Advances in Microengineered Platforms for Skin Research

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The skin plays a critical role in human physiology, acting both as a barrier to environmental insults and as a window to environmental stimuli. Disruption of this homeostasis leads to numerous skin disorders. Human and animal skin differ significantly, limiting the translational potential of animal-based investigations to advance therapeutics to human skin diseases. Hence, there is a critical need for physiologically relevant human skin models to explore novel treatment strategies. Recent advances in microfluidic technologies now allow design and generation of organ-on-chip devices that mimic critical features of tissue architecture. Skin-on-a-chip and microfluidic platforms hold promise as useful models for diverse dermatology applications. Compared with traditional in vitro models, microfluidic platforms offer improved control of fluid flow, which in turn allows precise manipulation of cell and molecular distribution. These properties enable the generation of multilayered in vitro models that mimic human skin structure while simultaneously offering superior control over nutrient and drug distribution. Researchers have used microfluidic platforms for a variety of applications in skin research, including epidermal–dermal cellular crosstalk, cell migration, mechanobiology, microbiome–immune response interactions, vascular biology, and wound healing. In this review, we comprehensively review state-of-the-art microfluidic models for skin research. We discuss the challenges and promise of current skin-on-a-chip technologies and provide a roadmap for future research in this active field.

Keywords: In vitro culture, Microfluidics, Organ-on-a-chip, Skin models

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INTRODUCTION

The skin is arguably the largest organ in the human body and plays an active role in body temperature homeostasis, vitamin synthesis, microbiome regulation, and somatosensory function (Bikle, 2011; Gallo, 2017; Romanovsky, 2014). Thus, disorders that affect skin integrity can lead to life-threatening situations. In this context, skin diseases have a broad pathophysiology, ranging from genetic alterations to autoimmune disorders and skin cancer. According to global disability-adjusted life years (DALYs) (2013), skin and subcutaneous diseases rank the 18th leading cause of global disease burden, and skin diseases are the fourth leading cause of disability worldwide. The DALY report shows that skin conditions account for 1.79% of the global burden of diseases (Karimkhani et al, 2017). Overall, skin diseases impact >84 million people in the United States, causing $75 billion in medical and drug costs. Skin disease–related deaths are reported to occur 5 years younger (68.2 years) than the average age of death for all causes (Lim et al, 2017). Thus, there is a need for better therapies for numerous skin diseases. However, most agents and therapies that show promise in preclinical tests fail in human clinical trials, often simply owing to a lack of efficacy (Van Norman, 2019). This has prompted the United States Food and Drug Administration to recently announce that new medicines no longer require animal tests before human drug trials (FDA Modernization Act 2.0)

Traditional 2-dimensional (2D) cell culture consisting of a monolayer of cells (eg, epidermal keratinocytes, dermal fibroblasts) has been the cornerstone of cutaneous biology investigations for decades. A key limitation of 2D skin cultures is their inability to replicate the intricate interactions between cells and tissues (Jensen and Teng, 2020). In recent years, researchers have explored more advanced 3-dimensional (3D) culture methods such as the generation of organoids. These models can mimic some structural complexity observed in human skin. However, they still lack relevant biological structures such as vasculature, which play an important role in disease pathogenesis (eg, inflammatory sequalae, metastasis) and drug delivery. In addition, imaging 3D organoids is challenging even with state-of-the-art optical microscopy (eg, multiphoton microscopy) because of their multilayered architecture and thickness, which lead to severe light scattering (Graf and Boppart, 2010; Bhatia and Ingber, 2014) (Figure 1a).

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Abbreviations: 2D, 2-dimensional; 3D, 3-dimensional; BBB, blood–brain barrier; DALY, disability-adjusted life year; DRG, dorsal root ganglion; ECM, extracellular matrix; FFA, free fatty acid; HF, hair follicle; HSE, human skin equivalent; HSV, herpes simplex virus; HUVEC, human umbilical vein endothelial cell; IC-SoC, interface-controlled skin-on-a-chip; iPSC, induced pluripotent stem cell; LC, Langerhans cell; PDMS, polydimethylsiloxane; SoC, skin-on-a-chip; vHSE, vascularized human skin equivalent

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Animal models, specifically rodent models, have significantly advanced our scientific knowledge in skin biology. However, preclinical drug testing in these models costs around $2−4 million for each candidate agent, with a high attrition rate (Van Norman, 2019). These models are time consuming and labor intensive. Most importantly, mouse skin is significantly different from human skin. For example, the human epidermis is significantly thicker than its mouse counterpart (Yun et al, 2018) (Figure 1b). In addition, animal models pose an ethical challenge. In 2013, the European Commission imposed a full marketing ban on cosmetic products or drug candidates tested on animals on the basis of the 3R (replacement, reduction, and refinement) principle, which states that animal experimentation should be replaced, reduced, or refined (Gao et al, 2022). Therefore, to maximize the translational potential of these new therapies, tools and models used in preclinical research should capture the architecture and complexity of human skin. These new models should include aspects such as blood flow delivering nutrients, cytokines, and potentially cells in a way that closely mimics in vivo conditions. In this context, recent advances in microtechnologies have led to sophisticated microfluidic models for skin research. Microfluidic devices excel at controlling fluid flow and generating 3D culture systems that mimic tissue anatomy. Microfluidic models, also known as microphysiological systems or organ-on-a-chip models, could provide new tools to study the phenotypic changes in diverse skin pathologies and response to therapies. These models excel at mimicking tissue microarchitecture and cellular organization. In the context of skin research, microfluidics has been leveraged to generate in vitro systems that mimic human skin architecture, including the presence of a stratified epidermis with keratinocytes and melanocytes and a dermal compartment with human fibroblasts embedded in a 3D extracellular matrix (ECM) provided with perfusable vasculature. Consequently, microfluidic models

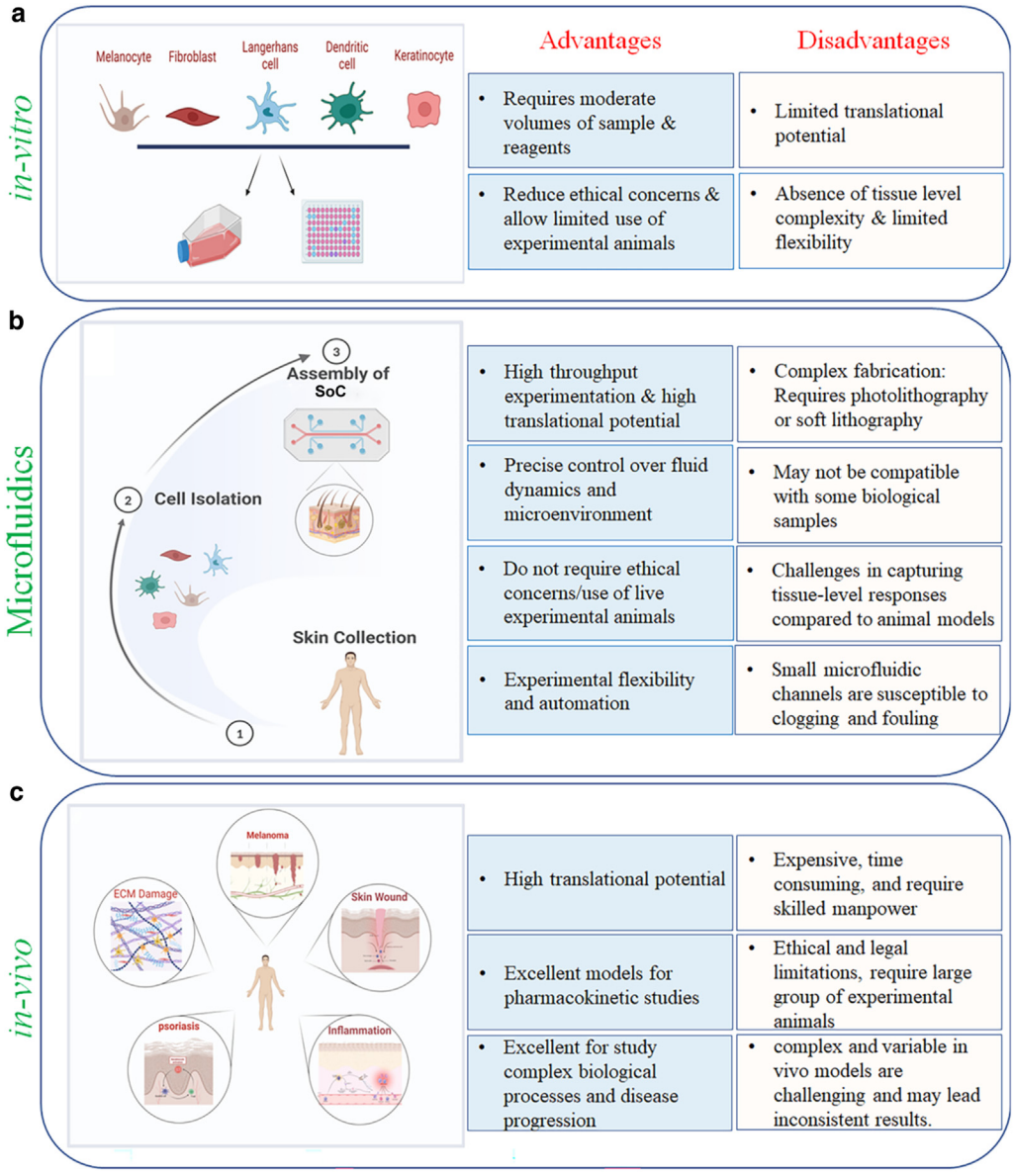


Figure 1. Different skin research platforms. Overview of preclinical and clinical skin research models used to study skin biology is presented. Shown is an illustration of multiple model systems currently used (in vitro/microfluidics/in vivo platforms) to study skin biology and skin pathologies with their advantages and disadvantages. (**a**) The illustration shows conventional 2D cell culture system with different skin cells, the advantages, and disadvantages of cell culture. (**b**) Schematic shows the construction of SoC platform using skin cells or direct skin biopsy and the advantages and disadvantages of microfluidic SoC platforms. (**c**) In vivo skin research models and their advantages and disadvantages. The schematic was created using BioRender. 2D, 2-dimensional; SoC, skin-on-a-chip.

offer a new approach to study diseases or pathophysiological situations where skin structure or skin cell communication is relevant, including wound healing, skin cancer, or skin immunology. Regarding skin cancer, microfluidic systems have been used to study how cellular crosstalk modulates tumor progression, showing that keratinocytes and fibroblasts play an active role in melanoma progression (Ayuso et al, 2021a; Sadangi et al, 2022). In addition, microfluidic models have been extensively used to study cancer cell migration, intravasation, and extravasation to other tissues, which could be leveraged to study melanoma metastatic cascade (Kim et al, 2022). The skin is an immunologically active organ, with immune cells trafficking in and out of the skin in response to pathogens or other factors (Zhang et al, 2022). In this context, microfluidic devices have been used to study a variety of aspects of the immune response, including immune cell extravasation, chemotaxis, antigen engagement, and pathogen or tumor cell clearance (Abizanda-Campo et al, 2023). Microfluidic devices could also be used to study skin autoimmune disorders such as sarcoidosis or other granulomatous diseases, offering a versatile tool to identify factors driving autoimmune reactions. Finally, microfluidic models also hold promising potential for skin genetic diseases such as epidermolysis bullosa, where genetic variants lead to skin fragility and blistering. These models will offer a versatile system to evaluate new therapeutic strategies such as gene replacement therapy or genome-editing therapy.

What is microfluidics?

Current 2D culture techniques and organoid cultures often lack fluid flow, and when they include it, it is under turbulent conditions (ie, chaotic mixing) as opposed to laminar flow conditions observed in in vivo capillaries. In the macroscale, most fluids exhibit turbulent flow, such as for waterfalls or water used from fire hydrants. Turbulent flow implies that molecules in the fluid move in irregular pathways, often leading to mixing and complex particle trajectories within the fluid stream. Turbulent flow is often leveraged in cell and molecular laboratories during pipetting steps, allowing easily mixing of multiple liquids reagents and substances. Conversely, microfluidics is the study of fluid dynamics at the microscale (submilliliter). At the microscale, fluid behavior becomes laminar, with particles moving in linear and predictable patterns. The highly predictable nature of laminar flow allows the user to finely control particle displacement and location by manipulating fluid flow through the system.

How microfluidic devices leverage laminar flow

Thus, researchers have used laminar flow in microfluidic devices to control cell and molecular (eg, proteins, GFs) patterning within microfluidic devices (Sackmann et al, 2014; Whitesides, 2006). This predictability has been leveraged to generate sophisticated microfluidic devices that include multiple cell types and ECM proteins spatially organized in a pattern that mimics the human tissue architecture, which has led to the concept of organ-on-a-chip model (Li et al, 2012; Ma et al, 2021) (Figure 1c). Microfluidic devices present several critical advantages compared with other traditional in vitro models such as transwells or organoids. In vivo, cells are often organized in thin layers of dozens or few hundreds of microns (eg, epidermis, brain cortical layers), which facilitates cellular crosstalk and coordination. Although traditional in vitro systems (eg, transwells) offer limited control over layer thickness, the capacity of microfluidic devices to generate thin (ie, few μm) cellular and molecular layers becomes highly relevant. This property has been leveraged in numerous studies evaluating cell–cell signaling in a variety of applications such as tumor-induced angiogenesis and immune cell recruitment (Ayuso et al, 2021a). In addition, microfluidic devices allow us to control system geometry, generating cylindrical, rectangular, or trapezoidal structures. In this context, previous studies have demonstrated that cells can sense the curvature in their surrounding microenvironment, leading to noticeable biological effects. Endothelial cells secrete more proangiogenic factors when assembled in cylindrical microvessels as opposed to other spatial configurations such as the traditional flat monolayers used for decades. Microfluidic models also allow continuous medium perfusion to mimic the blood flow or interstitial flow that occurs in most tissues. Numerous mechanobiology studies have shown the importance of medium flow and shear stress (ie, forces generated by fluid flow) on multiple biological processes such as angiogenesis, lung breathing, tissue stretching, or microvilli biology. Traditional in vitro models struggle to control fluid flow owing to their turbulent flow, making microfluidic devices an attractive alternative for shear stress and mechanobiology studies. Finally, microfluidic devices are highly compatible with integrated circuits, allowing the incorporation of chemo-electronic sensors to monitor or even manipulate biochemical parameters within the system (eg, temperature, pH, oxygen, glucose) (Azimzadeh et al, 2021). Organoids can achieve high complexity (eg, brain neurospheres), but the user has limited capacity to control the spatial organization within the organoid, and cell monitoring is limited owing to organoid opacity. Microfluidic devices have a limited thickness (often a few hundred microns), offering a versatile alternative to generate complex 3D cultures where the spatial architecture is defined by the user, and optical transparency is preserved. Overall, microfluidic models have superior potential to recapitulate human tissue and organ-level structure while offering improved monitoring capability compared with traditional tools.

Emergence of microfluidic devices for cell culture applications

Early microfluidics studies began in the 1940s and 1950 with the development of optical lithography, and the first-generation microfluidic models were created using silicon or glass. However, these microfluidic devices were expensive, and their fabrication can be time consuming (Ziaie et al, 2004). However, it was in the 1980s when the implementation of soft lithography allowed widespread of microfluidic devices across engineering laboratories. Soft lithography relies on the use of polymers such as polydimethylsiloxane (PDMS), which are cheaper and easier to use and have been widely used to fabricate microfluidic devices, leading to the first experiments with cells in PDMS-based microfluidic devices in the mid-2000s. These microdevices are optically clear, allowing real-time imaging of cellular responses to

different environmental settings (Ren et al, 2014; Xiong et al, 2014). They are particularly well-suited to studying cell migration, metabolism, drug response, and functions that depend on tissue microarchitecture and perfusion (Figure 2). Over the past decade, numerous microfluidic devices have been developed to study a variety of organs and tissues such as liver, kidney, lung, heart, smooth muscle, bone, eye, skin, and brain and have even made attempts at developing multiorgan on chip systems. Yang et al (2023) engineered an advanced integrated gut–liver-on-a-chip platform to replicate the human gut–liver axis in a controlled in vitro environment. This device integrates human gut and liver cell lines through microfluidic channels within a closed-loop system. It was employed to investigate nonalcoholic fatty liver disease by exposing cells to free fatty acids (FFAs) for 1 and 7 days (Yang et al, 2023). The findings showed that when Caco-2 gut-like cells and HepG2 liver-like cells were cocultured, they exhibited protective apoptotic responses to FFAs. In contrast, the monocultured cells showed an induced apoptotic response. Interestingly, both gut and liver cells exposed to FFAs accumulated lipid droplets and exhibited increased gene expression associated with copper ion response and endoplasmic reticulum stress.

Abulaiti et al (2020) have constructed an advanced heart-on-a-chip microdevice featuring dynamic 3D cardiac microtissues derived from human induced pluripotent stem cells (iPSCs) with microelectrochemical microfluidic systems. This microdevice allows visualization of pulsation dynamics of cardiac microtissues and enables the measurement of pressure, force, and fluidic output. The microdevice showed a strong correlation between particle movement and the frequency of electrical stimulation, mimicking patterns consistent with intracellular calcium signaling in cardiomyocytes. In addition, the device exhibited a pharmacological response to a β-adrenoceptor agonist isoproterenol (Abulaiti et al, 2020).

Ahn et al (2020) developed a microfluidic platform to emulate the structure and function of the human blood–brain barrier (BBB). This model offers a comprehensive 3D mapping of nanoparticle distribution in brain vascular structures. The device effectively replicates key BBB features, including cellular interactions, differential gene expression, limited permeability, and a 3D astrocytic network with reduced reactive gliosis and polarized AQP4 expression. Moreover, the model accurately depicts the distribution of nanoparticles at the cellular level and demonstrates receptor-mediated transcytosis through the BBB (Ahn et al, 2020).

Aceves et al (2022) have successfully developed a 3D model of the proximal tubule that allows for perfusion. This model was created using kidney organoid-derived epithelial cells. The platform comprises cylindrical channels embedded with an ECM, promoting the formation of a continuous layer of proximal tubule epithelial cells. Adjacent to each proximal tubule is a second perfusable channel that simulates basolateral drug transport and uptake. This innovative setup has resulted in a notable enhancement in the activity of transporters such as organic cation (OCT2) and organic anion (OAT1/3), consequently leading to improved drug uptake and

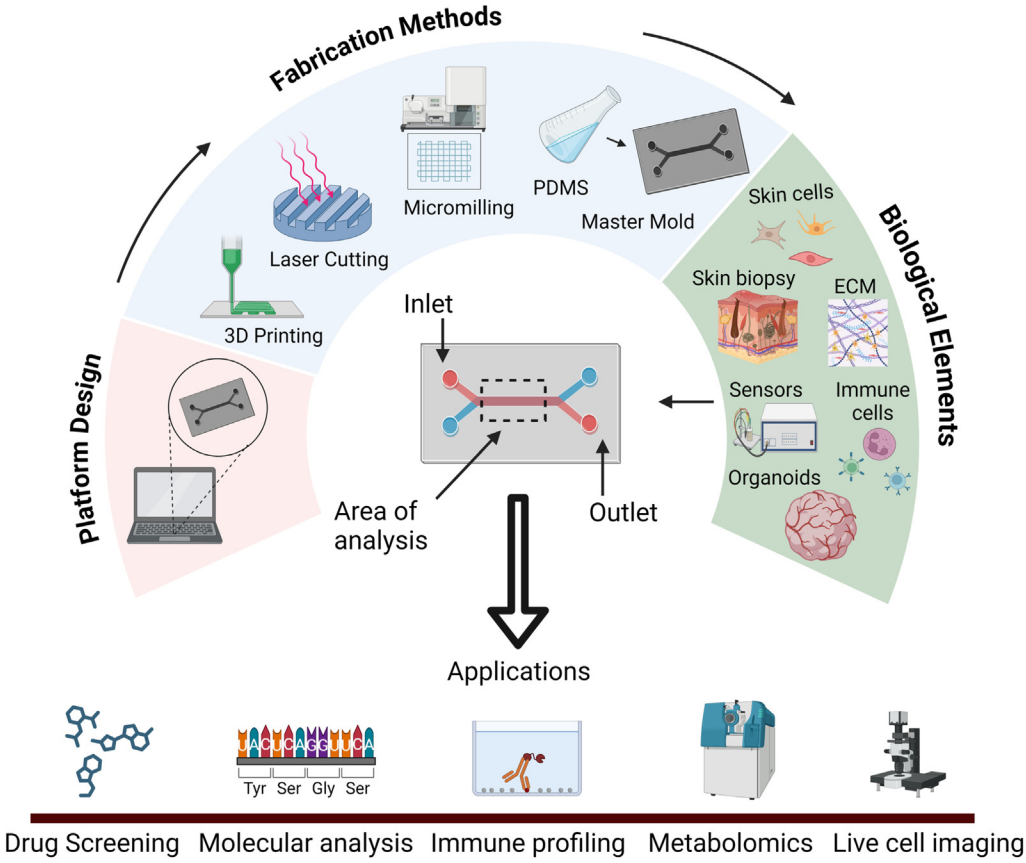


Figure 2. Microfluidic device design, fabrication, and operation. Simple microfluidic devices can be designed in user-friendly software such as Adobe Illustrator or MS PowerPoint, whereas devices with complicated designs and intricate 3D features often require specialized software used in engineering (eg, AutoCAD, Solidworks). Once the microdevice design is finalized, there are several fabrication techniques that can be used, including 3D printing, micromilling, or optical lithography. Each technique provides a unique set of advantages and limitations. Finally, the desired biological elements such as cells or ECM proteins are loaded into the microfluidic device by pipetting them through the inlets. The microdevice is now ready for biological analysis using a broad range of techniques such as optical microscopy (eg, fluorescence, confocal, multiphoton) or molecular analysis (eg, RNA-seq, mass spectrometry, nuclear magnetic resonance). 3D, 3-dimensional; ECM, extracellular matrix; MS, Microsoft; PDMS, polydimethylsiloxane; RNA-seq, RNA sequencing.

observed increase in lactate dehydrogenase release in response to cisplatin and aristolochic acid (Aceves et al, 2022).

MICROFLUIDICS MODELS FOR SKIN RESEARCH: DESIGN AND FABRICATION

Design considerations

In the past decade, microfluidic platforms have become promising tools for overcoming the limitations of cell culture and animal models for human skin research. Microfluidic devices that mimic human skin allow effective high-throughput research with less reagent usage and superior monitoring capability. Microfluidic devices can be designed with considerable flexibility, allowing them to be adjusted to the requirements of the desired cell types, implement cellular cocultures on the same chip, and better simulate the natural tissue milieu of the skin. Nonetheless, there is an increasing need to create microfluidic devices that model complex systems such as the tumor microenvironment in the skin and cellular and architectural complexities in other skin disorders. An important consideration in terms of microdevice design is the size of the structures that will be loaded in the microfluidic device. If large structures such as tissue biopsies are loaded in the device, the design needs to accommodate the size of such structures. Conversely, cells are relatively small compared with tissue biopsies, imposing less limitations in terms of the device size. However, previous studies have shown that monolayers of human keratinocytes must have their apical side exposed to air to induce differentiation into a stratified epithelium (process known as terminal differentiation). During this process, keratinocytes in the basal layer (ie, stratum basale) proliferate, whereas those located on the upper layers gradually undergo several key changes (water loss, lipid content increase, DNA degradation) until they generate multiple strata. Thus, most microfluidic devices for skin research include the presence of an air—liquid interface to induce keratinocyte differentiation. Researchers have leveraged capillary forces to design microfluidic devices that maintain this air—liquid interface at the keratinocyte level, avoiding keratinocyte submersion in media or dehydration due to evaporation at the interface. Capillary forces are often described by the Young—Laplace equation, which considers surface tension and device geometry to predict air—liquid interface behavior within the microfluidic device. Using this approach, keratinocytes are exposed to air to induce the generation of a stratified epithelium, whereas the dermal layer, including dermal fibroblasts and vasculature, remains submerged in the media. This approach allows microfluidic devices to control the geometry and orientation of the air—liquid interface while also achieving similar results in terms of keratinocyte stratification compared with traditional in vitro methods such as air lifting done in transwell plates and other similar platforms (Arnette et al, 2016).

Microdevice fabrication

Regarding microdevice fabrication, there are multiple techniques available, including soft lithography, computer numerical control milling, hot embossing, injection molding, or 3D printing. Each of these techniques presents several advantages and limitations in terms of material choice, fabrication speed, mass production capability, and price (Scott and Ali, 2021). UV/soft lithography presents the highest spatial resolution among the techniques discussed, allowing the generation of features (eg, channels, wells, pillars) of a few micrometer while keeping the costs relatively low. The 3D printing offers rapid prototyping, allowing the user to quickly translate changes in the microfluidic design into the manufactured product. However, 3D printing presents lower spatial resolution, requiring expensive equipment (up to hundreds of thousands United States dollar) to achieve resolution comparable with that of UV/soft lithography (Niculescu et al, 2021). Finally, injection molding offers unparalleled potential for mass manufacturing, generating dozens or even hundreds of devices in a few hours, but the spatial resolution remains limited, and once the injection mold is fabricated, changing the design is complicated (Guckenberger et al, 2015). Overall, selecting the optimal fabrication technique depends on multiple factors and must be considered carefully.

MICROFLUIDIC DEVICES FOR SKIN BIOLOGY: SKIN-ON-A-CHIP MODEL

Numerous microfluidic models have been developed using different technical approaches to study diverse aspects of dermatology, leading to the concept of skin-on-a-chip (SoC) model (Bal-Öztürk et al, 2018; Jeong et al, 2023; Jones et al, 2022; Ponmozhi et al, 2021; Risueño et al, 2021; Sutterby et al, 2020; Varga-Medveczky et al, 2021). The earliest iterations of SoC system relied on directly culturing skin explants (ie, fragment of skin obtained from a tissue biopsy) in microfluidic devices (technique commonly known as transferred skin). In recent years, advances in microfluidic device fabrication have led to the generation of more sophisticated models that use cell suspensions and ECM proteins to generate in vitro systems that mimic the skin microstructure (in situ SoC). Thus, the SoC systems can be divided in transferred and in situ depending on whether they rely on tissue samples or cell suspensions to generate the tissue construct (Figure 2).

Transferred SoC

The early SoC platforms were based on culturing skin tissue fragments within the microdevice. Wagner et al (2013) designed a transferred SoC platform using human skin biopsies and liver micro tissues cocultures exposed to fluid flow. They used human prepuce samples that were cut into 2-mm height, 5-mm diameter skin biopsies. These biopsies were placed in the chambers of the microdevice with the epidermis facing upward. The microfluidic device contained a series of microchannels that run underneath the dermis of the tissue biopsy, allowing the researchers to perfuse medium and study the crosstalk with liver cells. The metabolic crosstalk was assessed by measuring the consumption of liver tissue—produced albumin by the skin tissue in 14-day cocultures. Apoptosis and proliferation were validated using TUNEL and Ki-67 staining. The dose-dependent response to the toxic substance troglitazone indicated that this platform can predict drug response and toxicity (Wagner et al, 2013).

Despite the capacity to maintain various cells of human origin over longer culture periods and the ability of artificial

liver microtissue to mimic some functions, this system does not fully reproduce the functional complexity of human liver. The artificial liver tissue lacks nonparenchymal Kupffer (native immune cells) and sinusoidal endothelial (maintain vascular tone and metabolic homeostasis) cells. Incorporating additional cell types such as Kupffer and endothelial cells into a skin–liver coculture platform would contribute to recreating more physiologically relevant models. In this context, it is worth noting that Kupffer cells play a critical role in particle scavenging and phagocytosis in the liver (Wen et al, 2021), which in turn could affect compound or drug metabolism.

Transferred SoC for applications in skin immunology. Infectious skin diseases, such as impetigo, cellulitis, and abscesses, are very common and often can lead to life-threatening situations such as necrotizing fasciitis. Kim et al (2019) cultured skin biopsies on microdevices to test neutrophil migratory responses to *Staphylococcus aureus* infection and response to penicillin treatment. The PDMS device consists of 3 main components: a column loading channel, a blood loading channel, and a migration region. The epidermal and dermal skin biopsy tissues were aseptically obtained from healthy patients undergoing elective abdominoplasty, with multiple-edged 23G needles inserted into the skin tissue.

Each biopsy was transferred and used on the same day or frozen for downstream use. Bacterial infection was simulated by preincubating the skin biopsy in *S aureus* suspension before loading the skin biopsy into the microfluidic platform. Next, whole blood was loaded into the SoC, and neutrophil migration in response to *S aureus* and penicillin treatment was monitored. Their study showed that antibiotic treatment of *S aureus*–infected skin reduced neutrophil migration toward the skin. This study is relevant to both human skin and soft tissue infections (Kim et al, 2019). Studying the migration of neutrophils in response to bacterial infection may not be sufficient to capture the complexity of the immune cascade in the skin. Multiple studies have shown that Langerhans cells (LCs), epidermis-resident macrophages, play an important role in skin inflammation (Rajesh et al, 2019). Coculturing LCs with neutrophils could have provided better knowledge of the skin's immune response to cellulitis. Overall, skin immunology is a complex process that involves a variety of cell types trafficking in and out of the skin. Thus, SoC with intact skin samples may provide a versatile technology to monitor these complex cellular and molecular interactions that occur within the skin or between the skin and other organs.

Transferred SoC in multiorgan microfluidic models. A complex multiorgan-on-chip model was constructed using human skin, intestine, liver, and kidney coculture on a microdevice platform. Human prepuce biopsies (4.5-mm diameter, 2-mm thickness) were obtained by punching tissue samples with biopsy needles. Skin biopsies were stored at 4 °C in PBS for no more than 4 hours before their use in the microfluidic device. Skin biopsies were placed with the epidermal side facing upward into 96-well Corning Transwell inserts and subsequently positioned into the designated culture compartment of the multiorgan system. This complex coculture model showed high cell viability, functional crosstalk, and fluid dynamics over the entire 28-day coculture period independent of donor background. This is a useful model for testing the systemic toxicity of drug candidates (Maschmeyer et al, 2015). However, the transferred SoC has certain limitations. Despite the advancements, current SoC platforms still need to address a few critical factors to fully recapitulate human skin cellular and functional characteristics such as vascular perfusion. Moreover, the skin biopsy collection remains an invasive procedure and may cause scarring to the donor. Finally, models relying on skin biopsies often have limited potential to modify the biochemical and physical parameters of the tissue construct because these are defined by the skin biopsy donor.

In situ SoC

This method relies on generating a skin-like system using a bottom-up approach, where individual cell types are cultured in a microfluidic device to capture features of intact human skin. Lee et al (2017) created a gravity-driven perfusable SoC made of PDMS with a chamber for the 3D culture of human dermal fibroblasts, a microfluidic channel for human umbilical vein endothelial cells (HUVECs), and a supporting membrane to model the vascular structure. The culture conditions revealed that flow is critical in cell viability after 10 days of air exposure. In a following study, Song et al (2017) used the same platform to define how collagen from various sources affected human primary keratinocyte and fibroblast differentiation, showing that rat tail collagen was superior to porcine and duck feet collagen. Abaci et al (2015) developed a sophisticated microfluidic platform engineered to support the long-term maintenance of full-thickness human skin equivalents (HSEs), encompassing both the epidermal and dermal layers (Figure 3a). Their microdevice included 2 vertical PDMS chambers separated by a porous membrane to separate the dermal and epidermal compartments culture medium and nourish the dermal cell. Cells cultured in the epidermal upper compartment were exposed to air to induce keratinocyte maturation and terminal differentiation into a stratified epithelium. Their platform allowed media recirculation through the bottom compartment to mimic blood flow, and the HSE was maintained for at least more than 3 weeks in culture. The authors used the model to evaluate the effect of genotoxic compounds on skin cells and skin structure (Abaci et al, 2015). Overall, these models focused on mimicking the structure of normal skin, providing new in vitro platforms for development and toxicology.

In addition, the in situ SoC platforms advanced our understanding of cellular behavior in pathological situations such as cutaneous melanoma. Ayuso et al (2021b) and Sadangi et al (2022) demonstrated that multiple secretory factors from human dermal fibroblasts and keratinocytes modulated primary melanoma cell metabolism, migration, and senescence on a PDMS microdevice. The platforms discussed in this section focused on keratinocyte and dermal fibroblast biology, with some of the studies also including melanocytes in their models. However, human skin also includes other cell types and biological structures such as

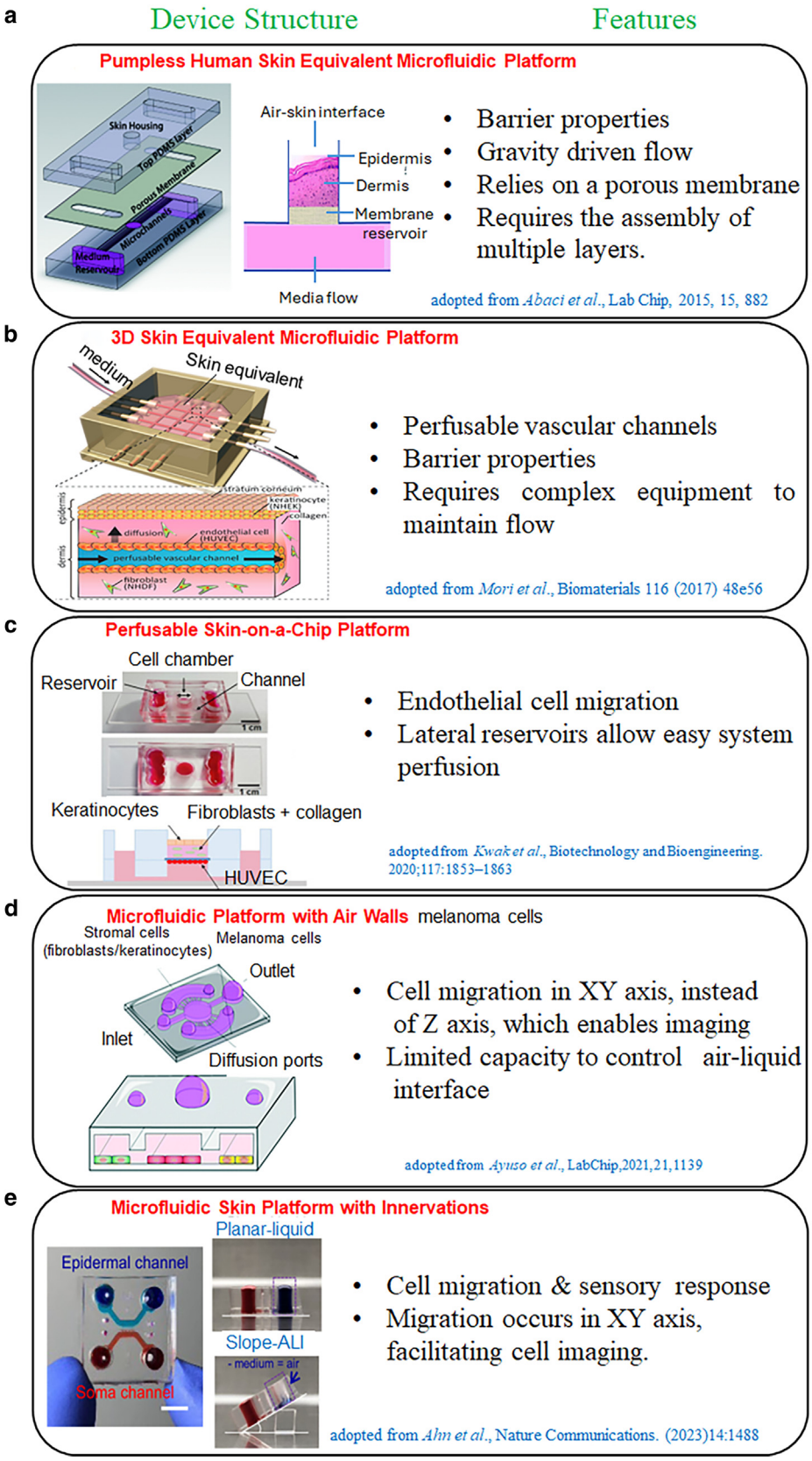


Figure 3. Different microfluidic skin platforms and their features. The schematic diagram shows different types of microfluidic platforms (**a**–**e**) used for different skin research studies. The diagram highlights different features of the platforms that are helpful for studying different skin pathologies.

vasculature, immune cells, nerves, sweat glands, or hair follicles (HFs). Thus, in the following sections, we discuss other SoC platforms that integrate some of these complex features, which would help advance our understanding of skin pathophysiology.

Modeling dermal vasculature

Skin vasculature plays a critical role in both normal skin physiology and disease pathogenesis. These roles include metabolic support, immune cell circulation, cancer metastasis, and wound healing (Detmar, 2000; DiPietro, 2016). Modeling skin in microfluidic devices with vasculature improves their general and translational utility. Groeber et al (2016) generated a vascularized skin equivalent by combining a biological vascularized scaffold, seeded with human fibroblasts, keratinocytes, and microvascular endothelial cells, and a bioreactor system. After 14 days with the air—liquid interface, histology revealed human skin—like architecture with dermal—epidermal junctions (Groeber et al, 2016). In another study, Marino et al (2014) engineered human blood and lymphatic vessels in tissue grafts using 3D hydrogels. Similar to normal lymphatics, these capillaries showed branching after transplantation onto immunocompromised rodents (Marino et al, 2014). The disadvantage of these methods is that these vascular components cannot be perfused with an external pump.

This limitation was resolved by Mori et al (2017), who reported fabrication of perfusable vascular channels coated with endothelial cells within a cultured skin equivalent by connecting the platform to an external pump (Figure 3b). These vascular channels mimic the morphology of human skin vasculature with tight junctions and served as a nutrient-delivery system. The cell density and viability in perfused skin equivalents were higher than those in nonperfused skin equivalents (Mori et al, 2017). Jones et al (2022) also showed similar results with vascularizing SoC platform.

Recently, Rimal et al (2021) constructed a more advanced vascularized HSEs (vHSEs) using a dynamic flow culture system. They tested the effect of dynamic flow on durability of vHSEs using a custom 3D bioreactor. It was concluded that dynamic flow culture restored tissue homeostasis and improved skin barrier properties, epidermal differentiation, and ECM modulation. Flow culture improved wound healing over static cultures (Rimal et al, 2021). As discussed earlier, the vascular system provides immune cells with a route to traffic into and out of the skin. Establishing methods to incorporate vascular structures in microfluidic models would significantly expand the potential of SoC platforms to study immune cell trafficking in and out of the skin. This is a critical requirement for investigations on autoimmune skin disorders, infection, and skin cancer.

Incorporation of immune components. Numerous skin disorders have an underlying local or systemic immune component. Therefore, understanding the reciprocal relationship between immune cells and skin cells in the pathophysiology of inflammatory skin disorders remains a highly active area of research (Nguyen and Soulika, 2019). The first immunocompetent skin construct with LCs was developed by Facy et al (2004) to recapitulate LC response to UVR. Later, Kwak et al (2020) developed a perfusable microfluidic skin platform by coculturing immune cells with human dermal fibroblasts, keratinocytes, and vascular endothelium. In this system, neutrophil transmigration could be seen upon stimulation with chemicals and UV irradiation (Kwak et al, 2020) (Figure 3c).

More complex skin constructs were developed recently by combining immune and stromal components (Figure 3d) as well as vasculature to recapitulate human skin response. In this direction, Ren et al (2021) successfully developed a PDMS-based microfluidic 3D SoC model that relied on confining a collagen hydrogel in a microfluidic chamber to subsequently seed endothelial cells and skin epithelial cells on each side, respectively. Human immortalized keratinocytes (HaCaT) and HUVECs were loaded into each side of the hydrogel to generate endothelial and epithelial layers, respectively. Inflammation was simulated perfusing TNF-α through the epithelial side (ie, skin chamber), which induced a chemotactic process in human T cells. Their platform enabled quantitative studies of transendothelial and transepithelial migration of chemokine-induced human T lymphocytes (Ren et al, 2021). The major limitation of this platform was the use of HaCaT cells, which are immortalized keratinocytes that show certain phenotypic changes compared with normal keratinocytes. These phenotypic changes include differences in their capacity to establish a cell barrier, which is arguably the primary function of primary keratinocytes. The interaction of other immune cells with the skin is unclear.

Quan et al (2022) constructed a biomimetic interface-controlled SoC (IC-SoC) system with an air—liquid interface using skin ECM and skin cells to test the drug candidates for an inflammatory response during skin infections. Their results demonstrated that IC-SoC improved skin differentiation and barrier function. Treatment with polyphyllin H antagonized *Propionibacterium acnes* infection, inhibited innate immune response, and improved barrier function (Quan et al, 2022). Furthermore, Sun et al (2022) constructed a complex vascularized 3D SoC capable of immune-cell and drug perfusion. Herpes simplex virus (HSV) infection resulted in the production of IL-8 and rapid transendothelial extravasation and directional migration of neutrophils. Importantly, perfusion with the antiviral acyclovir inhibited HSV infection in a dose- and time-dependent manner (Sun et al, 2022). These recent advancements could help better understand the immune system involvement in various inflammatory skin disorders and skin cancers.

Innervation and nervous system. Interactions between the nervous system and different skin cells are critical in normal skin physiology and pathophysiology. Incorporation of sensory neurons into in vitro skin constructs is necessary for modeling wound healing and generating better skin grafts (Laverdet et al, 2015). Early attempts at innervating skin-like constructs relied on collagen hydrogels, which had limited innervation potential (Biedermann et al, 2013). Collagen-chitosan tissue-engineered skin constructs have shown improved innervation after being transplanted into nude mice (Gingras et al, 2003). However, chitosan-based engineered skin presents critical differences with human skin in terms of mechanical properties (eg, lower Young's modulus, inability

to create an impermeable barrier to minimize water loss) and often requires the addition of exogenous GFs to support cell growth and vasculature establishment. In addition, the immunogenic potential of chitosan-based tissue-engineered skin can result in adverse immune reactions after transplantation. Overall, chitosan-based cultures present interesting advantages, but there are some technical challenges that remain to be addressed.

Martorina et al (2017) developed a fully innervated, in vitro human skin model with sensory functions in response to external stimuli. This engineered tissue consists of a fibroblast-derived dermis, a differentiated epidermis, and a network of rat dorsal root ganglion (DRG) neurons. The neuronal connections within the dermal–epidermal layer responded sensitively to capsaicin, an agonist of the transient receptor potential vanilloid, a cationic channel critical for sensory responses in the peripheral nervous system. The quantification of calcium currents demonstrated signal transmission along the neurites and functional dermal–epidermal crosstalk (Martorina et al, 2017).

Because skin–nerve crosstalk is critical for treating certain skin pathologies, recently, skin microfluidics researchers have focused their attention on the growth of the nervous system and innervation of in vitro skin tissue. Ahn et al (2023) constructed a 3D sensory neuron–keratinocyte coculture model on a microfluidic platform using the slope-based air–liquid interfacing culture, which provides an air–liquid interface necessary for epidermal keratinocyte differentiation and spatial compartmentalization (Figure 3e). The hydrogel-based multichannel platform successfully reproduced more organized epidermal differentiation, cell–cell/cell–matrix interactions, and barrier properties. The platform comprises a series of physically separated channels that contain several cell types: a neuron soma channel, a keratinocyte epidermal channel, and axon-guiding microchannels. The axon-guiding microchannels allowed the neuron soma to connect with the epidermal channels, enabling localized axon–keratinocyte interaction studies (Ahn et al, 2023). The authors used this platform to study the effects of diabetes-induced neuropathy on the keratinocyte barrier function. In this study, the model relied on neonatal rat DRG neurons, which may be functionally different from their human counterpart. Future studies could use this technology to include other neuronal types (eg, sensory) from humans to study other interactions between the skin and the nervous system (Figure 4 shows the specific readouts and results obtained with the discussed microfluidic devices).

Neurocutaneous responses in skin tissue are critical during skin pathologies. For example, Wohlrab et al (2022) designed a proof-of-concept model for itch. They showed that Jak inhibitors could directly affect the function of the frontal cortex neurosensory system in response to itching (Wohlrab et al, 2022). The major limiting factor in developing skin models with innervations is the scarcity of human sensory neuron samples. In this context, advances in iPSCs could provide an alternative cell source for these models.

Skin appendages. Human skin appendages are essential in sensory reception, thermoregulation, and lubrication. Thus, skin appendages should be incorporated into the SoC platforms to understand human sensory and wound-healing properties and study conditions such as alopecia and chemotherapy-induced hair loss (Hosseini et al, 2022).

Ataç et al (2013) designed a SoC microfluidic system that provided dynamically perfused conditions. The authors cultured full-thickness HSEs grown in transwells, which were combined in series with the culture of human HFs. Their results showed that HF(s) retained their tissue structure for the duration of these experiments (14 days), with pan cytokeratin and collagen IV double immunostaining revealing that basement membrane, connective tissue, and dermal papilla remained intact compared with those at day 0. Overall, this model highlighted the importance of medium perfusion to maintain HSE and other skin appendages in culture for long periods of time (Ataç et al, 2013). Recently, Lee et al (2020) have successfully generated HFs from human pluripotent stem cells in a complex skin construct with stratified epidermis, dermis, pigmented HFs with sebaceous glands, and neurosensory system. These skin organoids are equivalent to the facial skin of human fetuses and can be successfully grafted onto nude mice. Their model provides a foundation for skin disease modeling and reconstructive surgery (Lee et al, 2020). The generation of fully functional human skin models with appendages is still in its infancy owing to the limitations in mimicking the in vivo stem cell and follicular environments.

Mechanical factors. Skin cells respond to mechanical forces, including external pressure and shear stress, by mechanosensing and translating mechanical forces into biochemical signals (eg, cytoskeletal modifications). These properties are critical in cell differentiation, motility, and apoptosis (Martino et al, 2018; Ohashi et al, 2017). Shear stress is a critical mechanical stimulus to consider in the SoC platform. Particle movement over the skin surface causes shear stress (eg, air and mechanical friction, etc) on skin cells and could have significant effect on human skin cell biology (eg, formation of callous tissue) (Ponmozhi et al, 2021).

Using a PDMS-based platform, Agarwal et al (2019) demonstrated flow-induced shear stress response in epidermal keratinocytes. They observed cytoskeletal modifications and increased junctional protein expression in keratinocytes response to low shear stress (0.06 dyne/cm), whereas higher shear stress (6 dyne/cm^2) disrupted the structure of keratinocytes. Their study, utilizing various mechanotransducer inhibitors, highlighted the critical role of the actin cytoskeletal network in the mechanosensitivity of keratinocytes (Agarwal et al, 2019). Similar studies were performed by Lei et al (2020) on shear stress–induced effects on fibroblasts on different surface topographies. Their findings revealed that a shear stress frequency of 0.6 Hz notably influenced fibroblast alignment, polarity, migration, and adhesion on a planar surface. Conversely, a frequency of 1.0 Hz enhanced the differentiation of fibroblasts into myofibroblasts and led to disorganized fiber deposition around implants (Lei et al, 2020).

In addition to the shear stress, human skin is exposed to cyclic stretch and relaxation resulting from internal factors.

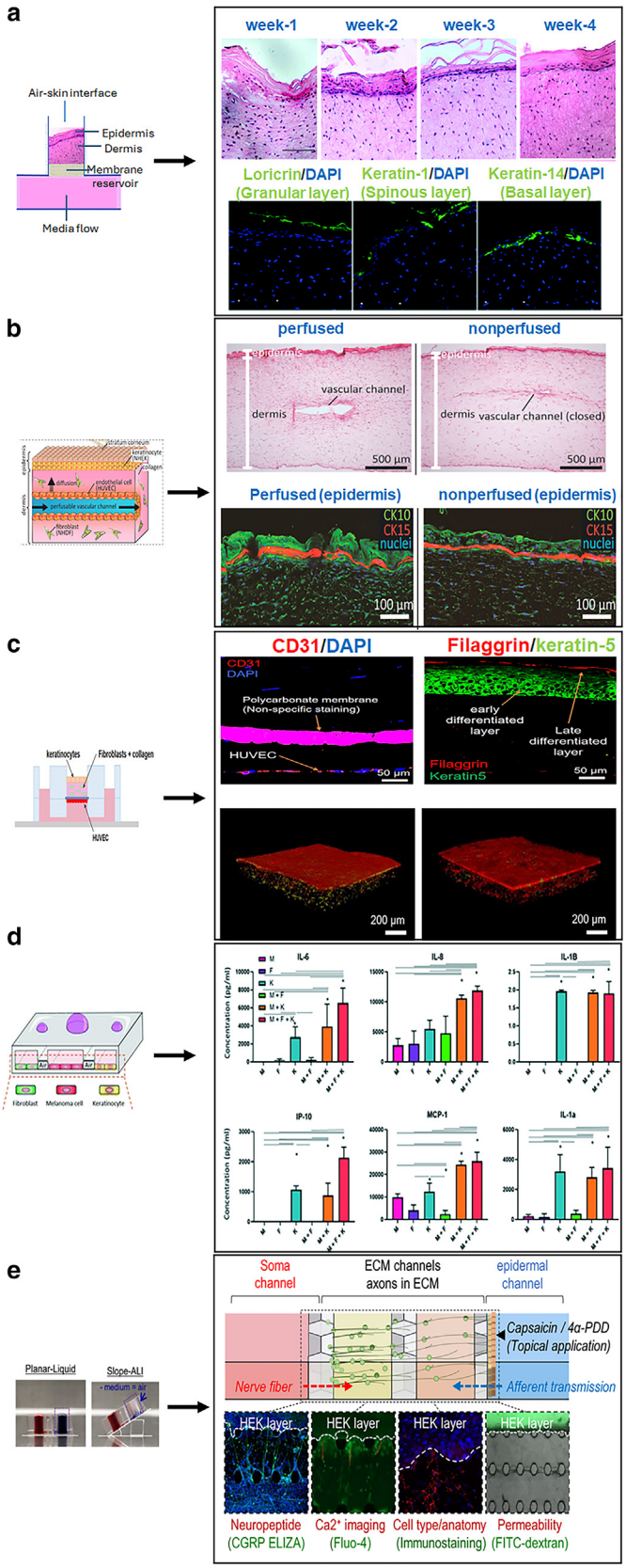


Figure 4. Biological functions of different microfluidic skin platforms. The schematic diagram shows the applications of different microfluidic platforms used for different skin research studies. The diagram highlights the biological function (staining and microscopy imaging) and outcome of different platforms. (**a**) Image representing HSE-on-a-chip platform comprised of fibroblasts and keratinocytes separated by a transmembrane. The rocking platform provides gravity-driven fluid flow and is useful for drug profiling. (**b**) The SoC with perfusable vascular channels comprised of keratinocytes, fibroblasts, and endothelial cells on the PDMS platform. The SoC device mainly helps in understanding barrier properties and metabolic changes. (**c**) The skin chip platform consists of epidermal

Lü et al (2013) showed asymmetric migration of human keratinocytes under mechanical stress (ie, stretching) when cocultured with human fibroblasts in a wound repair model. The asymmetric migration of human keratinocytes appears to be regulated by epidermal GF derived from fibroblasts subjected to mechanical stretch (Lü et al, 2013). The reports discussed in this section were limited to evaluating the effects of mechanical forces on 1 skin cell type, often cultured in isolation on a 2D surface, which neglects the structure of human skin. Future studies could leverage microfluidic models that include HSE(s) to study how human skin cells respond to mechanical stimuli in a physiologically relevant environment.

Sensor integration

Traditional tissue endpoint analysis involving destructive fixation/staining processes do not allow real-time monitoring. Incorporating analytical biosensors into microfluidic platforms allows real-time analysis of metabolic and molecular changes without affecting tissue integrity. For example, Zhang et al (2017) developed a multisensor organ-on-a-chip platform that can monitor pH, oxygen, temperature, and soluble protein biomarkers without affecting cell behavior.

In addition, researchers have integrated sensors with a variety of analyte detection techniques in microfluidic devices, including aptamer-based or immunoelectromichemical sensors to monitor changes in the protein or bioanalyte of interest (Boaks et al, 2023; Du et al, 2023; Kant, 2023; Marino et al, 2023; Yeganegi et al, 2023). Examples include sensors to detect cancer biomarkers such as prostate-specific membrane antigen, proinflammatory cytokines associated with a bacterial or viral infection, or the detection of regulated substances (Buttkewitz et al, 2023; Chen et al, 2023). Thus, integrating sensors in SoC could provide superior tractability to monitor treatment efficiency. However, these sensor-equipped microfluidic systems have yet to be used for skin studies, and they may require redesigning to accommodate the generation of an air—liquid interface. In this context, most microfluidic devices equipped with integrated sensors (eg, pH, glucose) have been designed to operate submerged in liquid (eg, culture medium) (Buttkewitz et al, 2023; Marino et al, 2023). Thus, although monitoring cells in the dermis and lower epidermis may be easier, making these sensors compatible with the air—liquid interface located at the top of the epidermis may present important challenges. Overall, biosensor integration into SoC platforms could provide real-time and actionable information about skin pathologies, although their development will probably require multidisciplinary collaboration between material and electric engineers, chemists, and cell biologists.

LIMITATIONS/DIRECTIONS OF MICROFLUIDICS MODELS

Despite the advances in the last few years, several engineering and biological challenges in the SoC field still need to be addressed to fully realize the potential of SoC platforms. Regarding engineering challenges, a significant proportion of these devices rely on PDMS and soft lithography. PDMS formulations often contain ethylbenzene and xylene in small quantities and can absorb hydrophobic molecules (<500 Da), which may interfere with cell behavior (Halldorsson et al, 2015; Palchesko et al, 2012). Furthermore, media perfusion is often desired or required for continuous nutrient and oxygen supply to the cells within microfluidic devices, which complicates the experimental setup. Arguably, biological challenges present more hurdles for SoC than engineering problems. As more cell types and biological structures are incorporated into these systems, balancing media composition and cellular crosstalk may become more difficult. In this context, keratinocytes, melanocytes, dermal fibroblasts, sensory neurons, and immune cells require specific media formulation to thrive in culture.

Another challenge is the ECM complexity required to mimic the in vivo architecture and responses. The ECM of human skin is mainly comprised of collagens, proteoglycans, elastins, and glycoproteins, which provide stability and plays critical role in numerous processes, including wound healing, cell differentiation, migration, and immune trafficking (Uitto et al, 1989; Widgerow et al, 2016). However, most SoC platforms rely on relatively simplistic ECM composition (eg, type I collagen hydrogel), which does not fully recapitulate the complex cell—ECM interactions in the human skin microenvironment. Some attempts to address this issue relied on seeding cells in the hydrogel and culturing them for some time to allow them to remodel their own ECM composition. However, this approach creates additional problems regarding hydrogel density and/or mechanical properties. Synthetic polymers are good alternative to hydrogels, but they also raise concerns about the capacity of the cells to degrade or metabolite such polymers (Geckil et al, 2010; Urbanczyk et al, 2020). Despite conceptual and technical advances, reconstructing cutaneous ECM that fully replicates the structural and functional complexity of ECM in vivo remains challenging.

Deregulation of resident immune cell migration and their immune responses often leads to opportunistic infections or autoimmune reactions. Understanding these complex cellular communication/interactions in real time could provide useful insights into various skin pathologies. However, the skin-resident and skin-infiltrating immune populations are very complex, encompassing numerous cell types (eg,

keratinocytes and dermal fibroblasts embedded in collagen, with endothelial cells at the bottom of the device separated from the rest of the stromal cells with the help of a porous membrane. This platform is mainly helpful for toxicology, inflammatory response, and myeloid cell infiltration. (**d**) The microfluidic platform with air walls was developed to understand the interaction between skin stromal cell roles in melanomagenesis. This device is mainly composed of skin stromal cells (fibroblasts and keratinocytes) in lateral chambers and melanoma cells in the central chamber. The liquid flow between 2 channels allows for communication/exchange of secretory factors. This platform is mainly useful in studying cancer cell—stromal cell communication and real-time imaging of cells as they move in lateral directions. (**e**) The PDMS-based microfluidic platform with innervated epidermis was comprised of epidermal keratinocytes in the epidermal channel and sensory neurons in the soma chamber separated by acellular dermal layer/ECM. This platform is useful in understanding cell—cell communication, cell—matrix communication, and sensory response (Abaci et al, 2015; Ahn et al, 2023; Ayuso et al, 2021b; Kwak et al, 2020; Mori et al, 2017). HSE, human skin equivalent; HUVEC, human umbilical vein endothelial cell; PDMS, polydimethylsiloxane; SoC, skin-on-a-chip.

LCs, T cells, and dendritic cells, etc) (Nguyen and Soulika, 2019; Quaresma, 2019). Most SoC models used for skin immunology have been limited to 1 or few immune cell populations. With ongoing advances in our knowledge of immune cell culture, we expect these models to incorporate additional cell types, leading to more holistic and physiologically relevant models.

COMMERCIAL POTENTIAL OF SoC MODELS

In the last years, we have seen the birth and growth of several companies (Genoskin) offering sophisticated in vitro models for skin research. Although not necessarily defined as microfluidic models, this new in vitro tool often leverages technological improvements to offer skin cultures or skin biopsies that allow the users to study skin cell biology in a more physiologically relevant scenario. Regarding adoption of microfluidic models by the biomedical community, most microfluidic devices still remain a niche tool, with their use limited to the laboratories that develop these technologies (Ayuso et al, 2022). This is partly due to the technical complexity required to operate them (Lee, 2020). In addition, the presence of air bubbles often compromises the integrity of the system and remains a challenge in the field. Finally, most microfluidic devices have been designed ad hoc for a given application (eg, generation of logarithmic gradients) (Ayuso et al, 2022) but tend to pose challenges when are repurposed to a different experimental configuration (eg, linear gradient), which in turn limits their broad adoption in the community (Micheli et al, 2022). As researchers continue to improve microfluidic designs, we anticipate a progressive adoption of these technologies by the community, coexisting with other in vitro tools. In this context, entrepreneurs have started to explore the potential of microfluidic devices for commercial applications. Biotechnology companies such as

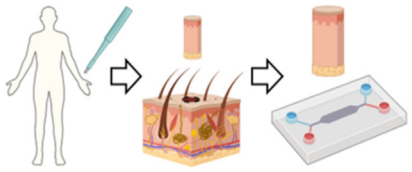
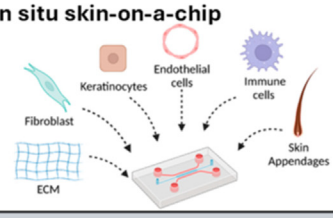
	Transferred skin-on-a-chip	In situ skin-on-a-chip
		
Cell/Tissue source	Requires a tissue sample (e.g., skin biopsy).	Requires one or multiple cell types (e.g., keratinocyte) and extracellular matrix proteins (e.g., collagen).
Design considerations	Requires large chambers to host the skin sample.	Requires the generation of a stable air-liquid interface to induce keratinocyte stratification.
Time required to establish in culture	Few hours.	Several days to allow for keratinocyte stratification.
Optical transparency/Microscopy compatibility	Moderate to low depending on the tissue thickness. Imaging structures at the top of the tissue sample (hair follicle) might be difficult.	High – bottom layer is often made of microscopy-grade glass to maximize optical quality.
Control over tissue structure and composition	Low – tissue structure and composition are imposed by sample donor.	High – tissue structure, dimensions, and cell composition/density are tunable.
System perfusion	System can be perfused by flowing medium underneath the tissue sample. Perfusion is not integrated with the tissue vasculature.	Endothelial cells can be assembled into perfusable vasculature. Better suited to study cell extra/intravasation and angiogenic response.
Potential to include skin appendages (e.g., hair follicle, sweat glands)	High - Functional skin appendages are present in the tissue sample although their distribution is defined by the tissue donor.	Moderate – skin appendages must be assembled by incorporating the required cells, which may required complicated or poorly established protocols.

Figure 5. Comparison of transferred skin-on-a-chip with in situ skin-on-a-chip platforms. Microfluidic devices for skin biology can be divided into 2 different categories. Transferred skin-on-a-chip relies of culturing skin tissue (eg, skin biopsy) in a microfluidic device that will provide culture medium, nutrients, drugs, or other cells (eg, immune cells) to the skin biopsy. This approach is preferred when the tissue integrity must be preserved and allows researchers to study complex skin structures such as sweat glands or hair follicles, which are still challenging to generate in vitro. The second category is in situ skin-on-a-chip, which relies on a bottom-up approach where the desired cells (eg, keratinocytes, fibroblasts, melanocytes) are assembled into a 3D structure that mimics the skin architecture. This method offers superior control over tissue configuration and provides the user with improved capacity to manipulate the tissue microenvironment. 3D, 3-dimensional.

AimBioTech offer several microfluidic platforms that allow the user to mimic specific properties of the human skin structure, including features such as an air–liquid interface or a stratified culture. AimBioTech IdenTx plate includes 3 microfluidic channels designed to inject a collagen hydrogel in the center, leaving the 2 lateral sides to seed endothelial or epithelial cells (eg, keratinocytes) (Ahn et al, 2023). However, an ideal model incorporating all skin components and that fully mimics the structure and function of human skin is yet to be built, and more biological and technological advances are needed before a widespread use of these technologies can occur.

FUTURE PERSPECTIVES

SoC platforms are promising tools to model physiological and pathological skin conditions, with transferred and in situ SoC platforms offering unique advantages and limitations (Figure 5). These platforms provide experimental flexibility and a physiologically relevant structure. SoC platforms could be a valuable tool to accelerate the identification of new therapeutics and drug development. Advances in iPSCs have led to the generation of a wide range of iPSC types using dermal fibroblasts, which are easily accessible and easy to culture (Scapini et al, 2016). Using iPSC protocols, dermal fibroblasts can be differentiated into other skin cell types such as keratinocytes (Sah et al, 2021), melanocytes (Ohta et al, 2013), or immune cells (Xue et al, 2023). Combining iPSCs with microfluidic platforms would allow researchers to generate patient-specific models that harbor relevant genetic alterations. In this context, there is a large variety of skin diseases with a genetic component such as incontinentia pigmenti, Gardner's syndrome, or epidermolysis bullosa. The rare nature of these diseases combined with challenges to generate animal models that mimic human pathophysiology makes the use of microfluidic models an enticing alternative. Patient-derived iPSCs harboring the relevant genetic variants in the *COL7A1* gene could be used to study potentially new therapies against dystrophic epidermolysis bullosa (Guide et al, 2022; Varki et al, 2007). In this context, gene replacement therapies are being explored, and the use of patient-specific microfluidic models could accelerate their implementation in the clinic (Bischof et al, 2024). The more complex SoC platforms can potentially offer high-throughput performance and multiplexing capabilities owing to their compatibility with sensor integration, which would allow monitoring of relevant biochemical and biophysical parameters regarding skin function (eg, barrier function, permeability). Although some challenges remain, the SoC is a promising new technology with potential across many areas of basic skin research and clinical applications.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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DECLARATION OF GENERATIVE ARTIFICIAL INTELLIGENCE (AI) OR LARGE LANGUAGE MODELS (LLMS)

The author(s) did not use AI/LLM in any part of the research process and/or manuscript preparation.

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