolation procedure. These methods strongly depend on the availability of antibodies which display high affinity and specificity towards the cognate antigens, necessitating the use of control antibodies. Moreover, optimal sample preparation is required to preserve even weak epitopes, such as transient phosphorylation events. Novel nucleic acid-based reagents capable of multiplexing which are optimized *in vitro* to bind to epitopes represent promising alternatives to antibody-based approaches [167].

3.2. Structural analysis

A very sensitive and informative method to interrogate 3D skin models is to study their architecture. Minute changes during the maturation of 3D models or treatment of mature models with external stimuli result in pronounced alterations in the model structure. One commonly used approach makes use of cellular staining using specific dyes, such as hemotoxylin and eosin (H&E) or Fontana–Masson. Other staining reagents such as phycoerythrin-conjugated phalloidin, which binds to F-actin, in conjunction with Hoechst 33342, which stains DNA, are being used to visualize the formation of cellular networks embedded in matrices [168].

Fluorescence microscopy comprises a variety of optical microscopy techniques which use fluorescence properties of molecules to generate images. Both widely used epifluorescence and laser scanning microscopy belong to this category. The latter enables dynamic quantitative analyses of 3D cellular objects at an excellent signal-to-noise ratio with optical sectioning capabilities, yielding good spatial resolution. Confocal laser microscopes are often the method of choice, even though they exhibit a limited penetration depth, cause photobleaching and induce phototoxicity, thereby limiting their use in analyzing large 3D objects [169]. Although they achieve a lower resolution, versatile two-photon and multi-photon microscopes overcome some of the above limitations. These microscopes provide a higher penetration depth and only generate photobleaching and phototoxicity in the close vicinity of the focal plane, thereby permitting long-term fluorescence imaging without comprising viability [170,171]. When studying objects up to millimeter-sized penetration depth over extended periods of time under minimal photobleaching and phototoxicity conditions, light sheet fluorescence microscopy is an attractive option. Serial sectioning allows 3D reconstruction of biological material, ranging from smaller sized cellular spheroids to tissue sections as well as whole zebrafish and *Drosophila* embryos [169,172,173]. All optical microscopes are subjected to limitations in resolution due to basic physical properties and the thickness and heterogeneity of specimen.

Fluorescence lifetime imaging microscopy (FLIM) allows investigation of the spatial resolution of a fluorescent molecule in conjunction with information about its microenvironment. Images are derived from the fluorescent lifetime of an exogenous or endogenous fluorophore, and not from its local concentration and/or intensity [174]. A multitude of cellular parameters, such as pH, ion and oxygen concentration, viscosity and cell metabolism, have been assessed using FLIM [175]. By combining FLIM with a microscopy technique enabling high 3D resolution, such as two-photon scanning microscopy, a highly resolved spatial distribution of a fluorescence lifetime dependent on its microenvironment can be generated, such as the epidermal pH gradient in a human 3D skin equivalent [175]. A limitation of FLIM concerns the analysis of dynamic events in living cells.

Second and third harmonic generation microscopy (SHG and THG, respectively) constitutes a multiphoton laser scanning approach which is not based on fluorescence. It allows label-free imaging with optical sectioning of life tissues to generate high resolution 3D reconstruction at high penetration depth, such as visualizing the migration of unlabeled leukocytes within explanted muscle tissue [176]. SHG and THG can be combined with other image approaches, such as fluorescence microscopy, allowing various combinations of cell and tissue imaging [177]. SHG is derived from materials that are structured in crystalline-like lattices, limiting its utility to a small number of structural proteins, such as fibrillar collagen. Signal-to-noise ratio and sensitivity are limitations encountered when imaging low abundance targets.

Electron microscopy is instrumental when biological specimens need to be studied at very high resolution at ultrastructural level. For this, samples have to be specially prepared for analysis, which rules out live cell analyses. Transmission electron microscopy (TEM) is used when investigating the effect of one cell type or external stimuli on the maturation and composition of 3D models, *e.g.* to determine the influence of keratinocytes on the maturation and elastin network present in the dermal compartment [178]. Scanning electron microscopy (SEM) is able to provide information about topography, morphology and composition of samples. This technique allows, for example, visualization of physical interactions between cells and a 3D matrix, such as fibroblast extensions and collagen fibrils [179]. However, the applicability of electron microscopy is subjected to several constraints: Live objects cannot be analyzed, the preparation of specimen is cumbersome and their analysis prone to image artifacts and the instrument is large, complex and requires expert training for operation.

There are several non-invasive methods described to study mechanical properties of skin, such as measurements of suction, torsion and traction. A rheometer can be used to quantitatively assess the shear strength of *e.g.* biomedical adhesives to skin models [180]. Atomic force microscopy indentation can be employed to measure the viscous and elastic behavior of individual skin layers [181]. In freshly excised mouse skin, the dermis revealed superior mechanical stiffness compared to the stratum corneum and the living cell layer of the epidermis (viable epidermis). On the other hand, the viable epidermis showed greatest viscoelasticity. These studies may allow development of improved micro-devices to act within the skin. A recently described biotribometer is capable of measuring the indentation cycle and frictional forces in a human dermal equivalent that is composed of a collagen–chitosan scaffold seeded with human dermal fibroblasts [182]. The results obtained by this approach show identical values of the Young's modulus E and the shear modulus G of the dermal equivalents and human *in vivo* skin, proving the emulation of mechanical properties of human skin by *in vitro* models. However, no comparative molecular characterization was performed in this model, and similar studies

have to be conducted on more complex 3D skin models and multiple dermal scaffold materials.

Most of the techniques and methods which are able to analyze an intact 3D structure are inappropriate for target screening in high throughput screening (HTS) approaches. In this context, the automation of cultivation and analytical procedures is also noteworthy. But it would be over ambitious to aspire to a high content and high throughput readout in one system. Generally, 3D skin models are very valuable for mode-of-action studies, carried out at lower throughput using hits which have been identified in heterologous systems. It still might be more efficient to screen for targets in simple 2D systems and apply more complex systems in the next phase of drug evaluation, due to the complexity of 3D model systems *per se* and the requirements on the readout systems. Another critical issue is data management. Multitarget profiling improves the productivity of drug discovery. Analyzing many cellular parameters, including gene and protein expression and metabolites generates powerful information, but also a huge amount of data.

Data interrogation is subject to several limitations. One concerns the availability of data *per se*. Most data are typically stored in individual non-public locations, *e.g.* qRT-PCR data as relative threshold values (Ct) for the expression of individual genes or immunohistochemistry data as processed images. Some datasets, *e.g.* microarray data, may be stored in publicly accessible databases, such as the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). Attempts are being made to couple complex datasets with complex standards for annotation and data exchange [183]. The immense amount of sequencing data obtained by RNA-Seq presents several challenges to bioinformation scientists, such as the development of efficient methods to store, retrieve and process data [154]. Another limitation is set by the difficulty of integrating diverse data output formats. Individual software solutions for specific data formats complicate comparative analyses. Furthermore, factors such as experimental setup, availability of control samples, number of replicates, data quality and choice of data analysis tools have a profound impact on data interpretation and the conclusions drawn. Therefore, integrated drug discovery approaches using multiple data sets generated from 3D skin models will require more holistic data analysis capabilities.

4. Discussion

Multiple issues have to be tackled in order to enable routine application of engineered human skin models for drug development. A great deal can be learned from the cosmetics industry, where pressure from legal authorities has provoked a vast sprouting of epidermal and organotypic models. But besides procedures for cell isolation, tissue production, and maintenance and subsequent characterization, more sophisticated analysis beyond cell viability tests by MTT and measurement of IL-1 release are required in the pharmaceutical industry. The increasing complexity of tissue equivalents is accompanied by similarly complex analytics at different levels. Although quite a broad spectrum of models covering different aspects of the skin as a biologically acting surface exists, the models are still single stand-alone approaches. In order to enable comprehensive application into drug development processes, the procedures need to be synchronized to allow cross-over comparison. For the successful, routine use of engineered human skin models in drug development, we have defined the following major requirements.

4.1. Requirements concerning production processes

In research and development (R&D) most of the tissue equivalents are produced manually except where the production procedure itself is the subject of investigation. The traditional way of creating organotypic skin models is to seed human dermal fibroblast into or onto a scaffold mainly composed of collagen I. After a period of 5–7 days, in which the fibroblasts adhere and contract the collagen gel, human keratinocytes (and possibly other cell types) are seeded on top of the gel. The step of collagen contraction is an uncontrollable event, depending on the condition of the fibroblasts, serum concentration and collagen source. Attempts to standardize this process step have been realized by drawing out the water straight after polymerization [187] (RAFT system). Under submerged conditions, the keratinocytes adhere and proliferate to result in a confluent cell layer covering the entire gel surface. In order to induce epidermal differentiation, the keratinocytes are lifted to the air–liquid interface and the medium is changed to differentiation medium [64]. To accomplish the air-lift, cost intensive cell culture inserts have to be used. In effect, it is possible to generate a stratified epidermal equivalent under submerged conditions, but these models contain more free water and less natural moisturizing factors than their counterparts that are raised while exposed to air [188]. The development of the epidermal structure in conventional air-lifted models takes about a further two weeks. The window for test performance is around three to four days, as the *in vitro* models are devoid of desquamation [189,190] and stratum corneum increases over physiological height. One important issue for a correct desquamation process is the water level [191], which is typically higher in *in vitro* engineered skin compared to *in vivo* skin [67]. Another important feature is the dynamic cultivation (applying pressure and shear forces) that is still lacking in *in vitro* approaches. Nevertheless, long-term cultures were also established which could be cultured over 12 weeks and showed growth patterns and keratin expression more similar to physiological levels. This was achieved by a modified dermal scaffold (Hyalograft-3D) that was colonized with skin fibroblasts, producing genuine dermis-type matrix [192]. Moreover, conditions supporting a prolonged lifespan of the *in vitro* culture resulted in a marked increase in lamellar bodies at ultrastructural level, confirming an advanced maturation of the epidermal lipid barrier [193]. Nevertheless, this promising approach failed to achieve the collagen-based dermal scaffolds that still represents the gold standard in human skin engineering. Even in new and technically challenging production approaches such as in the fully automated "Tissue Factory" developed at the Fraunhofer institute, collagen I is chosen as the dermal matrix scaffold. In many cases, automated production processes are still similar to manual procedures. But there are also more innovative approaches which are still in the fledgling stages, *e.g.* ink-jet bioprinting [194] and laser-assisted BioPrinting [195]. Although promising results have been gained for the dermal compartment and keratinocyte cell lines (HaCaT), the establishment of a stratified epidermis has still to be shown.

To summarize, an efficient and standardized production process of 3D skin models could augment the comparability of tissue equivalents.

4.2. Requirements for understanding drug metabolism in the epidermal layer

Potential drug metabolism in the skin is an important issue to consider. Different organotypic skin models were already compared in respect of cytochrome P450 (CYP) isoenzyme expression on mRNA and protein level as well as the inducibility of these enzymes by aryl hydrocarbon receptor ligands [196]. The data provided evidence for the expression of CYP types 1A1, 1B1, 2E1, 2C and 3A5 in organotypic skin models. Moreover, the expression of CYP1A1 and CYP1B1 was highly inducible by treatment with liquor carbonis detergens. These findings reveal the capability of *in vitro* created models for xenobiotic metabolism, but fail to compare their relevance with *in vivo* skin. A deeper look into gene expression of enzymes for xenobiotic metabolism showed that the expression of 87% of the genes was consistent between a reconstructed epidermal model (EpiDerm™) and human skin [16]. This is a clear indication of the presence of similar metabolic pathways, suggesting analogy in function. Though the basal expression of CYPs in particular was low in EpiDerm™, significant induction of CYP1A1/1B1 activity was observed following treatment with 3-methylcholanthrene.

In order to determine the localization of CYP26AI expression in normal skin and three-dimensional skin models, the enzyme was detected by immunofluorescence after stimulation with all-trans retinoic acid [197]. In contrast to cell culture monolayers, in which CYP26AI was only weakly detectable, strong constitutive expression of CYP26AI *in vivo* and in organotypic culture was found. Moreover, the expression was restricted to basal epidermal keratinocytes as well as eccrine sweat glands and sebaceous glands. These studies underline the validity of three-dimensional tissue models to evaluate metabolism and toxicity of cutaneous exposures to xenobiotics. The collected data correlate very closely to data obtained from human skin, rather than results from 2D systems.

To generate more data of different model systems would be an advantage to understand the impact of microenvironment concerning drug metabolism.

4.3. Requirement to correlate in vitro to in vivo models

Most studies which were performed with organotypic skin models were correlated to data collected with human skin biopsies, which is entirely entitled. In this respect, a human 3D skin model has been developed, that allows experimental interrogation of drug combinations containing the cell killing proteasome inhibitor bortezomib [198]. However, in cases where data in the process of drug development are gained using animal trials or excised pig skin, it is important to correlate the data compiled with *in vitro* models to the appropriate data of *in vivo* models with a certain pre-defined set of standard compounds, where the range of dose-dependent action is known. Some murine models already exist that enable the incorporation of transgenic keratinocytes and allow deeper insight into molecular mechanisms underlying epidermal morphogenesis [199–201]. More of such investigations and approaches are needed in order to fill the gap between pre-clinical animal trials and application on human skin and to clarify the relevance of organotypic models in respect to predictability and mode-of-action. Some lessons can be learned from the field of pharmaceutical toxicology, where false positive and false negative acting drugs are tested alongside known positive acting drugs in appropriate 3D hepatic models of animal and human cells [202].

4.4. Requirement to clarify the impact of drug formulation and application mode

There are multiple applications for which 3D skin models increasingly represent attractive systems for drug discovery, such as functional analyses of genetically modified cells, aging, skin immune system disorders and carcinogenesis, as described above [203]. For transdermal and topical drug delivery, model systems are required that address the skin permeation of compounds in order to support drug formulation. As noted previously, human organotypic 3D skin culture models are suboptimal for penetration studies due to poor barrier function [204]. Hence, multiple efforts were undertaken in the past to increase the barrier function of 3D models to lift their predictive power in relation to human skin. Concerning drug formulation testing, 3D wound healing models have been reported to be valuable for assessing solid lipid nanoparticle-mediated drug release on wound closure [205]. Furthermore, 3D human skin models serve as functional readout systems to assess the applicability of potential wound dressings, such as functionalized hydrogels, for the treatment of chronic wounds [206]. When it comes to the application of soluble substances, which should be assessed in a dose-dependent manner, the choice of the vehicle is critical and dependent on chemical properties of the substance, *e.g.* cLogP. But not all vehicles might be usable in the concentration needed, as they influence the viability of the tissue models [14]. Moreover, it turned out that action profiles might alter in case of different vehicles used for application, and some may act as penetration enhancers (personal communication). Therefore, systematic studies have to be performed with a set of pre-defined vehicles to acquire a full picture of how a vehicle or formulation can influence a specific skin model. Not only chemicals can modulate drug penetration into the skin, but also physical treatments. It has been shown that external stimuli can actively alter skin permeability, and thus facilitate the mass transfer of therapeutic agents. Notably, the use of external stimuli (*i.e.* ultrasound [207,208], electric fields [209,210], shear forces [211]) is able to increase cell membrane permeability and can accelerate drug permeation. As such stimuli were applied in microfluidic systems, they represent a reasonable extension in respect to "organ-on-a-chip" approaches that will be discussed in Chapter 4.7.

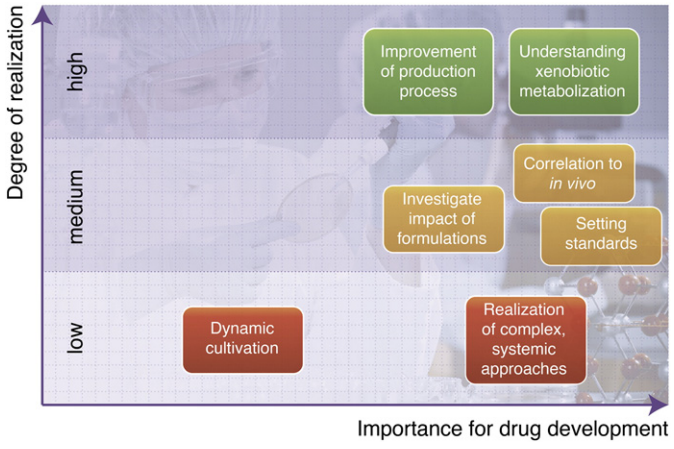

Fig. 7. Requirements for further development of 3D *in vitro* skin models arranged corresponding to their importance for drug development.

4.5. Requirement for setting standards

As stated above, a pre-definition of standard compounds for the different fields of application has to be drawn up and accepted in the appropriate community. Here again a look at validated models can offer guidance as a defined set of standards and expected ranges of data exist for corrosion, irritant testing and permeation analysis. As for psoriasis, where calcipotriol emerged as the standard drug, clear and consistent agreement for other disease models on standards and thresholds has to be defined.

4.6. Requirement for dynamic cultivation

Under physiological conditions skin cells are subjected to endogenous (proliferation, differentiation, migration) and exogenous (touch, itch) mechanical stimuli. Upon mechanical stimulation, keratinocytes react with activation of mitogen-activated protein kinases (MAPKs) and increased cell proliferation [212]. Mechanically stressed skin areas develop thicker epidermis and hyperkeratosis. This response is regulated *via* induction of calcium influx, phosphorylation of EGFR, and ERK1/2. The mechanical stabilization and stimulation of human skin equivalents are issues that may be indispensable for drug development. But for the simulation of skin diseases and disorders such as ulcers or blister formation, the integration of a mechanical element is necessary. Today's existing skin models delaminate at the transition of the SC to the SG (stratum granulosum)– and the basal membrane interfaces at relatively low shear stresses when separated from the surrounding cell culture insert (personal experience). This fact is comprehensible as the SC-epidermis as well as the epidermal–dermal junction differ in both biochemical and morphological aspects from *in vivo* skin. The epidermal–dermal junction *in vivo* is characterized by a papillary structure, leading to a manifoldly increased contact area between the epidermal and dermal layers. Such structures do not appear in organotypic skin equivalents generated *in vitro*. Moreover, only little is known about the

impact of mechanical impulses on the developmental processes in skin tissue and the effects of mechanical load on healthy and diseased skin.

The application of mechanical stimuli during the formation of 3D *in vitro* skin models is quite a new approach. Powell et al. [213] presented engineered skin constructs of keratinocytes and fibroblasts on electrospun collagen scaffolds to which uniaxial strain was applied. The strain application resulted in a significant increase of epidermal cell proliferation and enhanced epidermal differentiation. Improved strength of the 10% and 20% strain groups confirmed the assumption that mechanical treatment of developing epithelium enhances extracellular matrix production as well as epidermal differentiation.

It was shown that stretched keratinocytes show an increased expression level of cytokeratin 6 (K6) and a reduced level of cytokeratin 10 (K10), which is characteristic for the tissue present in wound edges [214].

Proinflammatory cytokines play a crucial role in the development of ulcers due to prolonged pressure application in distinct skin areas. The significant release of IL-1α, IL-1RA, TNF-α and CXCL8/IL-8 after pressure application to organotypic skin models has already been shown [144,149]. A minimum pressure of 75 mmHg is needed to induce cytokine release. Further studies, which focused on the release kinetics of cytokines, revealed that IL-8 and TNF-α were released into the surrounding medium while the IL-1α and IL-1RA remained in cell cytoplasm. Another marker for keratinocyte stress response is Tenascin-c. This glycoprotein, which is present in the extracellular matrix, is poorly expressed in healthy, adult human tissue. Expression in the skin is considerably increased by inflammation and epidermal hyperproliferation. Tenascin-c modulates proliferation, differentiation, migration and adhesion of cells in surrounding tissue and suppresses the immune response [215]. Epidermal keratinocytes produce Tenascin-c *in vivo* during wound healing. In human epidermal keratinocytes *in vitro* Tenascin-c expression is induced by TNF-α [216], and fibroblasts also express Tenascin-c during the process of tissue regeneration due to dynamic stimulation [217].

Hence, the integration of biomechanical stimulation could be a promising step to optimize the existing skin models.

4.7. Requirements for systemic approach

In order to deflect the systemic effects of a drug applied topically to the skin, the complexity of the system has to be increased by enabling the connection and communication to other organotypic systems. Especially in terms of drug testing, barrier tissues such as the epithelium of the gastrointestinal (GI) tract, the lung epithelium, the skin, and the blood–brain barrier can significantly reduce the bioavailability of drugs that are absorbed from the outside [218]. A research group of the Technical University of Berlin established a dynamic 2-tissue microcirculation chip, which integrated a human skin explant into a chip system next to a human artificial liver model. The tissues integrated accounted for 1/100 000 of the biomass of their original human organ counterparts [219]. Inside the system the tissues were either exposed to a fluidic flow or shielded from it by a transwell system. This system was observable over a period of 28 days, making it usable for repeated compound treatments. Applying the same culture mode, liver microtissues showed sensitivity at different molecular levels to the toxic substance troglitazone during a 6-day exposure and thereby proved the relevance of the system for drug development [219,220]. Human full thickness skin models have also been integrated into the multi-organ-chip. After a 9-day-culture under dynamic conditions, rearrangement and compression of the dermal matrix structure were observed. Using the multi-organ-chip (MOC) bioreactor platform the researchers found an improved spatiotemporal control of cellular microenvironments compared to traditional *in vitro* assays [221]. Moreover, biological vasculature was also integrated into this multi-organ-chip approach, coming even closer to an *in vivo* microenvironment by contributing to the need of dynamic cultivation, as defined in the previous section. Mechanical profiles of shear stress enable human organismal homeostasis at microscale [220].

Other approaches to combining multiple organs in a miniaturized manner are ongoing with already significant results [222,223]. These approaches are reviewed in informing articles, demonstrating the demand and relevance of human multi-organ systems especially for drug development [218,224–226].

One of the most popular devices is the human lung-on-a-chip developed by Donald Ingber and team at the Wyss Institute for Biologically Inspired Engineering (Harvard University, Boston), for which the mechanical aspect during breathing was also taken into account [227]. We may have to wait several years until the new instruments mimicking the whole body physiology are able to accelerate assessment of drug safety and efficacy or even replace animal-based methods and shorten clinical trials. However, this research has proven to be so significant that the Defense Advanced Research Project Agency (DARPA) awarded the Harvard researchers $37 million to push their innovation by building 10 different human organs-on-chip. All in all, the National Institutes of Health (NIH) and the DARPA are together investing up to $132 million in total for creating systemic “organ-on-a-chip” systems with the aim of increasing the productivity, safety and effectiveness of drug candidates. With reference to *in vitro* skin models, among the funded projects is one which focuses on the establishment of organotypic skin models based on induced pluripotent stem (iPS) cells in order to produce an unlimited supply of disease-specific cells for use in skin constructs (Angela Christiano at Columbia University Health Sciences, New York City). It might be misguided to expect a complete renovation of the biotech industry, but by using these new devices we will learn a good deal about pharmacokinetics, pharmacodynamics, as well as mechanical and biological constraints.

5. Conclusion

At the beginning of this review we raised the question concerning the applicability of today's *in vitro* engineered human skin models for drug development. As described, models already exist that serve as predictive tools to investigate new therapeutically active agents and some already proved their feasibility in the prediction of drug action. But all the results from different studies and trials which were performed so far are mainly from isolated approaches. These need to be consolidated, particularly in respect of *in vitro* skin disease model agreements concerning markers of disease status, levels of minimal compound concentrations to be applied to induce an effect of a known active substance as well as control and standard substances. The method of drug application (leave on/rinse off, static/dynamic) should also be considered. Once certain agreements are in place and standards are defined, comparison of drug efficacies will clearly be simplified, allowing up-scaling of screening performance.

In this review we highlighted different requirements, which are important for the routine application of 3D human skin models for drug development. But not all of these requirements are equally important. Some of them are already partly fulfilled or are of lower relevance for drug development. In Fig. 7 the previously defined requirements are arranged in respect to our valuation concerning current realization and overall importance for development of drugs.

In conclusion, the 3D constructs, mimicking critical features of the healthy or diseased human skin, represent valuable tools with great potential. Future efforts for successful and broad implementation of these models have to be focused on

1. Establishment of more complex and systemic approaches to enhance the predictability as already shown in Fig. 4.
2. Definition of appropriate and relevant standards and high reproducibility among repeated experiments; correlation to *in vivo* data and systematic investigation of impact on drug application and formulation.

3. Improvement and adaptation of production and cultivation processes to accomplish native-like tissue structures.
4. Adaptation and implementation of analytical methods, which allow the processing and data alignment of multiple models using preferably low- or non-disruptive approaches.

The balance between biological complexity reflecting the *in vivo* situation and suitable procedures to handle and analyze the 3D system is a critical element. The increase in complexity and possibly reliability escalates accordingly to the operating effort and analytical readout. High complexity is sometimes less important than flexibility, ease of use and business economics. Therefore, we believe that the merit of new approaches should be taken on a case-by-case basis concerning the requirements for implementation.

But we are convinced that 3D skin models offer benefit to both basic biological research and drug development. If we are able to tackle the issues mentioned above in a focused and synchronized manner, the routine application of 3D skin models is realistic. The 3D models will become an indispensable tool for the development of drugs acting on human skin and to evaluate active compounds contributing to cure skin diseases.

Acknowledgments

We gratefully thank Dr. Epifania Bono for her help in allocating publications on 3D disease skin models and Sanaz Afrouznia-Taleb and Joëlle Sprunger for histological data of skin models.

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