Minireview

Next generation human skin constructs as advanced tools for drug development

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Impact statement

Skin is a complex tissue that hosts various specialized cell types and performs many roles including barrier, immune, and sensory functions. For human-relevant drug testing, there has been a growing interest in building more physiological skin constructs by incorporating different skin components, such as vasculature, appendages, pigment, innervation, and adipose tissue. This paper provides an overview of the strategies to build complex human skin constructs that can faithfully recapitulate human skin and thus can be used in drug development targeting skin diseases. In particular, we discuss recent developments and remaining challenges in incorporating various skin components, availability of iPSC-derived skin cell types and *in vitro* skin disease models. In addition, we provide insights on the future integration of these complex skin models with other organs on microfluidic platforms as well as potential readout technologies for high-throughput drug screening.

Abstract

Many diseases, as well as side effects of drugs, manifest themselves through skin symptoms. Skin is a complex tissue that hosts various specialized cell types and performs many roles including physical barrier, immune and sensory functions. Therefore, modeling skin *in vitro* presents technical challenges for tissue engineering. Since the first attempts at engineering human epidermis in 1970s, there has been a growing interest in generating full-thickness skin constructs mimicking physiological functions by incorporating various skin components, such as vasculature and melanocytes for pigmentation. Development of biomimetic *in vitro* human skin models with these physiological functions provides a new tool for drug discovery, disease modeling, regenerative medicine and basic research for skin biology. This goal, however, has long been delayed by the limited availability of different cell types, the challenges in establishing co-culture conditions, and the ability to recapitulate the 3D anatomy of the skin. Recent breakthroughs in induced pluripotent stem cell (iPSC) technology and microfabrication techniques such as 3D-printing have allowed for building more reliable and complex *in vitro* skin models for pharmaceutical screening. In this review, we focus on the current developments and prevailing challenges in generating skin constructs with vasculature, skin appendages such as hair follicles, pigmentation, immune response, innervation, and hypodermis. Furthermore, we discuss the promising advances that iPSC technology offers in order to generate *in vitro* models of genetic skin diseases, such as epidermolysis bullosa and psoriasis. We also discuss how future integration of the next generation human skin constructs onto microfluidic platforms along with other tissues could revolutionize the early stages of drug development by creating reliable evaluation of patient-specific effects of pharmaceutical agents.

Keywords: Skin constructs, drug testing, microphysiological systems, skin-on-a-chip

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Introduction

The skin is a complex organ that serves many essential functions for human survival. It protects against environmental pathogens, avoids dehydration, and enables thermoregulation and sensation. Various skin-resident cell types carry out these functions, rendering the skin a challenging organ to model *in vitro*. The outermost layer of the skin, the epidermis, is principally made of keratinocytes and hosts many stem cell niches (epidermal, hair follicles (HF) stem cells).[1] Skin appendages (HF), sebaceous glands (SG), and sweat glands), mainly perform sensory and body thermoregulation functions. The bidirectional epithelial–mesenchymal crosstalk and the extra-follicular factors in the microenvironment are the main drivers for HF morphogenesis and the hair cycle.[2,3] This necessitates the inclusion of various cell types in engineered skin constructs. Melanocytes are residents of the epidermis and responsible for skin and hair pigmentation, playing a crucial role in UV protection.[4,5] Fibroblasts are found in abundance in the dermis and are implicated in many processes of skin homeostasis, the most important being to produce the extracellular matrix (ECM).[6,7] Resident immune cells

(macrophages, Langerhans cells, T cells, dendritic cells) also participate in skin homeostasis and regeneration and can trigger skin and/or hair disorders (lupus, alopecia, vitiligo).[8–11] Finally, the skin is highly innervated and vascularized, providing soluble factors to interact with other organs.[12] Each of these components plays a role in skin homeostasis and is involved in skin pathologies.

Although animal models (e.g. mice, pigs) are widely used to perform drug screening for skin disease, they are not representative of the physiology or structure of the human skin, resulting in high drug attrition rates at later stages of drug development.[13] Clearly, there is a need for human skin disease models that allow for reliable and high-throughput drug screening. Most of the human-relevant drug testing studies have involved the use of adherent cultures of skin cells (mainly keratinocytes), or co-culture of keratinocytes with other cell types including immune cells and dermal fibroblasts. However, these models often do not represent a sufficient level of complexity to recapitulate various cell–cell and cell–ECM interactions in the 3D microenvironment. Most of these limitations can be circumvented by using human skin constructs that are typically composed of dermal fibroblasts encapsulated in a gel and differentiated layers of keratinocytes forming the epidermis. This is a very useful *in vitro* model to study the phenotypic changes in keratinocytes or release of cytokines by these cell types in response to various drugs. On the other hand, most of the complex skin diseases, such as alopecia areata, epidermolysis bullosa, psoriasis and melanoma, involve additional cell types (e.g. immune cells), skin components (e.g. HF), and/or other organs.

The aim of this paper is to provide an overview of the strategies to build complex human skin constructs that can faithfully recapitulate human skin, and thus can be used in drug development targeting skin diseases. In particular, we discuss recent developments and remaining challenges in incorporating various skin components, availability of iPSC-derived skin cell types, and *in vitro* skin disease models. In addition, we provide insights on the future integration of these complex skin models with other organs onto microfluidic platforms as well as potential readout technologies for high-throughput drug screening.

Towards complex human skin models

Incorporation of various skin components

There is a growing interest in building more physiological skin constructs by incorporating different skin components, such as vasculature, appendages, pigment, innervation, and adipose tissue (Figure 1). These efforts are being made both for improving skin replacement therapy and generating more reliable *in vitro* models of skin. To date, studies focused on regenerating these components *in vivo* have generally been more successful than those developing the same components *in vitro*, mainly due to technical challenges in recapitulating the native 3D microenvironment. These technical challenges include optimization of co-culture conditions, control over spatial organization of cells, and availability of specific cell types. Here, we will mainly focus on the recent progress and prevailing challenges to develop *in vitro* models of complex human skin, as summarized in Table 1.

Vascularization. Vascularization of bioengineered skin substitutes has been the focus of interest since it is necessary for proper and long-lasting structure and function of skin constructs for clinical applications. It is also required for 3D *in vitro* skin models particularly for the evaluation of systemic drug delivery. Although the transport rate of drugs administered topically is primarily controlled by the epidermal barrier, systemic delivery or release of drugs to/from skin highly depends on the endothelial barrier function. Vascularization of skin constructs has been repeatedly studied by co-culturing endothelial cells (ECs) within the dermal compartment and stimulating capillary formation extrinsically by addition of growth factors, such as VEGF and FGF.[21–24] Using a similar strategy, Marino et al.[24] recently demonstrated the induction of lymphatic vessels in skin constructs. Despite the success of these studies for promoting the viability and functionality of engineered skin grafts, they do not allow for studying systemic delivery of drugs simply due to the lack of ability to perfuse these spontaneously formed capillary structures *in vitro*. This capability has been enabled by two recent breakthrough studies employing two different innovative tissue engineering strategies. Groeber et al.[25] combined a recellularized vascular scaffold and a tailored bioreactor system to generate a perfused vascular network in skin constructs. The scaffold is a decellularized segment of a porcine jejunum that mainly consists of collagen type III and type I. After reseeding this scaffold with human ECs, they were able to generate a closed perfusable vascular network. They showed that this vascular network can further be connected to a bioreactor perfusion system to mimic subcutaneous circulation. Our group has taken a different approach to this problem by using a sacrificial layer of alginate channels embedded in the dermal compartment of skin constructs.[14] We employed 3D-printing technique to accurately control the vascular pattern, allowing for creating a physiologically relevant structure and uniform distribution of blood vessels. Using both primary and iPSC-derived ECs, we were able to achieve an endothelial barrier that exhibited permeability properties comparable to that of the subcutaneous vasculature. In addition to enabling the study of systemic delivery of drugs to/from skin, these recent advancements will also pave the way to connect skin with *in vitro* models of other tissues of interest for building more comprehensive drug screening platforms.

Pigmentation. Human skin pigmentation consists of epidermal melanin units and relies upon (i) the production of melanin pigment by melanocytes and (ii) the transfer of melanin from melanocytes to keratinocytes through dendrites. These units provide photoprotection against harmful solar radiation and determine the unique color of the skin and hair. Clinical trials of engineered full-thickness skin substitutes conducted in the U.S.[26] and Japan[27] both reported a significant mismatch in the pigmentation of the grafted skin and pointed to hypopigmentation as a major

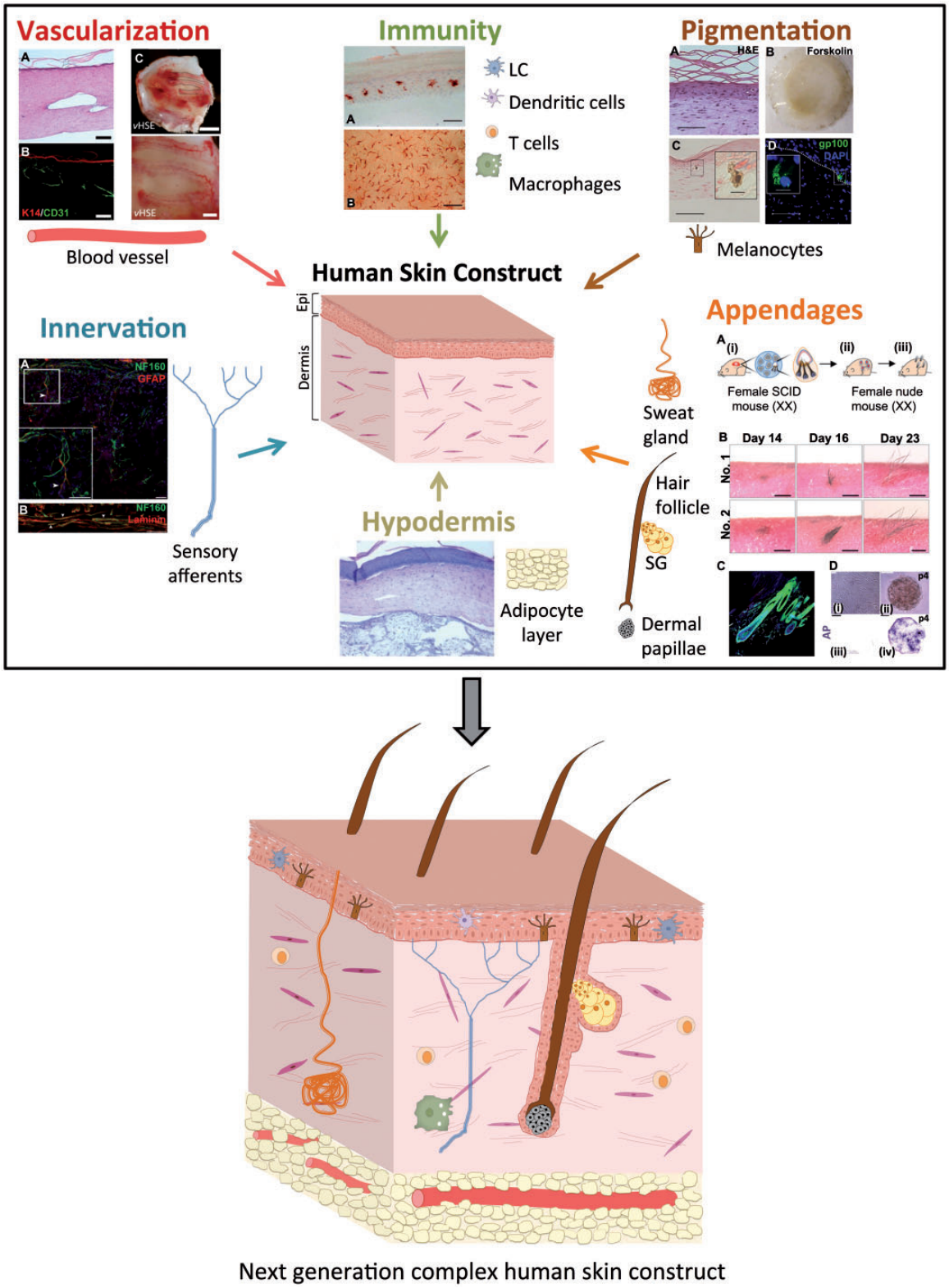

Figure 1 Ongoing studies towards building a complex human skin construct model. Top: Schematic of current skin constructs. Epidermis (Epi) is a stratified epithelium containing differentiated keratinocytes, which lies over the dermis made from fibroblasts mixed in a collagen matrix. *Vascularization*: (A) H&E and (B) immunofluorescent staining of histological sections of vascularized skin constructs generated using iPSC-derived ECs. The sections were immunolabeled with K14 (red), and CD31 (green) to evaluate epidermal integrity and endothelial coating in the microchannels. (C) The effect of vasculature pattern on the host neovascularization. Picture of newly formed host vasculature following the micropatterned human iEC-containing microchannels in vascularized skin constructs. Scale bars: 250 μm (with permission from Abaci et al.[14]). *Immunity*: Immunohistochemical analysis of reconstructed epidermis containing Langerhans cells. Histochemical analysis of reconstructed epidermis containing Langerhans cells was performed using anti-Langerin staining (peroxidase/AEC reaction) with hematoxilin counterstaining was performed on cryosection to localize Langerhans cells inner the epidermal layers (the brown cells are the Langerin-positive cells) and Langerin immunostaining (B) on epidermal sheets visualized the dense network of the dendritic Langerhans cells. Scale bar: 50 μm (with permission from Facy et al.[15]). *Pigmentation*: Skin constructs produced from iPSC-derived fibroblasts, keratinocytes, and melanocytes have normal anatomy and are functional. (A) Hematoxylin and eosin staining,

(Continued)

Table 1 The potential benefits of incorporating individual skin components into 3D skin constructs.

			Beneficial outcomes	
Skin component	**Skin diseases or conditions**	**Experimentation**	**Clinical therapies**	**Drug development**
Vasculature	■ Wound healing ■ Multiorgan diseases (e.g. melanoma) ■ Vascular malformations ■ Angiosarcoma	Perfused skin constructs	Skin grafts with improved viability	Systemic drug delivery to/from skin
Pigmentation	■ Hyperpigmentation ■ Vitiligo ■ Melanoma	Photoprotection, melanin transfer	Skin grafts with natural aspect and photoprotection	Photoprotectant, discoloration correctors
Hair follicles	Alopecia (AA, AGA, chemotherapy induced)	Hair follicle differentiation	Skin grafts with improved aesthetic	Alopecia treatments
Sebaceous glands	Acne, seborrhea	Sebaceous gland differentiation	Moisturized skin grafts	Sebo-regulating drugs
Sweat glands	Body thermoregulation	Sweat gland differentiation	Thermoregulation	Cosmetic testing (perspirants)
Immunity (macrophages, dendritic cells, T cells)	■ Sensitization ■ Atopic dermatitis, psoriasis ■ Autoimmune diseases (e.g. vitiligo, AA)	Immuno-competent skin	Skin grafts with resident immune cells	Drug side effects, allergen testing
Innervation	■ Loss of skin sensitivity (burns, age)	Sensory inputs, itch, pain	Skin grafts with improved sensation	Drug side effects, anti-itch/pain drugs
Hypodermis (adipose tissue)	■ Wound healing ■ Hair regeneration	Tri-layer skin constructs (hypodermis-dermis-epidermis)	Tri-layer skin constructs (for deep wounds)	Drug side effects

AA: alopecia areata; AGA: androgenetic alopecia.

shortcoming. The first pigmented skin construct has been demonstrated in 1985 by addition of foreskin melanocytes onto skin constructs.[28] Since then, there have been various modifications and improvements on regulating the long-term viability of the constructs and melanin transfer to keratinocytes.[29] More recently, our group has achieved pigmented skin substitutes using iPSC-derived melanocytes.[16] In this model, we showed the capability of iPSC-derived melanocytes to transfer their melanin into keratinocytes. This holds therapeutic potential to address the issues of hypopigmentation by providing an autologous source of melanocytes. In addition, pigmented skin models are of great interest for high-throughput drug screening studies, since drug-induced skin pigmentation accounts for 10–20% of all cases of acquired hyperpigmentation.[30] Therefore, the level of pigmentation constitutes an easy and comprehensive readout of drug side effects. A high number of common drugs can cause human skin hyperpigmentation such as certain antibiotics, diuretics, anti-inflammatory drugs, and pain relievers.[31] Skin discoloration can also be stimulated in complex diseases, such as vitiligo, or by hormonal changes, such as during pregnancy, and post-inflammatory conditions. Therefore, generating a standardized pigmented skin model with an enhanced control over the uniformness and the level of pigmentation will be pivotal for developing reliable high-throughput drug screening platforms.

Figure 1 Continued
(B) Forskolin-treated iPSC-derived skin constructs after 14 days at the air–liquid interface. (C) Fontana-Masson and (D) Immunofluorescence of gp-100 (green) and nuclei (blue) staining in skin construct. Scale bars: 100 μm. (with permission from Gledhill et al.[16]). *Innervation*: Detection of laminin and myelin sheaths in the reconstructed connective tissues enriched with Schwann cells. (A) Double immunofluorescent staining of neurons (160 kDa neurofilament, green) and Schwann cells (GFAP, red) on day 14. Schwann cells are colocalized with neurites (arrowheads). (B) Double immunofluorescent staining of neurons (160 kDa neurofilament, green) and laminin (red). The 160-kDa neurofilament staining colocalized with the laminin staining (arrowheads). Scale bars: 50 μm. (with permission from Blais et al.[17]) *Appendages*: Transplantation of the bioengineered IOS. (A) Schematic representation of the methods used for the generation and transplantation of iPS cell-derived hair follicles. Cystic tissue with hair follicles was isolated and divided into small pieces containing 10 to 20 hair follicles. The small pieces were transplanted into the back skin of nude mice using a follicular unit transplantation (FUT) method developed in humans; (i) *In vivo*-organized integumentary organ system derived from male iPS cells (XY) (ii) Intra-cutaneous transplantation (iii) Orthotopic hair function. (B) Macromorphological observations of two independent engraftments into the dorsoventral skin of nude mice showing the eruption and growth of iPS cell-derived hair follicles. Scale bars: 1 mm. (C) Immunohistochemical analyses of stem/progenitor cells in the follicles of natural pelage and enhanced green fluorescent protein (EGFP)-labeled iPS cell-derived bioengineered hair follicles (with permission from Takagi et al.[18]). (D) (i) Conventional culture systems enable the growth of cells in a 2D format. (ii) Growth of dermal papilla cells in hanging drop cultures results in the formation of dermal spheres. (iii) Alkaline phosphatase (AP) activity is not detected in passage four cultured dermal papilla cells. (iv) In dermal spheres, AP activity is strongly observed (with permission from Higgins et al.[19]). *Hypodermis*: Full-thickness skin constructs using silk and collagen biomaterials. H&E of constructs cultured in 1:1 skin:adipose media, which had more physiologically relevant features (with permission from Bellas et al.[20]). Bottom: Schematic of next-generation complex human skin constructs hosting all the different skin cell types as normal skin *in vivo*

Appendages. Many of the biological functions of the skin epithelium are mediated by its appendages. HF, SG, and sweat glands (SwG) are essential to maintain a healthy protective barrier by lubricating the skin and allowing for thermal regulation. The lack of skin appendages in skin grafts constitutes a major limiting factor to the improvement of regenerative wound healing therapies and the engraftment of large injured areas. HF would also allow for improved aesthetic properties of skin grafts for patients with severe wounding. Pilo-sebaceous units (HF and SG) are often considered as mini organs that are home to regionalized stem cell niches.[32] The presence of pilo-sebaceous units together with SwG in dermal-epidermal constructs offer many advantages for drug screening since they are well suited to study stem cell dynamics, morphogenesis, lineage commitment, tissue regeneration, and wound healing. In particular, some studies have been investigating the participation of HFs in transdermal drug penetration.[33] Furthermore, there is a growing interest for alternative methods to hair transplantation focusing on enhancing hair growth or preventing hair miniaturization leading to baldness (androgenetic alopecia), making the skin appendages of relevance for such studies. The lack of *in vitro* models for severe alopecia as well as chemotherapy-induced hair loss also reflects a major unmet clinical need. Similarly, SG in skin constructs are critical to perform assays aiming at regulating sebaceous secretions (acne, seborrhea, skin dryness). Current models are principally animal models where epidermal or HF stem cells and dermal cells or dermal papilla cells are mixed together after mono-layered or 3D cultures then reintroduced into the skin via different trichogenic assays (chamber, patch, flap) either ectopically (subrenal capsule) or orthotopically (nude mice dorsal skin).[18,34] Many studies have shown that mechanical forces of the 3D microenvironment influence cell properties.[35] We have previously demonstrated that recapitulating the 3D spheroid culture of dermal papilla cells by the hanging drop method is crucial to partially restore their gene signature and most importantly their hair inductive capacities.[19,36] Once placed between separated foreskin epidermis and dermis and grafted onto SCID mice, these human DP spheroids were able to induce *de novo* HF. These results are fundamental to design potential hair bearing human skin constructs. The possibilities for drug screening on hair inductivity with human cells in 3D are mainly restricted to assays using DP spheroids,[37] single hair or *ex vivo* skin cultures,[39,39] which disregard the HF microenvironment and thus introduce bias into drug testing.

Very little is known about the development of SwG, though a recent study using lineage tracing in mice revealed that they are derived from multipotent epidermal basal progenitors.[40] After injury, the skin cannot regenerate these structures, but tissue engineering is now bridging the gap to create these structures in complete skin constructs. Indeed, human epidermal keratinocytes have been shown to invade collagen gels and form eccrine duct-like structures in the presence of fibroblasts, and growth factors.[41,42] A recent study further demonstrated that fibroblasts in the microenvironment can increase the number of tubular-like structures by secreting Shh.[43] Bone marrow mesenchymal stem cells have also been shown to differentiate into sweat gland-like cells *in vitro* and to be functional once transplanted into deep burn injury patients.[44]

Despite these major breakthroughs in skin tissue engineering, many challenges remain before a fully functional human skin model is constructed to include skin appendages. Firstly, the reconstitution of the skin appendages' anatomy and their regionalized stem cell niches will be one of the limitations to overcome. Skin models need to mimic the intra-follicular microenvironmental as well as the extra-follicular macroenvironmental factors. To achieve this, such construct must have the capacity to organize different cell types in such way that the native epidermal–mesenchymal or dermal interactions will be recapitulated (e.g. keratinocytes/DP, SG/fibroblasts, sweat glands/fibroblasts). Some models have successfully generated HF *in vivo*[19] or have only been able to produce vellus hair-like structure *in vitro*,[45] but none have been able to reconstruct a fully differentiated HF in a skin construct *in vitro*, nor to produce SG. The biggest technical challenge is to recapitulate the 3D organization of the pilo-sebaceous unit niches. Incorporating other cell types in these constructs (innervation, immune cells, and ECs) will also increase the likelihood of a drug response more comparable to human skin *in vivo*. In terms of the origin of the cells, being able to use adult epithelial cells becomes an important contributor to making relevant artificial skin for pharmaceutical compound testing. This also requires defining cell culture conditions that are compatible with all cell types, while inducing the HF differentiation process.

Immunity. The skin represents the interface between the body and the environment and provides the first layer of defense against both physical and chemical insults. Therefore, it is no surprise that skin immunity is well developed and enriched in specialized immune cell types localized both in the epidermis and dermis. The epidermal compartment of skin is colonized by specialized dendritic cells called Langerhans cells (LCs) and dendritic epidermal T cells, while the dermis is populated mostly by myeloid and lymphoid immune cells that dynamically traffic in between the skin and vascular/lymphatic circulation upon their activation during an immune response.[46] Many medical conditions and side effects of drugs manifest themselves through skin symptoms. Most of the skin toxicity and immunological studies are carried out using animal models, although it is clear that these animal models often poorly represent the human immune system. Such concerns led to the development of *in vitro* models of immune response in a range of different complexities and settings.[46] On the high complexity end of these models are the skin constructs containing various immune cells, such as dendritic cells, LCs or T cells. Following the first study that successfully demonstrated the incorporation of LCs into skin constructs,[15] LC-containing skin constructs were used to recapitulate responses of LCs to UV radiation and several known allergens.[47] Recently, it was shown that the LCs derived from

MUTZ-3 progenitor cell line migrated through the basal membrane to the dermis in response to various irritants and drugs.[48] The migratory response demonstrated in this study is an important indicator of the immunocompetency of skin constructs, since it could be a simple and valuable read-out for hazard identification and testing allergic and inflammatory responses against therapeutic candidates. In addition to LCs, macrophages,[49] dendritic cells,[50] and T cells[51] were also added into skin constructs to study inflammation and T-cell activation. Ramadan and Ting[52] developed a microfluidic platform that allows for co-culture of keratinocytes and dendritic cells and for online assessment of trans-epithelial resistance (TEER) as a measure of the epidermal barrier function. Although these isolated steps of the skin immune responses such as LC migration or release of inflammatory cytokines are important indicators of the onset of a potential immune response, there is still a need for a complex model of skin immunity that can take into account the collective cascade of events during immune response and provides end-point readouts for high-throughput drug screening.

Innervation. The skin is a highly sensitive organ, densely innervated with different types of sensory neurons, which allows for discrimination between pain, temperature, pressure and touch. Restoring sensory perception could substantially improve quality of life and enhance would healing.[17] Moreover, skin innervation is implicated in epidermal homeostasis and wound healing by stimulating signaling pathways and secretion of neuromediators[12,53] making the incorporation of sensory neurons in complex skin models essential. Furthermore, as the most common forms of allergic drug reactions are skin side effects (skin rashes and itching), innervated skin constructs are necessary to advance drug screening *in vitro* models. It has previously been demonstrated that innervation of collagen hydrogel-based skin constructs was slow, starting only after eight weeks, and limited to the dermis.[54] Using collagen sponges with interconnected pores instead of hydrogels improved the innervation process.[55] Similarly, there is a growing body of evidence that neurons can be polarized and neurite ingrowth can be guided by microchannels, as shown using both 2D microfluidic platforms and 3D porous hydrogels.[56] There is also an emerging consensus that filling the nerve guidance microchannels by functional biomaterials, such as laminin, can be an important strategy permitting innervation of deeper tissues.[57] Although recently available microfabrication methods have enabled embedding microchannels in 3D hydrogels, they have not yet been utilized for innervation of engineered grafts. Overall, the innervation of engineered skin constructs is yet to be accomplished mainly due to unavailability of human sensory neurons and lack of robust protocols to promote innervation *in vitro*.

The hypodermis. The hypodermis is beneath and attached to the dermis via collagen and elastin fibers. The hypodermis mainly consists of subcutaneous adipocytes and functions as an energy reserve and a heat insulator. Adipose tissue is important for shock absorbing, protecting bones and joints against pressure and friction, minimalizing shocks from impacts to the skin. The fat cells of the hypodermis also produce many hormones. The hypodermis has blood and lymphatic vessels traversing in the region to circulate the blood and lymph to the dermis. Moreover, cutaneous nerves, SWG, SG, and anagen hair bulbs are present in the hypodermis.

Despite the important functions of the hypodermis, it is relatively one of the most understudied components of the skin.[58] Inclusion of the hypodermis in skin constructs could represent an improved human skin surrogate for cosmetic and pharmaceutical development, as well as more functional skin grafts for wound therapy.[59] Indeed, hypodermis-containing skin constructs have been constructed in several studies.[20,58–60] In addition, adipose-derived stem/stromal cells (ASCs) have gained attention due to their implication in repair of different organs.[61] Since adipocyte tissue engineering can be used to model obesity and to develop drugs to treat the disease,[62] the hypodermis may also be useful to elucidate the role of adipocytes/adipose tissue in maintenance of epidermal and dermal cell homeostasis and hair cycle.

iPSC-derived cell sources for skin tissue engineering

The skin has a complex structure with appendages and many cell types. To faithfully mimic human skin for physiological modeling and drug development, this complexity will need to be recapitulated. However, it is impossible to completely capture the degree of complexity with primary cells, due to their limited availability and/or growth potential.[63] The development of iPSC technology can circumvent the limitation of cell availability, since it enables to obtain cells from noninvasive sources, such as blood,[64] and uses a limited number of somatic cells to generate a large amounts of cells with unlimited growth potential.[65] These highly proliferative iPSCs can then be differentiated into many cell lineages to reconstitute different organs including skin. Our successful construction of 3D human skin entirely from iPSC-derived fibroblasts, keratinocytes and/or melanocytes[16,66] proves the reliability of iPSC-based technology for human skin models. Moreover, we differentiated iPSCs into ECs, which were incorporated into skin constructs and demonstrated their functionality.[14] Differentiation of other cells types, such as sensory neurons and dermal papilla cells have also been reported.[67] With the increasing number of iPSC-derived cell types, the opportunity to model the degree of skin complexity has become greater.

iPSCs technology still have noteworthy limitations that may constrain the scope of iPSC applications.[63] These limitations include low reprograming efficiency,[68] retention of an epigenetic memory of their parental cells,[69] variability among iPSC lines[70] and genomic instability.[71] Attempts have been made to seek alternative technologies that avoid the limitations associated with iPSCs and ethical issues intrinsic to embryonic stem cells (ESCs). To this end, somatic cell nuclear transfer (SCNT) has been developed to convert somatic cells into stem cells by transferring human nucleus into a nucleus-free human egg.[72] This

technology is technically challenging, time-consuming and costly, which is why there is growing interest to directly convert cells from one type to another. For example, fibroblasts have been converted into sensory neurons[73,74] and ECs.[75] However, sufficient cell number may not be always available for conversion.

Many of these limitations related to iPSCs may be circumvented. Reprogramming efficiency may be increased by targeting signaling pathways that modulate cellular reprogramming.[76] Parental epigenetic memory may be erased by extensive passaging, and variability among iPSC lines may be avoided by using a population of many iPSCs colonies instead of picking single colonies. Genomic instability and tumorigenicity raise safety issues for clinical applications. However, many reagents can be used to obtain adequate reprograming efficiency without the need for overexpression of myc and abrogation of p53 pathway, which have been commonly used.[77] Defined xeno-free, integration-free and feeder-free systems have also been developed for iPSC reprogramming and differentiation, to address regulatory issues related to clinical applications.[78] iPSCs can also be generated using small molecules,[79] although currently only successfully from mouse cells. Small molecules have great potential, because they can be easily manipulated and can avoid the variability associated with DNA-, RNA- or protein-based reprogramming methodologies.[79] Progress in this field will enable generation of safe iPSCs at low costs, for cell therapy, disease modeling and drug development.

Skin disease models: Generation of *in vitro* models of genetic skin diseases, such as epidermolysis bullosa and psoriasis using iPSC technology

Epidermolysis bullosa. iPSC technology opens a new avenue for possible medical applications, including cellular therapies for genetic skin diseases, and full-thickness skin modeling to study disease pathomechanism and drug screening. Here, we discuss the promising advances that iPSC technology offers in order to generate *in vitro* models of skin diseases, including Mendelian disorders such as epidermolysis bullosa (EB) and complex disorders like psoriasis. EB is an inherited skin fragility disorder which is characterized by mechanical-induced blistering and scarring within the cutaneous and mucosal membranes due to mutations in genes encoding for proteins involved in dermal-epidermal adhesion.[80] One of the most severe forms is the dystrophic EB (DEB). DEB is caused by mutations of *COL7A1* encoding type VII collagen (COL7) which is an ECM protein secreted as homotrimers at the dermal–epidermal junction (DEJ) by keratinocytes and dermal fibroblasts. In healthy skin, both cell types secrete procollagen VII, which undergoes proteolytic processing into COL7, and self-assemble into anchoring fibrils (AF). AF provides skin stability by attaching the epidermis to the underlying dermis, which is impaired as a consequence of loss of function mutations in *COL7A1* in DEB patients' skin.[81,82] Therefore, sufficient and long-lasting restoration of COL7 resulting in AF formation at the DEJ is a major goal of effective therapy for this orphan skin disease. In the last decade, emerging progress has been made in proof of concept studies aiming at the development of new generation therapies for recessive DEB (RDEB) using gene, protein, and cell-based therapies.[83]

A novel therapeutic strategy to treat DEB envisages combining iPSCs derived from patient's somatic cells, with gene-editing, and tissue engineering technologies. iPSCs established by integration-free reprogramming[84] can readily be differentiated into different skin cell types including keratinocytes and fibroblasts, and be used to produce autologous skin constructs. This method will avoid immune rejection due to allogenicity, thereby extending the lifespan of the skin constructs and allowing the local production of missing protein (e.g. COL7 for DEB). In addition, patient-derived iPSCs can easily be corrected using emerging gene-editing tools such as CRISPR/Cas9, because of their highest self-renewal and proliferation capacities to endure gene manipulation, and thus can be used to develop personalized therapies targeting specific mutations. Our group and others have already demonstrated that iPSCs derived from DEB patients' cells that have undergone CRISPR gene correction are able to differentiate into keratinocytes and fibroblasts.[85–89] In addition, these cells were used to build skin constructs, demonstrating the physiological functionality of the reconstructed skin *in vitro* using iPSCs.[66]

The proof of principle that genetic correction of epidermal stem cells was safe and effective to correct EB was first established for junctional epidermolysis bullosa JEB.[90] Moreover, currently two approaches are being developed to treat RDEB patients, and are undergoing clinical trials. One approach is based on transplantation of autologous epithelial sheets made of genetically corrected keratinocytes of RDEB patients (clinicaltrial.gov/NCT01263379). The other utilizes the transplantation of fibrin-based skin constructs composed of autologous genetically corrected epidermal keratinocytes and dermal fibroblasts using a self-inactivating retroviral vector. This treatment strategy is undergoing a phase I/II clinical trial for RDEB patients (http://www.Genegraft.eu). Expanding these clinical studies by including a vascular system into skin constructs would potentially have longer lasting effects with better efficacy of this skin replacement therapy for RDEB patients. Moreover, including a dermis containing gene-corrected cells would potentially prolong the life-span and efficacy of this skin replacement therapy for the patients.

In addition to skin replacement, there have also been other treatment strategies for EB patients such as cell therapy and chemical drugs, for which next generation human skin constructs can serve as a powerful testing platform. At the moment, cell-based therapies are at the cutting edge of translational research, and numerous studies have demonstrated positive and promising benefits for RDEB patients.[91–101] Since the requirements for efficacy and safety issues (especially for autologous cell-based products) are very high, it is still challenging to deliver data that are relevant for regulatory agencies. Indeed, using animal model or simplified reconstructed human skin onto immune deficient mice, is not sufficient to replace clinical trials.[94,101] However, using complex autologous skin

constructs built from RDEB patients' own cells (derived from iPSC) would allow to recapitulate the physiological context to test not only the efficacy of the treatments, but also potential immunoreactions.[85] In the recent years, it also became clear that the RDEB phenotype results from many complex cellular processes that are impaired due to COL7 deficiency. Previous *in vitro* and *in vivo* studies have shown migratory disruption in human cells and wound healing defects in human and mice.[102] Moreover, the work on the hypomorphic mouse model, which closely recapitulates the RDEB phenotype, indicates that transforming growth factor-β (TGF-β) is upregulated due to the absence of COL7. This has an impact on the transition of dermal fibroblasts to myofibroblasts, and leads to the overproduction of ECM resulting in scarring and fibrosis.[103,104] These results paved the way to the notion that TGF-β would be a good therapeutic target to treat RDEB patients. Recently, it has been shown that treatment of the hypomorphic mouse model with losartan, an angiotensin II type 1 receptor antagonist, reduces TGF-β-mediated fibrosis.[105] These results are very encouraging for potential clinical trials to treat RDEB. To this end, full-thickness skin models of EB developed by iPSCs from patient's own cells would provide a physiologically relevant platform for preclinical screening of these potential drugs and further allow the identification of new patient-specific drug formulations to treat EB patients with higher precision.[85,86]

Psoriasis. Many complex inflammatory diseases, such as psoriasis, can also benefit from iPSC technology. Psoriasis is an organ-specific autoimmune disease triggered by an activated immune system, which leads to a disturbed differentiation process of keratinocytes causing abnormal epidermal differentiation.[106,107] The development of new therapies for psoriasis requires better *in vitro* model systems for specific drug screening in order to design personalized treatments. Moreover, psoriasis does not exist as a spontaneous disease in the skin of lower animals[108] limiting the reliability of the animal models for this disease. An *in vitro* model of psoriasis has been developed by Barker et al.[109] by generating skin constructs using keratinocytes and fibroblasts of psoriatic patients. In their model, they did not observe a difference in the expression of the classical differentiation markers of keratinocytes compared to healthy skin constructs, but found a crucial difference in the pro-inflammatory gene expression and keratinocyte proliferation, suggesting an inflammatory phenotype resembling psoriatic conditions despite the absence of T cells. The integration of immune cells, such as dendritic cells and T cells in this psoriasis model, will constitute a valuable *in vitro* platform to dissect the underlying mechanisms in this complex disease and develop targeted therapies. Subsequently, building 3D personalized psoriasis models using iPSCs technology will allow for the performance of precision drug development to more effectively treat patients with psoriasis or other chronic skin diseases. Since diseases may display different clinical presentation, response and tolerance to treatments, designing new approaches of personalized medicine using iPSCs technology is a new perspective for future medicine.

Future directions

Integration on multicell type microfluidic platforms

The development of a platform that accommodates and connects skin with other tissues/organs of interest would be highly beneficial in terms of (i) recapitulating interactions of skin with other organs; (ii) detecting unknown side-effects of drugs; (iii) evaluating the effects of drugs metabolized by other organs (e.g. liver); (iv) estimating pharmacodynamics/pharmacokinetics of topically administered drugs; and (v) studying diseases that involve multiple organs (e.g. melanoma metastasis to brain).

Microfluidics technology provides the capability to precisely control the fluidic connections, and therefore the communication between different tissue chambers at small-scale, making these platforms also considerably cost-effective for drug screening applications. Our group recently transferred skin constructs onto a microfluidic culture platform that allows for the long-term maintenance of skin constructs at physiologically relevant nutrient supply rates.[110] This skin-on-a-chip platform has a unique capability to re-circulate the medium at desired flow rates without a need for a pump or external tubing. Using this platform, we demonstrated, as a proof-of-concept study, that the cancer drug, doxorubicin, may have direct toxic effects on keratinocyte proliferation and differentiation. In addition to our work, other groups focused on co-culture of skin tissue biopsies with other organs, such as liver, intestine and kidney on a microfluidic platform in a transwell format.[111,112] They have conducted several proof-of-concept studies demonstrating the long-term maintenance, function, and response of these biopsy cultures to drug toxicity. These multiple organ studies using tissue biopsies are very important for identifying the co-culture conditions and will offer significant insights for the maintenance of multiple engineered tissues on a single platform.

One of the most important steps in this line of research will be the validation of the physiological relevance of these platforms by recapitulating the already-known effects of a large set of pharmaceuticals targeting different diseases. Some challenges that may arise, limiting the predictive capability of these platforms, include determining the right strategy for relative scaling of the organs, choosing reliable interpretation techniques or models, and generating engineered tissues with reproducible levels of function. There have been different approaches to scaling the organs including allometric,[113] residence time-based,[114] and functional scaling.[115] While organ-scaling will be of great importance to achieve a physiologically relevant interaction between organs (cytokines, soluble factors); the prediction of drug efficacy and kinetics will rely also on the interpretation methods that account for levels of function of the organs.

Developing reliable read-out techniques

Complex skin constructs with well-differentiated epidermis will faithfully mimic human skin, but miniaturization

(scaling) may indeed be required for drug development. Miniaturization will allow us to reduce the costs, perform high-throughput screening and minimize the variability between different skin constructs. Downsizing engineered skin constructs with high levels of complexity may be technically challenging. Multicellular spheroids have been used for tumor modeling and drug development[116] and have been used to screen antimelanogenic agents.[117] However, it may be difficult to construct spheroids with well-developed epidermis, which is typical of mature human skin. It is likely that drug candidates screened using spheroids may not be all applicable to human skin, but it may be cost-effective to use them for initial drug screening, followed by validation in skin constructs containing well-differentiated epidermis, and ideally, in the presence of other organs.

Human skin is the barrier between the body and the environment, defending humans from environmental and pathogenic assaults. Skin integrity is therefore important for this function and will eventually be perturbed by many drugs. Dye diffusion across the epidermis may then be used to estimate the skin integrity. While this technique is useful in the context of topical drug delivery studies, it may not always be adapted for high-throughput screening, as some drugs may cause effects on skin structures at later stages.

Drugs often cause skin dyspigmentation and are responsible for 10–20% of acquired hyperpigmentation.[30] Therefore, as previously mentioned, melanocytes should be included in the screening to detect any pigmentation-related adverse effects by drugs. It is possible to use fluorescence-based[117,118] or luciferase-based assays[119] to target melanogenesis. However, the assessment of the end effects by quantifying the pigmentation[120] may be more inclusive than measurement of the expression of a particular gene in the melanogenic pathways.

Mitochondria exert critical functions by producing 90% of the cell energy and controlling cell survival via apoptosis mechanisms.[121] Therefore, drug-induced mitochondrial dysfunction can result in dire consequences. Many drugs are withdrawn from the market due to newly discovered subtle adverse effects that cannot be detected by histopathology. For some of these drugs, the development of mitochondria-based assays revealed the correlation between impaired mitochondrial function and adverse effects.[121] This underlines the necessity to include mitochondrial toxicity assays in drug development. Drugs should be assessed for three general mitochondria-related adverse effects: disruption in energy generation, elevated free radical production and abnormal apoptosis. Many reagents are commercially available to investigate the mechanisms of actions underlying the effects of drugs on mitochondria. Depending on the screening purpose, different reagents may be compared to select the most appropriate candidates.[122] If possible, systems with multiple dyes may be included for effective screening.[123] Inclusion of mitochondria assays in the process of drug development will enable the detection of potential drug side effects at early stages, thereby preventing unnecessary expenses.

Concluding remarks

Although it has been left out of the scope of this review, it is worth noting that in this line of research, the development of biomimetic biomaterials recapitulating the natural ECM[124] and the implementation of emerging biofabrication techniques such as bioprinting,[125] will unquestionably enhance the reproducibility and efficiency of engineered complex human skin models. Building the next generation skin constructs by including various skin components and patient-specific cells, while optimizing miniaturization methods, integration with other organs and of biomarkers/biosensors for high-throughput readouts will revolutionize the early stages of drug development by creating reliable evaluations of patient-specific effects of pharmaceutical agents.

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DECLARATION OF CONFLICTING INTERESTS

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