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Diversity of human skin three-dimensional organotypic cultures

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Recently, significant strides have been made in the development of high-fidelity skin organoids, encompassing techniques such as 3D bioprinting, skin-on-a-chip systems, and models derived from pluripotent stem cells (PSCs), replicating appendage structures and diverse skin cell types. Despite the emergence of these state-of-the-art skin engineering models, human organotypic cultures (OTCs), initially proposed in the 1970s, continue to reign as the predominant *in vitro* cultured three-dimensional skin model in the field of tissue engineering. This enduring prevalence is owed to their cost-effectiveness, straight forward setup, time efficiency, and faithful representation of native human skin. In this review, we systematically delineate recent advances in skin OTC models, aiming to inform future efforts to enhance *in vitro* skin model fidelity and reproducibility.

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Introduction

The skin, as the largest organ in the human body, serves crucial roles in immunity by safeguarding against pathogens, maintaining body surface hydration levels, and acting as the primary barrier against diverse environmental threats [1,2]. Furthermore, skin moderates homeostatic balance, including sensation and thermoregulation. Given its critical involvement in various bodily processes, skin-related ailments rank as the fourth leading nonfatal disease burden globally, affecting approximately one-third of the population [3,4]. Although these conditions typically do not lead to fatalities, their associated stigma and profound effects on self-esteem and mental well-being should not be overlooked. Consequently, in addressing diseases associated with the loss of skin integrity, animal models and two-dimensional (2D) *in vitro* culture have been extensively employed to investigate the skin disease mechanisms and validate therapeutic interventions. However, challenges such as interspecies variability in animal models [5] and the imperative to adhere to the 3R (replace, reduce, and refine) strategy [6] underscore the limitations of animal uses. Moreover, 2D monolayer cultures are constrained by their inability to replicate the stratified epidermis and lack of 3D cell-to-cell/ECM (extracellular matrix) interactions [7]. Hence, considerable efforts over a span of 40 years have been devoted to the development of *in vitro* cultured 3D skin models, specifically focusing on skin organotypic cultures (OTCs), with the primary aim of faithfully replicating *in vivo* human skin-like structures and functions. This concerted effort is driven by the imperative to facilitate both research investigations and clinical applications in the field.

Full-thickness skin equivalents were delineated in the 1980s [8,9], building upon pioneering co-cultures of keratinocytes (KCs) on fibroblasts (Fibs) at the air–liquid interface (ALI) [10]. By the close of the last century, differentiated KC cultures were successfully cultivated on various substrates, including collagen gels [11], nylon mesh [12], inert filters [13], lyophilized collagen-GAG membranes crosslinked by chemical agents [14], and human de-epidermized dermis (DED) [15]. In these models, living skin OTCs are nurtured in an ALI, evolving into a multilayered stratified epidermis with discernible epidermal cell layers. In the early 2010s, Itoh et al. [16,17] developed the protocols for differentiating human induced pluripotent stem cells (hiPSCs) into both KCs and Fibs, as well as 3D skin equivalents fully reconstituted from hiPSCs, representing another major breakthrough. Patient-derived or genetically modified skin cells have emerged as pivotal components in the development of OTC models designed to target a wide range of diseases [18]. This development significantly enhances the relevance of OTC models in clinical research pursuits.

Notable progress has been achieved in the development of skin spheroids and hPSC-derived organoid models. These organoids are constructed from a nearly complete *in vitro* self-organized skin system differentiated from hPSCs, forming a hierarchical skin organoid that faithfully recapitulates a stratified epidermis, fat-rich dermis, and pigmented hair follicles equipped with sebaceous glands [19–21]. Despite the significant attention received by hPSC-derived skin organoids, the ALI-based OTC model persists as a prevalent platform extensively utilized not only in skin development research but also in mitigating the limitations associated with PSC-derived skin organoid cysts. However, because of the planar structure and limited diversity of cell types, most current human skin OTC models are predominantly 3D layered skin substitutes devoid of appendages. Hybrid constructs that combine hPSC-derived cyst-like skin organoids with subsequent ALI culture techniques represent OTC models capable of recapitulating multiple appendage structures. Development of an *in vivo*–like skin organoid through the activation of the Wingless-related integration site (WNT) signaling pathway results in larger organoids devoid of off-target cartilage differentiation [22]. Employing an ALI-based OTC model, skin organoids are obtained featuring a stratified squamous epithelium, more closely resembling adult human skin [22]. Similarly, the application of OTC-based upscaling was also demonstrated in a human conjunctiva organoid model [23].

Alongside skin OTC models, four additional types of skin models also serve as significant components in *in vitro* 3D skin bioengineering (Figure 1a), which have been comprehensively discussed elsewhere, including skin spheroids, PSC-derived skin organoids, as well as advanced technologies such as 3D bioprinting and skin-on-a-chip systems. These models collectively contribute to the current landscape of *in vitro* cultured 3D skin models and hold great promise for various applications in research and clinical practice. Interestingly, 3D bioprinting is a technique on the rise that can be applied to various existing skin models, significantly expanding their application scenarios, including skin OTC models that often serve as the basic setup, which is then enhanced through bioprinting. As skin OTC models remain a cornerstone in skin bioengineering and are widely used in research (Figure 1b), our focus will be on providing a synthesis of existing literature pertaining to human skin OTCs.

Versatility of human organotypic culture models

Thorough characterization of skin OTC models is imperative for precise modeling utilizing the ALI culture method, which closely mimics the physiological complexity of human skin tissue, including its multilayered structure that comprises three distinct layers: the epidermis, dermis, and the innermost hypodermis [9,24]. In general, these models are typically categorized into three types based on their structural complexity (Figure 2): human epidermal equivalents (HEEs), human skin equivalents (HSEs), and advanced human skin equivalents (aHSEs). Different OTC models do not follow a simple linear evolutionary relationship. Although the complexity increases from HEE to aHSE models, their fidelity and consistency do not necessarily improve with increasing complexity. As a result, each model has its unique applications and advantages.

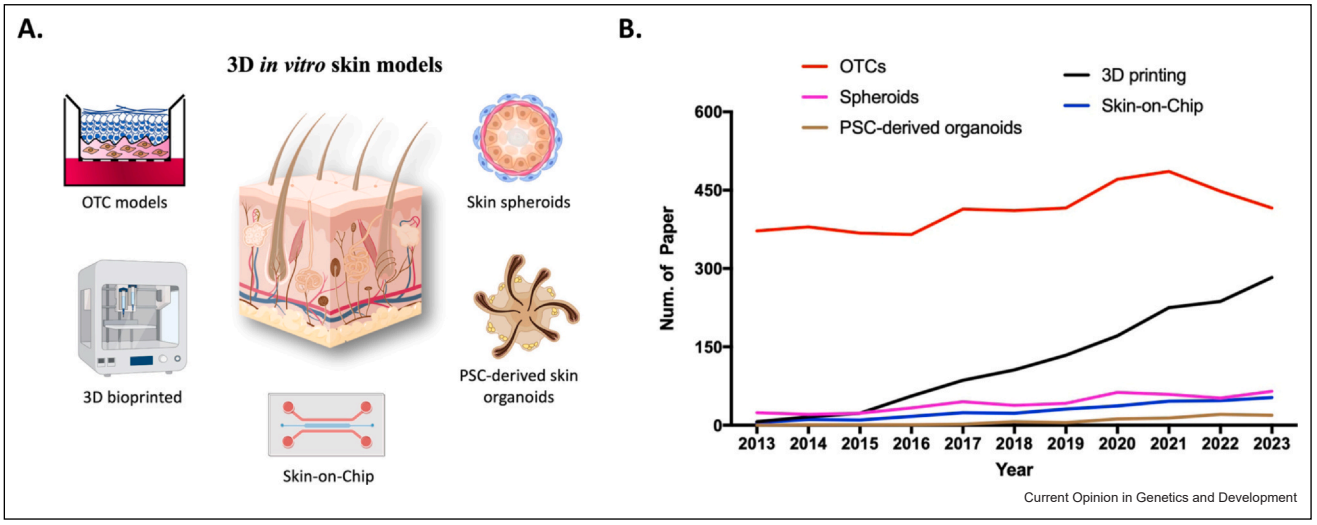
Human epidermal equivalents

HEEs represent the simplest form of skin tissue, composed exclusively of KCs. Initially seeded into transwells, these cells undergo brief cultivation under submerged conditions before transitioning to the ALI. This transition facilitates the stratification of the epidermis, complete with identifiable epidermal cell layers. Although a weakness of HEEs is the simplicity of its makeup, which does not allow for cell-type interactions, they do possess barrier properties akin to native human skin and is an ideal option for investigations focusing on areas where heightened complexity is unnecessary [25]. For instance, the HEE models offer dependable substitutes for *in vitro* permeation testing studies, a domain historically plagued by the unpredictable availability and exorbitant cost associated with excised human skin [26]. Owing to its cost-effectiveness and reproducibility, the HEE model is also implemented in hazard assessments and regenerative medicine, where it is now commercially available from numerous companies [27,28]. Moreover, recent studies underscore its significance in skin barrier research and disease modeling. Δ*TFAP2A*-HEEs generated via CRISPR/Cas9 have been used to investigate whether *TFAP2A* knockout and the consequent loss of KC differentiation gene expression lead to morphological alterations and epidermal barrier impairments [29]. Additionally, cultured human KCs and HEEs have been used to establish a preclinical model of Darier disease (DD) to better understand disease pathogenesis. Building upon the SERCA2-deficient HEE model, Mitogen activated protein kinase kinase (MEK) inhibition was shown as a potential targeted therapy strategy for DD [30]. Pigmented HEE models can be used to assess the effect of melanin following ultraviolet (UV) irradiation [31].

Human skin equivalents

Contemporary skin models predominantly comprise two discernible layers: the epidermis and dermis. This design allows for the optimal differentiation of the epidermis and the replication of the complex interactions between KCs and Fibs, which are crucial for maintaining skin homeostasis [32]. In their most rudimentary form, these reconstructed skin models are composed of an ECM-based biomaterial, such as collagen or DED, which is primarily populated by Fibs and overlaid with a stratified epidermis. This structural arrangement ensures

Figure 1



Different 3D *in vitro* skin models and their popularity in research. **(a)** An overview of different 3D *in vitro* skin models. Graphics generated, in part, using Biorender. **(b)** The 10-year trend (2013–2023) of research interest in 3D *in vitro* skin models. Various models were investigated within the PubMed database through independent search queries: "(skin) AND ((equivalent) OR (organotypic) OR (organotypic equivalent))", "(skin-on-chip) OR (skin-on-a-chip) OR ((skin) AND (microfluidic devices))", "(skin organoid) AND ((PSC) OR (pluripotent stem cells) OR (iPSC) OR (induced pluripotent stem cells) OR (embryonic stem cells))", "(skin) AND ((bioprinting) OR (3D printing))", and "(skin) AND (spheroid)". OTCs, organotypic cultures; PSC, pluripotent stem cells; iPSC, induced pluripotent stem cells.

that the dermal layer remains in direct contact with the culture medium, while the epidermis is exposed to the air. The dermal component of HSEs may be scaffold-free, formed through cell-self-secreted ECM or cell sheets [33,34], utilizing natural scaffolds such as native skin-derived acellular DEDs [35] and collagen, or employing synthetic scaffolds like polymerized hydrogels [36], electro-spun nanofibers, and porous substrates [37]. Significantly, collagen- and DED-based HSEs are increasingly recognized as promising skin models in skin bioengineering owing to their supportive cellular environments and low antigenicity [38,39].

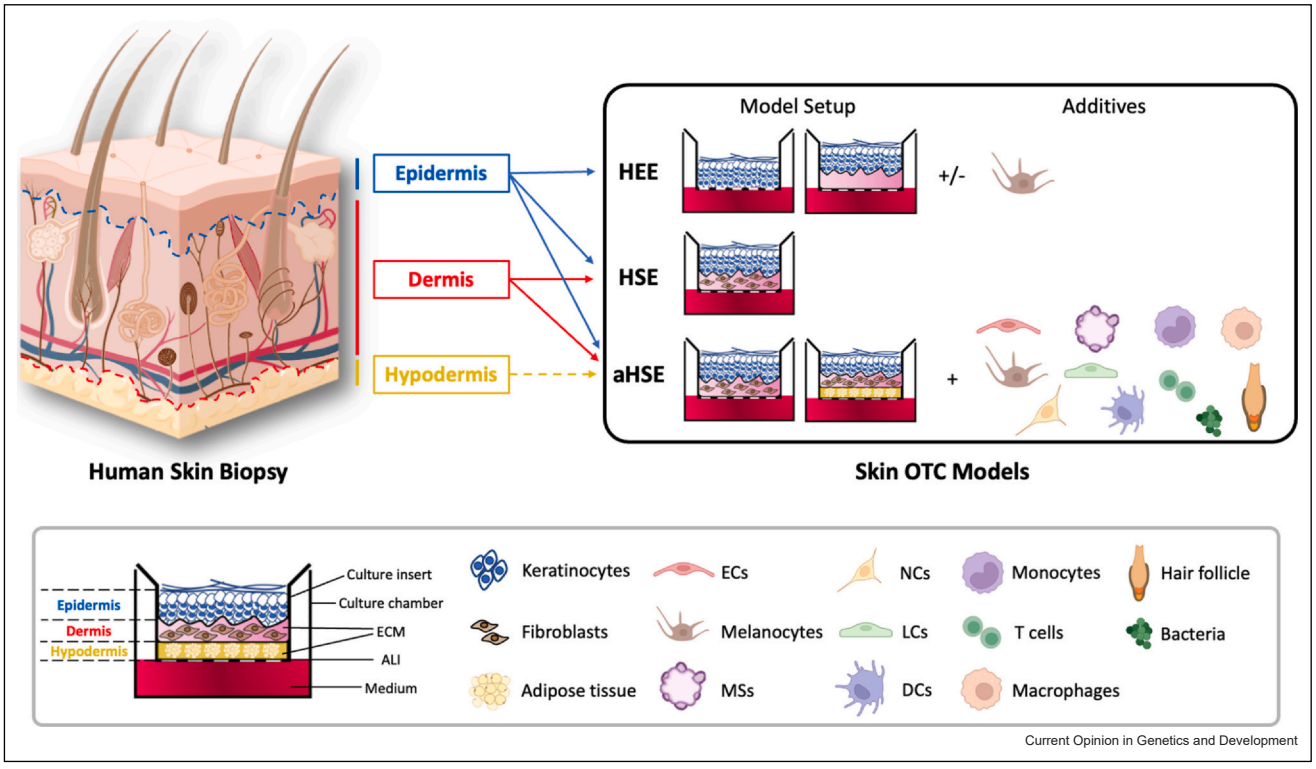
HSEs are used to investigate various aspects of normal and abnormal skin biology, including wound healing [40,41], aging [42,43], and the study of various diseases [18]. Additionally, they have been employed directly in studies and as 'hybrid' models, where humanized HSEs are grafted onto immunodeficient mice [44]. Furthermore, in response to challenges associated with donor variability, conventional primary cell-based HSEs have transitioned to more standardized and reproducible *in vitro* culture models. These models utilize either immortalized cell lines [45] or cells derived from hiPSCs [17]. Despite advancements and the ability to replicate various characteristics of native human skin and disease-specific phenotypes, full-thickness HSE models face limitations due to the absence of vasculature, appendages, and immune system. This deficiency complicates the simulation of systemic inflammation and pathogenesis associated with various appendages, such as folliculitis.

Advanced human skin equivalents

Extensive efforts have been dedicated to engineering aHSEs capable of integrating additional cell types. These include endothelial cells to vascularize the dermis [46] and melanocytes to introduce pigmentation [47]. Furthermore, neuronal cells [48,49], lymphatic cells [50], immune cells (e.g. dermal dendritic cells [51], monocytes [52], T cells [53], and macrophages [54]), adipocytes and adipose tissue [49,55,56], pluripotent stem cells [22,36,57], and skin appendages such as hair follicles or sweat glands [18,58] have been incorporated. aHSE models offer a high degree of customization, facilitating control over organotypic cell populations, genotypes, and culture conditions, thereby enabling meticulously controlled studies on tissue-level biology [59]. This expansion enhances the application of OTC models for investigating potential therapeutic techniques [18,60], particularly in mimicking inflammatory skin diseases like psoriasis and atopic dermatitis [59], while also studying skin-related bacterial adhesion and infection [22,45].

Given their high customizability and potential for significant variation in complexity depending on the intended application, 3D printing technology has been effectively integrated into aHSE models. Notably, the

Figure 2



An overview of *in vitro* skin OTC models. The upper part illustrates the relationship between human skin and *in vitro* OTC model. The biophysiological structure of native human skin comprises three distinct layers: epidermis, dermis, and hypodermis, as depicted by a human skin biopsy. Different layers of the skin correspond to various types of OTC models. The HEE model exists in two formats: epidermis-only and pseudo-full-thickness, which includes an acellular dermal component; the HSE model consists of both epidermis and dermis, representing a full-thickness bilayer structure; the aHSE can incorporate additional cell types beyond KCs and Fibs, combine with 3D printing technology, or integrate mechanical features. It may have a bi- or tri-layered structure. "+/–" denotes inclusion or exclusion of the specified additive; "+" signifies inclusion of at least one of the displayed additives. The lower part displays the figure legend. OTC, organotypic culture; HEE, human epidermal equivalent model; HSE, human skin equivalent model; aHSE, advanced human skin equivalent model; NCs, neuronal cells; ECs, endothelial cells; MSs, melanoma spheroids; LCs, lymphatic cells; DCs, dendritic cells. Partial credit for figure generation is attributed to Biorender.

development of large-scale personalized edgeless wearable human skin grafts was further vascularized by skin-specific endothelial cells, resulting in enhanced deposition of the ECM, improved mechanical properties, and site-specific differences in cellular and ECM organization [61]. Meanwhile, aHSEs can be created with rete ridges between their epidermal and dermal layers using 3D-printed stamps coupled with the micromolding method [62]. The produced rete ridges comprised rounded features of controlled geometry and periodicity in the dermal layer, advancing the current HSE model to a more skin-like state.

While advanced and capable of representing a broad range of native human skin characteristics and disease pathology, aHSE models present challenges in terms of development, being more time-consuming and complex compared to classical full-thickness HSE models. The heightened complexity not only raises the specialty for their widespread adoption but also escalates costs, particularly when utilizing cells of human origin or PSC-derived cells. Consequently, striking a balance between model stability and complexity is crucial in the design of studies focusing on skin-related research.

Illuminating the fidelity of skin organotypic culture models via single-cell omics

ALI-based planar OTC models for skin offer a robust platform enabling researchers to manipulate various types of skin cells and their microenvironments artificially. Traditionally, skin bioengineering studies have relied on phenotypic readouts. The planar format and ample size of skin OTC models theoretically enable the adaptation and implementation of assessment approaches utilized on native human skin. Unlike low-throughput methods such as quantitative polymerase chain reaction or immunofluorescence, highly sensitive RNA sequencing (RNA-seq) empowers researchers to simultaneously analyze the expression levels of all genes within a sample. This capability facilitates the

comparison of gene expression or predicted biofunction profiles across different samples or experimental conditions.

In the wave of technological evolution from bulk to single-cell level omics, single-cell RNA sequencing (scRNA-seq) has become a routine method for studying human skin development. It enables both profiling of gene expression measurements at a single-cell resolution and identification of reliable cellular heterogeneity, allowing for the identification of previously unrecognized levels of cellular heterogeneity, revealing regulatory relationships between genes, and tracking the trajectories of distinct cell lineages in the same or different developmental stages [63–66]. Although single-cell research related to *in vitro* skin models is relatively sparse and still in its early stages, some interesting conclusions can be drawn from these studies that would be difficult to obtain otherwise. For instance, scRNA-seq of human KCs was compared to holoclone signatures, and the resulting analyses were able to clearly distinguish epidermal holoclone-forming cells from other epidermal cell states and identify a continuous hierarchical trajectory, showing that holoclone-forming cells generate meroclone- and paraclone-forming cells [67]. hPSC-derived skin organoids, with their enhanced complexity, resemble a more fetal developmental stage [19], with their mouse counterparts forming competent morphogenetic units that can initiate hair growth after transplantation using epidermal IFNr to induce apical-basal polarity, dermal-Tgfb to induce basement membranes, and dermal-Vegf to mediate dermal cell attachment to the epidermal cyst shell [68]. Finally, a comparison of HEEs, HSEs, xenograft HEEs, and *in vivo* epidermis indicates that these systems also resemble a more fetal-like developmental state similar to the PSC-based organoids and contain all the cellular states as their *in vivo* counterpart but may exhibit defects in the basal and terminal differentiation programs depending on how they are cultured [44]. These results also reaffirmed the presence of cellular stress in *in vitro* models, offering important insights for future research in tissue culturing and engineering.

Conclusion and perspectives

The versatile skin OTC-based platform is ideally suited for investigating a broad range of physiological and pathological scenarios, presenting significant potential for advancing our understanding of skin developmental biology, disease modeling, and applications in regenerative medicine [69–71]. Hence, skin OTC models function as a crucial intermediary between animal models, traditional 2D cell cultures, and human skin biopsies, highlighting their adaptability and versatility within the realm of skin biology.

Reproducibility of skin organotypic cultures

The importance of standardized protocols for ensuring experimental reproducibility cannot be overstated. A major challenge in achieving reproducibility in skin culture systems stems from the absence of uniform, standardized protocols, which can lead to variations in factors such as culture medium, ALI duration, and key cellular parameters (e.g. fibroblast presence, cell seeding density, passage number, etc.). This lack of standardization complicates the comparison of studies performed under different culture conditions. However, the choice of appropriate cell sources for model development holds significant potential for enhancing the reproducibility of OTCs. For example, using PSC-derived cells or immortalized cell lines may offer advantages over primary cell sources, which are susceptible to interdonor variability. Nonetheless, determining which cell source provides the highest fidelity remains unclear and requires further investigation.

Future directions

In the realm of skin OTCs, the evolution of *in vitro* models is diverging along two promising paths. One focuses on replicating the full complexity of human skin, aiming to recreate its architecture and functionality *in vitro*. This path seeks physiological relevance by approximating the intricacies of living skin. The second approach emphasizes specialized models tailored to investigate specific skin features or functions. Irrespective of the chosen trajectory, single-cell analytical techniques are crucial for thorough characterization, ensuring the functional and mechanistic insights necessary to validate these models.

Within regenerative medicine, autologous skin grafting remains the gold standard for treating skin defects. However, its clinical limitations, particularly the restricted availability of donor sites, underscore the need for alternative strategies. In response to this pressing demand, numerous OTC-based cultured epidermis and skin substitute products have become commercially available (e.g. Commercially Available Skin Substitute Products [72]; Skin and Soft Tissue Substitutes [73]). Nevertheless, no artificial skin substitute currently achieves full functional equivalence to autologous grafts. Addressing these challenges, Nagano et al. recently succeeded in generating semi-autologous skin *in vivo* through niche encroachment, paving the way for large-scale human skin graft production in livestock animals [74].

In investigations concerning skin development, skin is frequently delineated as an intricate network of four symbiotic barriers: the physical, chemical, immune, and microbiotic layers [75]. Addressing these aspects, contemporary research is channeling resources into enhancing skin models from simplistic bilayer constructs to elaborate systems that incorporate both immune cells

and active surface microbiota. Hybrid constructs, amalgamating PSC-derived skin organoids with subsequent ALI culture methods, emerge as a promising foundational approach for the assembly of integrated skin systems.

To conclude, the advancement of *in vitro* skin OTC models is steadfastly trending toward enhanced complexity and functionality. Harnessing innovations in biotechnology, skin models on the horizon hold immense potential to revolutionize both scientific inquiry and practical applications.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare no conflict of interest.

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- • of special interest
- •• of outstanding interest

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Stabell and colleagues conducted the first and only single-cell-based skin OTC modeling studies to date. Their research undertook a comparative analysis of *in vitro* HEEs, HSEs, xenograft HSEs, and in vivo epidermis. This study provided a detailed single-cell-level resemblance between these models and *in vivo* conditions, while also highlighting cellular stress within *in vitro* models. These findings contribute critical insights for future research in skin tissue engineering.

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By incorporating melanocytes into the basal layer of the epidermis, the authors successfully recreated skin pigmentation *in vitro*. This study assesses both pigmented HEE and HSE. The presence of fibroblasts, which is crucial for the support of melanocytes, has been shown to influence melanocyte physiology and, consequently, skin pigmentation. The authors have utilized a pigmented HSE model to explore the modulation of skin tone and the protective effects against UV-induced damage. This demonstrates the significant role of the pigmented HSE model within the broader scope of skin biology research.

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Pappalardo et al. reported the development of large-scale personalized edgeless wearable human skin grafts. In their shape-guided hand skin model, the skin graft was further vascularized by skin-specific endothelial cells, leading to enhanced deposition of the ECM, improved mechanical properties, and site-specific differences in cellular and ECM organization. This study provided the first imitation of the skin ' s overall coordination and regional specificity, offering crucial and valuable insights for future large-scale skin transplantation and regeneration.

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Lewis team developed human skin models featuring distinct rete ridges between the epidermal and dermal layers by employing 3D-printed stamps in conjunction with a micromolding technique. The resulting rete ridges exhibited rounded attributes with precisely controlled geometry and periodicity within the dermal layer, thereby enhancing the current HSE model toward a more authentic skin-like configuration.

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As the first comprehensive multiomic reference atlas of human prenatal skin (7–16 post-conception weeks), this study characterized the skin ' s microenvironmental cellular organization at early developmental stages. Moreover, the authors benchmarked the hPSC-derived skin organoid model, confirming its high fidelity in mimicking fetal skin and hair follicle development from early to late time points, which provides valuable reference resources for future applications of hPSC-derived skin organoids. Notably, the authors demonstrated through experiments the crucial role of macrophages in early embryonic vascular development.

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In addition to incorporating traditional cellular complexity and engineered elements, it is crucial to consider the role of the human skin microbiota. Galvan et al. conducted a comprehensive review of the existing human skin models, discussing their advantages and limitations. They emphasized how integrating an appropriate microbiota into an *in vitro* human skin model could significantly enhance its ability to replicate *in vivo* conditions more accurately.