Tissue-Engineered Human Skin Equivalents and Their Applications in Wound Healing

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7.1 Introduction

The skin is an essential organ as it protects internal organs, enables sensation, and allows absorption, excretion, and expression, as well as plays a critical role in maintaining body temperature and metabolism [1]. It is comprised of three layers, namely the epidermis, dermis, and hypodermis (Figure 7.1) [2–4]. The epidermis is the outermost layer and made of predominantly keratinocytes; other cell types are also found such as Langerhans cells and melanocytes [3, 5], which protect the body against infection and moisture loss. Immediately below the epidermis is the dermis, which contributes to the elastic nature and mechanical properties of skin. The dermis contains vascularized extracellular matrix (ECM) in which collagen, elastin, and glycosaminoglycans are abundant [3, 4, 6]. Cell types found in the dermis are fibroblasts, endothelial cells, smooth muscle cells, and mast cells [3, 6]. The hypodermis is situated beneath the dermis and acts as an energy source. The main components of the hypodermis are adipose tissue and collagen [2, 3, 5].

Any kind of failure in skin integrity can interfere with its ability to function, result in infection, and cause pain and discomfort. The healing of a large area of an adult skin wound is considered a highly complicated biological process that requires the synergistic functions of various cell types, ECM, as well as extracellular and intracellular signaling [7, 8]. Regeneration of perfect skin with full skin functions (protection, regulation, and sensation) is challenging and has recently become a major aim in wound healing [9].

Skin grafting is one of the most promising approaches to heal extensive wounds [10]. In skin transplantation, autografting represents the gold standard. However, autografts are not always available, particularly in burn patients. Cadaveric allografts are the next best option, even though these mostly serve only as temporary wound coverings to allow native wound healing. However, allografts are not commonly available due to the longer time periods needed for harvesting skin donations as compared to other organs, as well as concerns of lasting defect [11]. Xenografts from frog skin, bovine, and porcine sources have also been used.*

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clinically, but immunological issues and risks of pathogen transfer exist [12, 13]. Collectively, there is clearly an acute shortage of transplantable skin and overwhelming demand for alternative sources. Types of different skin grafts are summarized in Figure 7.2.

When tissue engineering was formally introduced in the late 1980s, possibilities of growing transplantable tissues in the laboratory [14] and developing off-the-shelf, tissue-engineered skin substitutes (TESs) became an attractive solution to treat acute and chronic cutaneous wounds. The common method in skin tissue engineering involves seeding a biodegradable matrix or scaffold with
cells, typically epidermal keratinocytes, dermal fibroblasts, or stem cells. The 3D matrix/scaffold provides a supportive environment for skin cell growth and is typically comprised of natural biomacromolecules including type I collagen, glycosaminoglycan, and chitosan, or synthetic polymers such as lactide-based aliphatic polyesters, glycolide, and caprolactone [15]. ECM is produced by cells as they proliferate and differentiate. As the matrix/scaffold degrades, it is gradually replaced by cells and ECM, which ultimately leads to the formation of the functional skin [14].

Successful isolation and cultivation of human epidermal keratinocytes were first reported in 1975 [16], and since then TES has evolved from simple epidermal substitutes to complex full-thickness skin with various appendages (Figure 7.3a) [5]. In a full-thickness skin, the upper thin epidermal keratinocyte layer provides protection by inhibiting infection and fluid loss. A much thicker dermal layer underneath the epidermal layer forms the basal body of TES to enhance mechanical properties and is comprised of dermal fibroblasts and ECM proteins. A pre-vascularized layer is positioned underneath the dermal layer in order to increase the chance of TES survival when applied into wounds. Skin appendages are implanted in order to mimic the appearance and functioning of normal skin. For example, melanocytes are implanted into the epidermal layer for pigmentation and hair follicles into the dermal layer for sensation.

In recent years, significant progress has been made in developing highly complex TESs that closely resemble natural skin by improving their durability,
elasticiy, biocompatibility, functionality, and clinical efficacy. We introduce in this chapter the major milestones in TES development as well as their applications in wound healing.

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TESs can generally be categorized as (i) epidermal, (ii) dermal, or (iii) full-thickness models (Figure 3b). The technology for epidermal replacement (a confluent epithelial cell layer attached to a petroleum gauze carrier) was developed in the 1970s. Unfortunately, the rate of epidermal engraftment was less than ideal and even successful cases demonstrated poor durability and weak epithelium [17]. It was recently shown that the replacement of connective tissue could enhance the mechanical properties of healed skin [18, 19]. Consequently, dermal substitutes have been created to incorporate dermal fibroblasts into collagen scaffolds [20–22]. Since both the epidermis and dermis are essential for normal skin function and appearance, it is reasonable to replace both elements at the same time. Experience has demonstrated this to be true, as cellular interactions between dermal and epidermal elements improve epithelium maturation [23]. Bilayered TES (epidermal + dermal layer) are developed as a result, which permit the reconstruction of full-thickness skin, unlike either individual single-layered substitute. Advances in skin tissue engineering have further resulted in highly sophisticated TESs with very similar architecture and function as their natural counterparts, for example, full-thickness skin implemented with hair follicles [24, 25], a capillary network [26, 27], sensory innervation [28], adipose tissue [29], and pigment production [25, 30]. In the following sections, we give detailed explanations of each type of TESs, commercial or laboratory-engineered.

7.2.1 Epidermal Models

The epidermis is considered the outermost component of the skin and primarily comprised of keratinocytes, a specific type of epithelial cells. Application of an epidermal layer provides early reestablishment of a functional barrier, which is vital in the prevention of excessive transepidermal water loss and infection [28]. Moreover, re-epithelialization is crucial in the healing of cutaneous wounds, as it precedes repair in the dermis and accelerates the process of wound healing [29, 31]. In order to produce an epidermal skin replacement, the epidermis is usually separated from a skin biopsy, 2–5 cm² in size, and keratinocytes are subsequently cultured on fibroblasts [32]. Epidermal TESs are sold by a number of companies such as Genzyme’s Epicel® (Cambridge, MA, USA). Epicel® is intended for grafts of burn wounds and consists of cultured epithelium using autologous epidermal cells. Laserskin® (Fidia Advanced Biopolymers Srl, Italy), is another epidermal TES model designated for the treatment of deep second-degree
burns and chronic ulcers. TES is comprised of a benzyl esterified hyaluronic acid derivative that forms a biodegradable matrix and is laser-perforated with microholes intended for autologous keratinocytes to grow inwards and proliferate [33].

7.2.2  Dermal Models

Dermal substitutes have also been created in addition to epidermal substitutes. Dermal skin substitutes provide higher mechanical stability and prevent wound contraction. They consist of collagen- and fibril-containing loose connective tissue that firmly secure the dermis to the epidermis [34]. Moreover, these substitutes are populated with fibroblasts and macrophages, which enable interactions between the dermis and epidermis and trigger synthesis of ECM components as well as keratinocyte cell growth and differentiation.

To date, there are many commercially available dermal wound products. For example, Transcyte® is a non-living wound dressing produced by Advanced Tissue Sciences, Inc. (La Jolla, CA, USA), which cryopreserves human dermal fibroblasts on a polymeric scaffold and has been established as a viable temporary wound dressing for excised burn wounds [35]. Dermagraft® is a derivative of Transcyte® made by Advanced Biohealing, and has demonstrated the potential for treating diabetic foot ulcers (DFU) [36]. It is manufactured by culturing fibroblasts isolated and expanded from human neonatal foreskin on a biodegradable polygalactin mesh. The produced dermal substitute is a 3D matrix containing human proteins created over a 3-week culturing period during which matrix proteins, such as human dermal collagen, and other soluble factors are secreted [32]. Integra® Dermal Regeneration Template (Intergra® DRT, Integra Life Sciences Corp., Plainsboro, NJ, USA) is a commercial composite skin graft consisting of a thin silicone film outer layer and an inner layer of complex cross-linked fiber matrix. After dermal layer regeneration, the silicone film can be replaced with an epidermal graft. Integra® DRT has shown promising results in burn wound treatment [37].

Nevertheless, the currently available single-layer skin substitutes (epidermal or dermal) all face various drawbacks since a skin layer made of only keratinocytes cannot fulfill functional requirements of fibroblasts, and vice versa. For instance, the fragile nature of Epicel® leads to instability without mechanical support from dermal substitute, whereas Intergra® is susceptible to infection due to the absence of stratum corneum from epidermal layer, which helps fend off foreign invasions [1]. In order to tackle these problems, bilayered skin substitutes with anatomical and functional resemblance to normal skin have been developed.

7.2.3  Bilayered Models

Epidermis and dermis are combined to form a bilayer structure in order to develop a full-thickness TES model, and keratinocytes and fibroblasts, either autologous or allogeneic, are employed in such processes [38]. This strategy,
apart from improving mechanical properties of the construct, is mainly intended for combining the roles of keratinocytes and fibroblasts in the wound-healing process, as they function in synergy to recruit cells that are necessary for complete wound closure and bestow unique properties that are vital for tissue homeostasis and wound healing [39]. Interactions between fibroblasts and keratinocytes have resulted in optimal keratinocyte proliferation and differentiation, where keratinocytes showed close resemblance to natural epidermis [40].

An early example of full-thickness TES was developed using a collagen lattice laced with fibroblasts and covered with epidermal cells [41]. Because of this breakthrough contribution, Organogenesis (Canton, MA, USA) became a pioneering company in the field of tissue engineering. Organogenesis later established a living-tissue-engineered, bilayered skin model named Apligraf® by seeding keratinocytes in allogeneic skin fibroblasts, sourced from human foreskins, and soluble type I bovine collagen gel (see Apligraf® in Figure 7.4). Apligraf® has been implemented in the treatment of surgical wounds [43] and venous ulcers [44]. PermaDerm® (Regenicin, Inc.) is a promising new product with keratinocytes and fibroblasts seeded onto a collagen sponge that can indefinitely cover large skin burns and injuries [31]. In a more recent study, Zonari et al. fabricated a bilayered poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) construct with a 2D thin nanoporous membrane on top and a 3D porous scaffold at the bottom (Figure 7.5) [23]. The thin, nanoporous membrane mimicked the epidermis, while the scaffold simulated the dermis. Results showed minimal water loss, suitable mechanical properties, and desired susceptibility to enzymatic degradation. In addition, when cocultured with human fibroblasts, the seeded human keratinocytes differentiated and rearranged to form a multilayered structure as opposed to monolayer in the homotypic case, proving the construct to be a promising autologous skin graft.
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(b) Human keratinocytes organization

Figure 7.5 (a) Schematic of bilayered poly(hydroxybutyrate-co-hydroxyvalerate) skin construct seeded with human keratinocytes in the epidermal layer and fibroblasts in the dermal layer. (b) The keratinocytes grew and rearranged in a multilayer structure with proliferative cells in the basal layer and differentiated cells in the upper layer. (Zonari 2014 [23]. Reproduced with permission of John Wiley & Sons.)

7.2.4 Multifunctional Skin Models

Recent progress in skin biology has resulted in highly sophisticated and innovative approaches to reconstruct 3D skin equivalents with architecture and function very similar to those of their natural counterparts. The bilayered epidermal–dermal TESs have been implemented with various appendages, including hair follicles [24, 25], a capillary network [26, 27], sensory innervation [28], adipose tissue [29], and pigment production [25, 30].

For example, to develop a bilayered TES with hair follicles, Atac et al. fabricated a dynamically perfused chip-based bioreactor platform that was able to apply varying mechanical shear stress and significantly prolong culture periods [24]. This has resulted in ameliorated culture conditions that benefit numerous factors including biopsies of single hair follicular units. Overall, notable hair-fiber elongation from the epidermis was observed in the hair follicle cultures in the chip. These experimental findings at the miniature scale are promising indicators for greater skin emulation in engineered equivalents and hair follicle biology, as the hair follicle plays a role in skin metabolism and contains several stem cell lineages with regenerative capacity.

In addition, as is known, insufficient vascularization is a major threat for the clinical use of TES, as it can cause the TES to loosen, become susceptible to
infection, or experience partial necrosis. To circumvent this problem, Liu et al. fabricated a bilayered TES with capillary network by coculturing dermal fibroblasts with dermal microvascular endothelial cells [26]. Interactions of the fibroblasts with the endothelial cells formed a fibrous sheet, and after a 20-day period of coculture, capillary-like structures were observed. In order to build a bilayered tissue, epithelial cells were seeded on the fibrous sheet. Immunostaining demonstrated that epithelium promoted the formation of structures reminiscent of capillary. These structures were confirmed to be typical microblood vessels through transmission electron microscopy (TEM). The resultant blood vessels were believed to supply the cells in tissue graft center with nutrients and oxygen after implantation with improved clinical efficacy.

Moreover, the nerve component of the skin accountable for pain, temperature, and sensory perception can be destroyed by burns. Patients that receive standard bilayered TESs often suffer from reduced discriminative sensibility, hyperesthesia, and dysesthesia. To rescue this, Blais et al. integrated neurolemmocytes, which are also known as Schwann cells and are the principal glia of the peripheral nervous system, in reconstructed skin (RS) [28]. Specifically, Blais et al. demonstrated that Schwann cells stimulated an increase in the number of sensory neurites migrating in the 3D tissues by twofold as compared to the control with no Schwann cells. Moreover, through TEM, Schwann cells were found to colocalize along with neurites and to achieve in vitro myelin sheath formation. The effect of incorporating Schwann cells into in vivo nerve regeneration and function recovery was also studied through the transplantation of developed TES athymic mice. The researchers verified that the Schwann cells would survive the 25-day maturation period for the RS and that the addition of Schwann cells induced 1.81- and 1.71-fold increase in the amount of nerve fibers migrating in the graft at 60 and 90 days after the transplant, respectively. Such TES could be adopted as an effective strategy for improving nerve regeneration in wound healing [28].

Furthermore, absence of a subcutaneous fat layer in TESs may result in uncharacteristic mechanical and thermoregulatory properties as compared to normal skin. To address these issues, Monfort et al. fabricated a plasma-based trilayer TES, containing an epidermis, dermis, and hypodermis, that could generate a transplantable cell sheet [29]. Bone-marrow-derived mesenchymal stem cells (BM-MSCs) or adipose-tissue-derived stromal cells in a human plasma hydrogel were incorporated in the trilayer model and kept open to adipogenic clues for a 3-week period. Occasional feeding of fresh keratinocyte complete medium allowed continued viability of in vitro engineered adipocytes within the medium. The adipocytes were found to survive for a 2-year period and ∼50% of the cells differentiated into mature adipocytes [29].

Another current challenge in skin grafts is the absence of pigmentation, which leads to noticeable cosmetic implications. Pigmented TESs may solve these problems through the incorporation of hair follicle melanocytes. Liu et al. isolated hair follicle melanocytes and keratinocytes from the human scalp and seeded them onto a chitosan–gelatin membrane to produce pigmented TESs [30]. The produced constructs were successively used to resurface skin defects in nude mice and found to be successfully restored after 4 weeks by observing a complete
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epidermis structure and cuticular layer in the restored skin. This study demonstrates a successful example of using hair follicle cell sources to fabricate pigmented TES. The developed pigmented TES can find applications in the treatment of depigmentation diseases, skin deficiencies, and skin lacking pigmentation ability [30].

One more monumental achievement in TES development is the understanding of the role played by mesenchymal stem cells (MSCs) in skin regeneration. Besides their potential in morphogenesis, recent evidence suggests that BM-MSCs work in synergy with epidermal stem cells to accelerate re-epithelialization and have higher therapeutic potential in inducing formations of blood vessels and hair follicles than solely epidermal stem cells [45]. Similar to BM-MSCs, adipose-tissue-derived stem cells (ADSCs) can also differentiate into a number of skin cell types that can contribute to the wound-healing process [46]. One recent study in a bilayered TES system demonstrated that a mixture of dermal fibroblasts and ADSCs (1:1 ratio) had a better performance compared to that of each individual cell type in inducing keratinocyte proliferation and differentiation [47].

The aforementioned examples show promising progress in developing sophisticated TESs with architecture and function similar to those of natural skin with appropriate combinations of various cell types. Other factors associated with TESs to aid wound healing include delivery of beneficial growth factors and cytokines, which trigger proliferation of cells and synthesis of new matrix at the wound bed. The growth factors that have attracted significant attention include the epidermal growth factors (EGFs), transforming growth factors β (TGF-β), fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), granulocyte macrophage colony-stimulating factors (GM-CSFs), platelet-derived growth factor (PDGF), and connective tissue growth factors (CTGFs) [48]. During the inflammatory phase of wound healing, upregulation of pro-inflammatory cytokines (e.g., interleukin (IL) IL-1 and IL-6, and tumor necrosis factors-alpha (TNF-α)) is observed [48]. For instance, IL-1 is secreted by monocytes, macrophages, and neutrophils. IL-1 is also produced by keratinocytes to aid wound healing. In addition to its paracrine effect, IL-1 also functions in an autocrine manner to facilitate keratinocyte migration and proliferation [48]. Barrientos *et al.* have made a summary of growth factors and cytokines involved in the wound-healing process [48]. *In vitro* cell culture bioreactor is another feasible tool to facilitate cell proliferation and differentiation in TES due to its ability to trigger biochemical and mechanical cues as well as to regulate tissue development [49]. There are three main components in bioreactors: (i) metabolically active cells that have different phenotypes and produce ECM; (ii) polymeric scaffolds that provide favorable 3D matrices for cell adhesion, tissue growth, nutrient/oxygen/waste exchange, as well as biological and mechanical stimuli; and (iii) an environment that simulates *in vivo* conditions in which cells and polymers form complexes which subsequently develops into natural skin-mimicking tissues [50].

With the evolving resemblance to native human skin, the developed TESs are expected to have improved durability, elasticity, biocompatibility, functionality, and clinical efficacy when employed to aid wound healing. In the following
sections, we will discuss the recent progress of clinical applications of developed TESs in wound healing and the uses of different TES models *in vivo* and *in vitro* to study the wound-healing process.

### 7.3 Application of TESs in Wound Healing

The development of RS models that bear greater resemblance to normal skin leads to success in burn and chronic wound treatments as higher graft take rates are achieved. This development also enables the creation of a variety of products including vascularized, pigmented, and adipose RSs, and promotes laboratory research and applications in the skin cosmetics and pharmaceutical industries.

The following paragraphs will explore how the developed TESs translate to facilitate cutaneous wound healing. The focus will be on clinical applications as well as *in vivo* animal models thereof. Toward the end, *in vitro* wound-healing models will be touched upon in an effort to illustrate how improving our fundamental understanding of the wound-healing cascade may guide more sophisticated and targeted approaches to wound repair and regeneration.

#### 7.3.1 Clinical Wound-Healing Applications

##### 7.3.1.1 Epidermal Skin Regeneration

Being a source of constantly renewing keratinocytes, the regenerative capacities of epithelial populations have fast garnered popularity as demonstrated by several commercialized epidermal substitutes including Epicel® and Laserskin®. Epidermis-derived keratinocytes cultured into monolayers have previously been shown to successfully regenerate an epidermis over full-thickness wounds [51–53]. Similar results have been found in clinical trials where the use of carrier dressings seeded with autologous keratinocytes improved the healing rates of DFU [54].

The commercialization of several epidermal skin grafts has provided realistic and clinically viable alternatives for extensive burn wound coverage. For example, Epicel® (Genzyme Biosurgery, Cambridge, MA, USA) consists of keratinocytes obtained from a single, small, full-thickness biopsy obtained from the wounded individual. Coculturing with irradiated murine fibroblast feeder layers achieves a stratified epidermis, which is transferred to the patient via a petrolatum gauze backing. Clinical feasibility was demonstrated in 22 extensively burned children; compared to conventional treatment consisting of meshed split-thickness autografts, cultured epithelial autografts (CEA) demonstrated an organized, fully differentiated epidermis with rete ridges and a vascularized neodermis 6–12 months after application. Average initial and final engraftment rates were acceptable at 79% and 84%, respectively [55]. A retrospective review of 30 patients with large-surface-area burns (mean of 78%) demonstrated an even higher initial survival rate of CEA at 90% [56]. This allowed a significantly smaller need for autologous skin harvesting, which is particularly important when healthy skin is scarce.

Laserskin® (Fidia Advanced Biopolymers Srl, Italy) is another commercially available CEA product, which is intended for either deep second-degree burns
or chronic ulcer treatments. A pilot noncontrolled study of 14 patients with nonhealing foot ulcers secondary to type 2 diabetes mellitus treated with CEA revealed complete healing of 11 lesions with an average healing time of 41 days [32]. Despite the lack of control, nonrandomization, and small sample numbers, these results suggest that CEA may be effective in accelerating the treatment of DFU. The main advantage of CEA is its immunological safety profile, as only autologous keratinocytes are transferred to the wound. On the other hand, high cost, short shelf-life, delicacy, and time spent on mandatory custom preparation are some of the many disadvantages. It is primarily intended for burn treatment with high body surface area because of the difficulty of obtaining CEA, but has yet to find extensive applications in chronic wound settings. More extensive controlled clinical studies are required to assess its efficacy as well as superiority over conventional treatment.

7.3.1.2 Dermal Substitutes
Besides the previously mentioned disadvantages of CEA, one major limitation remains, namely the lack of a dermal component that is frequently associated with wound contraction and poor graft vascularization, resulting in delayed or incomplete healing. Dermagraft® (Smith & Nephew, Inc., Largo, FL, USA) is a cryopreserved human fibroblast-derived dermal substitute approved for the treatment of chronic (>6 weeks) DFU. Fibroblasts isolated from human neonatal foreskin are cultured on a bioabsorbable polygalactin mesh, resulting in the formation of a 3D matrix suitable as a dermal replacement [57]. Earlier studies had indicated fast incorporation and vascularization into the wound bed [22, 58]. Interestingly, multiple applications of Dermagraft® onto nonhealing DFU over several weeks resulted in a cumulative effect and faster wound closure compared to conventional treatment with gauze [36]. Weekly treatment was found to be superior to 2-weekly or less frequent application, which is likely due to the continued constant levels of growth factors and cytokines necessary for graft vascularization [59]. Transcyte™ is another fibroblast-containing dermal substitute that acts as a substitute for cadaveric skin to temporarily cover burn wounds after surgical excision [60, 61]. A prospective, randomized comparison study on 14 patients with partial-thickness burns demonstrated faster wound healing with Transcyte™ compared to conventional treatments using topical antimicrobial agents in combination with repeated wound debridement and subsequent wound dressings [62]. Faster re-epithelialization of wounds treated with Transcyte™ was achieved because of continuous wound coverage (albeit temporary), which allowed undisturbed healing. This not only resulted in faster wound closure rates but further reduced hypertrophic scarring, which complicated the control group exposed to repeated debridement.

More often than not, however, clinical and laboratory-based in vivo skin regeneration models employ a bilayered structure composed of a top epidermal layer and a bottom dermal layer, which may naturally provide paracrine signaling factors, thus rendering external growth factor supplementation redundant. This, in combination with the emergence of genetically modified animals, is hoped to shed more light on the complexities of cutaneous wound healing.
7.3.1.3 Dermo-Epidermal Skin Substitutes

Akin to natural skin, most studies investigating skin regeneration focus on the development of cellular dermal–epidermal constructs. These constructs mimic the structure of natural skin in order to benefit from paracrine signaling, which reduces the risk of over- or under-dosing of exogenous signaling molecules. The dermal layer is of biological, synthetic, or combined origin and frequently encapsulates cells with or without additional growth factors.

Eaglstein et al., for example, used a bilayered construct made of human fibroblast-seeded bovine collagen matrix with an overlying sheet of stratified human epithelium Apligraf® to cover excisional wounds in 15 patients [43]. Clinical evaluations were suggestive of reasonable graft take. However, the lack of controls and subjective, nonblinded assessments lessen the significance of these findings. A subsequent trial investigating the benefits of repeated applications of Apligraf® onto chronic rather than acute wounds was conducted in a larger cohort consisting of 233 patients [63]. Recalcitrant venous ulcers were successfully treated over 8 weeks with up to three separate applications of Apligraf® compared to simple compression therapy.

Larger wounds (>50% of total body surface area), particularly burn wounds, suffer from delayed and ineffective wound closure, partly due to a lack of autologous or allogeneic skin grafts. Boyce et al. applied a bilayered skin equivalent similar to Apligraf® but using the patients’ own cells (PermaDerm™) on 40 burn patients and compared wound-closure rates with those treated using split-thickness autografts [31]. Compared to the significant donor-site morbidity inflicted by skin harvesting, PermaDerm™ merely requires a punch biopsy, which accelerates recovery and is more acceptable among patients. Despite promising results using dermo-epidermal replacements, one major disadvantage prevails – the lack of important skin appendages such as hair follicles, capillary networks, sensory innervation, and pigmentation. More recent progress in skin biology has enabled the reconstruction of 3D skin equivalents which not only resemble natural skin in terms of architecture but also emulate skin’s innate functions.

Delayed vascularization is a major contributor in skin engraftment failure, particularly in patients with extensive burns. Rapid vascularization and hence nutritional and oxygen provision to the graft are prerequisites for intermediate to long-term graft survival. In order to demonstrate the benefits of pre-vascularization strategies in terms of graft take rates, Tremblay et al. cultivated keratinocytes, fibroblasts, and endothelial cells in a collagen sponge to create a human endothelialized reconstructed skin (ERS) model, which was grafted onto excisional wounds in nude mice [64]. Macroscopic and immunohistochemical results demonstrated blood vessel formation and anastomosis between graft and host vasculatures, and this took place in 4 days compared to 14 days for non-vascularized RS (Figure 7.6).

In another example, Chan et al. demonstrated clinical feasibility with vascularized skin substitutes based on collagen–poly(ethylene glycol)–fibrin-based bilayer hydrogels seeded with autologous ADSCs from human burn tissue (Figure 7.7) [65]. Immunocytochemical analysis showed differentiated
Figure 7.6 Immunohistochemical characterization of revascularization of the RS and ERS. Sections were immunostained with a marker of the cell nucleus (Hoechst, in blue), a marker of endothelial cells (in green), and with an antibody specific to mouse red blood cells (in red) (arrows). White dotted lines indicate the dermal–epidermal junction. In the RS, 4 days after graft, no endothelial cell tube containing red blood cells was observed under the epidermis, while some capillaries connected to the blood flow were detected in the bottom-half part of the graft thickness. Nevertheless, 14 days after graft, a homogeneous endothelial cell network filled with red blood cells was detected under the epidermis of the RS. Four days after graft of the ERS, human capillaries (arrows), which contained red blood cells, were observed close to the epidermis. (Tremblay 2005 [64]. Reproduced with permission of John Wiley & Sons.)

Figure 7.7 Schematic illustration of the development of different skin layers using surgically debrided adipose stem cells (dsASCs) and hydrogel scaffolds. (Chan, http://www.hindawi.com/journals/sci/2012/841203/. Used under CC-BY-3.0 http://creativecommons.org/licenses/by/3.0/.)

stromal, vascular, and epithelial cells, which indicated that debrided skin can become a source of autologous stem cells for the development of vascularized skin constructs with no need for culture expansion or exogenous growth factors.

Most skin equivalents only inadequately address the regeneration of skin appendages such as hair follicles or melanocytes. Numerous studies have demonstrated the successful regeneration of hair follicles in animal models [66–68]. However, de novo regeneration of human hair follicles has yet to be accomplished. It is hypothesized that this may be due to the loss of key inductive
properties of human dermal papilla cells upon culturing, which removes both contextual as well as positional cues from the surrounding epithelial cells. Aggregations of specialized mesenchymal cells located within the dermal papilla are known to control the cyclical growth activities of hair follicles [69], and dispersal of these cells results in a disruption of hair follicle development [70]. More recently, Higgins et al. succeeded in partially restoring such inductive capacities using an organotypic 3D spheroid culture system capable of growing human dermal papilla cells, thus paving the way for human hair follicle restoration [71].

Hypopigmentation, secondary to pigmentation disorders or after grafting of skin substitutes lacking melanocytes, is a common problem which can be particularly striking in darker skinned individuals. Apart from the obvious cosmetic implications, melanocytes are partly responsible for protection against UV irradiation, and therefore the absence of these cells within skin substitutes may have more serious implications especially where large areas of skin are affected [72]. A follow-up study of 132 patients suffering from leucoderma (an acquired condition with localized loss of pigmentation) demonstrated that treatment with autologous cultured melanocytes resulted in 100% re-pigmentation in select cases (stable leucoderma) [73]. Similar results were obtained in several other cases of post-burn leucoderma treated with melanocytes [74, 75].

Despite such promising results, there is no clinically available standard treatment for skin wound healing capable of perfectly regenerating skin including all its appendages. Thus, in an attempt to evaluate novel wound-healing therapies, scientists have created both in vivo and in vitro wound models that emulate human pathologies such as acute burn wounds [76, 77], chronic nonhealing wounds [78, 79], and skin conditions such as vitiligo [80, 81].

### 7.3.2 In vivo Wound-Healing Applications

Skin-humanized small animal models have emerged to mimic human skin healing as the model of choice for researchers because of multiple reasons including cost effectiveness, availability, and so on. Such models can be used to investigate various human wound conditions, for example, to investigate the mechanistic particulars of certain aspects of healing [82–84]. For example, Escamez et al. used transplanting dermo-epidermal equivalents containing fibroblasts and keratinocytes from immunodeficient mice as a base to develop a skin-humanized mouse model, which exhibited features of human wound healing [85]. regeneration of human skin on the backs of nude mice was achieved by fabricating a fibroblast-encapsulating fibrin sponge, which served as a dermal equivalent, and culturing keratinocytes on top of it. Transplantation of this construct into mice and subsequent creation of full-thickness wounds enabled the study of human wound healing in an animal model. This is particularly useful, as ethical considerations and heterogeneous nature of the disease limit the advancement of the knowledge in diabetic wound healing. In another study, Martínez-Santamaría et al. demonstrated that the application of fibroblast-seeded, plasma-derived fibrin dermal scaffolds improved wound healing in diabetic skin-humanized mouse models when compared to acellular fibrin gels [86]. This was attributed to faster
re-epithelialization, early granulation tissue maturation, and increased vascularization (Figure 7.8). The research findings using the preclinical wound-healing model can be further used to develop new therapeutic strategies for clinical uses.

### 7.3.3 In vitro Wound-Healing Models

TESs can also be applied as *in vitro* wound models to study the wound-healing process in detail. Such bench-based wound-healing models are particularly advantageous for preliminary investigations because of being easily manipulated and highly time and cost efficient compared to animal- or human-based studies and may eventually avoid animal testing [76]. Additionally, *in vitro* models enable the isolation and study of individual aspects of wound healing, which is
of fundamental importance in the elucidation of cellular and molecular cascades involved in the wound-healing process.

One of the simplest methods of assessing wound healing involves the creation of a 2D cell monolayer. For example, fibroblasts seeded into culture dishes and grown to confluence are scratched by running the sharp end of a razor blade or pipette tip along its surface, thereby removing a thin strip of the cell layer [87, 88]. This model is particularly well suited for relatively simple tasks involving the study of cellular migration and proliferation, as well as cytokine release profiles in response to injury [89]. Walter et al., for example, utilized scratch assays to investigate the effects of a cell-conditioned medium on different rates of migration of dermal fibroblasts and keratinocytes [90]. Ojeh et al. demonstrated the inhibitory effects of caffeine on wound healing and epithelialization using a keratinocyte scratch assay [91]. The simplicity of scratch assays has enabled the isolated study of the effects of bioactive compounds on wound healing. For example, Ghazi et al. demonstrated that a scratched keratinocyte monolayer exposed to hyaluronic acid of different molecular weights healed quickest when exposed to medium molecular weight hyaluronic acid. These results may represent a facile topical therapeutic strategy to promote wound healing [88]. However, more sophisticated in vitro investigations must be performed prior to drawing conclusions regarding the molecular mechanisms of re-epithelialization and wound healing.

Bellas et al., for example, created an in vitro trilayered skin equivalent based on silk and collagen seeded with ADSCs, endothelial cells, fibroblasts, and keratinocytes to study skin biology in a physiologically relevant and sustainable system (Figure 7.9) [92]. The combination of four different and spatially organized cell types is believed to create an optimal tissue microenvironment, which may serve as a human preclinical surrogate system to study the effect of different drugs on wound healing. By using the trilayered construct, rosiglitazone, an activator of the adipogenic program, was found to cause hyperproliferation (bracket) at the basal layer of the epidermis after 9 days of culture (Figure 7.9). This may be because the drug rosiglitazone slowed the inflammatory process, resulting in the hyperplasia of the epidermis [93].

![Figure 7.9 Schematic illustration of a trilayered, full-thickness skin model based on silk and collagen seeded with ADSCs, endothelial cells, fibroblasts, and keratinocytes. The trilayer constructs were found to be responsive to the adipogenesis stimulator drug rosiglitazone, which caused hyperproliferation (bracket) at the basal layer of the epidermis after 9 days of culture. (Bellas 2012 [92]. Reproduced with permission of John Wiley & Sons.)](image-url)
Several other studies have subsequently used *in vitro* human skin models to study wound healing; a proof-of-concept study conducted by Maione *et al.* utilized human fibroblasts derived from DFU to recreate 3D chronic wounds which were characterized by stunted angiogenesis, delayed granulation tissue maturation, hyperkeratinization, reduced re-epithelialization, and impaired ECM deposition [94]. In addition to reflecting the clinical attributes of DFU, a correlation was established between the wound-healing potential of DFU fibroblasts and *in vivo* wound closure in mice models. Therefore, the reported 3D DFU models are biologically relevant tools for exploring cell–cell and cell–matrix interactions related to chronic wound pathogenesis and may further enhance clinical efficacy through translation of *in vitro* results.

Unfortunately, the currently developed *in vitro* models may suffer from general drawbacks including lengthy fabrication protocols, short-term viability, and significant variability in terms of study protocol standardization and performance evaluation, rendering systematic comparisons between studies difficult. While *in vitro* wound-healing models are preferred for the study of isolated actions of growth factors, cytokines, or genes on individual cell types, *in vivo* models are frequently deemed invaluable in elucidating the downstream effects of these factors on wound healing in models that closely mimic the physiology of human wounds. It is therefore vitally important to make continuous efforts to understand the complex biological processes, the interactions between various cell types, ECM, as well as extracellular and intracellular signaling during wound healing. Further technical advances may eventually lead to the production of new TESs resembling natural human skin.

### 7.4 Conclusions and Future Directions

The range of skin substitutes available and their successful application for *in vivo* and *in vitro* wound healing are a demonstration of advancement in the tissue engineering field. The terminal goal of skin tissue engineering is to regenerate a fully functional skin using TESs. The functional skin should possess all the skin appendages, including hair follicles, sweat glands, and sensory organs, as well as different skin layers including the epidermis, dermis, and fatty subcutus. In addition, rapid formations of vascular and nervous networks, as well as integration with the surrounding host tissues, are other key performance indicators of TESs. The regenerated skin should ideally have all the normal skin functions including barrier function, pigmentory protection against UV rays, temperature regulation, suitable mechanical properties, and esthetic appearance.

Existing TESs can already fulfill some of the aforementioned requirements. For instance, cultured keratinocytes applied on fibrin or matrix, either as cells or a cell layer, can quickly restore barrier function after skin burn or trauma. Another example is that keratinocytes seeded on a dermal substitute (e.g., collagen/fibronectin mixture with/without fibroblasts) are able to restore the mechanical properties partially. However, to date, issues of nonrapid availability and low commercial potential for greater scale have inhibited the creation of an
autologous bilayered skin substitute, that is, a substitute without the risk of host rejection. Heterologous cells such as poorly differentiated cells and stem cells do not have risks of graft rejection and their genetically stable nature inhibits tumor formation; these features are promising in terms of offering an off-the-shelf therapy that is commercially scalable. Applying the current knowledge of embryonic development and adult regeneration to develop a process of engineering such cells is critical for improving upon the existing TES. The in-depth understanding of differentiation mechanisms and means of cell manipulation require continued research. Elucidation of the fundamentals of stem cells could lead to the use of cellular therapy to accomplish local tissue repair.

In addition, TESs themselves are highly complex, for example, biomaterial scaffolds with controlled release of various signaling and differentiation molecules, as well as protein domains for promoting cell migration and adhesion. As advancement leads to the engineering of different tissues, a greater need for tissue-specific scaffolds will develop and require further research. Future scaffolds are anticipated to become more sophisticated and fulfill all the needs of various cells. Moreover, inclusion of intrinsic activity, mediated by cytokines or immobilized peptides, into scaffolds derived from biomaterials is another developing research area. In order to understand cytokine interactions on a cellular and molecular level, further fundamental research must be pursued. Similarly, the prevention of adverse host reactions at the immunological level is a subject that requires further investigation.

On the other hand, the substitutes also need to be simplistic for easy handling and to serve as carrier (e.g., hydrogel or electrospun fibers), which, upon non-inflammatory degradation, allows cells to interact and to regenerate skin structures akin to natural skin. In this case, the cells are first undifferentiated prior to delivery, and then the substitutes rapidly disappear upon implantation. The creation of next-generation TES requires further understanding in the disciplines of embryonic development, stem cell biology, and biomaterial engineering.

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References


