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Current and Future Perspectives on Skin Tissue Engineering: Key Features of Biomedical Research, Translational Assessment, and Clinical Application

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Conflict of Interest

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Abstract

The skin is responsible for several important physiological functions and has enormous clinical significance in wound healing. Tissue engineered substitutes may be used in patients suffering from skin injuries to support regeneration of the epidermis, dermis, or both. Skin substitutes are also gaining traction in the cosmetics and pharmaceutical industries as alternatives to animal models for product testing. Recent biomedical advances, ranging from cellular-level therapies such as mesenchymal stem cell or growth factor delivery, to large-scale biofabrication techniques including 3D printing, have enabled the implementation of unique strategies and novel biomaterials to recapitulate the biological, architectural, and functional complexity of native skin. This progress report highlights some of the latest approaches to skin regeneration and biofabrication using tissue engineering techniques. Current challenges in fabricating multilayered skin are addressed, and perspectives on efforts and strategies to meet those limitations are provided. Commercially available skin substitute technologies are also examined, and strategies to recapitulate native physiology, the role of regulatory agencies in supporting translation, as well as current clinical needs, are reviewed. By considering each of these perspectives while moving from bench to bedside, tissue engineering may be leveraged to create improved skin substitutes for both in vitro testing and clinical applications.

Keywords

clinical perspectives; skin regeneration; tissue engineering; wound healing

1. Introduction

1.1. The premise of Tissue Engineering

The tissue engineering field was originally established 25 years ago by Langer and Vacanti in the aim of combining engineering design principles with our understanding of biological mechanisms to replace or regenerate damaged tissue.[1] Since then, tissue engineering has been leveraged for a variety of biomedical applications including disease modeling, resource sustainability, novel clinical therapies, and has also facilitated the development of powerful technologies such as gene editing, bioreactor culture, and 3D bioprinting among many others.[2]

Central to this field is the principle that successful tissue formation involves the synergistic activity of many cell types, not just the isolated effects of any single population. Furthermore, these cells communicate with each other in a 3D system through both living and nonliving components. This overarching theme of combining cells, 3D scaffolds, and environmental signals represents a promising strategy to create tissues for studying or treating diseases, but—like many other biomedical technologies—it often faces challenges in translation into clinically effective therapies. Examples of such challenges include the accurate recapitulation of tissue physiology, scalability to meet clinical needs, and financial cost.[2] These requirements are particularly important for skin tissue engineering, where there has been high demand driven by clinicians as well as the cosmetics and pharmaceutical industries for personalized, functional, and cost-effective skin substitutes. Current strategies

for fabricating such constructs will be discussed in detail, accompanied by recent noteworthy examples pertinent to clinical regenerative applications and in vitro skin models. We also include an analysis of current challenges and potential breakthroughs from the perspectives of major contributors to this field.

1.2. Normal Skin Structure and Function

As the outermost layer of the body, the skin is the first line of defense against external mechanical, biochemical, and environmental factors. Mammalian skin comprises multiple stratified layers, broadly the epidermis, dermis, and subcutaneous fat tissue (often referred to as the hypodermis).[3] The thinnest and most external layer of skin—the epidermis—is avascular and composed of multiple layers of keratinocytes. This layer also contains pigment-producing melanocytes as well as antigen-presenting Langerhans cells that play a role in the host immune response.[3-6] The underlying dermis is rich in blood vessels, nerve endings, and various glands, and is composed mainly of fibroblasts that synthesize type I collagen for the extracellular matrix (ECM).[3-8] Lastly, the hypodermis, which may be considered part of the endocrine system, consists mostly of adipose cells that function in energy storage and thermoregulation.[3,4] The hypodermis is often dismissed in skin models as simply a system of fat storage, but it also functions as a complex lipid barrier rich in stem cells, hormones, and growth factors.[6,7,9-14] As this tissue layer provides the nerves and blood vessels that permeate into the upper layers, the hypodermis plays a key role in re-epithelization, wound healing, and angiogenesis.[9,15-17] The structure and major components of normal human skin are summarized in Figure 1 and Table 1 below.

2. Relevant Applications of Tissue Engineering in the Development of Skin Substitutes

The purpose of tissue engineered skin is to replace or model skin tissue with a construct that mimics native physiological form or function. Such a construct could be used in research and product development to examine the potential effects of various stimuli on skin without using animal models. Alternatively, tissue engineered skin constructs could have potential application as wound dressings or skin substitutes in cases of severe skin injury, where patient survival and clinical outcome are highly dependent on restoring the skin's normal barrier function in a timely manner.[18-20]

2.1. Use and Development of Skin Models for Industry

Generally speaking, the field of tissue engineering remains in its early stages of development. It relies heavily on academic research advancement and, while startup companies are sprouting up and developing worldwide, successful clinical outcomes have not been consistently achieved and large-scale industrial production is often unattainable. However, the case of skin is unique. Advances in skin tissue engineering and modeling have been chiefly led by large commercial entities in the last several decades, particularly the cosmetics and pharmaceutical industries. Therefore, it is important to highlight their role in the advancement of this field. Skin care products, cosmetics, and other topical agents have been traditionally tested in animal models; publications assessing skin corrosion and irritation (such as the Draize rabbit skin irritation test) date back to as early as the

1940s.[21,22] These testing methods have since evolved, in large part thanks to investments into developing alternative models to in vivo animal and ex vivo human skin approaches. A critical turning point occurred in the early 2000s with the introduction of the EU's 7th Amendment to the Cosmetics Directive. This amendment prohibited animal testing of finished products or cosmetic ingredients, introducing a marketing ban regardless of the availability of alternative nonanimal tests.[21-23] Industry was thus forced to find alternatives or develop new methods.

The economic muscle behind the cosmetics industry energized efforts in developing living skin equivalents that could recapitulate part or all of the skin's natural structure. These skin equivalents typically consist of allogeneic skin cell populations that are grown in layers and seeded on scaffolds derived from ECM proteins. Patents for this type of approach have been registered since the 1990s,[24-27] but the technology was expedited into commercialization in large part due to the push from the cosmetics industry to develop suitable alternatives to animal testing. For instance, one of the most prominent manufacturers of tissue engineered skin is a wholly owned subsidiary of the cosmetics company L'Oreal. Their Episkin product line and other similar products are examples of reconstructed skin models that are widely available and extensively used as in vitro substitutes for human skin (referenced more than 565 times in scientific literature, as self-reported in L'Oreal's literature database).

These efforts led by industry have resulted in major scientific advances in skin tissue engineering, particularly in the development of skin models, living tissue equivalents, and protocols to assess skin properties. These include, among others, reproducible, in vitro assays using engineered human skin constructs to assess chromosomal damage from topically applied agents,[28,29] full-thickness skin equivalents to serve as complex skin models,[30] compromised skin assays to study chemical penetration through wounded skin, [31] and skin models to study the use of LED light for acne therapy.[32] Furthermore, since 2004, the Organization for International Cooperation and Development (OECD) has developed several in vitro methods for testing dermal corrosivity and irritation based on commercially available products.[33] Several US and EU agencies recognize these alternate test methods as a way to reduce animal testing and increase global harmonization.[34] Such efforts have also extended to precompetitive cooperation between major companies. For example, Proctor & Gamble, L'Oréal, Johnson & Johnson, GlaxoSmithKline, Unilever, and Novartis, among others, have combined their efforts to develop alternative models to animal testing, which have resulted in several joint publications.[21,35-37] Overall, the innovation and R&D departments of major companies have produced substantial advancements in the use and development of skin models, some of which are further highlighted below (Table 2).

2.2. Wound Healing

Perhaps an even more obvious application of skin tissue engineering is to augment or develop replacements for skin grafts used to treat patients with serious cutaneous injuries. In the clinical setting, skin grafts may be used to treat extensive tissue defects by restoring normal barrier function while stimulating wound repair responses. However, if normal tissue healing is impaired, or if there are insufficient amounts of healthy donor tissue available, tissue engineered constructs may be necessary.[38] While some products have been shown

to reduce morbidity and improve clinical outcomes after injury, no single skin substitute currently on the market has been demonstrated to fully restore normal skin structure and physiological function.[19,39]

When the skin is extensively injured, it loses its ability to prevent bacterial infection and regulate temperature or fluid transport[5,40] The natural response to severe skin injury in adults, involving tissue granulation and re-epithelialization, is characterized by a rapid proliferation of fibroblasts that deposit randomly oriented collagen fibers to fill the tissue defect, followed by the migration of keratinocytes and contraction of myofibroblasts that restore the barrier.[41] This collection of disorganized tissue results in a fibrotic scar, and is often accompanied by lack of a sensation and elasticity as well as flawed features—in effect, "healing" does not restore native skin function, histological structure, or aesthetics.[4,5,40,42]

Another point to consider is that other cell types normally present in the skin may be slower to regenerate, or do not grow back at all. For example, even if sebaceous glands are transplanted in skin grafts, normal secretory function typically does not occur for months[38,43] Similarly, sensory and autonomic nerves present in neighboring areas of healthy skin may ingrow to eventually re-innervate the wound area, but the process is slow and never fully complete.[38] This leads to patches of skin that may experience abnormal sensation or sweat function. Finally—and perhaps even more importantly to patients—the loss of melanocytes leads to changes in skin pigmentation, which can be disfiguring and difficult to treat with current cosmetic techniques.[44]

2.2.1. Current Strategies for Acute Wounds—Cutaneous wounds may be classified as acute or chronic, depending on the etiology. Some of the most common causes of acute skin injury include mechanical trauma, burns, or the surgical excision of skin malignancies.[19,44] The current gold standard for treating such wounds is autologous skin grafting, which—while able to cover the tissue defect and restore barrier function using the patient's own skin tissue—suffers from the same limitations as described above in that the wound site experiences significant contraction and haphazard tissue remodeling[4,5,45,46] Furthermore, the procedure is restricted by the availability of appropriate harvest sites from the patient, as well as the fact that the donor site becomes another wound requiring management. Studies have also indicated that hypertrophic healing and keloid formation may occur unpredictably, especially among those who already have a genetic bias.[4,41]

The availability of autologous skin is also a limitation in cases where a patient's wounds exceed more than 60% of their total body surface area; in these cases the injuries cannot be adequately covered by autografts due to the lack of enough harvestable tissue.[46,47] Treatment thus requires the use of alternative startegies, most commonly cadaveric allografts.[19] These function mainly as a temporary dressing to protect and stimulate healing in the wound bed before an autograft can be placed.[19,44]

2.2.2. Current Strategies for Chronic Wounds—In contrast to acute skin injuries, chronic wounds develop due to a deviation from the normal wound healing process.[48] Examples include diabetic ulcers, venous leg ulcers, and pressure sores. In each of these cases, an underlying comorbidity prolongs inflammation and delays the closure of an open

wound, leading to an increased risk of infection. Difficulty in healing is often further compounded by tissue ischemia or continual pressure on the site.[48,49]

Treatments for chronic wounds usually involve addressing the underlying condition, mechanically offloading the affected area, and debriding infected sites. In extreme cases, amputation may be indicated.[49] To try to prevent this, a wide range of clinical products to aid in the rate of wound closure and tissue granulation have been developed, although their use is generally limited due to unproven clinical efficacy, high cost, and extensive time required for in vitro cell expansion.[19,39] Examples of these products include biologic dressings, cultured epithelial autografts, and composite skin substitutes.[19,43]

3. Advanced Tissue Engineering Approaches to Regenerate Skin

At the most basic level, a successful tissue engineered skin construct ideally captures the complexities of the native 3D structure and fulfills the functions of natural skin tissue. Furthermore, it should support vascularization and provide supportive cues to cells present in the local environment. Last, if implanted in vivo, it must also be capable of integrating into the host with minimal scarring while generating a controlled inflammatory response.

In recent years, a diverse variety of strategies have been developed to try to achieve these goals. Many involve the delivery of cells or cues capable of stimulating or participating in tissue repair. The goal of these substitutes may be to directly replace cells previously lost at the defect site, deliver stem, or progenitor cells that differentiate into the native tissue type, or stimulate prohealing behavior by other cells already present in situ.[20,48]

3.1. Scaffolds to Guide Regeneration

The reconstruction of skin in tissue engineering has for the most part been focused on the development of stratified constructs mimicking the bilayered structure of the epidermis and dermis.[50-59] Early approaches used synthetic components to minimize fluid loss and mechanical stress while maintaining structural stability at the wound site. Nylon and silicone composites proved popular and could be further coated with biomolecules and skin cells, leading to the emergence of products such as Biobrane, Transcyte, and Integra.[60-63]

Scaffolds using only natural materials have also gained popularity because they contain protein motifs that facilitate cell adhesion, and demonstrate better compatibility and degradation in vivo, particularly when incorporating biomolecules already naturally part of the skin ECM.[43] Proteins such as collagen[53,64] gelatin,[65] plasma-based fibrin,[56,66] keratin,[67-69] chitosan,[70] and dextran[71] have been used both separately or in combination to culture fibroblasts and keratinocytes in efforts to mimic the dermis and epidermis, respectively. In general, these naturally derived biomaterials are used to produce porous, soft substrates by a variety of methods including self-assembly,[66] chemical crosslinking,[65] freeze-drying,[67,72] electrospinning,[55,70] and knitting.[55] These constructs may also incorporate growth factors and cells of interest (generally fibroblasts, keratinocytes, or stem cells grown in vitro) in order to facilitate native cell ingrowth or the proliferation of seeded cells from autologous or allogeneic sources. Such growth factor- or cell-laden hydrogels are widely used to study skin properties such as immunoreactivity,[53] wound

closure,[64] epithelialization,[65,66,70] angiogenesis,[65,67] or hair growth.[67,71] The inclusion of specialized cells and growth factors in scaffolds, as well as their immunomodulatory roles, will be further described in subsequent Sections 3.2 and 3.4.

ECM-based scaffolds are commonly used in vitro for modeling aspects of skin physiology and transport phenomena to take advantage of characteristic properties found in protein-based materials. While these models are helpful for addressing specific properties of native skin tissue, they are generally not comprehensive in that they take a narrow approach toward a singular goal while neglecting the complexity of skin physiology as a whole. For instance, Uchino et al. developed a cell-laden 3D human skin model containing vitrified collagen that supported the culture of dendritic cells in a layered construct.[53] In another recent publication, Sakamoto et al. used a pliable gelatin hydrogel sheet that sustained the release of basic fibroblast growth factor and conformed to the shape of the wound.[65] This construct was shown to accelerate epithelialization, granulation tissue formation, and angiogenesis in mice. In these and other similar publications, there is thorough characterization and careful study of a specific property—in these cases, formation of either stratified or vascularized tissue—yet to be successfully translated for clinical wound healing and tissue regeneration, such models must be further developed to study both these factors and more simultaneously.

As compartmentalized as these models may be, they have justified the use protein-based scaffolds in clinical trials, which have generally reflect the positive trends observed in vitro. In 2016, for example, Loan et al. published a clinical cohort study on the use of keratin-based scaffolds for superficial and partial thickness burn injuries.[69] When compared to the current clinical standard of care, keratin-based products provided faster re-epithelialization rates, reduced scarring, as well as improved clinical parameters such as reducing healing time, inpatient time, outpatient appointments, and antibiotic use.

While many other commercialized clinical ECM constructs, including Dermagraft, Apligraf, Integra, AlloDerm, MatriStem, MatriDerm, PriMatrix, and PELNAC have been marketed as dermal equivalents or degradable dressings that aid in accelerating wound closure, the cosmetic results still typically remain poor.[73] This perhaps reflects the heavy focus of skin regeneration research on detailed cell behavior and molecular pathways. Translation from a series of cellular functions to the macroscopic processes of scarring and wound contraction is often difficult to achieve. However, as fabrication techniques and biomaterial options continue to expand, skin substitutes that are functional both at the micro- and macroscale may be expected to emerge in the near future.

3.1.1. Engineering Multilayered Tissue—Products like EpiSkin,[74-77] EpiDerm[28,57,78] MatriDerm,[78]and Apligraf[79] are bilayered scaffolds designed to mimic the stratified structure of human skin. A typical production process usually includes cultivating fibroblasts inside a hydrogel (generally type I collagen), upon which a layer of keratinocytes is seeded. The constructs are then submerged in growth media until the populations are mature, and the level of media eventually decreased to expose the keratinocytes to an air–liquid interface. This stimulates the cells to proliferate, stratify, and keratinize to form the epidermal layers.[80] Living skin equivalents are used throughout literature as in vitro skin barrier models and have also been used clinically with generally

positive results in re-epithelialization[78] and wound closure,[79] although, as before, scarred skin is the norm. These engineered scaffolds typically recapitulate only the epidermis and dermis, making them ideal for addressing injuries such as first- and second-degree burns. As such, third- (involving the epidermis, dermis, and hypodermis) and fourth-degree burns (affecting all the layers down to the muscle and bone) are less often considered.

While not currently used for clinical applications, a number of trilayered constructs featuring a hypodermis-like layer have been developed for in vitro models of human skin.[81,82] Air–liquid interface culturing is typically utilized to develop these constructs, which are used to study skin tissue properties such as barrier function or cell behavior.[82,83] Some examples include, among others, the use of these trilayered models to investigate the role of adipocytokines in inhibiting fibroblast proliferation and scarring,[62]the regenerative potential of adipose-derived stem cells,[81] and the role of the hypodermis in drug absorption and metabolism.[30,82]

The fabrication and regeneration of the hypodermis layer have not yet been fully explored and thus carries great potential both in the lab and the clinic for skin growth and regeneration. Past studies have reported that incorporation of other cell types such as mesenchymal stem cells (MSCs) within scaffolds may support these endeavors.[84,85] As will be discussed in the following section, MSCs are highly advantageous owing to their potential for multipotent differentiation, their immunomodulatory effects, and ease of patient isolation and expansion in vitro.

3.2. Cell Therapies

The use of autologous or allogeneic cell populations to aid in replacing or regenerating tissue has long been a common strategy for skin tissue engineering.[2] However, the current methods that strive deliver them while maintaining high tissue complexity often require extensive time and effort to generate the construct, and therefore have limited use in point-of-care settings.[19,39] To address this, one potential alternative to the current clinical products that deliver cells in flat sheets or scaffolds is an autologous skin cell suspension spray commercially marketed as RECELL. This product uses noncultured autologous cells harvested from a patient, which are subsequently suspended in solution and sprayed on the wound, allowing the cells to adhere to the target tissue surface.[86] The cell suspension predominantly contains keratinocytes (>64%), but also includes significant populations of fibroblasts and viable melanocytes.[87] Holmes et al. completed a comparative clinical study of RECELL and autologous split-thickness skin grafting in the treatment of acute burns, where the clinical outcomes of the former were as effective as the latter while requiring almost 40 times less donor tissue.[88] Patients in this study reported significantly less pain at the donor sites, perceived greater improvement in wound appearance, and expressed overall higher satisfaction than patients receiving skin grafts. It is important to note, however, that RECELL is not a standalone solution for regenerating functional skin and does not directly address the 3D positioning of the transplanted cell types or the multilayered nature of skin tissue. Additionally, RECELL can potentially exhibit interuser variability, as it is a manual point-of-care process, and there is a steep learning curve for physicians. As will be discussed, patients typically have very favorable opinions of innovative skin

tissue engineering technologies based on the desire to improve their out-comes. However, there are often differing opinions within the clinical community based on various factors including clinicians' familiarity with the new technologies or amount of experience in other techniques.

Besides the active replacement of skin cells or structure using major cell types present in skin tissue, other cellular therapies under development instead aim to use stem cells or cytokines to induce natural tissue regeneration. One of the most popular choices for this strategy is mesenchymal stem cells. MSCs were originally isolated and characterized from mouse bone marrow by Friedenstein et al. in 1970, and categorically defined by the International Society for Cell Therapy in 2006.[89,90] They have been shown to readily differentiate into osteoblasts, chondroblasts, and adipocytes when exposed to various stimulating factors.[89,91] Studies also indicate that MSCs have the potential to differentiate into other cell types outside of the mesodermal germ layer including endothelial cells, keratinocytes, and skin appendage cells.[92-94] These cells are still most commonly derived from adult bone marrow (BM),[95-99] but they can also be isolated from many other tissues in the human body such as adipose tissue,[100] umbilical cord blood,[101] or peripheral blood.[102,103] While they are perhaps most well known for their use in cartilage and bone repair therapies, MSCs have also been extensively researched for immunomodulatory applications.[90] The diverse range of activities either induced directly by MSCs or indirectly stimulated by their prohealing cytokines is summarized in Figure 2.

In their undifferentiated state, MSCs exhibit immuno-privileged properties and have been previously leveraged for allogeneic implantation in multiple human clinical trials, as unprimed MSCs have a tendency for immune homeostasis and exhibit little immunomodulatory activity unless triggered.[104]On the other hand, MSCs can also be primed or licensed to become either pro- or anti-inflammatory based on their micro-environment.[105,106] These cells produce a wide variety of cytokines and growth factors, many with immunomodulatory functions. This has made them an interesting research topic for adjunct to tissue engineered constructs and skin regeneration cell therapies; rather than replacing the host cells, MSCs used in this way can affect or facilitate a therapy simply by their presence.[94,107-109]

Unsurprisingly, the cytokine production, immunomodulatory behavior, and differentiation potential of MSCs have long been investigated for beneficial effects on wound healing. In the mid-2000s, several groups showed that healing in various types of cutaneous wounds (e.g., excisional, burn, radiation damage) could be accelerated and improved with application of autologous bone marrow MSCs.[110-112] These prohealing effects may even persist over significant periods of time—studies have shown that, when introduced systemically, exogenous MSCs can localize in damaged areas and maintain viability for up to 6 years after implantation in humans.[113,114] However, the responsiveness of MSCs to immune signaling is mostly localized to their microenvironment, requiring either induction of endogenous MSCs migration, or direct placement of exogenous cells at the site to maximize therapeutic benefits.[115] Many hydrogel and polymer scaffolds have been thus been developed to induce activity and maintain MSC viability to promote the production of angiogenic, immunomodulatory, matrix remodeling, or other regenerative cytokines.[116,117]

In skin tissue engineering in particular, MSCs can function to promote wound healing when immobilized in hydrogels placed over the wound site or when added as an intermediary layer in split-thickness skin graft procedures.[118,119]

Consequently, the function and benefit of adding MSCs or MSC-conditioned media directly to a tissue engineered construct is an ongoing research topic. Their angiogenic, immunomodulatory, and paracrine signaling functions are also of immense interest, as well as their multipotent differentiation capabilities. However, while promising, the general efficacy of MSC-based therapies is often difficult to determine due to the phenotypic variation of cells that occurs both between donors and even within the same individual.[120,121] This perhaps contributes to the fact that according to data reported by the US National Institutes of Health (http://www.clinicaltrial.gov), there are 624 ongoing or completed clinical trials using MSCs as of November 2018, yet none have so far been able to successfully bring a product to market. Nevertheless, as techniques for assessing MSC phenotype and understanding their capabilities become more advanced, specific and therapeutically active populations of cells may be isolated and used to develop clinically efficacious procedures.

3.3. 3D Printing and Biofabrication of Skin Tissue Constructs

Conventional fabrication techniques such as manual dispensing, molding, freeze drying, and porogen leaching have been used extensively in skin tissue engineering for the fabrication of cellular scaffolds.[122-125] Novel approaches have used free-form deposition[54] or modeling on PDMS chips[52] to miniaturize in vitro models. Although easy to implement, they lack the engineering control required to fabricate architecturally complex tissues. 3D printing is an additive manufacturing technique that enables precise layer-by-layer deposition of materials to fabricate complex designs in a highly repeatable manner. Bioprinting refers to the 3D printing of biological materials and cells for the generation of living tissues.[126] Owing to the highly stratified and complex structure of the skin, bioprinting offers unique advantages for developing clinically relevant skin constructs that capture native heterogeneity and architecture. Several reviews have extensively covered the main aspects of 3D bioprinting and its relevance to tissue engineering and skin regeneration.[127-130] Here, we will recap the key aspects as they pertain to skin bioprinting as well as some of the latest advances in this field.

3.3.1. The 3D Printing Process—In order to print a physiologically relevant and transplantable skin construct, many design criteria have to be met. For example, it needs to recreate the necessary dermal layers and components, maintain the flexibility and elasticity observed in native skin tissue, and spatially conform to irregularly shaped wound surfaces with varying dermal layer requirements. Additionally, the graft has to mimic protective barrier functions of the stratum corneum,[131] integrate into the host, and exhibit low immunogenicity.[128] Consequently, regardless of whether the skin substitute is used as an in vitro model for pharmaceutical testing or as a graft for clinical use, choosing the right combination of geometry, compatible bioinks, printing technique, and post-printing maturation are critical.

3.3.2. Imaging and 3D Model Design—As a preliminary step toward the printed skin construct, a 3D computer-aided design (CAD) model of the geometry with the desired internal architecture and spatial position of the bioink components is created. Conventional parametric mechanical and product CAD software or a variety of 3D printing-specific design software packages can create the desired geometries. However, parametric programs cannot easily create complex accurate, patient-specific models that are sometimes needed for clinical implantation. Noninvasive imaging techniques such as magnetic resonance imaging (MRI),[132] computed tomography (CT),[133] ultrasound,[134] and optical coherence tomography (OCT)[130] are used to scan patient features and map the architecture to be printed.[127] MRI and ultrasound imaging are more commonly used for soft tissue components due to their ability to distinguish between the various skin layers.[127] Patient imaging can then be integrated with software into a digital 3D reconstruction of the skin tissue.[135] New techniques such as active dynamic thermography (ADT) are constantly being developed and tested for accurate surface measurements.[136,137] These imaging techniques are particularly important when recreating challenging shapes and features such the contours of the face and digits,[138,139] mapping texture and depth,[139,140] or accurately determining skin color and pigmentation levels.[141,142] The medical images are subsequently converted to the 3D printable stereolithography (STL) format where specific bioinks can be assigned for each printed layer or section.

The efficacy of these techniques relies upon factors such as image resolution, depth penetration, as well as cost in order to be uniformly applicable for patients. However, the accuracy with which multilayered structures can be scanned and translated into printable grafts will undoubtedly improve with time and will play an important role in the development of personalized tissue engineered treatments in the future.

3.3.3. Bioinks and Material Selection—A number of biomaterials, both natural and synthetic, have been examined and reviewed in literature as potential skin substitutes.[48,127,128,143] Naturally derived polymers such as collagen, gelatin, alginate, fibrinogen, and chitosan have the advantage of being biodegradable, decorated with functional peptides, and structurally similar to native ECM. Due to the abundance of collagen and proteoglycans in native skin tissue, they are a popular choice for skin grafts. However, their poor mechanical properties and rapid degradation rate limit the long-term stability and applicability of the graft.[128] Electrospinning is a 3D printing technique commonly used for fabricating composite scaffolds with biofunctionality augmented by synthetic polymers. These polymers, including polylactic acid (PLA),[144] poly(ε-caprolactone) (PCL),[145,146] and poly(lactic-*co*-glycolicacid) (PLGA),[147,148] exhibit superior mechanical properties and are commonly integrated with the softer, natural components described previously. However, the exact choice of biomaterials can also determine the printing technique being used, as they consequently affect the incorporation of cells within the scaffolds. For example, the harsh solvents used in electrospinning are not conducive to cells or some naturally occurring polymers such as collagen.[149] An alternative approach for bioink material selection explored by Kim et al., involves the use of decellularized porcine skin (S-dECM).[150] A significant advantage offered by dECM bioinks is the retention of matrix proteins critical to cell functionality and an improved cellular response to native cytokines and growth factors.

Here, the researchers formulated a printable S-dECM bioink that was laden with fibroblasts for the dermal layer and inkjet-printed keratinocytes for the epidermal layer. Extensive in vitro analysis revealed favorable mechanical and rheological properties of the S-dECM bioink. Notably, minimal construct shrinkage was observed, a problem often associated with collagen. Functional evaluation of the cell-seeded constructs revealed improved cell attachment, higher expression of proteins such as fibronectin, decorin, and type I collagen, as well as thicker dermal and epidermal layers compared to collagen controls. Similar results were observed in vivo where 3D-printed skin patches of S-dECM laden with adipose-derived stem cells and endothelial progenitor cells were grafted on mice for a cutaneous wound healing model. Overall, the cell-laden skin patch promoted neovascularization and re-epithelialization while accelerating wound closure. These results highlight the importance of proper bioink selection for optimal clinical translation.

The choice of cells within the bioink also has a significant impact on the functionality of the 3D printed construct. Keratinocytes, which deposit keratin and are a major component of the epidermis, are the most abundant cell type being investigated for most skin tissue constructs.[128] However, a fully functional skin substitute uses additional cell components to develop aspects of native physiology such as ECM deposition, vascularization, pigmentation, and gland formation. Fibroblasts are routinely used for their high proliferative capacity and their broad-spectrum matrix deposition (collagen, elastin, proteoglycans).[151] Cubo et al. presented 3D printed plasma-derived fibrin scaffolds for the standardized production of skin equivalents. Using a custom-modified open source 3D printer (Printrbot), the researchers achieved systematic, layered deposition of human fibroblasts and keratinocytes, obtained from skin biopsies of healthy donors, human plasma, and calcium chloride in a single continuous print. The 3D printed skin construct consisted of a dermis (plasma-derived fibrin loaded with fibroblasts) and an epidermis (keratinocytes) layer that were matured in vitro. The ability to fabricate large skin constructs (up to 100 cm^2) rapidly using this platform is an important advantage from a clinical perspective. When grafted on to the backs of immunodeficient mice, these scaffolds showed promising results with respect to skin morphology, the various layers characteristic of healthy skin tissue (stratum basale, spinosum, granulosum, and corneum) as well as the presence of keratin and organized collagen fibrils binding the dermis and epidermis. Additionally, neovascularization of the 3D printed skin construct indicated full functional recovery and integration with native tissue. The ability to manufacture stratified skin tissue rapidly that has a large surface area and using human-derived cells has great potential for successful in vivo integration and clinical translation.[56]

Incorporation of melanocytes (for desired skin pigmentation and color),[141,152] adipose derived stem cells and adipocytes (for adipose tissue development),[153] and endothelial cells (for vascularization)[154] are currently active areas of research and poised to have a significant impact on the final outcome of tissue engineered skin scaffolds. The promise of advanced skin tissue regeneration to facilitate wound repair or in vitro model development relies on the successful application of such multicellular, multimaterial bioink.

3.3.4. 3D Printing Techniques—A number of 3D printing techniques are currently available for biofabrication.[126] Latest trends include ink-jet deposition and laser-assisted

bioprinting, which allow for tight control over microstructures and spatiotemporal deposition of biomaterials and cells—especially stem cells—for biomimicry and miniaturization studies.[143] Micro-extrusion-based printing is more cell friendly and allows for incorporation of biological molecules, but is limited to a printing resolution of >100 μm for cell-based inks.[155] Techniques such as electrospinning, and more recently, scaffold-free spheroid-based fabrication have also been explored.[156,157] The various 3D printing techniques are summarized below (Figure 3). The large number of available printing technologies has provided multiple options to optimize layers at the micro- and nanoscales. Choosing the appropriate printing technique for the structure and application can be a key part of conceptualizing a project or product. Xiong et al., for example, reported the extrusion-based 3D printing of gelatin microporous scaffolds coated with silk fibroin (SF) and its sulfonated derivative.[158] These composite scaffolds, designed to sequester and concentrate fibroblast growth factor (FGF-2), resulted in pore sizes of 100 to 200 μm when coated with pure SF and pore sizes of 400 to 500 μm using sulfonated SF. Scaffold performance was evaluated both in vitro and in vivo in a full-thickness rat skin defect model. FGF-2 incorporated scaffolds exhibited higher rates of cell proliferation, migration, and favorable morphology in vitro, particularly in sulfonated SF coated scaffolds. Similarly, in vivo analysis revealed improved would repair and vascularization after 28 d compared to control groups. High-magnification imaging indicated that surface roughness could also be varied using this method. A higher collagen content with organized collagen fibers as well as significantly thicker re-epithelialization was observed in these scaffolds. Overall, the scaffolds promoted full-thickness skin healing, and the incorporation of FGF-2 enhanced cell proliferation rate, tissue morphology, collagen fibril assembly, and vascularization.

Extrusion printing was also used by Kim et al. to produce artificial skin phantoms mimicking human skin as catalogued by color and tone (Fitzpatrick skin types I-VI) in order to match the corresponding optical and mechanical properties in laser tattoo removal applications.[159] Epidermal-dermal phantoms were printed using gelatin and agar with different concentrations of coffee and TiO_2 added to mimic the melanin variations responsible for skin color and tone. Bilayered scaffolds were designed to match the thicknesses of epidermis and dermis, 150 μm and 1 mm, respectively. The resins were successfully extruded into 30 μm thick layers, producing overall a 138 μm thick epidermis and 810 μm thick dermis with optical properties emulating various tones of the human skin.[159] This addresses an important aspect of skin tissue engineering regarding the complete recapitulation of native skin pigments and texture which is discussed further in the clinical section. Nevertheless, would closure and healing remains the priority.

Other novel systems for micro-3D printing of skin include the Integrated Composite tissue/organ Building System (ICBS) presented by the Cho and co-workers and the laser-assisted Bio-Printing (LaBP) system reported by the Chichkov group.[160,161] These systems are reported to be capable of producing layered epidermal-dermal scaffolds with high spatial resolutions, and also organizing sequential layers of human primary dermal fibroblasts (HDFs) and epidermal keratinocytes (HEKs) with their respective ECM compositions. Using the LaBP technique, researchers fabricated stratified layers of fibroblasts and keratinocytes embedded in a collagen gel to engineer a 10 mm x 10 mm scaffold with a full thickness of 2 mm (Figure 4a).[161] The layers were printed on a sheet of Matriderm as a proof of concept

for post-print clinical translatability. Cell viability, construct structure, and cell junctions were maintained over a period of 10 d in vitro. More importantly, the presence of laminin suggested the potential for the formation of the basal lamina, an important facet of skin tissue. Although recapitulating the dermal and epidermal layers is critical for a successful skin graft, a full-thickness skin model consisting of the hypodermis more closely mimics native skin physiology and functionality. Recent efforts by Kim et al. have attempted to recapitulate native skin architecture by 3D printing perfusable, vascularized skin constructs consisting of all three layers.[162] Building upon their previous transwell platform,[160] they successfully co-printed a PCL transwell chamber with sequential layering of the hypodermis (preadipocytes-embedded adipose-derived dECM-fibrinogen bioink), vascular channels using sacrificial bioinks (endothelial-cell embedded gelatin with thrombin), and the dermis (human dermal fibroblast (HDF)-encapsulated skin-dECM-fibrinogen bioink) to create a vascularized skin construct. The epidermal layer consisting of inkjet-printed keratinocytes was added after 7 d of construct maturation. Histological analysis revealed the presence of distinct layer-specific markers for the hypodermis, dermis, and the epidermis, as well as laminin representative of a basal layer (Figure 4b).[162] The vascular channel was capable of supporting the underlying hypodermis while enabling an interface with the dermis. Improved epidermal stratification and higher expression of p63, a skin stemness marker, was also observed compared to a two-layered scaffold lacking a hypodermis or vascularization. Overall, such a full-thickness, vascularized skin construct that closely mimic native skin physiology holds great promise as an in vitro diagnostics platform or for investigation of skin pathologies.

Unlike in vitro printing, in situ 3D printing would bring the printer into the operating suite to build a construct directly on the patient. The idea proposes a portable solution that is on-demand for clinicians, while circumventing the time required for tissue maturation in patients with immediate needs. This was first explored by Binder et al. where full-thickness skin defects were first created on the dorsa of athymic mice.[163] Human keratinocytes and fibroblasts embedded in collagen/fibrinogen hydrogel precursors were printed layer-by-layer directly on the skin defect using a modified 3D printer after the defect topography was mapped. The study showed promising results with complete closure of the wound by 3 weeks, as well as the formation of an organized dermal collagen layer with a fully formed epidermis. A follow-up of this technology investigated the use of either amniotic fluid-derived stem cells or MSCs as bioink components for immunomodulatory effects. Similar to the previous work, improved wound site recovery and neovascularization was observed.[164] The development of the Biopen handheld surgical device by O’Connell et al., is another example of rapid construct translation.[165] Here, the researchers devised a custom-made tool capable of bioprinting cell-laden gelatin methacrylamide/hyaluronic acid–methacrylate hydrogels and demonstrated that it could dispense adipose stem cells in a manual, direct-write fashion while maintaining high cell viability. Despite the exciting possibilities of in situ printing, creating a wound bed-following, precisely shaped contour may ultimately be less important than developing methods for reducing the maturation time between printing the scaffold and achieving a useable construct.

3.3.5. Construct Maturation and Bioreactor Culture—The final step in the biofabrication process is construct maturation prior to testing or implantation. Common strategies to achieve the air–liquid interface for developing the top-most keratin layer are prematuration of the cell-laden scaffold in the appropriate medium, followed by deposition of the keratinocytes on the top layer. The scaffold is then raised out of the culture medium until the surface is exposed to air.[54,166] However, this strategy limits the construct thickness based on its capacity for diffusive mass transfer of oxygen and other nutrients. Although a number of bioreactor types are used in tissue engineering,[131] the challenge in maintaining an air–liquid interface makes perfusion-based bioreactors the most well suited for this purpose. Using a perfusable vascular channel system, Mori et al. cultured a skin equivalent that exhibited epidermal and dermal morphology as well as the formation of endothelial tight junctions within the vascular channels. The skin construct was also successfully tested for preliminary barrier function, and maintained greater thickness than non-perfused controls (Figure 5).[167]

Bioreactors are also popular tools for developing vascularized tissue constructs, as flow-induced shear stress is known to promote vascularization.[168] Consequently, the presence of dynamic flow is key to developing a full-thickness skin tissue with the required degree of vasculature for rapid in vivo integration. However, one challenge in maintaining the required air–liquid interface is the dynamic scaffold contraction during the process of maturation. This leads to inconsistent interface levels that could potentially hamper the tissue maturation process. Achieving a static interface requires advanced capabilities and active fluid level monitoring, which have not yet been fully explored.

Although skin is a highly elastic tissue, it is not under constant or cyclic loads in contrast to other tissues such as bone, muscle, or cartilage. Early work by Atala and co-workers used bioreactor systems for the expansion of living skin matrices to increase the surface area of skin available for reconstructive purposes, while simultaneously demonstrating that the mechanical properties of native skin were not adversely affected using this method.[169] Following trials showed similar success in patient-derived skin samples, promising a path for clinical applications.[170] Interestingly, upon a 5 d stimulation of cultured skin constructs (epidermal keratinocytes and dermal fibroblasts on porous silicone sheets) via stretching, Tokuyama et al. observed that the constructs exhibited a thicker epidermal layer and higher expression of ECM proteins compared to non-stimulated controls. The basement membrane structure was also more developed, underlining the impact of mechanical stimulation on skin physiology.[171] Although promising, the materials typically used for skin tissue engineering —mainly collagen, fibrin, gelatin—are not mechanically durable in in vitro conditions, and therefore there is limited work done with the dynamic culture of skin constructs.

3.4. Delivery of Immunomodulatory Cues

Another element of tissue engineering involves delivering biochemical cues to constructs to stimulate tissue regeneration. This may be promoted by drugs, cytokines, or growth factors, or mediated by the material properties of the scaffold itself[172,173] In the physiological process of wound healing, a critical factor that dictates the outcome is the host’s immune response. When the skin is injured, the body will attempt to heal the wound by engaging

inflammatory and regenerative processes in an ordered sequence.[41] In the first 24–48 h postinjury, neutrophils infiltrate the tissue and play a critical role in early host defense by clearing necrotic tissue and bacteria from the site. Circulating monocytes then enter the tissue and differentiate into macrophages. These macrophages may further polarize to different phenotypes—M1 macrophages are proinflammatory but necessary for early host defense, while M2 macrophages are anti-inflammatory and stimulate tissue healing via cytokines such as IL-10, TGF-β, and VEGF.[174] In chronic wounds, however, the polarization is predominantly M1, resulting in the secretion of cytokines such as IL-1β and TNF-α that maintain a state of chronic inflammation and prevent M2-mediated tissue healing from occurring.[49,175,176] Thus, the presence of underlying comorbidities may affect a wound's ultimate outcome, for instance prolonging inflammation and inducing chronic ulceration instead of closing the wound.

Major research efforts have focused on the use and release of signaling ligands or small molecule analogues to modulate the behavior of the immune system locally and over extended periods of time.[177] One such example is sphingosine-1-phosphate (S1P), whose receptors are highly expressed on monocytes and macrophages. This sphingolipid plays a major role in their proliferation, phenotype, and migration in both the central nervous system and peripheral blood.[178,179] When exposed to S1P in vitro, these cells preferentially adopt anti-inflammatory phenotypes and display a reduced secretion of proinflammatory cytokines if stimulated.[174,179] Lim et al. used S1P in combination with the antifungal agent ciclopirox olamine, which also displays proangiogenic activity.[180] The group found that injection of the two agents into a polyvinyl alcohol sponge implanted in diabetic fatty rats supported endothelial migration and the formation of functional vessels. Similarly, fingolimod (also known as FTY720 and Gilenya) is a small molecule drug that acts as an agonist for several S1P receptors and has been previously shown to support endothelial cell function and stabilize microvasculature.[178,181] Past work in a muscle ischemia model using thin films of PLGA to control fingolimod release showed that the drug preferentially recruits anti-inflammatory monocytes and M2 macrophages via stromal cell derived factor-1 alpha-(SDF-1α) mediated chemotaxis and supports local arteriogenesis.[182]

As mentioned previously, physiological wound healing is a multistep process. While these and many other immunomodulatory strategies focus on influencing the cellular effectors of the host immune response and their downstream effects on tissue regeneration, other have aimed to directly combat sources of inflammation and the key biochemical signals that prolong this response. For instance, curcumin, a naturally occurring polyphenol found in turmeric, has gained some interest as a potential agent for stimulating wound healing. Although its full mechanism of action has not yet been elucidated, it has been previously shown to demonstrate some anti-inflammatory as well as antimicrobial potential.[177,183] Tong et al. developed a cellulose nanocrystal film to release curcumin.[184] The group demonstrated that this system was able to inhibit bacterial growth when applied topically to streptozotocin (STZ)-induced diabetic rats with full-thickness skin defects. Furthermore, the treatment resulted in a significant increase in wound closure rate compared to controls, and regrowth of skin layers as well as glands and hair follicles. Likewise, resveratrol, another natural polyphenol, has also been investigated for its anti-inflammatory and bacteriostatic properties. Berce et al. fabricated chitosan-sodium hyaluronate-resveratrol sponges that were

shown to support the formation of granulation tissue with reduced neutrophilic infiltration in mice.[185] Furthermore, the construct displayed a lack of bacterial contamination compared to the control, and supported local angiogenesis and re-epithelialization.

Beyond the delivery of factors to stimulate wound healing, another potential strategy may be to instead locally inhibit signals for inflammation and tissue damage at the wound site. Toward this aim, Kasiewicz and Whitehead used lipidoid nanoparticles loaded with siRNA targeting TNF-α.[186] Transfection with these nanoparticles decreased TNF-α production by macro-phages as well as MCP-1 by fibroblasts in co-culture. Although this strategy was only tested in an in vitro co-culture model, it represents a promising method for reducing inflammation locally, especially since systemic anti-TNF therapy carries a risk of global immunosuppression and opportunistic infection.

Lastly, scaffold material properties may directly influence immune cell response and the resulting effects on tissue regeneration. For instance, Waters et al. investigated the in vitro response of macrophages cultured on oxidized keratin isolated from human hair.[188] The group found that this material induced polarization to an M2-like phenotype as characterized by both surface marker expression and cytokine production. Similarly, Sun described the use of a dextran-isocyanatoethylmethacrylate-ethylamine (DexIEME) hydrogel to stimulate skin regeneration in both porcine and mouse models.[187] This dextran-based, bioabsorbale hydrogel was also shown to promote healing at pre-existing scar sites and promote the formation of hair follicles. The author examined macrophage polarization in response to DexIEME macromers and found that this predominantly led to M2 polarization, suggesting that the material is able to modulate the behavior of macrophages to affect wound outcome.

Other scaffold factors such as topographical patterning and surface chemistry may also alter the microenvironment of immune cells to directly influence their phenotype[173,190] Additionally, peptide motifs may be used to create immunomodulatory scaffolds, as evidenced by the self-assembling hydrogel composed of substance P and other bioactive peptides fabricated by Kim et al., which was shown to recruit MSCs and facilitate wound closure in a diabetic mouse model.[189] Characterizing the immune profile of chronic wounds and determining how various biochemical and material factors may modulate it to promote wound healing represents a promising direction of research that has yet to be fully explored.

4. Current Perspectives and Future Directions of Skin Tissue Engineering

As might be expected, the criteria for successfully developing a skin substitute and translating it to patient use varies widely depending on the perspective of the evaluator. Although most would agree that regenerative efforts must focus on achieving wound closure and proper tissue healing at a suitable rate, there are further considerations to be made in order for a tissue engineered construct to be deemed effective and equally accepted by the biomedical research community, regulatory agencies, industry, clinicians, and patients. These will be discussed in detail below and are briefly summarized in Figure 6.

4.1. The Biomedical Research Community

To academic institutions and industrial R&D, the underlying goals of biomedical research are not entirely dissimilar and in fact quite frequently overlap. Both strive to harness fundamental biomolecular mechanisms in order to develop novel technologies that can be translated into clinically effective therapeutics. In the case of skin tissue, this pertains to expanding our current understanding of how various microenvironmental factors affect cell phenotype, ultimately leading to epidermal–dermal stratification and tissue regeneration.

As previously discussed, the stratified structure of the skin is critical for its function. Blood vessels and neural networks grow through the interface of the hypodermis and dermis forming the deep vascular plexus; capillaries spread into the layers providing metabolic and gaseous exchange, while oxygen and nutrients will only reach the epidermis by diffusion.[5,10] A lack of healthy, synergistic layer development would result in unviable nerve growth or vascularization, which would impair the sensing and thermoregulatory functions of skin.[3,6] Irregular interfaces between the layers could also lead to improper adhesion, fluid collection (blisters or bullae), or separation.[3] Therefore, when developing strategies for regenerating skin in vitro, recapitulation of normal tissue architecture is critical. Techniques for monitoring tissue development and structure almost always involve histological assessment using stains such as hematoxylin/eosin and Masson’s trichrome. Epidermal stratification can also be tracked via expression of layer-specific markers such as involucrin, filaggrin, loricrin, and cytokeratins 5, 10, and 14.[56,191]

In recent years, the role of the host immune response has also emerged as a vital topic for consideration. As previously discussed, major efforts have focused on modulating cell behavior in order to facilitate a normal sequence of inflammatory, tissue granulation, and remodeling processes. These cell types include circulating monocytes, macrophages, and T cells—especially regulatory T cells—that perform direct effector functions in addition to maintaining a complex milieu of regulatory growth factors and cytokines.[192,193]

One further consideration for the industry involves the logistics of scaling up to mass-production.[194] This includes ensuring that consistency can be maintained between lots—for example, non-autologous components (e.g. ECM-based scaffolds) should exhibit similar mechanical properties between batches. Likewise, the procedures used in manufacturing, processing, and characterizing the products must show repeatable and reliable results. Finally, if autologous cells are to be included in the therapy, consistent methods of harvesting cells from patients at high yields will need to be developed. These cells then need to be expanded with minimal manipulation in vitro, as extensive passaging may cause them to change their phenotypes or senesce to become less effective. To further complicate this process, this entire procedure must be carried out in the minimal amount of time possible so as to expedite patient treatment, and at minimal production costs so that the constructs can be marketable.

4.2. Regulatory Agencies

The timeline for approval of innovative solutions can be quite long, as translation from bench to bedside often requires regulatory approval to determine the safety and efficacy of

the therapy, as well as review of quality controls. In the USA, therapeutics containing human cells, tissues, or tissue-based products (HCT/Ps) intended for implantation, transplantation, infusion, or transfer to a human recipient are generally regulated by the FDA's Center for Biologics Evaluation and Research (CBER) (21 CFR 1271.3d). In 1998, the Center proposed regulations, implemented in 2005, that defined different types of HCT/Ps, product processing, and regulatory requirements for the manufacturers of those products (21 CFR 1271). Two parts of the Public Health Service Act govern the regulation of HCT/Ps—Section 361 aims to prevent the introduction, transmission or spread of infectious disease, whereas Section 351 provides FDA with the authority to regulate biological products. A tiered, risk-based approach governs which regulations apply to a given HCT/Ps. Very briefly, if a HCT/P is deemed to be minimally manipulated, be for homologous use, not combined with another article (with some limited exceptions), and depending on its systemic effect and dependence on metabolic activity of living cells, CBER only regulates the product so as to prevent the introduction, transmission, or spread of infectious disease.[195] Otherwise, the HCT/P is regulated as a drug, device, and/or biological product. These rules and regulations have been the subject of considerable discussion since their proposal.[196]However, as part of its commitment to the 21st Century Cures Act and recognition of the expanding nature of the field, FDA finalized guidance on the scope of these regulations in 2017to give additional clarity to where each HCT/P product falls in the regulatory framework.[195,197]

Many tissue engineered products, including the skin products described here, must go through regulatory approval processes: e.g., either as a Premarket Approval (PMA), Investigational New Drug (IND), or Biologics License Application (BLA). To help product developers navigate the regulatory system, CBER provides an overview of what is required for a review of their products, including preclinical trial design, assessment outcomes, and the progression through clinical trial phases.[198,199] There are also programs and designations available to expedite the development and review of eligible biological products through Priority Review and Accelerated Approval.[200] Stakeholders are also able to contact CBER early in their product development cycle through the INitial Targeted Engagement for Regulatory Advice on CBER producTs (INTERACT) program[201] before they are ready to submit a more formal meeting request. Early interaction in this way may help ensure that proposed testing yields the necessary information for a premarket submission.

4.3. Clinicians and Patients

The current gold standard treatment for closing severe skin wounds is skin grafting (full- or split-thickness), though for the past 40 years huge efforts have been invested into engineering an alternative solution. From a clinical standpoint, it is desirable to replace "like with like." In other words, the ideal scenario would be to treat skin defects with autologous skin or skin-like substitutes. These constructs would ideally include all the native components of skin: the epidermal and dermal layers, the tissue-specific combination of proteins and cells, as well as skin appendages such as hair follicles, sweat glands, and sebaceous glands.

Most tissue engineered scaffolds and skin substitutes, however, are still early in research and product development stages. These include 3D printed constructs and scaffolds developed or matured using lab-on-a-chip or bioreactor technologies, none of which are readily available yet for clinical use. Products such as Dermagraft and Apligraf are cellularized, matrix-cultured products that are wound healing adjuncts but should not be considered true skin scaffolds. Rather, they should be catalogued as “smart dressings” that are intended to rapidly degrade and induce primary healing by influencing the local cell population. Other products such as Integra, Alloderm, MatriStem, MatriDerm, PriMatrix, and PELNAC are bioengineered or decellularized matrices that incorporate into the wound and provide a template for cellular infiltration. However, based on clinical experience, there is considerable patient-to-patient and product variation in the results and outcomes. These products can be considered a step in the right direction—they provide a starting place and generally facilitate positive outcomes with proper clinical handling. But, as mentioned, they do not cover the requirements for functional, layered skin and still depend on additional grafting to fully reconstruct complex wounds. Clinical needs point towards a one-step technique, an off-the-shelf product that can recapitulate the original tissue structure (including its microenvironment).

As mentioned previously, autografting has the additional complication of adding a second injury at the donor site, which is painful and may sometimes heal with additional deformity. This quandary has generated intense clinical interest in the capabilities of 3D printing of skin substitutes to avoid donor site morbidity: it introduces a potential solution that does not require significant harvest from the patient. Bioprinting techniques can produce constructs in a wide range of sizes and material combinations. In the near future, this method could feasibly be leveraged to achieve perhaps as much as 500 cm^2 of full thickness composite skin. The direct printing of skin on a patient has also been introduced as a potential method, but—while an alluring idea—is generally questioned by clinicians due to its steep costs.

As might be expected in a burn or trauma center, patients may at times be incapable of making decisions for their treatment, so physicians must in these cases make a judgment on the techniques to be used. Many clinicians tend to avoid complex tissue engineered constructs because they are often quite expensive and not uniformly effective; in fact the clinical burn community has not fully embraced new technologies for decades. The surgical treatment of burns has changed little since the late 1970s and there have been arguably few major operative burn advancements since then. The first was Integra, which entered clinical use as a dermal template but not as the all-in-one skin substitute that clinicians hoped for. Next came Epicel, a cultured epidermal autograft intended for use only in deep, widespread burns that produces widely variable results.[44] The most recently approved burn treatment, RECELL, received approval in September 2018. It began clinical trials in 2010 and has since been used on approximately 250 patients in the US, either as part of the clinical trial protocol or under compassionate use.[88] Clinical trials for regenerative medicine therapies often require a long-time enroll enough subjects to meet the study requirements or long-term endpoints to ensure safety. Several measures have been taken to expedite the review of these therapies including the FDA’s INTERACT program discussed earlier and the regenerative medicine advanced therapy (RMAT) designation defined by the 21st Century Cures Act.[202]

When able to participate in coordinating their own care, patients in general seem more accepting of tissue engineered solutions than clinicians. This is perhaps due to the straightforward desire of wanting to get better faster, or the perception that newer technology will yield better outcomes. This outlook also extends to participation in clinical trials for tissue engineered products—the public appears to be generally optimistic of this field, and volunteers are frequently excited to be a part of the developmental process. From experience, many patients have also come forward asking for regenerative medicine solutions, often requesting that clinicians try these strategies after hearing about these strategies from the press or internet. However, there remains a high expectation for success fueled by misinformation, and thus it is important to explain the current capabilities of tissue engineering—what it is, what is not, and how it works.

None of the approaches discussed so far is a panacea for every burn or wound; they each are a tool in a fast-growing toolkit for clinicians. However, there are still a couple of tools missing. Aspects of skin tissue engineering research that could be further addressed include pigmentation, nerve regeneration, and mechanisms of adult human scarring. For example, skin pigmentation is a significant area that remains to be addressed in detail. Grafted skin—whether or not regenerative technologies are used—often looks different even if the texture of the skin appears normal without scars. Such pigmentation differences can be very apparent and may be more troublesome cosmetically to some patients than scars. Improving this outcome could make a substantial difference in the quality of life for some patients, especially when abnormal pigmentation might be considered disfiguring or not part of a patient's self-identity. Broadly speaking, there are no current consistently effective solutions to treat skin hyper- or hypopigmentation. While there are techniques to either bleach or tattoo the affected areas, these are generally incomplete and often generate unsatisfactory cosmetic results. Case series suggest that cell suspension products containing viable melanocytes (e.g., RECELL) are especially promising for addressing dark skin tones, as well as for the treatment of vitiligo.[203] However, there is still a disparity between case series using novel tissue engineered products versus robust, prospective, randomized clinical trials. Only by extensively studying and rigorously testing these products will their true efficacy be revealed.

5. Conclusions

Being able to meet or exceed the quality of current gold standard autologous skin grafts with off-the-shelf, composite, full-thickness constructs represents the "Holy Grail" of skin tissue engineering. For clinical applications, there is the added requirement of minimizing or altogether eliminating scar formation, as well as the need for broad effectiveness across a wide range of patient populations and wound types. Other qualities to the ideal skin substitute include the integration of functional appendages into these substitutes, as well as the ability match patient-specific pigmentation. The regenerated skin not only must look like native skin but also has to function appropriately; the clinical and physiological properties of the skin layers and structures have to be just right.

So what is the route to this "Holy Grail"? The potential for accelerated and complete skin regeneration from the field of tissue engineering has greatly expanded over the last

few decades, with many novel strategies and viable technologies reaching the product market. However, the current options available still remain limited in a number of ways—for instance, too often the tradeoff between efficacy and cost is too high for a product to be regularly used. Further efforts to achieve an ideal skin substitute will require continued communication and collaboration among researchers, clinicians, and regulatory bodies to ensure that the final product optimally attains the wide range of objectives discussed here. In this way, skin substitutes will become more widely accepted as a viable solution for reducing the number of animals used for commercial testing, or for improving the quality of life in patients with serious skin injuries.

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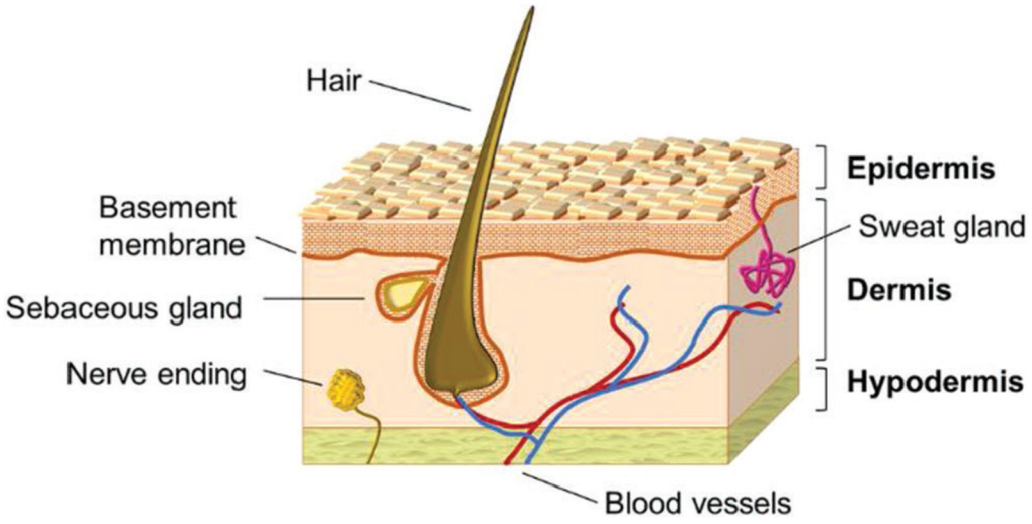

Figure 1.
Schematic representation of the major structures and layers of skin tissue that are necessary for normal skin function.

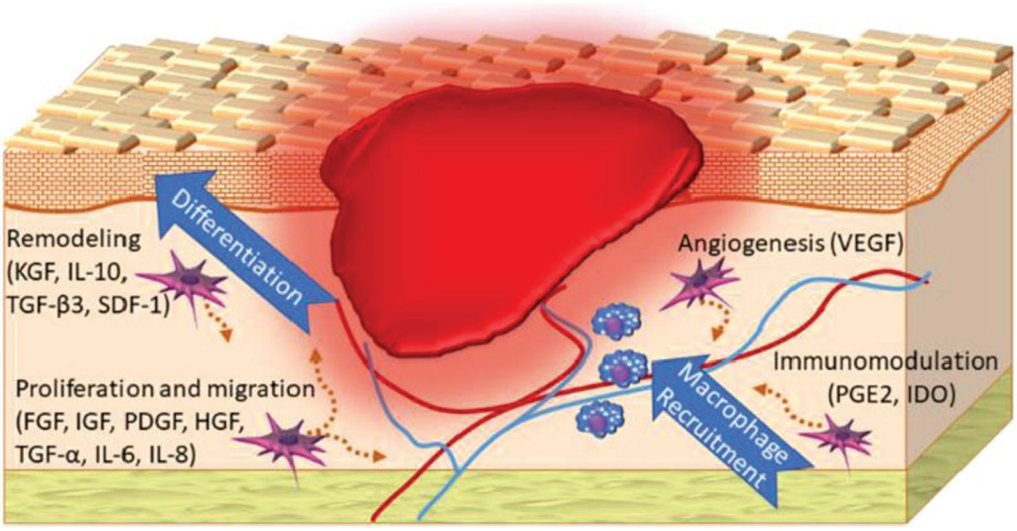

Figure 2.

Mesenchymal stem cells (MSCs) participate in many aspects of wound healing. They directly and indirectly promote several cellular functions by: (Clockwise from top) releasing proangiogenic cytokines; recruiting macrophages and producing immunomodulatory cytokines; releasing chemokines as well as factors for cell proliferation and remodeling; differentiating into fibroblasts and even skin appendage cells.

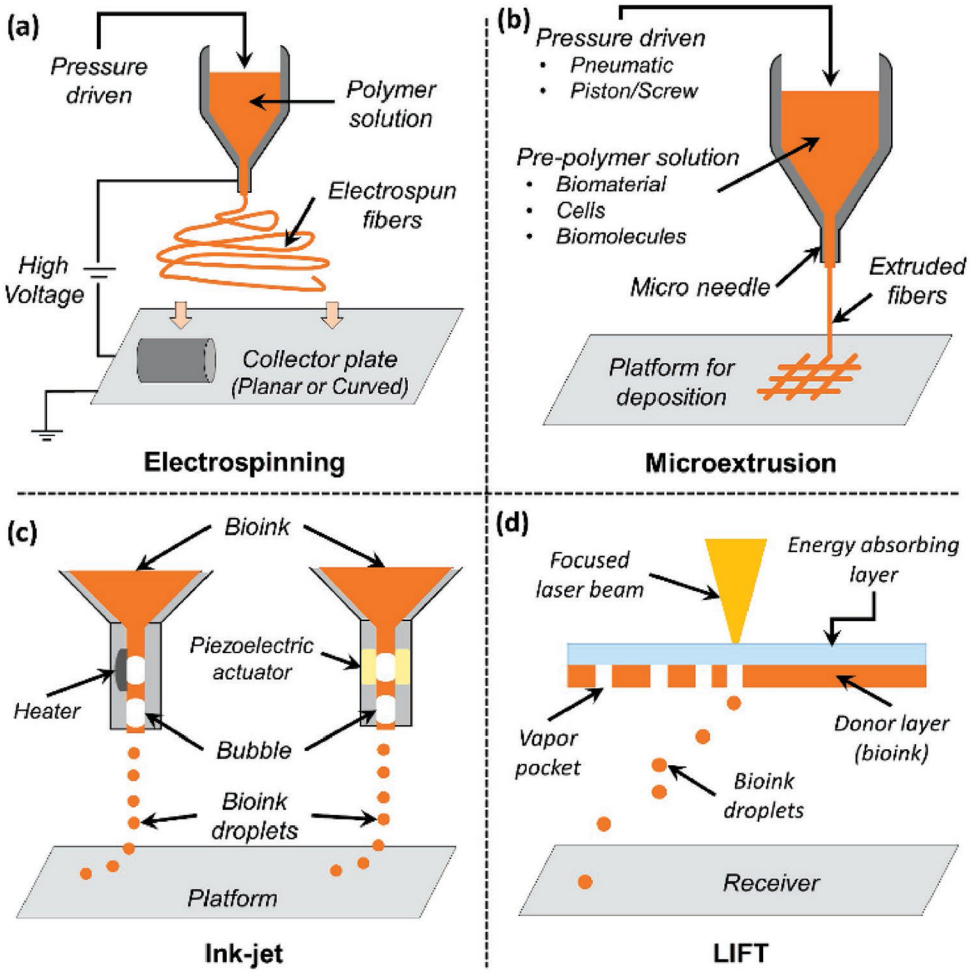

Figure 3.

Common techniques of 3D printing for skin tissue engineering. a) In electrospinning, the extruded polymer solution is subjected to voltage differences that generate filaments at the micro- or nanoscale, depending on the printing conditions. The fibers can be deposited into a planar surface or woven onto nonplanar structures. b) In microextrusion printing, the polymer solution containing cells and other biologics is extruded through a needle and deposited layer-by-layer on the platform. Multiple layers can be assembled by controlling the needle movement. c) Ink-jet printing enables the dropwise deposition of the bioink. Typically low viscosity solutions are used and the droplets can be generated via localized temperature or pressure variations. d) In laser-induced forward transfer (LIFT), also called laser-assisted bioprinting (LAB), a focused laser beam is pulsed on top of a donor layer containing the desired bioink formulation. Energy is transferred from the laser and through the energy absorbing layer (typically metal-coated glass) to create localized vapor pockets that dislodge the donor layer bionk in the form of droplets. Changes in laser position allow the generation of the desired pattern, making this a nozzle-free printing method.

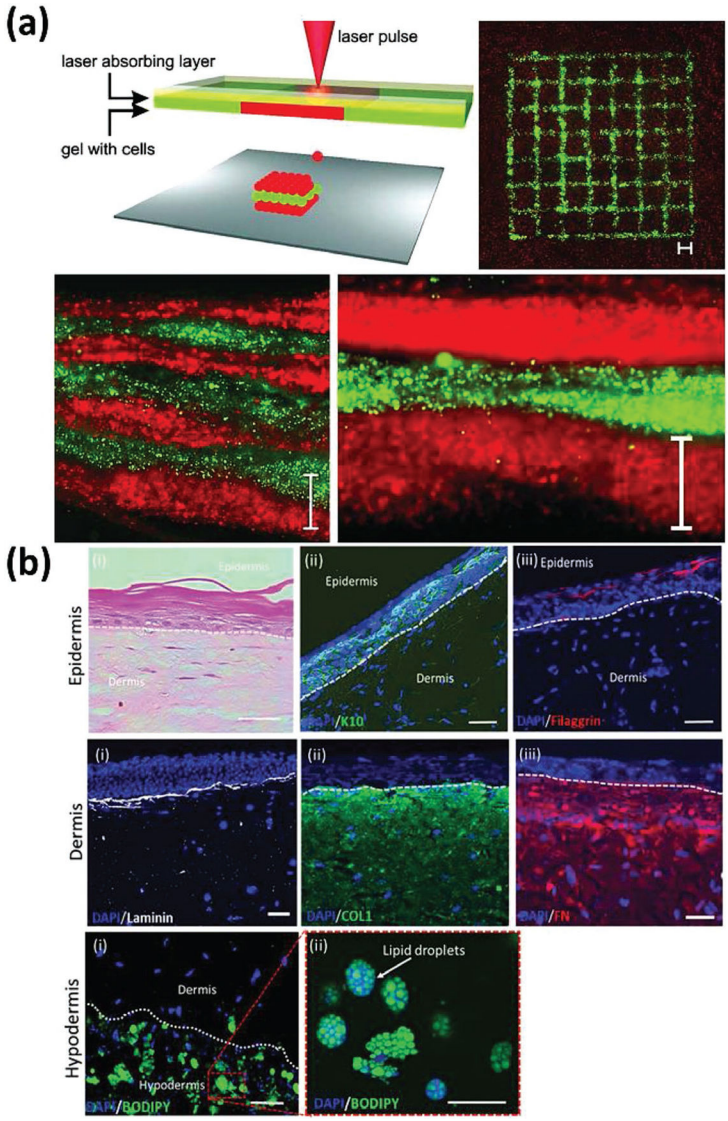

Figure 4.

Examples of 3D printed stratified skin constructs: a) 3D printed scaffold using the LaBP technique to fabricate a grid of fibroblasts (green) and keratinocytes (red) (top panel). Seven alternating layers of the cells can thus be printed with high precision for a total area of 10 mm × 10 mm and height of 2 mm (bottom panel). Scale bars (500 μm). Reproduced with permission.[161] Copyright 2012, Wiley Periodical Inc. b) Histological images of 3D printed skin tissue sections after in vitro maturation. Cytokeratin 10 (CK10) and filaggrin representing early differentiation and late differentiation of epidermis, respectively; Laminin representing the epidermal-dermal section, secreted ECM components (COL1: collagen type I, FN: fibronectin); Boron-dipyrromethene (BODIPY) staining representing lipid droplets of adipocytes in the hypodermis. Scale bars: 50 μm. Reproduced with permission.[162] Copyright 2018, Wiley-VCH.

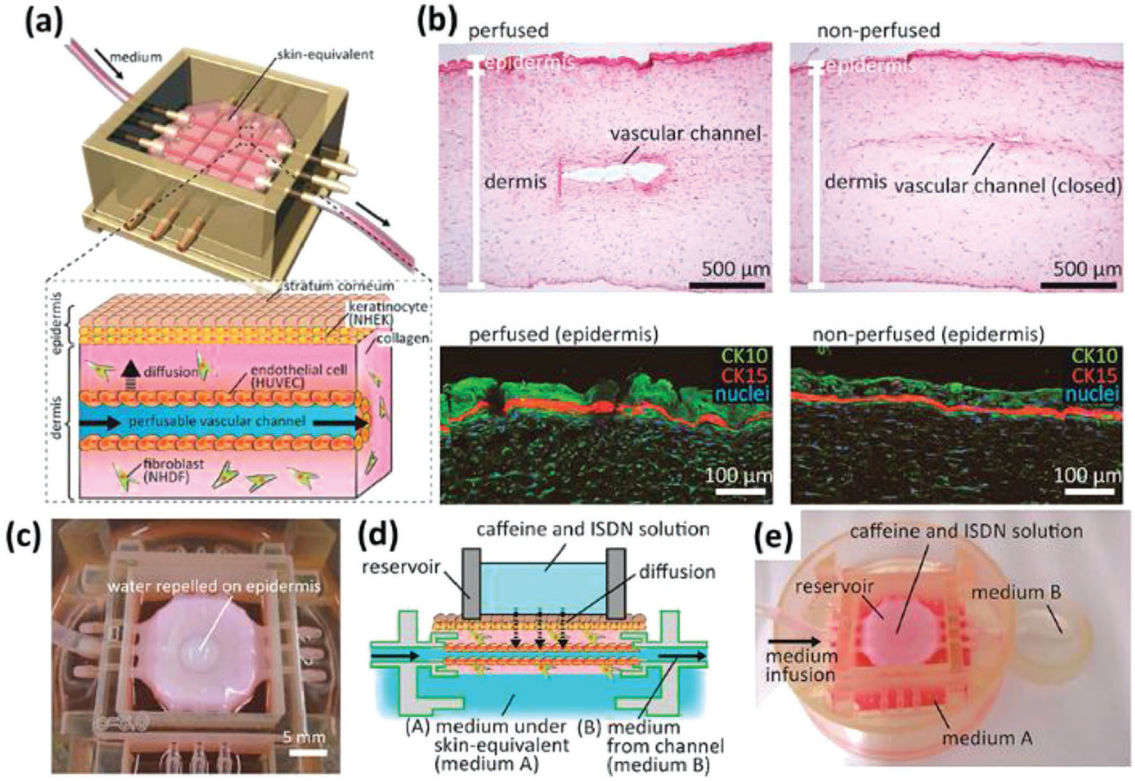

Figure 5.

Perfusable skin construct: a) Schematic and illustration of the skin perfusion culture device outlining the cell types and biomaterials used. b) Histology images of perfused and nonperfused cultures with vascular channels (top) and immunostained cytokeratin 10 (CK10), cytokeratin 15 (CK15). c) Skin barrier demonstrated by d) water repellance. e) Schematic and image demonstrating the application of the barrier function against caffeine and isosorbide dinitrate (ISDN). Adapted with permission.[167] Copyright 2017, Elsevier.

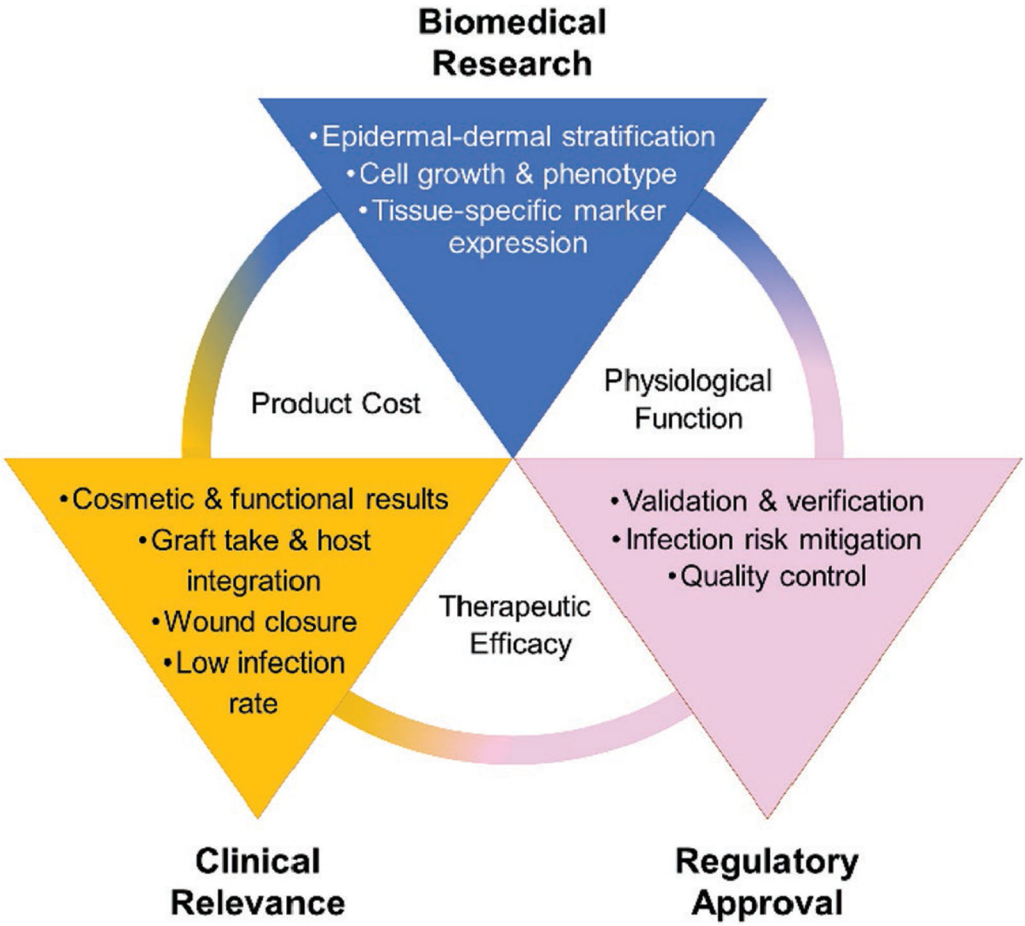

Figure 6.

Major considerations for a successful tissue engineered skin product from the perspectives of biomedical research organizations (e.g., academia and industry), regulatory agencies, and the clinic.

Table 1.

Major structures, cell types, and ECM components present in each layer of normal skin tissue.

Layer [a)]	Major structures	Major cell types	Major ECM components
Epidermis	Stratified squamous keratinized epithelium	Keratinocytes, melanocytes, Langerhans cells, Merkel cells	Keratin, type IV/VII collagen (basement membrane)
Dermis	Blood vessels, nerves, mechanoreceptors, hair follicles, sebaceous glands, sweat glands	Fibroblasts, endothelial cells, Langerhans cells, mechanoreceptor cells, smooth muscle cells, hair follicle cells	Type I collagen, elastin, proteoglycans, type IV/VII collagen (basement membrane)
Hypodermis	Blood vessels, nerves, hair follicles	Adipocytes, fibroblasts, endothelial cells, smooth muscle cells, hair follicle cells	Type I collagen, elastin

[a)]Besides multicellular structures and ECM, all skin layers also produce growth factors critical for tissue function. These include, among others, epidermal growth factor (EGF), transforming growth factor beta (TGF-β), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor 1 (IGF-1).

Table 2.

Select listing of tissue engineered skin models developed or used in industry.

Industry investigator	Skin model used	Research application
The Procter & Gamble Co.[28,29]	MatTek EpiDerm	In vitro nonanimal, skin-based genotoxicity assay for cosmetics
L'Oréal S.A.[204-207]	Episkin products	In vitro nonanimal, skin-based genotoxicity assay for cosmetics and other topically applied compounds
	AGE-modified collagen hydrogels	3D in vitro model of advanced glycation end (AGE) product accumulation in aging skin
	Primary keratinocyte cultures	Effect of resveratrol on keratinocyte proliferation and senescence
	Episkin RHE	Reconstructed human skin model to select cosmetics ingredients on the basis of metabolism, efficacy and/or safety
Stiefel— GlaxoSmithKline (GSK)[74,208]	SkinEthic RHE	In vitro model to study the effects of topical acitretin for treatment of severe psoriasis
	Primary human keratinocyte-derived living skin equivalent	Human living skin equivalent for identifying proteomic changes downstream of filaggrin deficiency relevant to the pathogenesis of atopic eczema
Johnson & Johnson Consumer Inc.[30-32]	Cell-seeded silk/collagen skin equivalent	In vitro trilayered skin equivalent (epidermis, dermis, and hypodermis)
	Franz-type cell chamber with skin compromised by tape-stripping	In vitro model of compromised skin for the study of chemical penetration through skin
	MatTek EpiDerm	Effect of retinol on hyaluronic acid production and skin moisture for anti-aging skin product development
	MatTek HEE	Effect of low-level red light therapy as treatment for acne
Novartis International AG[209]	SkinEthic RHE[a)] and Organogenesis Apligraf[b)]	Comparison of topical drug penetration into reconstructed human skin equivalents

[a)] Acquired by L'Oreal S.A. as an Epskin product

[b)] Previously known as Graftskin. RHE, reconstructed human epidermis; HEE, human epidermal equivalent.