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Skin equivalents: skin from reconstructions as models to study skin development and diseases

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Summary

While skin is readily available for sampling and direct studies of its constituents, an important intermediate step is to design *in vitro* and/or *in vivo* models to address scientific or medical questions in dermatology and skin biology. Pioneered more than 30 years ago, human skin equivalents (HSEs) have been refined with better cell culture techniques and media, together with sophisticated cell biology tools including genetic engineering and cell reprogramming. HSEs mimic key elements of human skin biology and have been instrumental in demonstrating the importance of cell–cell interactions in skin homeostasis and the role of a complex cellular microenvironment to coordinate epidermal proliferation, differentiation and pigmentation. HSEs have a wide field of applications from cell biology to dermocosmetics, modelling diseases, drug development, skin ageing, pathophysiology and regenerative medicine. In this article we critically review the major current approaches used to reconstruct organotypic skin models and their application with a particular emphasis on skin biology and pathophysiology of skin disorders.

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What's already known about this topic?

- Human skin equivalents are being used for different purposes from cell–cell interactions to modelling diseases.

What does this study add?

- We have critically reviewed the various methods for the reconstruction of epidermis.
- We provide an update of the different applications of skin equivalents in skin biology and dermatology.

The skin is the largest organ in humans and serves as a major barrier that, among other functions, prevents the invasion of pathogens, limits chemical and physical aggressions and regulates the loss of water and electrolytes.[1] It is a complex organ composed of the epidermis and its appendages (hair follicles, sebaceous glands and sweat glands), which are separated from the dermis by a basement membrane (BM) consisting primarily of laminins and collagens.[2] The epidermis is a highly dynamic stratified epithelium made principally from ectodermal-derived keratinocytes, which constitute about 90% of epidermal cells. New differentiating keratinocytes continuously emerge from the proliferative basal layer of the epidermis to replenish the upper layers, progressively differentiating into the external cornified and desquamating dead envelope. In addition to keratinocytes, the epidermis contains a minority of functionally important cells of nonectodermal origin, including neural crest melanocytes, bone marrow-derived Langerhans cells (antigen-presenting cells) and Merkel cells.

The dermis is a thick connective tissue providing tension, strength and elasticity to the skin through an extracellular

matrix (collagen, elastin and extrafibrillar matrix). In addition to nerve terminations and vessels, the dermis is composed of three major cell types: fibroblasts, macrophages and adipocytes. It also contains epidermal appendages (hair follicles, sebaceous gland and sweat glands).[3]

Various cell types constituting the skin have been studied individually using two-dimensional (2D) monolayer cultures. However, these monolayer tissue cultures fail to capture the relevant complexity of the *in vivo* microenvironment and cell–cell interactions that considerably affect the responses of cells to different stimuli. In fact, growing evidence indicates that there are significant differences in phenotype, cell migration, proliferative capacity, cell surface receptor expression, extracellular matrix synthesis, cellular signalling, metabolic functions and responses to various stimuli when the same cells are grown under 2D or three-dimensional (3D) culture conditions.[4–11] To approach *in vivo* conditions, skin histocultures (also called skin explants), which are obtained by putting the intact skin samples containing all resident skin cells (such as immune and neuronal cells) into growth medium either with a collagen gel support or simply free-floating, have been widely used and improved over time (e.g. histocultures that allow longer experimentation time than traditional explants).

Skin histocultures have many uses, ranging from testing drug sensitivity to designing effective individualized therapies for each patient.[12–19] However, this model has several drawbacks including (i) a limitation in the number and size of skin histocultures, (ii) the complexity owing to the presence of all resident skin cells resulting in their inherent heterogeneity, which cannot be experimentally controlled and (iii) difficulties in the uniform genetic manipulation. These limitations restrict the use of these models and indicate the need to develop a new 3D *in vitro* system that allows a more detailed study of the basic molecular processes affecting skin physiology and pathophysiology. Therefore, the reconstitution of 3D human skin equivalents (HSEs) using both dermal and epidermal components is a relevant strategy to answer the physiological questions that cannot be solved solely in the context of monolayer tissue culture or skin explants/histocultures. Furthermore, HSEs, which have been developed and improved over the last 30 years, present time- and cost-effective alternatives to the use of laboratory animals, especially mice, which have a skin architecture that is ill-suited to human studies. Indeed, mouse epidermis is much thinner than human epidermis (only three layers in adult murine epidermis compared with generally six to 10 layers in human epidermis). Secondly, mouse epidermis is densely packed with hair follicles, whereas human epidermis possesses larger interfollicular regions. Thirdly, mouse melanocytes reside mainly in dermal hair follicles, while human melanocytes are located in the basal layer of the epidermis. Fourthly, a cutaneous muscle layer (i.e. the panniculus carnosus) is present in mouse skin but absent from human dermis.[20]

In this article we critically review the various techniques for human skin reconstruction and their current and future fields of application to skin physiology and diseases.

Aims to constitute human skin in *ex vivo*

In vitro reconstruction of human skin

In 1960, Cruickshank *et al.* demonstrated that when the epidermal keratinocytes of adult guinea pig were seeded at high density, they grew in culture even in the absence of the normal underlying dermal connective tissue.[21] However, in subculture the cells had a tendency to differentiate. A critical step forward was made in 1975 when Rheinwald and Green generated cell colonies that had originated from an isolated founder keratinocyte. This was achieved by using lethally irradiated 3T3 fibroblasts as feeders.[22] This technology enabled the production of large quantities of keratinocytes and their expansion in *in vitro* cell culture conditions. Pioneering 3D epidermal reconstruction, Freeman *et al.* developed a method in which human epidermal cells were cultured on decellularized pig dermis.[23] A small piece of human skin containing both epidermis and superficial dermis was put on the top of the dead pig dermis used as dermal substrate. Only epidermal keratinocytes attached to the dermis, allowing the growth of pure keratinocytes associated with a good level of differentiation. A better differentiated epidermis was then produced by Lillie *et al.* and Fusenig *et al.* when they placed the cultured keratinocytes on collagen membranes at the air–liquid interface.[24,25] More complete differentiation was obtained by Prunieras *et al.* using human de-epidermized dermis (DED)[26] rather than dead pig dermis.[23]

Since then, the production of skin equivalents (SEs) at the air–liquid interface has been achieved by different methods in which the principal difference is the dermal equivalents, which can be acellular or cellular structures. An inert filter[27–30] or DED can be used as acellular dermal substrate.[26,31–36] Reconstruction of epidermis using acellular DED requires the removal of the epidermis that preserves the BM, followed by dermal sterilization with glycerol, ethylene oxide, alcohol or gamma irradiation. After sterilization, the formerly epidermal side of the DED is seeded with keratinocytes. Following an immersion period during which the cells proliferate, the dermis is placed at the air–liquid interface, thereby inducing keratinocyte differentiation and the formation of a stratified epidermis (Fig. 1a).[26,31–36] In the alternative techniques, a cellular substrate composed of fibroblast-populated DED or collagen matrix can be used as dermal equivalents.[37–41] With this technique, the dermal equivalent is prepared by mixing a collagen solution with human dermal fibroblasts or by seeding fibroblasts on the subcutaneous side of the DED. After contraction, this support is seeded with normal human epidermal keratinocytes. The culture is initially maintained in submerged conditions allowing the proliferation of cells, before being placed on a grid at the air–liquid interface resulting in keratinocyte differentiation and the formation of a horny layer (Fig. 1a). It should be noted that other cell types can be incorporated into the epidermal (including melanocytes and Langerhans cells) or the dermal compartments (including fibroblasts and lymphocytes) to obtain a more complete physiological system.[35,36,42–45] However, the

incorporation of immune cells is poorly reproducible. It is worth mentioning that epidermis reconstructed on an acellular dermal substrate contains only three to four viable stratified epidermal layers, so a successful reconstruction by this system needs the supplementation of medium with various growth factors including epidermal growth factor (EGF), keratinocyte growth factor, and/or insulin-like growth factor. The presence of fibroblasts in the dermal substrate stimulates the proliferation of keratinocytes, improves the epidermal morphology and enhances the formation of BM proteins.[41,46,47]

The dermal substrate used in the reconstructed epidermis (RE) has an important impact on experimental studies. One of the main differences between epidermis reconstructed on DED or collagen matrix is the way in which the BM is formed. In skin reconstructed with DED, an intact BM and a papillary morphology of the epidermal–dermal junction (EDJ) zone is preserved, whereas in skin reconstructed with collagen matrix, a BM is produced by the fibroblasts and keratinocytes during the culture process[48] and the EDJ zone remains horizontal without rete ridges. Another important difference is that the composition of DED (which may contain various growth factors, proteases and cytokines) is not clearly defined and differences among individual donors need to be considered, thereby justifying the use of a similar dermis for a set of experiments.

Long-term maintenance of human skin equivalents and humanized mouse models

Following the seminal discovery of the method allowing the growth of keratinocytes as stratified cultures,[22] their use as grafts was suggested[49] and they were introduced in 1981 for burn therapy.[50] Grafting epidermal sheets enriched in holoclone-forming keratinocytes provided evidence of the long-term survival of the grafts but failed to provide an immediate dermal replacement, resulting in possible wound contraction and graft fragility.[51] To solve this problem, grafting of SEs was promoted for the therapy of burns, but no real breakthrough has been made.[52–54]

In an attempt to maintain epidermal homeostasis and increase the lifespan of SE beyond 2–3 weeks, researchers implemented humanized skin mouse models. They consist of chimeric models in which skin of human origin is regenerated, vascularized and innervated by mouse vessels and nerves. For this purpose, two experimental protocols have been developed. The first is based on grafting either the RE (performed as described above)[55–57] or split-thickness human skin obtained from volunteers[58,59] on the back of athymic mice (Fig. 1b). The second is based on seeding cells directly on the back of mice. A silicon chamber is implanted on the back of severe combined immunodeficiency mice, then a slurry of keratinocytes and fibroblasts is placed in the chamber.[25,26,60] The chamber is removed 1 week after grafting and humanized skin is formed at the grafting site in 2 weeks (Fig. 1c).[60]

The main difference between these models lies in the development of BM. Compared with the RE engrafted onto the back of mice, the human skin reconstructed by an injection of slurry cells has greater numbers of keratin intermediate filaments within the basal keratinocytes that are connected to hemidesmosomes, and more numerous connections of collagen filaments and anchoring fibrils to the lamina densa on the dermal side.[60] While the grafting of human skin samples has the advantage of maintaining BM, the EDJ zone and rete ridges, its implementation is technically difficult for the following reasons: (i) establishing the model requires large keratome sheets or multiple punch biopsies from volunteers or patients; (ii) the grafting needs to be performed quickly in order to minimize graft ischemia and (iii) uniform genetic manipulation of grafts is difficult. Indeed, humanized skin mouse models that use an isolation step and cell amplification offer the possibility of using *in vitro* genetically manipulated human keratinocytes, fibroblasts and/or melanocytes, thus generating transgenic or knockout humanized skin.

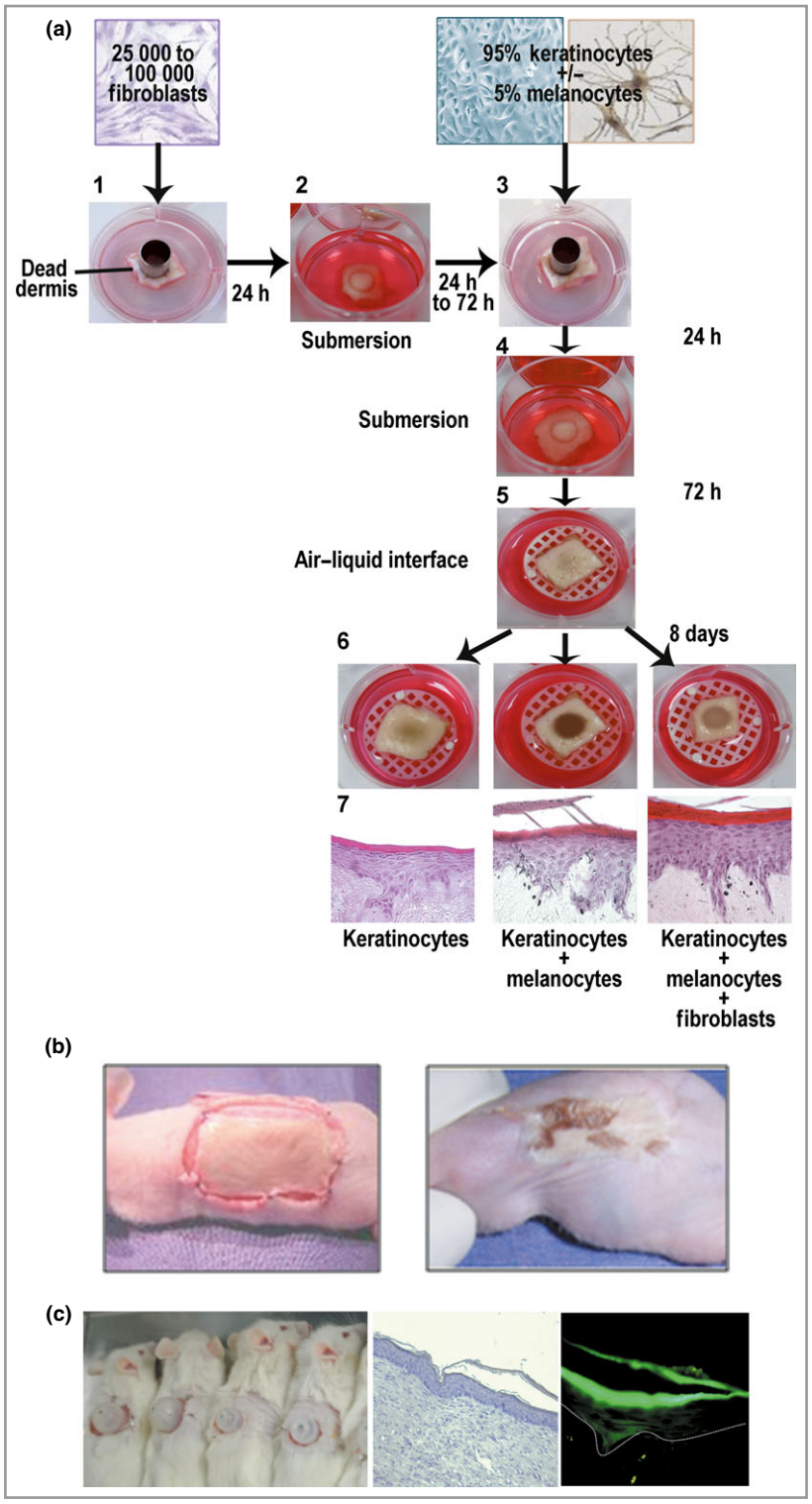
Applications of human skin reconstruction

The reconstruction of artificial HSEs has become an important tool for investigating skin physiology and pathophysiology. These models supply critical information about cell–cell interactions, the effects of the stromal environment on cell proliferation, differentiation and pigmentation. SEs are relevant models for testing dermocosmetics and drugs topically, and also for investigating photoageing and cancer. Furthermore, specific genetic alterations in either dermal or epidermal compartments, which can easily be achieved, facilitate mechanistic and signal transduction studies. These skin models also provide suitable alternatives to the use of laboratory animals. As a consequence, increasing numbers of commercially available skin models have reached the market, such as EpiDerm® (MatTek, Ashland, MA, U.S.A.), Episkin® (L'Oreal; SkinEthic, Nice, France), Apligraf® (Organogenesis Inc., Canton, MA, U.S.A.) and Labskin (Innovenn, Dublin, Ireland). The latter model uses a dermal component consisting of fibroblasts incorporated into a fibrin matrix and allows microorganisms to be grown on its surface, mimicking infection or the skin's natural microflora.

Skin equivalents as a model for studying the effects of cell–cell interactions on skin homeostasis

Keratinocyte–fibroblast interactions and skin homeostasis

Fibroblasts influence skin homeostasis by affecting BM formation, in addition to keratinocyte proliferation and differentiation. The EDJ zone, which provides an adhesive and dynamic interface, is composed primarily of laminin, collagen, nidogen and perlecan. Studies of BM formation during skin reconstitution have shown that keratinocytes alone are capable of synthesizing the major components of BM (such as type IV collagen, type VII collagen and laminin) and depositing them in a polarized manner into the EDJ zone.[48,61–63]



Fibroblasts affect BM formation through three mechanisms. Firstly, they secrete cytokines such as transforming growth factor (TGF)-β, which stimulates keratinocytes to synthesize BM components.[46] Secondly, fibroblasts are necessary for the assembly of an ultrastructurally complete BM. Thirdly, fibroblasts are capable of producing type IV collagen and type VII collagen, laminin-5 and nidogen, which also contribute to BM formation.[46,64] In addition to being involved in BM formation, fibroblasts are able to affect keratinocyte proliferation and differentiation.[46,64,65] This effect is based on fibroblast density where an increase in fibroblast numbers in dermal equivalents with a minimal and maximal threshold leads to an improvement in epidermal architecture.[41]

Fig 1. *In vitro* and *in vivo* reconstruction of epidermis. (a) *In vitro* reconstruction of epidermis. (1) Dermal equivalent is prepared by seeding fibroblasts on the subcutaneous side of the dead dermis. (2) Twenty-four hour later, culture is immersed for a few days (24–72 h) to allow contraction. (3) Following immersion, the formerly epidermal side of the dead dermis is seeded with keratinocytes and melanocytes. (4) Culture is then immersed for a few days (up to 72 h) to allow cell proliferation. (5) Epidermis is then placed on grid at air–liquid interface resulting in keratinocyte differentiation and formation of horny layers. (6) As seen macroscopically, if melanocytes are included in reconstruction, the reconstructed epidermis (RE) is more pigmented. (7) Histology and immunohistology can be done on formalin-fixed, paraffin-embedded RE to verify the architecture of the epidermis or assess the expression of various proteins/markers. The panels show the general morphology of epidermis reconstructed with keratinocytes, keratinocytes + melanocytes and keratinocytes + melanocytes + fibroblasts following staining with haematoxylin and eosin. (b, c) Skin humanized mouse models. (b) *In vivo* human epidermal reconstruction can be carried out by grafting either human skin samples or skin equivalents reconstituted using the methods explained above onto the back of mice. The left panel shows the graft of human skin samples on the back of mice. The right panel indicates the macroscopic aspect of chimeric skin 1 month after graft. (c) *In vivo* human epidermal reconstruction can also be performed by adding a slurry containing human keratinocytes and fibroblasts into a silicone chamber implanted on the back of severe combined immunodeficiency mouse muscle fascia (left panel). The middle panel shows histology of humanized epidermis formed on the back of mice. In this experiment human keratinocytes transduced with a viral vector expressing green fluorescent protein (GFP) have been used. GFP expression is detected in epidermal part of regenerated human skin (right panel).

Keratinocyte–melanocyte interactions and their effects on pigmentation

Melanocytes, which are responsible for skin colour, are maintained in the basal layer through their interactions with (i) BM components owing to the presence of discoidin domain receptor (DDR)1 and integrin α6β1 receptors (in the BM, these receptors interact with type IV collagen and laminins, respectively)[66,67] and (ii) keratinocytes owing to the presence of E-cadherin.[67] These interaction leads to the transfer of melanosomes into the keratinocytes via the melanocyte dendrites.

When skin melanocytes are incorporated into SEs, they are spontaneously located in the basa1 layer in close contact with multiple keratinocytes, a localization similar to that in normal human skin. Detection of the melanosomes in different layers of the RE, and notably in the granular layer when dark melanocytes are used, further indicates that melanocytes are functional in RE.[68,69] Using chimeric reconstructs (keratinocytes and melanocytes derived from phototypes II, III, IV, V or VI), melanocytes have been identified as the cells responsible for skin phototypes. Indeed, epidermal pigmentation is dependent on the phototype of melanocytes and is independent of the origin of keratinocytes.[36,45,70–72] Stimulation of the RE by applying pigment modifiers such as 3-isobutyl-1-methyl-xanthine, α-melanocyte-stimulating hormone or ultraviolet (UV) irradiation has shown that the epidermal melanin unit in pigmented reconstructs function exactly as in the *in vivo* situation in terms of pigment production.[73–78] Several studies have characterized the biological behaviour of melanocytes in skin reconstructs by focusing on melanin transfer, response to UV radiation exposure and DNA damage.[36,70,76,79]

Fibroblast–melanocyte interactions and their effects on pigmentation

Direct evidence of dermal influence on pigmentation comes from clinical observations of the outcome of epidermal suspension transplantation surgery in vitiligo in autologous recipient skin of different colours (e.g. nipple).[71] When incorporated into pigmented SEs, fibroblasts have been found to play a key role in the control of pigmentation.[71,80] In fact, when pigmented epidermal reconstructs are grafted onto the backs of mice, they develop a nonregular pigmentation pattern that is dependent on the presence of colonizing human or mouse fibroblasts.[71] In agreement with this finding, treatment of pigmented epidermal reconstructs with conditioned medium from UVA-irradiated aged fibroblasts mimicked senile lentigo pathology, a disorder characterized by hyperpigmented areas in photo-exposed skin.[81] Fibroblasts have been shown to affect pigmentation via various mechanisms. Fibroblasts secrete hepatocyte growth factor, basic fibroblast growth factor and stem cell factor, which bind to their receptors (i.e. c-Met, FGF-R and c-Kit, respectively) located on the plasma membrane of melanocytes, resulting in the activation of microphthalmia-associated transcription factor (MITF), the master regulator of pigmentation.[82–85] Fibroblasts can also play an inhibitory role in pigmentation via the secretion of TGF-β1 or Dickkopf1, a fibroblastic secreted factor that suppresses melanocyte growth and melanogenesis.[84,86,87] More recently, neuregulin-1, which is specifically expressed by fibroblasts from dark-skinned subjects, has been identified as a melanogenic dermal factor.[88]

Skin equivalents in studies of epidermal and dermal stem cells

Several studies in various tissues suggest that stem cell regeneration largely depends on the interactions between these cells and the environment where they reside and function. Specialized niches for various types of stem cells have been characterized in mammalian epidermis and dermis. These stem cells, which are distinct in marker expression patterns and growth properties, contribute differently to the homeostasis of epidermal and dermal compartments.[89–91] RE (*in vitro* or humanized mouse model) can be used to identify the stem cell populations and their localization. Moreover, it can be used to determine whether the epidermal and dermal cells derived from induced pluripotent stem cells (iPSCs) and embryonic stem

cells (ESCs) are morphologically and functionally similar to those of mature skin cells.

Epidermal stem cells and their regulation

At least three major stem cell populations are involved in epidermo-annexial regeneration: the hair follicle bulge, the sebaceous gland and the basal layer of interfollicular epithelium.[92–94] They can be distinguished on the basis of their marker expression patterns. Briefly, hair follicle bulge stem cells express K15, CD34, Lgr5 and K19, in addition to transcription factors Sox9 and Lhx2;[91] sebaceous gland stem cells express the transcriptional repressor, Blimp1[95] and interfollicular epithelium stem cells express elevated levels of α6 and β1 integrins and EGF receptor.[89] Because these stem cells are responsible for maintaining skin integrity and hair folliculogenesis,[92,96] the focus of recent studies has been the identification of stem cell populations and characterization of the molecular signals that govern regeneration of the epidermis and hair follicles. To this end, *ex vivo* transplant and grafting experiments on the backs of mice have been widely used. These experiments showed that the bulge area contains different stem cell populations that are able to generate hair follicles, sebaceous glands and epidermis.[97–99] Concerning the molecular regulation of hair stem cells, transplanted skin graft methods uncovered intrafollicular (short-distance niche) and interfollicular (long-distance niche including dermis, subcutaneous fat and adjacent follicles) microenvironments affecting the activity of these cells. In fact, stem cells are regulated by interaction between activators as well as inhibitors arising from the interfollicular and intrafollicular environment. Specifically, components of the wingless-type/β-catenin, sonic hedgehog, and TGF-β/bone morphogenetic protein pathways appear to be particularly relevant to epidermal stem cell function.[92,96,98,100–103] These experiments also showed that hair cycling in transplanted hair follicles was affected by the surrounding host skin, indicating that an interaction occurs between grafted skin, donor and host skin.[100]

Dermal stem cells

Recent studies suggest that a cell population within the dermal papilla of hair follicles, which expresses somatic stemness marker SOX2, may function as adult dermal stem cells (DSCs).[104–109] Rodent hair follicle-derived dermal cells can interact with local epithelia and induce *de novo* hair follicles in a variety of hairless recipient skin sites.[110–114] However, human dermal papilla cells failed to recapitulate this procedure under the same conditions, suggesting that human cells lose some key properties during the culture period. Recently, it was shown that if human dermal papilla cells are grown as spheroids, they can induce *de novo* hair follicles in human skin.[115] This study suggests novel treatment possibilities for cicatricial alopecia.

Li *et al.* have demonstrated a direct differentiation of DSCs into melanocytes. These melanocytes act in the same way as mature epidermal melanocytes upon incorporation into RE. They are able to migrate to the BM, produce pigment and express appropriate melanocytic markers such as MITF, tyrosinase, tyrosinase-related protein 1, gp100 (human melanoma black 45) and E-cadherin.[116,117]

Researchers have also demonstrated that perivascular sites in the dermis may act as a mesenchymal stem cell (MSC)-like niche in human skin.[118] SE models have been used to define the role of MSCs in epidermal regeneration. Ma *et al.* have reported that MSCs are able to proliferate and form a multilayered epidermis when seeded on top of a contractible fibroblast-embedded collagen gel.[119] Expression of two major epidermal markers (keratin 10 and filaggrin) revealed further similarity to epidermal lineage.[119] When incorporated into a dermal substitute, it has been shown that skin-derived MSCs are able to grow and proliferate efficiently[120] and provide a microenvironment that supports keratinocyte attachment, proliferation and differentiation similar to normal fibroblasts. However, these cells failed to transdifferentiate into epidermal lineages.[121]

Skin cells derived from induced pluripotent stem cells and/or embryonic stem cells

It has been shown that iPSCs and ESCs can be differentiated into keratinocytes, fibroblasts or melanocytes.[122–128] Seeded on dermal substitutes, ESC- and iPSC-derived keratinocytes have been shown to form a pluristratified epidermis.[124–126] Hewitt *et al.* have shown that iPSC-derived fibroblasts are similar to dermal fibroblasts in the establishment of the BM, which supports keratinocyte proliferation and differentiation upon incorporation into SE.[127] Both human ESC- and iPSC-derived melanocytes are able to migrate to the BM and produce melanin upon incorporation in RE.[128]

Skin equivalents in wound healing and clinical applications for patients with burns

SEs can be used to characterize some aspects of the wound-healing process. *In vitro* 'scratch' wound models using 2D monolayer cultures are, in fact, very limited for such studies. These cultures are helpful for studying only keratinocyte proliferation and migration in response to wounding, but cannot be used to investigate epidermal–dermal or epithelial–mesenchymal cross-talk during wound repair.[129–131] Growing evidence indicates that *in vitro* wound-healing models are similar to *in vivo* models in many aspects, including proliferation, migration and expression of growth factors (e.g. TGF-β1 and platelet-derived growth factor-β) in addition to cytokine secretion [e.g. interleukin (IL)-1α, IL-6, tumour necrosis factor-α].[129,132–136] Collectively, these results indicate that REs are valuable models for studying the chronology of re-epithelialization, cell proliferation, migration, differentiation and cell–cell cross-talk. Because these models can be used to modify various factors (e.g. medium components and types of incorporated cells),[137] they are very useful for understanding the roles played by individual cellular processes and the signalling pathways that drive tissue repair.

Although very valuable models, *in vitro* SEs lack the complexity associated with wound-healing mechanisms in native tissues. To study molecular mechanisms of tissue regeneration in a complex system, many researchers are now using rat and mouse models with limitations.[138] Several lines of evidence indicate that SEs grafted or implanted onto athymic mice represent more appropriate models for studying wound healing. Indeed, data obtained with this system showed that re-epithelialization progressed at a similar rate to human wounds[139–141] and that the phenotype of wounded keratinocytes is consistent with human wound-healing physiology.[142]

Covering the wound area as quickly as possible is one of the most crucial issues in improving the treatment of patients with deep burns. Acellular cadaver skin and fresh porcine skin have been used to treat large burn injuries.[143,144] However, the drawbacks of these strategies, notably the difficulties associated with their handling and disease transmission in addition to the possible risk of rejection and infection, have limited their use.[44,46,145] The application of skin reconstructs using collagen alone or collagen with skin cells has been shown to be of some benefit for patients with burns.[146–148] Guenou *et al.* reported that RE with human ESCs have a structure consistent with that of mature human skin 12 weeks after grafting onto immunodeficient mice, suggesting that this resource could be developed to provide temporary skin substitutes for patients awaiting autologous grafts.[124] Several studies have demonstrated that grafts of skin substitutes populated with MSCs accelerate wound healing.[149,150]

Skin equivalents in photodamage and photoprotection studies

As a relatively simple model that could mimic the *in vivo* situation, SEs are used to investigate skin responses to UV irradiation. As with human skin, the major effect of UVB on RE is the formation of sunburn cells,[151] while the disappearance of superficial fibroblasts due to the UVA-induced cell death is the major effect of UVA on dermis.[152,153] It has also been shown that keratinocyte differentiation markers are downregulated at early time points after UVB exposure. These downregulated markers are restored when SEs are maintained in culture for a longer period.[153] The involvement of melanocytes in photoprotection has been widely studied by using SE technology.[36,154,155] The possibility of using genetically modified keratinocytes, melanocytes and fibroblasts renders these SEs powerful tools for studying cell signalling pathways in addition to autocrine and paracrine factors regulating skin responses to UV irradiation.[33,156–160] SEs are also a resource for evaluating the protective effects of topically applied products such as sunscreens.[76,152,161–163] A point that should be considered, especially when the aim of study is the evaluation of a topical application or a repetitive treatment, is the absence of desquamation in *in vitro* SEs.[164]

One of the consequences of chronic UV irradiation is photoageing. SEs have been used to reveal biological modifications in epidermal and dermal compartments during this process. For instance, RE using preglycated collagen as a dermal substitute led to an increased expression of extracellular matrix molecules (e.g. type III procollagen), the synthesis of metalloproteinases [e.g. matrix metalloproteinase (MMP)-1, MMP-2, MMP-9] and BM molecules (e.g. type IV collagen), in addition to the modification of the expression patterns of α6 and β1 integrins in epidermis, which are highly relevant for the *in vivo* study of aged skin.[165,166] Interestingly, glycation is a nonenzymatic reaction that takes place between free amino groups in a protein and a reducing sugar, such as glucose or ribose. The formation of these bridges between dermal molecules is thought to be responsible for loss of elasticity and other properties of the dermis observed during ageing.[165,166] In agreement with this finding, when using *in vitro* skin reconstructed with keratinocytes derived from several human donors of different ages, the age of the donor was found to have a significant effect on the epidermal architecture and the expression pattern of p16INK4A, a biomarker of cellular ageing in human skin.[167–169]

Skin equivalents and modelling of diseases

Human reconstructed skin, which mimics many morphological and molecular characteristics of normal human skin, has been used for modelling various skin diseases such as psoriasis, genodermatoses (e.g. epidermolysis bullosa and xeroderma pigmentosum) and vitiligo.

- Psoriasis has been modelled by using keratinocytes from patients with psoriasis,[170,171] inhibition of transglutaminase,[172] addition of lymphocytes[173] or stimulation using proinflammatory cytokines.[174,175] Results indicate that *in vitro* reconstructed skin models, which display the molecular characteristics of psoriatic epidermis, are relevant for studying the biology of this disease and for screening antipsoriatic drugs.[170,174,175] Future studies including incorporation of other cell types such as T cells could provide important insights into the interactions between cell types in psoriasis.
- In recessive dystrophic epidermolysis bullosa, which is due to mutations in the *COL7A1* gene, *in vitro* REs composed of patient-derived keratinocytes and/or fibroblasts have been proposed for modelling this disease.[176] Patient-specific induced iPSCs have also been used for this purpose.[125,126] These SEs provide significant advantages for testing preclinical strategies including cell- and gene-based therapy.[177,178]
- For xeroderma pigmentosum (XP), a rare autosomal recessive disorder of DNA repair, SEs reconstituted with keratinocytes and/or fibroblasts taken from patients with XP mimic features of the disease (i.e. DNA repair deficiency, UV sensitivity, predisposition to cancer and deficiency in catalase activity).[160,179,180] Indeed, the XP model displayed repair deficiency with long-lasting persistence of UV-induced DNA damage and p53-positive nuclei upon exposure to UVB, unlike the normal SE, which efficiently repaired UVB-induced DNA lesions.[180] Furthermore, *ex vivo*

gene therapy for XP-C cells has been tested using *in vitro* SEs and SEs grafted onto mice. Results showed that corrected XP-C keratinocytes exhibited efficient DNA repair capacity and normal features of epidermal differentiation in both models.[181]

- Vitiligo, the most common depigmenting disorder, is an acquired disease characterized by progressive loss of melanocytes.[182] RE reconstructed with normal keratinocytes and melanocytes from vitiligo showed that vitiligo melanocytes present a defect in adhesion and that this deficiency is amplified if vitiligo keratinocytes are also incorporated into reconstructs.[183] This suggests an intrinsic defect in melanocytes and possibly in the keratinocytes of patients. Furthermore, some stress conditions (e.g. adrenaline or oxidative stress) could trigger the transepidermal loss of normal and vitiligo melanocytes.[183] As already mentioned, one of the mechanisms by which melanocyte basal location is ensured is DDR1–type IV collagen interactions. To test whether deficiency in DDR1 or the connective tissue growth factor (CCN)3, which regulates DDR1,[66] is implicated in vitiligo, their endogenous protein expression was inhibited using lentivirus-mediated expression of shRNA against DDR1 or CCN3. CCN3 downregulation prevented melanocyte attachment, unlike inhibition of DDR1 expression.[184] This model can reproduce hypo- and hyperpigmentary disorders associated with melanocyte defects and can highlight dermal influences on the pigmentary phenotype. SEs have been used in our laboratory for modelling *in vitro* the hypo- and hyperpigmentary disorders associated with lentigo senilis,[81] systemic scleroderma and melasma (Y. Gauthier, S. Lepreux, A. Taïeb, M. Cario-André, unpublished data).

Limitations of human *in vitro* and *in vivo* reconstructed epidermis and perspectives

With the improvements achieved in recent years, SEs have become an indispensable tool for investigative dermatology, especially for addressing (i) skin homeostasis and the molecular mechanisms that govern different cell types and their interactions, (ii) skin repair, specifically the signalling pathways that drive this process, (iii) skin regeneration, by understanding the properties and the behaviour of the skin stem cells residing in various niches and (iv) skin diseases, by modelling these diseases using cells from patients or genetically engineered ones to reproduce molecular defects.

However, these models are still perfectible for several essential reasons:

- As blood flow is not present in *in vitro* reconstructs, this negatively influences cell nutrition and metabolism, a bias limited by optimal medium composition and renewal. However, securing a vascular bed would provide an optimal environment for immune cells, which are difficult to maintain in SEs. Lastly, this vascular bed would facilitate the incorporation of MSCs in order to study their effects in skin biology.
- Having a functional immune system in RE remains a major objective. Although mouse models have efficiently advanced our knowledge about basic immunological mechanisms, they cannot address all questions concerning human skin immunology. Despite the many preserved features between the human and mouse immune systems, several studies have highlighted important differences.[185,186] This could explain why some protocols for treating autoimmune diseases or cancer immunotherapy work well in mice but have not been successful in human trials.[187] The incorporation of immune cells in *in vitro* RE has been tried with some success by various groups,[163,188–190] but a well-established and reproducible protocol is not currently available. Therefore we need to make greater efforts to overcome this obstacle in the future.
- Epidermal reconstructs lack neuronal cells. The sensitivity of the skin is due to the presence of sensory neurons that transmit tactile, proprioceptive, chemical and nociceptive sensations. Several reports have shown that the density of sensory neurons ending in the epidermis is increased in immune skin diseases such as psoriasis and atopic dermatitis.[191–194] Therefore, incorporation of neurons would be of great benefit for investigating skin reactions in detail after specific stimulation and the interconnection between all resident skin cells.

As humanized mouse models will not be useable for many research aspects for ethical reasons, cell-scaffold technology with its new generation of skin substitutes is an emerging field. This technology has the potential to meet the engineering challenges posed by the complexity of skin.

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