Evolution of Skin-on-a-Chip Platforms

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The increased demand for physiologically relevant in vitro human skin models for testing pharmaceutical drugs has led to significant advancements in skin engineering. One of the most promising approaches is the use of in vitro microfluidic systems to generate advanced skin models, commonly known as skin-on-a-chip (SoC) devices. These devices allow the simulation of key mechanical, functional and structural features of the human skin, better mimicking the native microenvironment. Importantly, contrary to conventional cell culture techniques, SoC devices can perfuse the skin tissue, either by the inclusion of perfusable lumens or by the use of microfluidic channels acting as engineered vasculature.

reconstructed skin models | tissue engineering | skin-on-a-chip

1. Introduction

Skin and subcutaneous disorders affect approximately one-third of the global population and are associated with a burden encompassing psychological, social and financial dimensions [1]. In particular, chronic skin diseases, such as psoriasis, eczema and atopic dermatitis, result in significant morbidity and affect patient quality of life [2]. On the other hand, malignant skin diseases, such as malignant melanoma, are frequently fatal [3]. The high prevalence of skin diseases combined with the emergence of new technological advancements in drug development is fueling the growth of the skin-targeted drug delivery market.

Skin-targeted drug delivery systems include topical, dermal and transdermal approaches. In topical drug delivery, the active substances are intended to remain on the skin's surface (e.g., barrier creams, sunscreens and repellents) whereas dermal delivery targets active substances into the relevant skin layers (e.g., corticosteroids and antibiotics). In parallel, transdermal drug delivery research has also seen an upsurge in recent years. This approach could circumvent the complications of oral and intramuscular drug delivery and achieve controlled systemic delivery of drugs. Recent innovations in this field include a transdermal patch indicated against symptoms of Parkinson's disease [4] and a skin-targeted vaccine against COVID-19 [5].

The successful development of skin-targeted drug delivery systems requires careful consideration of the human skin's anatomy, physiology and physicochemical properties. This evaluation is important to achieve the desired drug effect and avoid adverse reactions such as skin sensitization and/or irritation. The development of new pharmaceutical drugs would greatly benefit from biomimetic in vitro skin models that could replicate the key components of the in vivo healthy and diseased human skin.

Conventional preclinical drug testing relies on in vitro cell cultures and animal models. Most commonly, in vitro cell culture relies on two-dimensional (2D) cell culture systems, typically monolayers of epidermal keratinocytes and/or dermal fibroblasts. While these models offer a rapid, reproducible system to study drug responses, they are not good predictors of the complex interactions seen in vivo [6]. The lack of a 3D physiological tissue environment greatly minimizes the models' physiological relevance and applicability. On the other hand, in vivo animal models offer information on systemic effects but cannot replicate human skin anatomy and physiology. During the development of pharmaceutical skin-targeted formulations, mouse models are often mandatory for in vivo translational research. However, mouse skin is structurally and functionally different from human skin; it is thinner, contains more hair follicles, includes fewer keratinocyte layers, presents decreased barrier function and greater absorption [7]. Moreover, animal models suffer from low throughput and interspecies variability [8]. These flaws in the conventional testing methods result in a lack of correlation between the input (drug candidates) and output (approved drugs), contributing to the R&D decline [9].

From an ethical perspective, the replacement of animal models satisfies a growing societal concern regarding animal experimentation. Ethical guidelines dictate that, where possible, animal experimentation should be replaced, reduced, or refined (3R principle). The cosmetic industry has been greatly affected by the restrictions imposed on animal testing. Since 2009, the European Commission has been approving regulations on cosmetics, establishing a testing and marketing ban: a prohibition against testing finished cosmetic products or ingredients on animals and commercializing any cosmetic product or ingredient that has been tested on animals within the European Union [10]. The European Centre for the Validation of Alternative Methods (ECVAM) was established in 2010 as a reference laboratory for researching and validating alternative methods, following 3R principles [11]. In 2013, a full marketing ban was put in place for all human health effects tested in animals, including repeated-dose toxicity, reproductive toxicity and toxicokinetics, irrespective of the availability of alternative non-animal tests.

The combinatory effect of the high prevalence of skin diseases, R&D decline and restrictions on animal testing pressured the development of physiologically relevant skin models that could replace conventional, inefficient approaches. Recently, 3D cell culturing techniques have improved the relevance of the available models and demonstrated the synergistic effects that different cell types have on each other [12]. These models can be assembled into complex structures to simulate more physiologically relevant conditions. Both reconstructed human epidermis (RHEm) and full-thickness skin models (FTSm) have been used for many applications including basic, pharmacological and cosmetic research. Innovative techniques such as 3D printing and scaffolds are promising approaches to increase the relevance of these models. However, current tissue-engineered skin models still fall short of the desired controllability and are deficient in several essential key components of the in vivo skin. In particular, their lack of vascularization results in restricted nutrition supply, cell–cell and cell–extracellular matrix (ECM) interactions.

The need for physiologically relevant and functional tissue models led to new technologies for cell cultures such as organ-on-a-chip (OoC) or microphysiological systems. This modern technology aims to surpass the limitations of the 3D cell-based culture platforms and increase the predictive power of in vitro models[13]. These systems have the potential to achieve experimental controllability and reproducibility similar to 2D cell culture while allowing for

increased physiological relevance and complexity. In the last few years, OoC technology has been used to recreate advanced biomimetic skin models, known as skin-on-a-chip (SoC) models [14].

2. Two-Dimensional Skin-on-a-Chip Models

One approach to mimic the human skin's structure and functional responses consists of culturing layers of 2D cells on-chip, mimicking different skin compartments. Using this approach, Wufuer et al. proposed a model consisting of three layers (epidermal, dermal and vascular) co-cultured inside a SoC device [15]. A porous membrane separated each layer to allow interlayer communication. A model of skin inflammation was generated by perfusion with tumor necrosis factor (TNF-α), followed by measurement of proinflammatory cytokines levels and tight junction analysis. The efficacy of the drug dexamethasone was evaluated using the developed inflammation model. The study demonstrated that this drug could attenuate the effects of TNF-α including endothelial barrier dysfunction.

In the same year, Ramadan et al. described a SoC to develop an immune-competent in vitro skin model [16]. The model included a confluent layer of a human skin keratinocyte cell line (HaCaT), cultured on a porous membrane acting as a model of the epidermis and immune cells (leukemic monocyte lymphoma cell) positioned beneath the membrane. Silver/Silver chloride (Ag/AgCl) wires were inserted into the platform to measure the TEER, thereby monitoring the cell layer integrity through the course of cell culture and in response to chemical/physical stimuli. The group found that the perfusion-based culture promoted the barrier function and the lifespan of the cellular system. Furthermore, they investigated the effects of lipopolysaccharides (LPS) and the effects of UV radiation stimulus on the developed model. These experiments allowed the understanding of the role of the human keratinocyte layer as a protection barrier.

Sasaki et al. developed a photolithography-free device to culture an HaCaT monolayer and perform permeation assays [17]. The group developed a simple platform without using complex microfabrication techniques, which could be a barrier to some researchers. A porous membrane was sandwiched between branched microchannels and bonded using a PDMS mortar. The group tested the effect of potassium dichromate on the permeability of the cell monolayer by introducing fluorescein isothiocyanate-dextran (FITC-Dextran) solution on the top channel.

In the previously referred 2D SoC models, the skin cells were cultured directly in the microfluidic chip (in situ) to simulate the different skin components. These models have been successful in simulating diseases and their interaction with the immune system. However, they do not represent the complexity and 3D architecture of the native human skin.

3. SoC Models Based on 3D Cell Cultures

In the last years, various groups developed OoC devices for 3D skin tissue formation inside the platform. For this, two different approaches have been used; one based on the construction of dermal compartments with perfusable lumens, mimicking the vascular networks and the other based on the use of channels, usually designed on a PDMS layer, below the skin tissue.

3.1. Models with Perfusable Lumens

FTSms with perfusable lumens have been generated using multiple 3D patterning techniques such as 3D printing, templating and sacrificial molding as well as cell-based approaches. The first reconstructed FTSm with a perfusable network was generated by Groeber et al. [18]. The group combined a biological vascular scaffold (decellularized segment of porcine jejunum) with a tailored bioreactor system to form a differentiated FTSm. Additionally, endothelial cells lined the walls of the development vessels to generate in vivo-like vasculature. However, the use of an animal organ limits the use of this model in large-scale production.

Abaci et al. developed a FTSm with vascular networks micropatterned by 3D printing sacrificial channels of cross-linked alginate [19]. These microchannels were embedded in the dermal compartment composed of collagen type I and were subsequently removed using sodium citrate after epidermal cornification. The group introduced either endothelial cells derived from iPSCs or HUVECs to cover the inner surface of these channels. These cells made it possible to recapitulate the endothelial barrier function, decreasing the permeability and diffusivity of the channels when compared to the control. Furthermore, the group grafted the vascularized human skin models to immunodeficient mice. It was possible to obtain neovascularization during wound healing, showing its potential for the treatment of cutaneous wounds.

Mori et al. fabricated a culture device using 3D templating techniques [20]. The group developed a device with anchoring structures and nylon wires strung across the connectors. A collagen structure was fixed into the device and perfusable vascular channels were created by removing the nylon wires. The group could recreate some features of the dermal/epidermal morphology and in vivo tight junctions on the vascular channel. However, this technique resulted in only one microchannel and lacked a microvascular network in the dermis. Moreover, the contraction of the collagen used for the dermal compartment affected the permeation assay, which had to be restricted to the central portion of the model.

An alternative approach for generating perfusable vascularized human skin models consists of the use of 3D bioprinting. Kim et al. used this technique for engineering a complex vascularized 3D human skin composed of epidermis, dermis and hypodermis [21]. For vasculature generation, a bioink composed of gelatine, glycerol, and thrombin with embedded endothelial cells was printed onto the hypodermal compartment; once finished, the construct was incubated at 37 °C, eliminating the gelatine and leaving hollow tubes inside the tissue. Proper tissue formation and maturation were reported, along with good vascular permeability properties. Still, the vasculature in the model was limited to one microchannel and lacked microvascularization.

Recently, Salameh et al. used 3D templating techniques to develop a vascularized FTSm that includes a more complex vasculature system than the previously described models [22]. The technique used to produce hollow channels inside the collagen matrix was similar to the work by Mori et al., using nylon wires [20]. However, to induce vasculogenesis, an additional step was included by seeding HUVECS transduced with Turbo-RFP lentiviruses (RFP-HUVECS). After removing the nylon wires, the hollow channels were seeded with HUVECS, and perfusion was initiated using a peristaltic pump. It was possible to generate a differentiated epidermis, perfusable vascular

channels with angiogenic sprouts and an adjacent microvascular network. Furthermore, the potential of this model for topical and systemic applications was validated. Two compounds (caffeine and minoxidil) were topically applied to measure skin permeability and a pollutant (benzo[a]pyrene) was systemically applied.

3.2. Models with Basal Perfusion

More recently, multiple SoC devices used microfluidic-based techniques to develop FTSms with basal perfusion. Lee et al. created a device with a simple structure and gravity-induced flow system to generate 3D FTSms [23]. The chip consisted of two layers of PDMS assembled on top of a glass base. The bottom PDMS layer consisted of the fluidic chamber, and the top PDMS layer had a central chamber for the skin model formation. A polycarbonate membrane was bonded between the bottom and top layers. The skin model was composed of collagen and primary human dermal fibroblasts mimicking the dermal compartment and primary human epidermal keratinocytes representing the epidermal layer. In addition, HUVECs were cultured on the bottom-side surface of the membrane to represent the vascular structure. The group observed comparable expression of keratin 5, involucrin and filaggrin when compared with conventional, transwell-based skin models. However, the *stratum corneum* of the perfused skin was less homogeneous than the conventional model.

In the same year, Song et al. used the described platform to compare the shrinkage of collagen between static and dynamic systems [24]. The group also studied the expression of key marker proteins (fibronectin, collagen IV and keratin 10). They observed that the contraction of the hydrogel was lowest in the dynamic system. However, the markers were less expressed in the SoC model. A similar platform was also used for studying skin aging and the effect of different drugs and formulations. By inserting permanent magnets into a dedicated cavity on the PDMS layer and applying an external electromagnetic field Lim et al. could stretch the membrane attached to the polymer and develop a wrinkled skin [25].

Strüver et al. developed a perfusion platform to apply mechanical forces and shear stress to in vitro skin models [26]. They used bovine collagen and primary fibroblasts to develop the dermal compartment followed by the addition of primary keratinocytes on top of the matrix. After 1 day under submerged conditions, the skin models were transferred to the developed platform and grown for 6 days at the ALI. The device was constructed to be compatible with 6-well cell culture inserts. To assess the barrier function of the skin models, skin permeability studies were performed using reference substances (testosterone and caffeine). The perfusion resulted in the thickening of the *stratum corneum* and a higher compaction of the dermis equivalent. Furthermore, the group observed increased expression of filaggrin and involucrin following dynamic perfusion. However, it was not possible to observe improved barrier function on the dynamically cultivated skin by performing skin permeability. The group concluded that the perfusion of skin models might not be sufficient to produce an enhanced barrier function similar to in vivo skin.

Most of the previously mentioned studies included the use of animal-derived collagens to produce the dermal compartment. Consequently, they reported the poor mechanical properties resultant from the fibroblast-mediated matrix contraction and matrix degradation. These phenomena decreased reproducibility and limited the SoC

lifespan as well as its applicability. Moreover, the groups also reported a lack of attachment of the culture to the membrane and chip walls due to acute shrinkage.

Recently, to overcome these problems, chemical and physical modifications of the matrix adding synthetic or natural polymers were proposed. Sriram et al. developed a model based on the use of fibrin that could overcome the typical limitations (e.g., low mechanical stability and contraction) of current SoC devices that use animal-derived hydrogel for the dermal compartment [27]. As both pure collagen and fibrin present poor mechanical stability, the group developed a modified biomaterial by combining fibrinogen with PEG. This mixture was combined with fibroblasts and the gelation was initiated by adding human thrombin. The volume of the final mixture to be pipetted into the SoC device was optimized to produce a flat surface for keratinocyte seeding. The device was composed of a multi-chamber microfluidic chip consisting of two fluidic compartments separated by a permeable microporous membrane. The experimental setup also included interchangeable lids and insets to switch from a bioreactor to an in vitro analysis system. Using the developed device, the group generated a stratified epidermis with an enhanced basement membrane. In particular, the deposition of collagen IV, VII and XVII were enhanced when compared with the control. The TEER values were also higher than the controls, pointing to an enhanced barrier function.

Recently, a different approach for the development of a stable and physiologically relevant dermal compartment was presented by Zoio et al. [28][29]. The group used rapid prototyping techniques to develop a modular device with integrated electrodes for TEER measurement. The reported approach combined the production of a fibroblast-derived matrix (FDM) with an inert polystyrene porous scaffold integrated on-chip, excluding exogenous hydrogels and membranes. TEER was measured in situ during skin culture and to evaluate the impact of a benchmark irritant onto the skin barrier. The dynamic flow resulted in increased synthesis and deposition of FDM proteins (collagen I and fibronectin). The developed SoC presented increased thickness and enhanced barrier function compared to the controls.

Valencia et al. developed a 3D skin model including a dermal and epidermal compartment using an innovative approach [30]. The group developed a controlled parallel flow method to generate a bilayer tissue by using syringe pumps. The HaCaT cell line and HFs were used to generate the model. In a similar approach to Sriram et al., the group used human fibrinogen to form a fibrin hydrogel for the dermal compartment. Thrombin and tranexamic acid were added to the fibrinogen to prepare a pre-gel (non-gelled fibrinogen solution). The device was fabricated using an edge plotter and included two PMMA layers, one PDMS layer, vinyl upper and lower chambers and an integrated polycarbonate membrane. Contrary to conventional systems where cells are manually pipetted into the culture channels, the group was able to inject all components (cells and ECM) directly into the channels using syringe pumps. With the developed approach, it was possible to obtain a 3D dermal compartment with HFs and HKs on top to simulate the epithelial compartment. Although this is a promising approach that could increase automatization and standardization, no differentiated epidermis was produced, and no air-liquid interface was established.

Finally, Rimal et al. developed a 3D vascularized FTSm with basal dynamic flow using a custom-built 3D-printed bioreactor [31]. The group generated a scaffold-free dermal compartment using cell coating techniques. Primary fibroblasts were coated with a thin layer of ECM (fibronectin and gelatin) and mixed with endothelial cells. Primary keratinocytes were added on top of the generated dermal compartment. The group reported enhanced epidermal barrier properties and ECM deposition (fibronectin and collagen I) under dynamic flow. Furthermore, flow culture resulted in a significant increase in skin thickness compared to static cultures. The group tested the applicability of the developed model by performing 3D wound healing assays in the absence and presence of flow. A faster wound healing in flow culture was observed.

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