Evaluation of in vitro human skin models for studying effects of external stressors and stimuli and developing treatment modalities

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Abstract
Skin is exposed to a variety of potential stressors and stimulators that may impact homeostasis, healing, tumor development, inflammation, and irritation. As such it is important to understand the impact that these stimuli have on skin health and function, and to develop therapeutic interventions. Animal experiments have been the gold standard for testing the safety and efficacy of therapeutics and observing disease pathology for centuries. However, complex ethics, costs, time consumption, and interspecies variation limit the transferability of results to humans and reduce their repeatability and reliability. Furthermore, traditional 2D cell studies are not representative of human tissue. Skin tissue is a dynamic environment, and when cells are isolated in unphysiologically stiff, static petri dishes their behavior, and phenotypic expression is altered. Increasingly complex in vitro models of human skin, including organoids, 3D bioprinting, and skin-on-a-chip platforms, present the opportunity to gain insight into how stressors affect tissue at a cellular level in a controlled and repeatable environment. This insight can be leveraged to further understand pathological skin conditions and better formulate and validate drugs and therapeutics. Here, we will discuss the application of in vitro skin modeling to investigating the effects of mechanical, electromagnetic, and chemical stressors on skin.

Keywords
3D bioprinting, drug development, in vitro, organoid, skin model, skin-on-chip
INTRODUCTION

As the largest organ in our body, skin serves as a barrier and interface between us and our environment. This external surface is exposed to a plethora of stimuli from electromagnetic radiation (EMR) (e.g., UV, Wi-Fi), thermal variation, and pathogens, to directly applied chemicals and drugs and a wide breadth of materials, including fabrics, wound dressings, woods, metals, plastic, food, pets, and physical cues as demonstrated in Figure 1. In addition, the skin’s external surface area serves as a platform to express our individuality and diversity through cosmetics and tattoos.

Our skin is tasked with interacting with such a variety of stimuli while maintaining homeostatic balance, but what happens when these stimuli - or indeed our own immune system - upsets this balance? Irritation, inflammation, redness, and itchiness are common responses of skin to certain stimuli and a common occurrence. Skin eruptions are a common symptom of infectious diseases such as chicken pox and measles and have been linked to SARS-CoV-2. In addition to being a symptom of infectious disease, particular diseases that specifically target the skin permeate throughout the population, with a reported one in three individuals diagnosed globally and ranking as the fourth leading cause of disability worldwide. While not typically fatal, skin disease presents a significant burden on the individuals quality of life and mental health. As such, there is a need to study the effect of various stresses on skin to understand the occurrence and progression of pathological conditions and develop intervention and treatment strategies.

A variety of traditional and non-traditional approaches to skin modeling have been employed to investigate the impact of external stimuli on skin function including 2D and 3D cell studies, organoids and organotypic models, 3D bioprinting, and skin-on-a-chip platforms. With varied degrees of complexity, advantages and disadvantages, there is no one model that is able to recapture the complete function and response of human skin. The goal of this review is to provide insight into the use of in vitro skin models for investigating the effects of mechanical, electromagnetic, and chemical stresses on human skin.

HUMAN SKIN PHYSIOLOGY

As the largest organ in the human body, skin’s primary function is to separate us from the external world. However, skin is more than a simple barrier. Instead, it is a complex, multilayered organ that interfaces with a...
multitude of biological systems by providing sensory information to our nervous system, synthesising vitamin D for the endocrine system, alerting the immune system when foreign pathogens are identified and aiding in water retention. [5] The skin is able to achieve these different functions due to its highly organised, complex and multilayered structure.

The composition of skin is heterogeneous with variation in thickness, density, and cell types at different locations. [6] However, all locations contain three distinct layers: the epidermis, dermis and hypodermis. The outermost layer, the epidermis, is approximately 0.1–0.2 mm thick, composed primarily of keratinocytes and can be further broken down into distinct layers. [7] A highly proliferative basal layer generates new cells that are pushed toward the surface. As the cells migrate, they are flattened and move further away from the nutrient-rich dermis causing them to die. [8] The stratum corneum, the outermost layer of skin, is an excellent non-selective barrier owing to its composition of dead, flat corneocytes, and hydrophobic extracellular lipid matrix. Blood vessels, and therefore the nutrient supply, are located in the dermal and hypodermal layers. The dermis is approximately 2–6 mm thick and contains an extensive extracellular matrix (ECM) composed of collagen and elastin fibers as well as some ground substance including water, glycosaminoglycans, such as hyaluronic acid, proteoglycans, and glycoproteins. [7,9] The predominant cell type is fibroblast which synthesizes collagen and other ECM materials as well as play a critical role in wound healing. [10] The innermost hypodermal layer adds insulation and cushioning to the skin as it is primarily composed of fat storing adipocytes.

This complexity is challenging to recapitulate in vitro, hence simplified, single cell type models tend to be preferred. However, caution should be taken when selecting cell types to be included and the implications of such decisions considered. For example, different fibroblast subtypes, such as reticular dermal or papillary, have different characteristics with the former expressing high metabolic activity and the latter expressing low metabolically activity and high proliferation. [10] This variation is neglected by homogeneous cell cultures, and the use of different subtypes may impact experimental results. In addition, intercellular communication between the layers provides cues for coordinated skin function. [11–14]

3 APPROACHES TO SKIN MODELING

Tremendous progress can be observed in many industries over the past century, for example the telecommunications industry is unrecognisable from the first telephone invented in the late 19th century to the mobile phones that are ubiquitous in modern society. However, the same advancements have not been afforded to in vitro models of human skin which had up until recently remained largely stagnant among the technological revolution. Despite interspecies variability and complex ethical considerations, animal models have been the gold standard for testing compound efficacy or investigating the effects of various stressors. [11,15] However, low efficiency of drug development strategies and increasing pressure to reduce dependency on animal testing necessitate the development of alternate, physiologically relevant models of human skin. [16,17]

Numerous approaches and techniques have been adapted from conventional cell culturing, tissue engineering, 3D bioprinting and microfluidics to generate in vitro models of human skin. Typically, an increase in model complexity can be achieved with more advanced techniques resulting in enhanced biomimicry. This improvement, however, often comes at a cost of increased variability between models, decreased throughput and reliability, and increased training, with all these factors contributing to limited laboratory adoption. [18] While not exhaustive, Table 1 provides a snapshot of the application of different approaches to skin modeling applied to investigating the effects of stressors through representative references.

3.1 2D cell models

Two-dimensional cell studies can be dated back to the early 1900s and have remained the method of choice for the following century. [44] This reductionist approach involves isolating cells from tissue or the use of immortalised cell lines to produce adherent monolayer cultures of either keratinocytes or fibroblasts as models of the epidermis or dermis, respectively. [45,46] These basic studies have expanded our knowledge of how cells behave and respond to a variety of stimuli. However, there is a distinct lack of cell-cell or cell-matrix communication, substrates express unphysiologically stiff mechanical properties, and cell growth is constrained to 2D, forcing apical-basal polarity and "flattening" of cells altering their morphology and deviating behavior from native tissue function. [47] The results obtained when cells are isolated from their familiar dynamic environment and grown in stiff, static petri dishes tend to have low predictive power over clinical outcomes and as such are limited largely to cytotoxicity studies but are ineffective at elucidating efficacy in drug testing. Despite these limitations, 2D cell studies remain popular as the present simplified model with high reproducibility, throughput, and comparative literature.
| Approach/platform | Skin layers | Cell types | Purpose/outcome | Stimuli | Ref |
|-------------------|-------------|------------|-----------------|---------|-----|
| 2D skin model     | Epidermis   | NHEKs      | Notch1 contributes to viral-induced cervical cancer | Cocultured virus (HPV) | [19] |
|                   |             |            |                 |         |     |
|                   |             | HaCaT      | Progression model of UVB-induced carcinogenesis | UVB     | [20] |
|                   |             | HaCaT      | Demonstrated ability of ELF-EMF to decrease wound healing time | Extremely low-frequency electromagnetic fields (ELF-EMF) | [21] |
| Dermis            | Primary fibroblasts |            | Fat extract protects against UVB photodamage | UVB | [22] |
| 3D skin model     | Epidermis   | HaCaT      | Platform to investigate inflammatory response of skin to infection and validate antimicrobials | Wound E. coli | [23] |
|                   |             | NHEKs      | Immune competent model of virus-host interaction | Co-transfected virus (HPV) | [24] |
|                   |             | Langerhans cells | | | |
|                   |             | Primary keratinocytes & melanocytes | Melanin content increases skin resistance to UV | UV | [25] |
|                   | Epidermis+Dermis | Immortalized HDFs, Ker-CT Immortalized HKs, Naive CD4 T cells | Fibroblasts impede fungal infection | Fungi infection (Candida albicans) | [26] |
|                   | NHK          | NHDF       | Screen toxicity of tattoo ink | Tattoo ink | [27] |
|                   | NHK          | HDFs       | Permeation of fluorescein sodium | Fluorescein sodium | [28] |
|                   | Primary keratinocytes & fibroblasts | | Investigate influence of temperature on skin cell growth | Temperature | [29] |
|                   | Epidermis+Dermis | Primary keratinocytes, fibroblasts, and endothelial cells | Vascularised TGFβ-induced fibrosis model | Drug to treat fibrosis (nintedanib) | [30] |
| Organoid          | Epidermis+Dermis | WA25 hESCs and desmoplakin-mEGFP (DSP-GFP) iPSCs | Mimic hair growth | N/A | [31] |

(Continues)
| Approach/platform | Skin layers | Cell types                                      | Purpose/outcome                                      | Stimuli                                      | Ref  |
|-------------------|-------------|-------------------------------------------------|-------------------------------------------------------|----------------------------------------------|------|
| iPSCs derived from CBMCs | Epidermis | HDFs HEKs                                      | Generated organoid from CBMC iPSCs                  | N/A                                          | [32] |
| 3D bioprinting    | Dermis     | HDFs HEKs                                      | Scaffold-free 3D printed complex skin shapes        | N/A                                          | [33] |
|                   |            | Human epidermal melanocytes                     | Present pigmented skin model                        | N/A                                          | [34] |
| Epidermis         | Dermis     | HDFs HEKs                                      | Perfusable blood vessels                            | Shear stress                                 | [35] |
| Dermis Hypodermis |            | HUVECs HPAs                                    | Observed skin response to Lipopolysaccharide and UV | Lipopolysaccharide and UV                     | [36] |
| Skin-on-a-chip    | Epidermis  | HaCaT Human leukemic monocyte lymphoma cell line (U937) | Observed skin response to Lipopolysaccharide and UV, on-chip TEERS sensor | Lipopolysaccharide and UV | [36] |
|                   | Dermis     | PHKs PHFs                                      | Wrinkled skin equivalent                           | Cyclic uniaxial stretch                      | [37] |
| Epidermis         | Dermis     | HaCaTPHKsPHFs HUVECs HL-60                     | Neutrophil response to UV radiation                 | UV                                           | [38] |
| Dermis Endothelium|            | HaCaT HS27 fibroblasts HUVECs                  | Modeled and treated TNF-α -induced inflammation    | Drug to treat inflammation (Dex)             | [39] |
|                   |            | NDFs NHEKs HUVECs                              | Perfusable and stretchable 3D skin equivalent       | Cyclic stretch & shear stress               | [40] |
| Nonbiological     | Epidermis  | N/A                                            | Skin-irritation testing platform                    | Chemical irritants                           | [41] |
|                   |            | N/A                                            | Urban pollutants impact skin physiochemistry       | Urban pollutants                            | [42] |
|                   |            | N/A                                            | Modeled sweat glands                                | Contact adhesives                            | [43] |
3.2 | 3D cell models

Building on the foundations of 2D cell studies, 3D cell studies introduce distinct layers, an ECM to encourage spatiotemporal micronutrient distribution, and multidirectional cell growth analogous to native tissue conditions. Reconstructed epidermis models can be fabricated by exposing epidermal keratinocytes to the air-liquid interface (assisted via transwell plates or similar) or growth factors, encouraging differentiation to form an in vivo like stratified epidermis.\textsuperscript{[48]} In addition, the epidermal layer may be cultured on top of a cellular or acellular dermis equivalent. In native in vivo tissue, the dermis is comprised primarily of an ECM with fibroblasts as the dominant cell type. Thus, to effectively mimic the dermal layer, the inclusion and composition of the ECM plays a key role.\textsuperscript{[49,50]} ECMs can be derived from natural, synthetic, or mixed origins including decellularized ECMs (often derived from porcine tissue), hydrogels, polymers, chitosan, and collagen.\textsuperscript{[51–54]} Natural ECM materials tend to be structurally weaker, so it is often desirable to combine the advantages of synthetic and natural ECMs.\textsuperscript{[51]} Numerous fabrication techniques have been applied to generate ECM scaffolds including electrospinning, freeze-drying, photo crosslinking, melt moulding, solvent casting, and 3D printing.\textsuperscript{[55–57]} Cells may then grow and attach to the scaffold and release ECM materials as an effective 3D model. In addition to in-house 3D skin models, a number of prefabricated, single, and multilayered models are commercially available for grafting applications (with FDA approval) or in vitro toxicity and efficacy/permeation studies. Some common models include EpiSkin, EpiDerm, EpidermFT, StrataTest, Apligraf, and Episcel.\textsuperscript{[58,59]}

Recapturing intercellular communication and synergy is achievable in these more complex skin models that contain different cell types. In particular, communication between keratinocytes and fibroblasts has been shown to play a fundamental role in skin function. A comparative study between keratinocyte-fibroblast and keratinocyte-only full-thickness models demonstrated that in the absence of fibroblasts, keratinocytes behave abnormally.\textsuperscript{[11]} The keratinocyte-only model expressed impaired differentiation, increased permeability to caffeine and testosterone, deregulation of the skin barrier and tight junction protein expression, and a decreased lipid/protein ratio compared to the keratinocyte-fibroblast model. These observations suggest that keratinocytes’ interaction with fibroblast is essential in maintaining the normal physiology of the skin. Additional neurological and immunological components should also be considered to achieve skin models with enhanced biomimicry.\textsuperscript{[60]}

By favouring 3D multilayered skin models, more complex experiments may be performed that aim to investigate stimuli interactions with the skin at both the tissue and cellular level.

3.3 | Organoids

The term organoid can be used to describe organ-like tissue that 1. contains multiple cell types, 2. replicates organ function and 3. is self-assembled. Generally, the term is reserved for organs grown from stem cells, although it is used to refer to any tissue that mimics organ function.\textsuperscript{[61]} To grow organoids, either pluripotent or adult stem cells are exposed to a cocktail of growth factors to guide skin formation by approximating natural development or tissue maintenance.\textsuperscript{[62]}

In an impressive breakthrough, Lee et al generated complex, hair-bearing skin organoids comparable to 20-week-old foetal tissue exclusively from human pluripotent stem cells.\textsuperscript{[31]} Over the 4–5 month incubation period, stepwise modulation of different growth factors encouraged differentiation of the skin organoid to achieve a stratified epidermis, dermis, pigmented hair follicles, sebaceous glands, and sensory neurons that mimic the neural circuitry associated with a human touch. The organoid was successfully grafted into nude mice to grow hair-baring skin. This self-assembled skin is suggested to provide utility for future studies of disease modeling, skin development, and skin graft applications.

When derived from pluripotent stem cells, organoids mimic foetal and young skin development may not be suitable for generating aged skin models. In addition, they are currently labor intensive and take months to develop, decreasing their throughput and accessibility to non-specialists. A lack of vasculature limits the maximum size tissue can be grown and maintained as nutrients will be unable to reach internal cells leading to cell death.\textsuperscript{[62]} Despite these drawbacks, organoids have very promising potential for generating truly biomimetic human skin equivalents and, in particular, present a very exciting pathway for personalised medicine.\textsuperscript{[63]}

3.4 | 3D bioprinting

Based on the same principles as 3D printing, 3D bioprinting or cell printing, involves the layer-by-layer extrusion of cells suspended in a bioink to form multilayer tissue constructs.\textsuperscript{[64–66]} Bioinks can be composed only of cells, but often an additional carrier material, such as a hydrogel, will be added to act as a molecular scaffold.\textsuperscript{[65]} Printing cells and biological matter presents a unique challenges
and complexity related to the sensitivities of living cells requiring precise material selection, growth, and differentiation factors and cell types.\[67\]

The number of cell types included using conventional manual seeding of 3D skin models is generally restricted to 1–2 (keratinocytes and fibroblasts). A key aspiration for 3D bioprinting is to facilitate the inclusion of multiple cell types, including adipocytes, to form a hypodermis layer.\[35\] In addition, complex structures can be printed, including blood vessels, that would otherwise present a challenge. Validating this potential is research by Kim et al who fabricated a vascularised, full-thickness human skin model including an epidermal, dermal, and hypodermal layer using 3D printing techniques.\[35\] The printed model exhibited a microenvironment that more closely replicates in vivo tissue compared to alternate techniques and proposed that this increased similarity may result in better predictive outcomes in drug, cosmetic, and general research experiments. In addition, pigmentation has been achieved by 3D bioprinting by alternating between keratinocyte and melanocyte laden bioinks while printing the epidermal layer on top of a layer of fibroblasts.\[68\]

Application of 3D bioprinted skin tends to be focused on high throughput regenerative medicine and skin grafting as opposed to investigating the effects of external stimuli, thus presents an opportunity for these more complex models to be utilised for research applications.\[65\]

### 3.5 Skin-on-chip

At the intersection of microfluidics and cell culture lies an exciting potential to develop organ models that are able to capture the complex structure and mimic the microenvironment of native human tissue, termed organ-on-a-chip or microphysiological systems.\[69–72\] Skin-on-chip (SoC) platforms facilitate the fine control of medium flow and substrate stiffness as well as imparting mechanical forces, including shear stress and cyclic stretch, to recreate the mechanical environment of human skin.\[73,74\] Key aspects of the cell-cell, cell-matrix and cell-environment can be replicated including the delivery of nutrients through blood vessels, waste removal, inclusion of multiple cell types and cell growth in 3D. These platforms do not necessarily aim to recapture the function of an entire living organ, rather the minimal functional units can be expressed and evaluated on a microchip. As such, a spectrum of complexities and customisation can be achieved, from simple models comprising of a single channel and cell type to multilayered structures. Typically, porous membranes are employed to divide the layers and replicate structures found in native human skin while facilitating intercellular crosstalk as well as enabling access to the contents of all layers, which is not possible in a standard petri dish.

3D skin models as previously described, can be cultured directly in the microchip or in transwell holders that can be transferred to the microchip as desired. For example, Kim et al combined 3D printing with 3D bioprinting to concurrently fabricate a full thickness, vascularised skin model inside a polycaprolactone transwell holder.\[35\] A peristaltic pump was then used to perfuse cell culture medium through the bioprinted vascular channel to provide nutrients to the skin models, mimicking native blood vessel function. This hybrid approach offers high throughput, automatisation, and customisation of skin models.

SoC modules can be fluidically linked to other organ-on-chip modules to create a human-on-chip for system-wide evaluation, pertinent to transdermal drug delivery.\[75,76\] Further modules can be fluidically linked to SoC platforms to continuously monitor for key biomarker secretion, temperature, transepithelial electrical resistance, pH, and oxygen that yield insight into aspects of cell health and function, including tight junction formation, cellular metabolism, respiration, and acidification.\[63,77,36\] The ability to create such microfluidic circuits may facilitate full characterisation and automation of testing, increasing throughput and decreasing workload.

By presenting researchers the opportunity to achieve fine control of biological processes and to design and customise their cell culture environment, SoC platforms may further our understanding of skin response to external stimuli.

### 3.6 Nonbiological

Synthetic, artificial, phantom or nonbiological models of human skin refer to materials that are fabricated with physical properties including stiffness and elasticity, water absorption, pH, swelling, and roughness that are akin to human skin.\[78\] A variety of materials may be used including liquids, gels, elastomers, epoxy resins, metals, and textiles; however, no single material replicates all functional aspects of native human skin.\[78\] Instead, the specific application should be defined, the key characteristics required identified, and then a material capable of meeting those requirements selected. For example, gels, elastomers, metals, and textiles are capable of mimicking the thermal properties of skin and thus would be suitable to observe the thermal heating effects of EMR. Such models have seen application as substitutes for studies on the frictional interaction and adhesion of skin with wound dressings, skin interaction with topical products, and the impact of urban pollutants on skin physico-chemistry.\[79,43,42,80\]
The use of polymers instead of tissue culture is particularly appealing for applications concerning the mechanical function of skin or interaction of skin with fabrics or adhesive materials where cell response is not being assessed or for modeling more complex structures. For instance, Hou et al fabricated an artificial perspiring skin model to mimic human sweat.\(^{[81]}\) The roughness, water contact angle, sweat pore size, and sweat pore density of human skin were reproduced using embossing with sweat pores (approximately 80 μm diameter) laser micromachined to enable the model to ‘sweat’. In a subsequent study, the perspiring model was used to assess the peel force required to remove different adhesives from the model at variable sweat rates. With the increasing popularity of wearable sensors, nonbiological skin models such as the one described could offer an accessible alternative for testing the adhesion and friction of such sensors without the need for cell culture knowledge, training, or laboratory access.

4 | SKIN MODELS FOR INVESTIGATING STRESSORS

Environmental stressors originate from many foundations and can induce either therapeutic or harmful effects on human skin. Some stimuli, such as solar radiation, are both necessary for survival but can also cause mutations leading to cancer. Thus, it is important to achieve a thorough understanding of the molecular mechanisms that drive skin-stimuli interactions, and in vitro skin models represent the most promising method to achieve this.

4.1 | Mechanical stimulation

While we have sensory neurons in our skin to alert us to physical sensations, mechanical stimuli can also be recognised by individual cells. This mechanosensitivity allows cells to sense and respond to their physical microenvironment, including stretch, shear stress, substrate stiffness, pulsed ultrasound, and pressure.\(^{[82]}\) These stimuli can modulate cell behavior, inducing morphological changes, proliferation, migration, and altering phenotypic expression leading to harmful or therapeutic impacts on skin health.\(^{[83,84]}\)

Substrate stiffness plays an important role in regulating cell morphology, adhesion, and phenotypic expression.\(^{[82]}\) It is common for 3D skin models to include epidermal keratinocytes cultured on either cellular or acellular hydrogels with mechanical properties representative of the dermis. Dermal materials can be fabricated from synthetic, natural or hybrid origins and include gelatin, collagen, fibrin, poly-acrylamide, hyaluronic acid, and polypeptides.\(^{[85]}\) Alternatively, decellularized ECMs may be derived from animal tissue and later inoculated with human fibroblasts.\(^{[86]}\) Zarkoob et al suggest that keratinocytes cultured on soft polyacrylamide gels (E = 1.2 kPa) display coordination in forming epithelial sheets and reduced spreading, increased migration, and increased colony formation compared to stiff polyacrylamide gels (E = 24 kPa).\(^{[87]}\) Despite the impact of substrate stiffness on cell expression, conventional 2D cell studies tend to be carried out on unphysiologically stiff plastic or glass substrates that deviate cell behavior away from native human tissue.

Wrinkled and aged skin can be modeled in vitro by exposing skin cells to cyclic stretch, as demonstrated by Lim et al.\(^{[17]}\) The developed skin-on-a-chip platform containing a 3D epidermal/dermal skin model utilizes attractive and repulsive forces from an internal fixed magnet and an external electromagnet to induce rapid, uniaxial stretch of 5.3 mm/s at a frequency 0.01 Hz for 12 h a day. Thinning of the stratum corneum and wrinkle formation were observed over 7 days of tissue culture. This wrinkled skin equivalent has proposed utility in testing the efficacy of anti-wrinkle compounds used in cosmetics. It may also be used to better understand the underlying mechanisms that cause skin compounds used in cosmetics. It may also be used to better understand the underlying mechanisms that cause skin compounds used in cosmetics.
ments and therapeutics.[37] In addition, physiologically relevant mechanical forces can be leveraged to improve the biomimicry of in vitro skin models. Microfluidic skin-on-a-chip platforms are appropriate for studies concerning mechanical stimuli as they offer flexibility and solutions for exposing cells to shear stress, cyclic stretch and provide tuneable substrate stiffness.[73]

4.2 | Electromagnetic stimulation

EMR is a specific form of the more general electromagnetic field, where energy is emitted and absorbed by charged particles as it travels through space in wave form. Accelerated atomic particles produce a time-varying electric and magnetic field. EMR has both electric and magnetic
Electromagnetic (EM) waves are typically described by any of the following three physical properties: frequency (f), wavelength (λ), and photon energy (e). Depending on their frequency (measured in cycles per second (Hertz)) and corresponding wavelength (measured in meters), EM waves are mapped onto the electromagnetic spectrum. The electromagnetic spectrum is divided into seven broad categories: radio waves, microwave, infrared, visible light, ultraviolet, X-rays, and Gamma rays. Some of these classifications are further divided into subcategories. The electromagnetic spectrum, as shown in Figure 3, covers the frequencies ranging from 1 Hz to above 10^25 Hz, which corresponds to wavelengths from thousands of kilometres...
down to a fraction of the size of an atomic nucleus. The frequency or its corresponding wavelength of the EMR determines its ability to travel through objects, its heating effects and effects on living tissue. EMR is described as a stream of mass-less particles called photons. Each photon has a certain energy level and is travelling in a wave-like pattern at the speed of light. Oscillation rate in Hertz defines the energy level of each type of photon. The rate of oscillation is inversely proportional to the distance each photon travels in meters. Higher photon energy means a higher frequency of oscillation and shorter wavelength. Thus, radio waves contain photons with the lowest energy level, while Gamma rays have the highest energy level in the spectrum.

Energy transmitted through EM waves is broadly divided into two categories: 1. ionising radiation (EM waves that carry enough energy to break molecular/chemical bonds between molecules and ionise atoms; and 2. non-ionising radiation (low-frequency EM waves that do not have sufficient energy to break molecular/chemical bonds and ionise atoms). Gamma rays and X-rays are examples of ionising radiation, whereas radiation from microwaves, radiofrequency fields, and low frequency (LF) fields (also known as magnetic fields) is found at a relatively long wavelength and classified as non-ionising radiation. The cut-off point between ionising and non-ionising radiation is approximately $3 \times 10^{11}$ Hz, with radiation below this frequency considered non-ionising and above considered ionising radiation. Ionising radiation is capable of causing lasting damage to DNA resulting in cancerous tumor development, photodamage, and immune suppression. Conversely, ionising levels of EMR may be used in radiotherapy to treat cancer and for medical X-rays to generate images of internal body structures. As the effects of ionising radiation are well characterised, the focus of this section will be on lower-frequency, non-ionising EMR is unable to penetrate deep into the body and thus primarily targets the skin.\[89\]

### 4.2.1 Radio waves and microwaves

Residing at the LF end of the electromagnetic spectrum are radio waves (RWs), 300 MHz - 3 kHz, and microwaves (MWs), 300 GHz - 300 MHz. These frequency bands are commonly used by the telecommunications industry, for specific types of spectroscopy, and for heating and power applications. As this band of radiation is non-ionising, short-term exposure is considered safe; however reports of health and biological effects from long-term exposures are conflicting. Health effects of RWs and MWs are generally accepted to be limited to thermal heating effects; thus, current safety guidelines are determined based on the threshold at which heating occurs.

Radiofrequency ablation, or fulguration, is a common treatment for dysfunctional tissue, including chronic pain, headaches, cancer, varicose veins, and Barrett’s esophagus.\[90-92\] It involves inserting needles into the target site to deliver targeted RWs that heat and ablate the tissue. More recently, MW ablation has been introduced as an alternative to radiofrequency ablation. The higher frequency, and thus higher energy, enables penetration of larger areas, higher temperatures, faster ablation times, and propagation through tissue with low electrical conductivity. While this additional power comes with an increased risk of collateral injury to non-target organs, it is considered safe and more suitable for some treatments including liver tumors.\[93\]

Low power millimetre wave irradiation, a subset of RF radiation (30–300 GHz), is believed to have therapeutic benefits and has been successfully applied in therapeutic practices. To validate the safety of this therapy, an in vitro 2D keratinocyte-only model demonstrated no observable effects on chemokine production, heat shock protein stimulation, or intercellular gap junctional communication when exposed to low power millimetre wave irradiation.\[94\] Additionally, Sanchez et al exposed a reconstructed human epidermis to RF radiation within the limit for local exposure and observed no effect on keratinocyte or fibroblast apoptosis, proliferation, or inflammation.\[95\] It was reported that heat shock protein (HSP) expression was altered in fibroblasts; however this is not likely to impact skin health.

Radiofrequency therapy has also been used as non-invasive treatment for the appearance of cellulite. The mechanism of action for this treatment is through heating and mechanical stimulation of the deep skin tissue (dermis and hypodermis) using RF radiation. While the effects have been well characterised and validated on animal models,\[96\],[97\] there is an underlying gap in our understanding of the molecular mechanisms responsible for the observed phenomenon. The use of full-thickness skin models including epidermal, dermal, and hypodermal adipose tissue, may be suitable to understand these mechanisms.

As previously discussed, mechanosensitive ion channels spanning the cell membrane act as molecular transducers, converting mechanical stimuli into electrical and/or biochemical intracellular signals which modulate cellular behavior. These mechanical forces include tension, compression, cyclic stretch, shear stress, and substrate stiffness, all of which have a role to play in influencing cell behavior. TRPV4, a non-selective cation channel expressed in epithelial and endothelial cells, can be activated by mechanical stimuli including hypotonic stress and shear stress and thermal heating or cooling. A recent study has found that MW radiation below thermal heating...
thresholds (1800 MHz and 17 dBm) is capable of activating TRPV4, indicating that MW radiation may be able to mechanically stimulate cells.198 Whether or not this biological effect translates into harmful or therapeutic, or will have no health effects is yet to be determined. Despite the importance of mechanical stimulation in modeling physiologically relevant in vitro cellular experiments, especially when investigating mechanical stimuli, these systems have seen limited application to investigations into potential health effects of RF/MW radiation.

4.2.2 Infrared and visible light

The term “light” is used inconsistently in the literature, with some references including IR and UV and others referring exclusively to visible light. Herein, we will use the latter definition. While light is not inherently different to other forms of EMR, it is the only part of the spectrum humans capable of seeing, and thus we assign specific terminology to refer to the actions of light with terms like phototherapy, photoaging, and photobiology used to describe the interaction between light and ourselves. The photobiological action spectrum, that is the range of light that interacts with skin, is dependent on the amount of energy stored in the light, the penetration depth and the availability of photoactive substances that can interact with the light. Light penetration depth is determined by its wavelength and reflectance, with longer wavelengths capable of deeper penetration and a larger refractive index lessening penetration.99 Photoreactive molecules, or chromophores, that are present in human skin are vulnerable to the effects of IR, visible light, and UV radiation and include melanin, DNA, urocanic acid, and amino acids.100

Contrary to the harmful effects of UV radiation on skin, visible light and IR are considered safe and have seen therapeutic applications including wound healing and sterilisation, acne, psoriasis and blemish treatment, anti-aging therapy, and reduction of scars.101–103

Red and blue lights are commonly used for a variety of different therapeutic strategies. Blue light resides closer to UV radiation on the EMR spectrum and has a shorter wavelength than red light; thus it contains more energy but is not capable of penetrating skin much deeper than the epidermis. Conversely, red light is able to reach deep into the lower dermis and hypodermis tissue.108 The small difference of 200–300 nm in wavelength between red light and blue light thus varies the impact on skin health and therapeutic application by targeting different layers, as shown in figure 3B. Blue light is commonly used as an antimicrobial modality in photodynamic therapy which involves exposing non-toxic photosensitizers to light to produce abundant destructive reactive oxygen species that fight infection.101,104 Photosensitizers may be manually applied site of infection, or they may be naturally occurring. Photodynamic therapy is particularly appealing as an antimicrobial treatment as worldwide antibiotic resistance increases.105 However, when investigating blue light at distinct wavelengths of 410, 420, 453, and 480 nm on a fibroblast-only skin model, Opländer et al reported intracellular oxidative stress and toxic effects at 410 and 420 nm, but no toxic effects at 453 and 480 nm.106 They suggested that blue light with shorter wavelengths may contribute to premature photoaging, but the same anti-proliferative mechanism may find utility in the treatment and prevention of keloids, hypertrophic scars, and fibrotic skin diseases. It should be noted that this work was conducted on a fibroblast-only model; however due to the limited penetrative ability of blue light, it is unable to act in the deep dermis tissue, so observed effects may not necessarily translate into clinical outcomes. Alternatively, when investigating the effects of 470 nm blue light on wounded human dermal fibroblasts at energy densities of 3, 5, 10 or 55 J/cm², Masson-Meyers et al concluded that blue light at low fluence does not impair in vitro wound healing.107

Contrary to blue light, red light and near IR radiation (around 600–1070 nm) is considered to reduce the effects of photoaging and aid tissue repair in human skin. Lasers and more recently LEDs have been adopted for use in photobiomodulation therapy, and while a consensus has yet to be reached on whether or not they have different effects, it appears that both are effective treatment options.108 It appears that photobiomodulation effects human dermal fibroblast by encouraging proliferation and migration, positively influencing mitochondrial function and ATP production, cell viability, and altering protein and gene expression.109 Although assumptions on the molecular mechanism of action have been made, the precise cellular and molecular mechanisms remain an enigma. At low intensities, it is believed that photochemical mechanisms rather than heating effects are responsible for the therapeutic benefits observed.110 In addition, energy density, or fluence, of the beam is thought to play a more critical role in clinical outcomes compared to varied wavelength within the red-IR band.109 It is common for fibroblast-only skin models to be utilised for photobiomodulation studies as the primary target is the dermis layer.111

Some studies have been conducted on full thickness models including the EpiDermFT (MatTek, Ashland, Mass) comprised of both epidermal keratinocytes and dermal fibroblasts.102 The model was irradiated by an LED array with a peak emission wavelength of 660 nm and an energy density of 4 J/cm². Owing to its biomimetic tissue architecture, the full-thickness model facilitated histological assessment and for local versus circulatory responses
to be quantified by investigating both the tissue and supernatant, respectively.

Despite the ever-increasing prevalence of photobiomodulation therapies in the cosmetic and health care industries, there is limited consensus of optimal parameters and a lack of understanding of the molecular mechanisms responsible for observed outcomes. Recent efforts to create standardisation and higher quality of reporting have culminated in the generation of a checklist of key photomedicine dose and beam parameters to be referenced by authors and reviewers. In addition to non-standardised, poorly reported emission characteristics, cell culture conditions have been shown to influence in vitro outcomes, but again are not standardised. Improved and standardised in vitro models of human skin are paramount to improving the reliability, validity, and transferability of research into clinical outcomes.

### 4.2.3 Ultraviolet radiation

The sun is essential for the survival of living organisms on earth, emitting heat, light, and energy as well as harmful radiation including UVR, which can impact the normal function of skin. UVR is commonly separated into three subgroups: UVA (315–400 nm), UVB (280–315 nm), and UVC (100–280 nm). UVC has the shortest wavelength and is absorbed by the atmospheric ozone before hitting the earth’s surface. The significant majority (~95%) of solar UVR falls within the UVA range; however, UVB is considerably more carcinogenic and therefore the primary culprit for skin cancer development, redness, and sun burns. Despite this, UVA is able to penetrate deeper into the skin, impacting the dermis and hypodermis, instigating premature skin aging and wrinkling.

All epidermal cell types can be affected by UVR; however, cell response is dependent on cell type. Melanoma, the deadliest form of skin cancer, arises from melanocytes, and is substantially less prevalent than non-melanoma cancers as skin contains comparatively few melanocytes compared with keratinocytes. Additionally, UVR can trigger skin cells to release cytokines and chemokine, activating an immune response. Functional skin models, particularly those containing melanocytes and immune cells, are a valuable tool for elucidating the cellular mechanisms responsible for UV-induced skin disease, screening the efficacy of sun protectants and for validating potential therapeutic applications of UVR. While they tend to be a strong focus on the harmful effects of UVR on skin health, there are some therapeutic applications in wound sterilisation and wound re-pigmentation.

Keratinocyte-only models are often suitable for studying the effects of UVR on non-melanoma tumor generation in the epidermis. Tyagi et al demonstrate that when exposed to UVB, HaCaT cells exhibit heightened proliferation rates, resistance to apoptosis, and altered morphology consistent with oncogenic transformation. When the irradiated and non-irradiated control HaCaT cells where transplanted to mice, all mice that received the irradiated cells developed tumors, while no tumors were observed in the control, indicating the carcinogenic transformation of HaCat cells exposed to UVB. Alternatively, pigmented skin models including melanocytes can be cultured to investigate the resistance of melanin-containing skin to solar UV damage.

In addition to cancer progression, the complex immune response of skin to UV radiation can be modeled and studied in vitro, as demonstrated by Ramadan et al. A 3D coculture of immortalized human keratinocytes (HaCaT) and human leukemic monocyte lymphoma cell line (U937) model the epidermis and dendritic immune cells, respectively. The model was exposed to shear stress through cell culture medium perfusion, improving tight junction formation closer to that of native human skin prior to UVB exposure. The effect of UVB radiation on barrier function was measured using transepithelial electrical resistance and was found to decrease barrier function by disturbing tight junction proteins. While insightful, the lack of a dermis or vascularisation limits the model’s ability to holistically recapture the immune response of skin to UV radiation as transmigration of Leukocytes cannot be observed.

An alternate model that addresses these limitations by incorporating a functional epidermis, dermis and endothelium are described by Kwak et al (Figure 4). By perfusing leukocytes through a recreated vascular endothelium, the platform mimics native transmigration of immune cells through blood vessel walls and into the dermis. The skin cells are housed in a PDMS chip with a porous membrane separating the epidermis and dermis from the endothelium, as shown in Figures 4A and 4B. Cell culture and leukocyte perfusion are facilitated by tilting the chip on a custom, gravity-flow device (Figure 4C). When exposed to UV radiation, an immune response is elicited by the release of cytokines and chemokines which initiate the inflammatory process and encourage leukocyte transmigration (Figure 4D). To recreate this response, first the model was irradiated at 75 mJ/cm², then differentiated leukocyte cells, HL-60, where incorporated into the cell culture medium and their response observed, as shown in Figure 4E. Greater leukocyte migration to the dermis occurred when the model was exposed to UV radiation when compared with a non-irradiated control, indicating that a stronger immune response was generated by the irradiated model. This was quantitatively confirmed by measuring the number of leukocyte cells observed in the...
dermis for the irradiated and non-irradiated conditions (Figure 4F). The utility of the platform in investigating skin inflammation and immune response to stimuli has been validated, and it may find utility in not only studies pertaining to EMR, but also in chemical irritation screening applications.

While efforts have garnered insight into the cellular impact of UVR on human skin, an in depth understanding on the molecular mechanisms responsible has yet to be achieved. Vascularised, immune-competent models such as the platform demonstrated by Kwak et al offer increased physiological relevance necessary for understanding skins immune response to EMR radiation. Furthermore, the majority of research has been conducted on pigmentless skin.\[122\] The fabrication of pigmented skin models that mimic different skin tones will aid in inclusivity and our understanding of how EMR interacts with all skin tones to formulate more effective, specialised treatment approaches.

### 4.3 Chemical stimulation

Chemical stimulation from our external environment can come from drugs (both topical and transdermally acting), cosmetics, urban pollutants, tattoos, and chemical
Cell culture medium specific to each cell type is perfused through the chambers, which are staggered in size to enable observation of cells from each layer at the edge of the chambers. The different cell types adhere to the porous membrane where they are grown to confluence as shown in Figure 5C. Inflammation and edema are induced by perfusing TNF-α through the chambers, then a drug which is known to treat inflammation and edema (dexamethasone) was applied to evaluate the efficacy of the SoC platform as a drug testing model. TNF-α inhibits endothelial cell tight junction formation; however, when dexamethasone is applied, tight junction formation appears more circumferentially continuous and closer to healthy endothelial cells, as shown in Figures 5D and 5E. Edema is modeled and evaluated by injecting FITC-dextran into the endothelial (vascular) layer and measuring transport to the dermal layer by collecting fluid from the middle layer. Results indicate that the permeability of the model exposed to TNF-α is 1.8 times greater than the control, while the model treated with Dex exhibited only 1.2 times greater permeability (Figure 5G). These results demonstrate that the platform may find utility in simulating edema, eczema, and other inflammatory skin diseases, to better formulate and test the efficacy of treatments as well as screening for toxicity in cosmetics.

Nonbiological platforms present several advantages including repeatability, throughput, and accessibility compared with their biological counterparts for studies concerned with the surface properties of skin. For example, the effect of urban pollution on skin was investigated using a silicon model coated with an artificial sebum. Squalene, a lipid naturally produced by skin, is an intermediate molecule found in human sebum that plays a role in hormone, cholesterol, and vitamin D biosynthesis and is highly susceptible to oxidation. Urban pollutants where shown to degrade the quality of squalene, which when applied to the artificial skin model, significantly increase skin hydrophilic and monopolar behavior. The physicochemical consequences of the squalene oxidated via pollutants was different compared to squalene oxidation caused by high temperature and help to explain and expand our understanding of the impact of pollutants on skin health.

Combining the relevance of induced pluripotent stem cells and immune cells with microfluidic SoC technology has a strong potential to improve preclinical drug development for both healthy and diseased skin states. Overall, skin models with improved biomimicry, particularly immune function, present a promising opportunity to reduce the low predictivity of current preclinical trials while reducing the dependency of animal experiments in line with the 3R principle of reducing, refining, and replacing the use of animals in the pharmaceutical and cosmetic industries.
CONCLUSION AND FUTURE OUTLOOK

A variety of techniques have been employed to develop in vitro models of human skin with increasing similarity to native human tissue. As the field moves beyond simple 2D cell culture, methods such as organoid generation and 3D bioprinting offer the promise of multilayered, multicellular tissue constructs with complex structures and appendages. SoC platforms offer the potential for fully integrated, self-sufficient evaluation of skin model health under different stressors. When integrated with multiple organ-on-chip platforms, systemic effects can be elucidated, and the platforms can be used in conjunction with discussed techniques including organoids and 3D bioprinting, making them a versatile option. Nevertheless, overelaboration can lead to excessively laborious models with low reproducibility and high variation. Simple solutions should be favoured; however, caution must be taken when simplifying skin models to avoid excluding relevant components. Of particular importance to many studies is the inclusion of vascularisation and immunocompetency, neither of which have achieved widespread adoption.

As the development of increasingly physiologically faithful in vitro models of human skin is still in its infancy, the application of such models to investigating the effects of stressors on skin have been limited. Furthermore, there is a pronounced lack of standardization and superficial reporting of key biological and physical parameters of the effects of phototherapy in both in vitro and clinical trials, making replication and comparison difficult.

One possible application of skin models that has yet to be realised is in validating wearable sensors or e-skin for healthcare monitoring and human-machine...
This technology presents a great opportunity in health care monitoring; however, products often fail to perform or cause irritation and discomfort to the user from the stiffness of materials used, adhesives, allergic reactions, or from heating. Nonbiological platforms and SoC are very promising for validating and wearable devices, and both may mutually benefit from progress in microsensor development.

While not covered by this review, it is worth mentioning in silico or computer simulations when considering the future of skin model development. Computer simulations may prove to be the most powerful tool in terms of throughput, flexibility, and repeatability. However, much like the restrictions faced by in vitro skin models, in their current state, they are not yet capable of more complex, multifaceted experiments. Instead, a single functional component of skin (i.e., mechanical properties, molecular dynamics or thermal transfer) can be simulated for specific applications. It is probable for in vitro moleculardynamics or thermaltransfer canbesimulated (e.g., in microsensor development).

A variety of approaches are available for modeling skin in vitro; however, there is no single platform that excels for every application. The specific experimental requirements and aspect of skin function being investigated should drive the decision on which technique or model is most suitable. Thus, rather than fabricating sophisticated skin models in isolation, they should instead be designed to meet the specifications of the target application.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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self-sufficient microfluidics, and microfluidic models to study the mechanobiology of human cells.

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