

Exploring the Potential of 3D Skin Models in in vitro Wound Recovery

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Student vypracuje literární rešerši na téma trojrozměrného modelu kůže jako průlomového nástroje ve výzkumu hojení ran *in vitro*.

Začlenění modelu kůže do výzkumu složité dynamiky hojení kožní tkáně může poskytnout přesnější pochopení komplexních buněčných interakcí a mikroprostředí ran.

Praktická část diplomové práce se bude zabývat přípravou kožního modelu s přístupem k reprodukovatelnému vyvolání jizvy a následného procesu hojení ran.

Pro charakterizaci tohoto modelu hojení ran *in vitro* bude provedena biologická charakterizace a histologická analýza.

Forma zpracování diplomové práce: **tištěná/elektronická**

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ABSTRAKT

Tato diplomová práce se zaměřuje na vývoj a hodnocení *in vitro* modelů lidské kůže pro studium procesů hojení ran, a to s důrazem na dvourozměrné (2D) i trojrozměrné (3D) přístupy. Práce je rozdělena na teoretickou a praktickou část. Teoretická část obsahuje čtyři kapitoly. První kapitola popisuje stavbu a funkce kožního systému člověka jako základ pro pochopení fyziologické složitosti kůže. Druhá kapitola se věnuje hlavním fázím hojení ran, včetně hemostázy, zánětu, proliferace a remodelace, a zdůrazňuje zapojené buněčné a molekulární mechanismy. Třetí kapitola představuje oblast tkáňového inženýrství se zaměřením na strategie konstrukce 3D kožních ekvivalentů v laboratorních podmínkách. Je zde rozebrána důležitost volby vhodných typů buněk, nosičů (scaffoldů) a kultivačních podmínek pro dosažení fyziologicky relevantní struktury kůže. Čtvrtá kapitola se zabývá současnými *in vitro* testy hojení ran, porovnává 2D a 3D modely, zmiňuje analytické metody používané ke sledování uzavírání ran a regenerace tkáně a pojednává o současných limitacích těchto modelů.

V praktické části byl proveden scratch test s použitím HaCaT keratinocytů pro hodnocení buněčné migrace ve zjednodušeném modelu hojení ran. Dále byly připraveny plně vrstvené 3D kožní modely ko-kulturovou lidských dermálních fibroblastů a HaCaT keratinocytů v kolagenové matrici. Biologická funkčnost těchto modelů byla hodnocena pomocí MTT testu a pro zobrazení jejich morfologie byla využita běžná technika barvení hematoxylinem & eosinem. Výsledky poskytují přehled o využitelnosti 2D a 3D *in vitro* modelů při výzkumu hojení ran a podporují jejich potenciál jako spolehlivých a etických alternativ k testování na zvířatech v dermatologickém a farmaceutickém výzkumu.

Klíčová slova: integumentární systém, hojení ran, tkáňové inženýrství, 3D model kůže, test hojení ran, histologie

ABSTRACT

This master's thesis focuses on the development and evaluation of *in vitro* skin model for the study of wound healing processes, with an emphasis on both two-dimensional (2D) and three-dimensional (3D) approaches. The work is divided into two main parts: a theoretical background based on literature review and an experimental section covering laboratory methods and analysis. The theoretical part consists of four chapters. The first chapter describes the structure and function of the human integumentary system, serving as a foundation for understanding the physiological complexity of the skin. The second chapter examines the key phases of wound healing, including hemostasis, inflammation, proliferation, and remodeling, highlighting the cellular and molecular mechanisms involved. The third chapter introduces the field of tissue engineering with a focus on strategies for constructing 3D skin equivalents *in vitro*. It outlines the importance of selecting appropriate cell types, scaffolds, and culture conditions to recreate physiologically relevant skin structures. The fourth chapter discusses current *in vitro* wound healing assays, comparing 2D and 3D model systems and analytical methods used to evaluate wound closure and tissue regeneration along with current limitations of these models.

In the experimental part, a 2D scratch assay was performed using HaCaT keratinocytes to assess cell migration in a simplified wound healing model. Additionally, dermo-epidermal 3D skin model was prepared by co-culturing human dermal fibroblasts and HaCaT keratinocytes in a collagen-based matrix. The biological functionality of the 3D models was evaluated using a modified MTT assay and the tissue models were visualized using the common hematoxylin & eosin staining technique. The results provide insight into the applicability of both 2D and 3D *in vitro* models in wound healing research, supporting their potential as reliable and ethical alternatives to animal testing in dermatological and pharmaceutical research.

Keywords: integumentary system, wound healing, tissue engineering, 3D skin model, wound healing assay, histology

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INTRODUCTION

The skin is the largest organ of the human body and serves as a vital protective barrier against external environmental factors. Its complex, multi-layered structure allows it to perform essential physiological functions, including thermoregulation, immune defense, and sensory perception. When the skin is damaged, the body initiates a highly coordinated wound healing process involving numerous cell types, signaling molecules, and extracellular matrix interactions. Understanding the mechanisms underlying wound healing is essential not only for the treatment of acute and chronic wounds but also for the development of effective topical therapies and cosmetic formulations (1, 2). Traditional studies of skin repair have relied heavily on 2D cell cultures and animal models; however, these systems are limited by their inability to fully mimic the physiological complexity of human skin and often raise ethical concerns related to animal use (3, 4).

In recent years, 3D *in vitro* skin models have emerged as promising alternatives. These models, often referred to as human skin equivalents (HSEs), are constructed by layering keratinocytes and fibroblasts onto biomimetic scaffolds, allowing for the formation of both epidermal and dermal compartments (2). Through cultivation at an interface with air and liquid, they can develop stratified and differentiated epidermis, closely resembling native skin architecture (5). The application of such 3D models in wound healing assays promises a physiologically relevant platform for studying cellular behavior, such as re-epithelialization, inflammation (6). Advancements in technologies like 3D bioprinting and microfluidic systems are further enhancing the complexity and functionality of these models, with ongoing research focused on improving their reproducibility, integration of vascular and immune components, and overall translational potential (7).

This master's thesis explores the potential of 3D skin models for *in vitro* wound healing research. The theoretical part provides a comprehensive literature review of skin anatomy, the physiology of wound healing, and the principles of tissue engineering and *in vitro* wound healing assay design. The experimental part focuses on the fabrication of a 3D wound healing skin model composed of keratinocytes and HDFs in collagen matrix and investigating its potential in wound healing. Moreover, the biological characterization of this model will be evaluated through histological analysis and a modified MTT assay. The work emphasizes the relevance of these models in replacing or reducing animal testing, in line with current regulatory and ethical trends in biomedical and cosmetic research.

I. THEORETICAL PART

1 THE INTEGUMENTARY SYSTEM

The integumentary system consists of the skin and its associated structures, which include hair, nails, and exocrine glands. The skin is a vast organ that envelops the entire body, forming its outermost protective layer. It is composed of three primary layers: the epidermis, the dermis, and the hypodermis. Covering approximately 2 m² in adults on average, it is recognized as one of the largest organs in the human body. In terms of weight, it accounts for about 16% of body mass. The skin's thickness varies based on anatomical localization and the individual's hydration, nutrition, and age (1, 8–10).

The skin represents a dynamic, metabolically active organ with numerous crucial functions for maintaining homeostasis and protecting the body. It acts as a barrier, forming the first line of defense against external chemical, physical, and biological agents, prevents excessive water loss from the body, and helps regulate body temperature. Through specialized sensory structures, the skin functions as a key sensory interface with the external environment. Additionally, it plays a crucial role in the endogenous synthesis of vitamin D (1, 8–10).

The surface of the skin is heterogeneous, furrowed into folds and grooves that create the skin relief – the division of the surface into small polygonal bodies (8). Human skin is generally categorized into two main types: hair-bearing and non-hair-bearing skin (11). Non-hair-bearing skin, also referred to as glabrous skin, is primarily localized on the palmar and plantar surfaces. It is distinguished by the absence of pilosebaceous units composed of hair follicles, arrector pili muscles, and sebaceous glands (10). Glabrous skin also exhibits distinct surface grooves with unique patterns known as dermatoglyphics. Their course is determined by the arrangement of dermal papillae and the configuration of collagen and elastic fibers in the dermis (8). In contrast, hair-bearing skin has pilosebaceous units. The structure, size, and density of hair follicles varies significantly depending on the anatomical location (11).

1.1 Epidermis

The epidermis is the outermost layer of the skin, designed to function as a continuously renewing protective barrier. Devoid of blood vessels, it consists of a stratified squamous epithelium primarily composed of keratinocytes. Melanocytes, Merkel cells (MCs), and Langerhans cells (LCs) are also present within the epidermis. The keratinocytes form the epidermis at a basement membrane, dividing and progressively moving the older cells

outwards. They differentiate throughout this process; thus, the epidermis can be divided into several distinct layers, as seen in **Figure 1**: *stratum basale*, *stratum spinosum*, *stratum granulosum*, *stratum lucidum* present only in thick skin, and lastly, *stratum corneum* (10–12). It takes about 2 weeks for the keratinocytes to migrate from the *stratum basale* to the top of the *stratum granulosum*, and another 2 weeks for the cells to cross the *stratum corneum* to the surface, where they are finally shed (13).

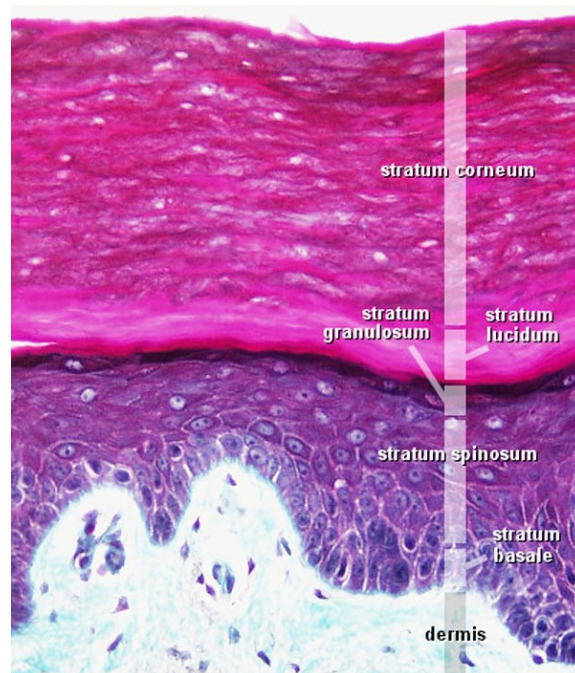


Figure 1: Thick adult skin epidermis, trichrome staining.

(source: (14))

As the keratinocytes migrate through the epidermis, they terminally differentiate in a process called keratinization, which ultimately results in forming the dead surface layer of the skin (15, 16). The cells undergo two phases. The first phase is called the synthetic phase and begins as soon as the cells divide in the deepest layer of the epidermis. One daughter cell remains in the basal layer, and the other begins progressing toward the skin's surface (10). This cell builds up a cytoplasmic supply of keratin, filaggrin and involucrin, in addition to undergoing morphological changes (16). During the second phase, cellular organelles are lost, and the cell's contents are combined into a mixture of filaments and amorphous cell envelopes. Hence, it's called the degradative phase. The resulting cell is called a corneocyte or horny cell and makes up the outermost layer of the epidermis (15).

The skin plays a critical role in protecting the body against environmental harm. When it comes to mechanical protection, such role is manifested by the intricate cytoskeletal

architecture of the keratinocyte of the epidermis. Keratin forms the major intermediate filament cytoskeleton of epithelial cells and provides them with structural resilience against mechanical trauma (17). Moreover, they aid in cell-cell adhesion through desmosomes and are a crucial component of hair and hair follicles (18). It is one of the most rigid biological materials, possessing exceptional hardness (19). In humans, more than 50 distinct functional keratin genes exist (18). They are usually co-expressed as a heterodimer between one acidic (type I) and one basic (type II) keratin. These heterodimers self-assemble into antiparallel, staggered tetramers, yielding intermediate filament through lateral and longitudinal interactions (17).

The keratins are expressed in highly specific patterns related to the epithelial type and stage of cellular differentiation. They are expressed by several cell types. In the epidermis, the major producent are the keratinocytes. As the cells differentiate, they show distinct patterns of keratin gene expression within adjacent epidermal layers. In the *stratum basale*, the main keratins involved are K5 and K14 and are crucial for the structural integrity of the basal keratinocytes. As these cells differentiate and move outwards, the keratin expression is switched particularly in the *stratum spinosum* and *stratum granulosum* to K1 and K10. Here, they play a role in the mechanical protection and barrier function of the skin. Further contributing to the keratinization process is the K2 expressed in the *stratum corneum* (11, 17, 18).

1.1.1 Keratinocytes

The deepest layer of the epidermis is made up by keratinocytes attached to the basement membrane which separates the epidermis from the dermis. Hence, they are called the basal keratinocytes, and this layer is called the *stratum basale*, or the basal cell layer. It is continuous and consists of a single row of cells interconnected by desmosome junctions (8, 15). These cells are small and cuboid-shaped, with large nuclei and a small cytoplasm volume containing many ribosomes and dense bundles of keratin intermediate filaments (11). Their connection to the basement membrane is secured by hemidesmosomes. Most of the basal cells in this layer are mitotically active, giving rise to keratinocytes that will later make up the upper layers of the epidermis. However, the *stratum basale* also contains clonogenic stem cells that have a long lifespan and progress through the cell cycle very slowly. They slowly generate other basal cells and can increase their replication rate during inflammation, disease, or injury (15).

Above the basal cell layer, the keratinocytes make up the *stratum spinosum* or the spinous cell layer. About five cells thick, it comprises a variety of cells that are different in shape, structure, and properties. The cells in the lower rows are polyhedral and have a rounded nucleus (15, 20). They actively synthesize intermediate keratin filaments, also called cytokeratins. These are anchored to the desmosomes that tightly adhere adjacent cells to one another, providing the epidermis with resistance to mechanical stress (21). The keratin filaments are organized around the nucleus and bound to the desmosomes at one end while remaining free at the other, closer to the nucleus (15). The cells also begin to flatten as they progress outwards (21). In the upper rows of this layer, the keratinocytes begin to gradually differentiate and smoothly transition into the *stratum granulosum*, or the granular cell layer (8). Moreover, secretory organelles called lamellar bodies are present in the cytoplasm of the cells that reside in the upper levels of the *stratum spinosum*. These are first produced in the Golgi apparatus of the basal cells, and as the cells enter the *stratum granulosum*, they migrate towards the periphery (10, 11). They secrete lipids, protease inhibitors, glycoproteins, antimicrobial peptides, and hydrolytic enzymes into the intercellular space. It is believed that they play a role in forming a barrier to prevent excess loss of fluids from the skin (10, 13, 15).

Aside from the lamellar bodies, the *stratum granulosum* also contains keratohyalin granules in the cytoplasm of the keratinocytes, which is characteristic of this layer. These consist of profilaggrin and involucrin proteins (13). Profilaggrin is a phosphorylated and functionally inactive precursor to filaggrin (22). Several enzymes that dephosphorylate and cleave it facilitate its conversion to filaggrin (23). As the name suggests, filaggrin further binds to intermediate keratin filaments and causes their aggregation, contributing to the mechanical strength of the cell's cytoskeleton. Because of this, the cells are progressively flattening (24). In the *stratum granulosum*, lysosomal enzymes induce the degradation of the keratinocytes' nuclei and organelles. The cells become flattened as they move up toward the skin's surface, the *stratum corneum* (SC) (10, 15).

The *stratum lucidum* is most noticeably developed in the thick skin of the palms and soles. It is readily visible by light microscopy after staining with hematoxylin and eosin (H&E) as it refracts light, appearing lucid. The cells here can be referred to as "transitional" as they gradually lose their nuclei and organelles, and the keratohyalin granules are formed into keratin (8, 11). The cells also contain eleidin, a transformation product of keratohyalin

and another precursor for keratin (20, 21). This layer passes with a precise boundary into the SC, where the life cycle of keratinocytes is completed (8, 20).

The SC is composed of flattened, tightly assembled hexagonal remnants of keratinocytes, also called corneocytes, with no nuclei or organelles (8, 11). They are stacked in vertical layers ranging from 15 to 25 layers on most body surfaces to as many as 100 layers on the palms and soles (13, 20). Under the influence of filaggrin, the protein component of keratohyalin granule, the keratin filaments align into disulfide cross-linked macrofibers (11). The corneocytes are then encased in a highly insoluble, cornified envelope within the plasma membrane, which is formed by the cross-linking of soluble protein precursors, including involucrin and loricrin (25). Filaggrin is ultimately broken down on the skin surface into a pool of free amino acids, from which histidine and glutamine are converted to, respectively, trans-urocanic acid and pyrrolidone-5-carboxylic acid (26). The filaggrin degradation products form the basis of natural moisturizing factors within corneocytes that help maintain adequate skin hydration and slightly acidic pH (23, 27). The corneocytes are held together with desmosomes and extracellular lipids, providing a semi-impenetrable structure that makes up an essential part of the skin's barrier function against the environment (10, 13). These lipids are primarily made up of three major lipid classes: ceramides, non-esterified fatty acids, and cholesterol (28).

Additionally, the SC can be separated into *stratum conjunctum* and *stratum disjunctum*. The former represents a deeper level of compact cells that continuously pass into the *disjunctum*, where they are gradually separated and shed (8). This process, called desquamation, is crucial for cell renewal in the epidermis and for maintaining the skin's barrier function. The corneocytes are gradually detached from the surface of the SC through the controlled degradation of desmosome junctions between the cells. The process involves enzymes like serine proteases and is strictly regulated by the interaction between these proteases and their inhibitors or skin pH (29).

1.1.2 Melanocytes

Melanocytes are specialized pigment-producing cells found in the skin, inner ear, choroid, and iris of the eye. Melanocytes in adult skin originate from embryonic precursor cells known as melanoblasts, which arise from a specific subset of neural crest cells and migrate to the interfollicular epidermis (a portion of the epidermis located between the orifices of hair follicles) and developing hair follicles during development (11). They are present at

a ratio of 1 melanocyte per 10 basal keratinocytes, though the frequency is greater in certain body areas, such as the shins, face, and genitalia (10).

Their principal role is making a pigment called melanin. It is synthesised and accumulated in the melanosome, which are submicroscopic oval-shaped bodies formed in the endoplasmic reticulum and Golgi apparatus of melanocytes (20). The melanosomes are transported to keratinocytes through dendritic protrusions that reach up to the middle of *stratum spinosum* (8). During this process, the melanosomes enlarge and mature in four stages, accumulating melanin and passing from the end of the dendritic protrusion to neighboring keratinocytes. Each melanocyte supplies, on average, 36 keratinocytes; their mutual connection forms a functional melanocyte unit (8, 20).

Two types of melanin pigments can be found in human skin and hair: brown to black eumelanin and yellow to red pheomelanin. Eumelanin has a protective role; it absorbs and scatters ultraviolet (UV) radiation and neutralizes reactive oxygen species. On the other hand, pheomelanin is photosensitizing and thus generates reactive oxygen species when exposed to UV-A radiation (11). The melanosomes containing the pigment aggregate toward the superficial side of the nucleus. Such placement helps shield the genetic information within the keratinocytes' nucleus from damage induced by the effects of UV radiation (15).

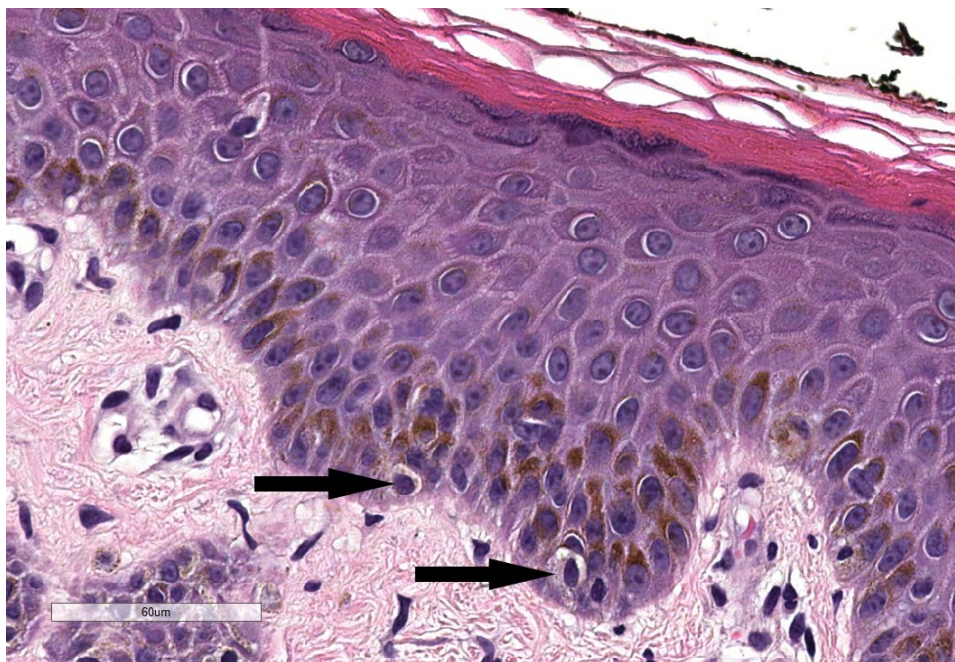


Figure 2: Melanocytes (black arrows) and melanin-carrying keratinocytes, skin from the cheek H&E stain.

(Source: (30))

1.1.3 Merkel Cells

MCs are post-mitotic cells scattered across the epidermis and constitute less than 5% of epidermal cells (11). They are primarily found in the *stratum basale* and concentrated in touch-sensitive areas, such as palms, soles, oral and genital mucosa, and nail beds (10, 31). The origins of MCs are yet unclear; nonetheless, there are two theories. One hypothesis suggests that they are derived from keratinocyte stem cells due to their location and the expression of cytokeratins, which comprise their intermediate filaments. Another theory proposes that they originate from neural stem cells that migrate during embryogenesis, similar to the development of melanocytes, due to their electrophysiological properties and ability to release neuropeptides (11, 20, 31).

These cells connect closely with myelinated sensory afferents, forming the MC-neurite complex thought to function as mechanoreceptors responsible for mediating gentle touch. Indeed, MCs are touch-sensitive cells that transduce mechanical stimuli. They are equipped with PIEZO2-dependent transduction channels, which are essential for converting physical stimuli into electrical signals by controlling Ca^{2+} influx to the MCs (32, 33). The unique structure of the MC-neurite complex allows for effective communication between the two cell types, thus allowing both MCs and sensory neurons to contribute to the encoding of mechanical stimuli (32). MCs contain neurosecretory granules positioned directly opposite the junctions with adjacent sensory nerve endings (11). When mechanical pressure is applied, the cells depolarize and secrete neuropeptides, presumably through these granules, thus modulating responses to mechanical stimuli (33). Moreover, it has been recently suggested that the ability of MCs to respond to mechanical stimuli extends beyond sensory perception and may also play a role in skin health and healing processes. They are able to interact with neuropeptides and other signaling molecules to influence wound healing and skin responses to injury (34).

1.1.4 Langerhans Cells

LCs are immunocompetent cells mainly found in the upper layers of the epidermis, particularly the *stratum spinosum* (35). In fewer numbers, they can also be found in the *stratum basale* (15). LCs constitute about 5% of the cell population in the epidermis and maintain a nearly constant distribution across the human body (20). They constitute the first line of immunologic defense in the skin, which is achieved by their functional role in innate and adaptive immune responses. Because LCs exhibit characteristics of tissue-resident

macrophages while functioning as antigen-presenting cells with features of dendritic cells, they are described as multifaceted in phenotype and function (36). LCs are distinguished by the presence of Birbeck granules, which are distinctive rod-shaped organelles likely involved in the processing and presentation of antigens, particularly those carbohydrate-bearing (37).

Within the epidermis, LCs form a dense network, acting as sentinels of the immune system. Their strategic placement enables them to interact with various skin components and respond to potential dangers encountered at the skin surface (35). LCs are uniquely equipped to survey the epidermal environment. In the absence of external stimulation, the cells display a distinctive type of movement known as dendrite surveillance extension and retraction cycling habitude (dSEARCH). This type of motion is characterized by rhythmic elongation and retraction of dendritic projections between intercellular gaps to sample the outermost layers of the skin (38). This allows them to sense the environment for antigens and determine the quality of the immune response (35). Pathological stimuli intensify dSEARCH activity, and direct contact between neighboring Langerhans cells increases. It is proposed that dSEARCH facilitates efficient antigen sampling by enabling the scanning of larger surface areas (38).

Additionally, under pathological conditions, LCs can pick up an antigen and travel through the lymphatic system to a nearby lymph node. During this journey, the LCs transform into lymphoid dendritic cells, losing their macrophage traits and Birbeck granules. Once there, within the paracortical area, the LCs display the antigen on its surface for recognition by T lymphocytes, which then undergo clonal expansion and initiate a primary immune response (11, 39). Increased LC migration during inflammation may partially deplete their numbers in the epidermis. Various murine models suggest that the loss is balanced by the entry of other LC subsets into the skin, such as peripheral monocytes, which quickly transform into temporary LCs. Meanwhile, precursors of the LCs either renew themselves or are replenished by bone marrow-derived cells (40).

However, this may not be the only way for LCs to interact with T cells. A recent study by Oka et al. found that LCs can directly interact with certain T cells in the human epidermis. According to this study, such LCs exhibit markers crucial for activating and regulating T cells, suggesting that their mutual interaction may also have a regulatory nature. (41)

1.2 Dermis

Beneath the epidermis lies a fibrous, richly vascularized layer called the dermis (8). The dermal-epidermal basement membrane separates the dermis from the epidermis and anchors the two layers together. This basement membrane is actively synthesised by basal keratinocytes and dermal fibroblasts, two cell types critical to the structural and functional integrity of the skin. Their cooperation indicates a mutual role in the development and regeneration of this interface (11).

Basal keratinocytes are anchored to the uppermost part of the basement membrane, the *lamina lucida*, through hemidesmosomes, type XVII collagen, laminin 332, and integrins $\alpha 6\beta 4$ and $\alpha 3\beta 1$ (42). These proteins form anchoring filaments that connect the hemidesmosomes to the lower layer of the basement membrane, the *lamina densa*, which interfaces with the dermal extracellular matrix (ECM) (10). In addition, anchoring fibrils composed of type VII collagen extend from the dermis to attach to the basement membrane, further stabilizing the connection (43). Another essential constituent of the basement membrane includes type IV collagen, which provides a scaffold to help maintain the structural integrity (42).

The dermis constitutes most of the skin's mass, with its thickness varying depending on anatomical location (10). It comprises a complex network of fibrous, filamentous, and amorphous connective tissue, creating a supportive environment for blood vessels that deliver nutrients and nerves that provide sensory input (15). Several epidermally derived appendages, including sweat glands, sebaceous glands, and hair follicles, partially exist in the dermis (21). Resident cell populations within the dermis are fibroblasts and immune cells, such as macrophages and mast cells. In response to infection or injury, other immune cells, like lymphocytes, plasma cells, and leukocytes, can migrate into the dermis (15).

The fibrous architecture of the dermis is predominantly shaped by dermal fibroblasts, spindle-shaped cells with extended cytoplasmic processes. Their primary function is the synthesis and maintenance of the ECM, which is essential for preserving the structural integrity of soft connective tissues such as the dermis. In addition, fibroblasts secrete a variety of cytokines and growth factors that mediate cell-cell communication, thereby regulating the behavior of neighboring fibroblasts as well as other cutaneous cell types. Moreover, the fibroblasts also play a principal role in tissue remodeling and wound healing (44, 45).

Unlike the epidermis, the dermis does not undergo a clearly defined process of cellular differentiation. However, the organization of its ECM components, particularly collagen and elastin fibers, varies with depth. Based on these structural differences, the dermis is divided into two distinct sublayers, as illustrated in **Figure 3**: the *stratum papillare* (papillary dermis) and the *stratum reticulare* (reticular dermis) (20).

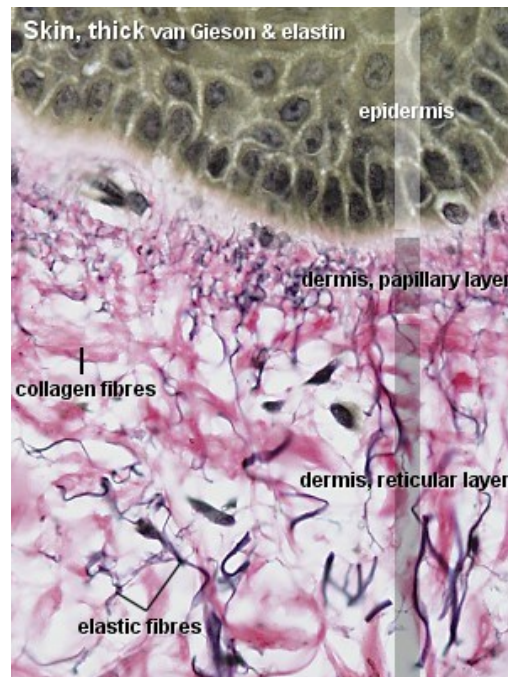


Figure 3: The dermis in thick skin, van Gieson's stain combined with an elastin stain.

(source: (46))

The papillary dermis represents the superficial region of the dermis and is situated directly beneath the epidermis. It consists of loosely arranged connective tissue, predominantly composed of collagen and elastin fibers. The fibers that constitute this sublayer are thin and consist mostly of type I and III collagen, which forms a loose and irregular network that serves a structural role. This network is surrounded by a gel-like material, a so-called “ground substance” consisting of glycosaminoglycans (GAGs), long unbranched heteropolysaccharides such as hyaluronates and dermatan sulphate. This material provides support for other skin components as well as maintaining proper moisture content essential for the proper skin function (45, 47).

Compared to the reticular dermis, the papillary dermis contains a higher density of fibroblasts, immune cells, and mast cells. This sublayer is also rich in capillaries and lymphatic vessels, essential for supplying nutrients to the epidermis and clearing metabolic waste (8, 9, 20).

Structurally, the papillary dermis is characterized by dermal papillae, which are finger-like projections that protrude upward into the overlying epidermis. This interlocking architecture increases the surface area of the dermal-epidermal junction, enhancing mechanical stability and molecular exchange. It is especially well developed in areas subjected to frequent mechanical stress, such as the palms and soles, where it provides increased resistance to shear forces (21, 45).

The reticular dermis, located beneath the papillary layer, comprises the deeper and more substantial portion of the dermis. It comprises densely packed type I collagen and elastin fibers that are highly organized into a robust network. This architecture imparts mechanical strength, structural support, and elasticity, allowing the skin to resist deformation when supporting the body. Compared to the papillary dermis, The previously mentioned skin appendages are embedded within the reticular dermis and contribute to essential physiological functions such as thermoregulation, barrier maintenance, and sensory perception (9, 20, 45).

1.2.1 The Role of Collagen

The primary ECM component synthesised by fibroblasts is collagen. Collagen is not a single protein but rather a family of structurally related, yet genetically distinct proteins. To date, 29 types of collagen proteins have been identified, comprising a diverse family of structurally and functionally distinct macromolecules. This family can be divided into several groups: fibrillar collagens (e.g., types I, II, or III) and non-fibrillar collagens (e.g., types IV, VI, or VII) (48). The primary distinguishing feature between these two classes is the ability of the fibrillar collagens to self-assemble into large fibrils. In contrast, non-fibrillar collagens generally associate with fibrillar networks and often serve as molecular bridges, stabilizing the organization of the extracellular matrices. For example, type XII collagen interacts with type I fibrils and other ECM components to maintain collagen suprastructure and absorb stress (49).

A hallmark feature of collagen is its triple-helical conformation, which arises from the repeating amino acid sequence glycine-X-Y in its polypeptide chains, where X and Y are other amino acids – often proline (Pro) and hydroxyproline (Hyp), respectively (11, 47, 50).

Three subunit polypeptide α -chains intertwine in a right-handed triple helix to form the fundamental trimeric structural unit of collagen, called procollagen (51). Among the amino acids composing these chains, glycine (Gly) is the most abundant, followed by Pro. These

amino acids are critical for the stabilization and compact packing of the triple-helix, with Gly facilitating tight helix formation due to its small size, and Gly-Pro hydrogen bonds contributing to helix cohesion. Other amino acids, such as lysine (Lys), alanine (Ala), aspartic acid (Asp), glutamic acid (Glu) are also present in smaller quantities (47, 48).

Furthermore, the collagen polypeptide chains contain significant levels of Hyp and hydroxylysine (Hyl) residues, two amino acids that are the result of post-translational modifications of Pro and Lys residues. The hydroxylation reactions are catalysed by prolyl and lysyl oxidases (LOX), respectively. The hydroxylation of Pro residues is critical for stabilizing the triple-helical structure, while hydroxylated Lys residues are essential for the formation of stable cross-links between collagen fibrils. Subsequently, Hyl residues undergo glycosylation, during which galactose and then glucose residues are transferred to the hydroxyl group of Hyl by galactosyltransferase and glycosyltransferase, respectively (11, 51).

Following the post-translational modifications, the procollagen molecules are subsequently secreted into the extracellular space through vesicles derived from the Golgi apparatus. Subsequently, loose termini are cleaved by specific proteases. Such structure is now called tropocollagen, and is further processed based on the type of collagen (51). For fibrillar collagens, such as type I collagen, this facilitates spontaneous self-assembly of tropocollagen into higher-order supramolecular structures, such as collagen fibrils. This organization is driven by electrostatic and hydrophobic interactions. The resulting fibers are further stabilized by intermolecular cross-linking, mainly involving interactions between Lys-Lys and Hyl-Hyl residues, giving mechanical strength and stability to the collagen fibers (11, 48). The formation of fibers is summarized in **Figure 4**.

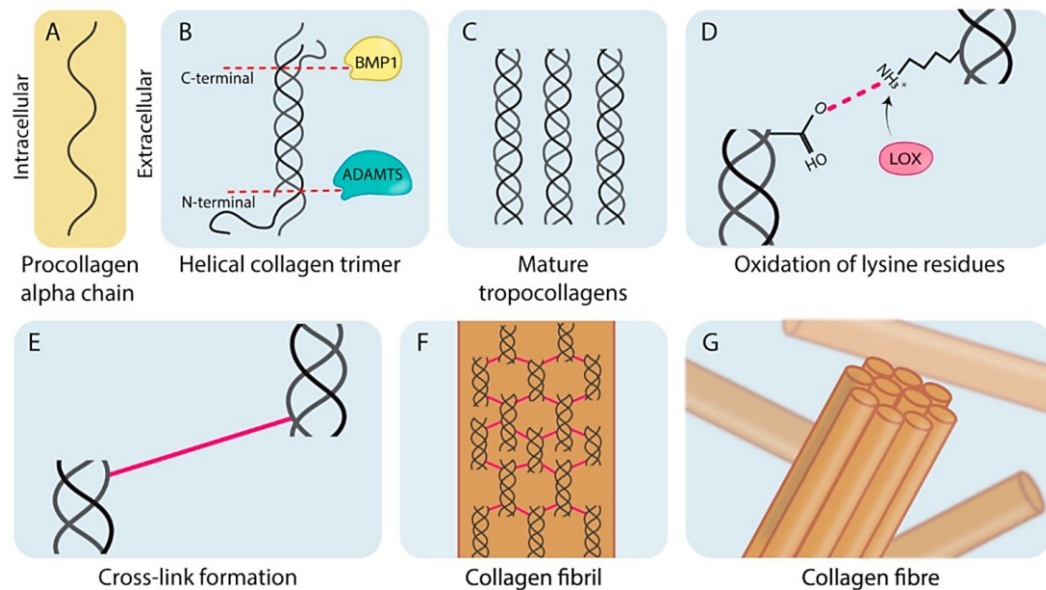


Figure 4: The organization of collagen fibrils into bundles of collagen fibers.

(source: (52))

The most common types of cross-link in type I collagen are derived from Lys and Hyl residues (11). An oxidative deamination reaction, catalyzed by LOX, removes the ϵ -amino group from the Lys, generating a highly reactive aldehyde group. This aldehyde then spontaneously condenses with non-oxidized ϵ -amino group of Lys in an adjacent collagen molecule, leading to the formation of intra- and inter-molecular covalent cross-links (53).

The primary collagens involved in skin architecture and function are predominantly type I and III, followed by fibril-associated collagens, such as types XII, XIV, XVI and VI (50). Type I collagen is the most abundant collagen in the human dermis. It consists of two identical $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain, forming a heterotrimer. In the dermis, type I collagen co-assembles with type III collagen to form broad, extracellular fibrils that contribute to the dermal matrix structure. The typical type I to type III ratio in human skin is approximately 8:1, although this proportion can vary with age and physiological condition (11, 47).

1.2.2 The Role of Elastin

Another integral component of the dermal connective tissue, primarily synthesised by fibroblasts, is elastin. Although it is a relatively minor component, accounting for only 2-4 % of the total dry weight of the dermis (54), elastin plays a crucial role in providing the skin with stretch and elastic recoil properties, thereby maintaining its physiological function.

Similar to collagen, elastin forms a fiber system that permeates the dermis. These fibers consist of a dense elastin core surrounded by a mantle of fibrillin-rich microfibrils (55).

Elastogenesis, the process of elastin formation, is most active during fetal and neonatal development. In adults, however, elastogenesis becomes minimal due to the downregulation of ELN gene transcription, which encodes elastin, in senescent dermal fibroblasts (56). Despite the reduced synthesis in adulthood, elastin is an extremely long-lived protein, with a half-life exceeding 70 years (57).

Initially, elastin is synthesised as a soluble, monomeric precursor polypeptide known as tropoelastin. Like collagen, tropoelastin is rich in Gly, which constitutes approximately one-third of its total amino acid content. However, unlike collagen, Gly in tropoelastin is not evenly distributed (11). Another difference is that the tropoelastin consists of two major domain types: hydrophobic and hydrophilic. The hydrophobic domains are rich in non-polar residues such as Gly, valine (Val) and Pro, typically occurring in repeating motifs. These alternate with hydrophilic domains enriched in Lys and Ala, where a characteristic feature is the presence of two Lys residues separated by two or three Ala residues (11, 58). The hydrophilic domains are involved in the cross-linking of elastin (55).

Tropoelastin is chaperoned to the cell surface by the elastin binding protein (EPB), which protects it from degradation and intracellular self-aggregation (57). Upon secretion, tropoelastin undergoes a process called coacervation – spontaneous self-association by hydrophobic interactions – which results in the formation of distinct globular aggregates on the cell surface. These aggregates are deposited onto a microfibrillar scaffold of fibrillin proteins to form elastic fibers. This is facilitated by specific regions on the microfibrils and supported by the auxiliary proteins fibulin-4, fibulin-5 (55, 59). The resulting elastic fibers consist of elastin molecules aligned in parallel bundles, guided by the microfibrillar scaffold, which contributes to fibers of relatively uniform diameter (11). The elastogenesis pathway is illustrated in **Figure 5**.

The structural integrity of elastic fibers is ensured by covalent cross-links between the Lys residues. The process, similarly to the cross-linking in collagen, is mediated by LOX. In elastin, however, cross-linking results in the formation of unique amino acids desmosine and isodesmosine. Desmosine, for example, is generated when three oxidized Lys residues spontaneously fuse together with a fourth unmodified Lys. These desmosine compounds form a covalent linkage that joins two tropoelastin molecules. Such specialized cross-linking gives elastin its defining ability to recoil after stretching (11).

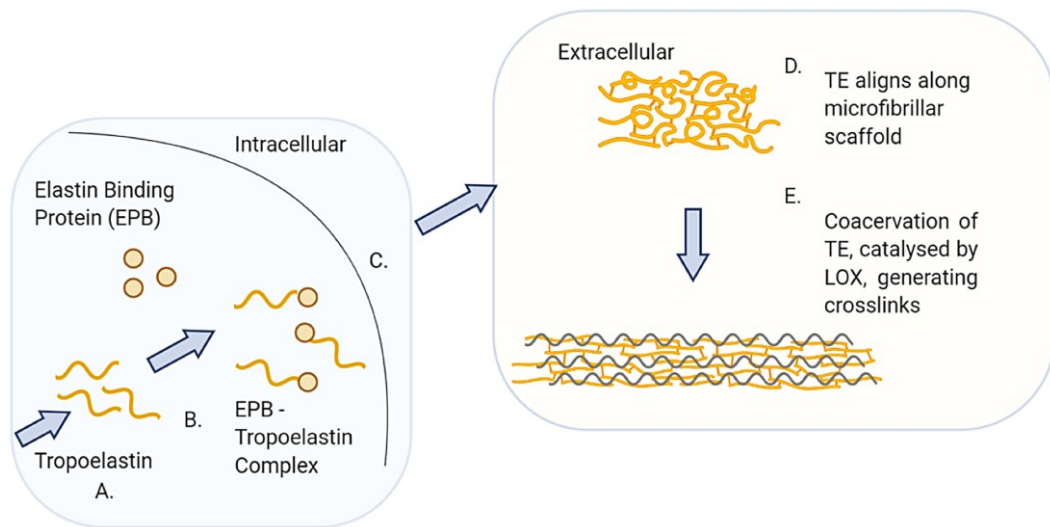


Figure 5: Elastin biosynthesis pathway.

(source: (60))

Three types of elastic fibers are recognized in the skin based on the amount of elastin present in association with microfibrils: oxytalan, elaunin, and mature elastic fibers. Oxytalan fibers are composed solely of fibrillin-rich microfibril bundles and are typically located at the dermo-epidermal junction. Elaunin fibers, which interconnect oxytalan fibers in the papillary dermis with elastic fibers in the reticular dermis, contain both fibrillin-rich microfibrils and amorphous elastin. Elastic fibers are in the reticular dermis and predominantly consist of elastin (57).

The orientation of collagen and elastin fibers within the dermis is critical to skin's structural integrity and mechanical properties. Traditionally, structural analysis of the fiber organization was limited due to the fibers' dense packing and intertwining. However, in a series of studies, Ueda et al., combined multiphoton microscopy and biaxial skin tissue extension to investigate fiber organization in human reticular dermis. Their findings revealed that collagen fibers exhibit a complex, multi-directional organization throughout the dermal layers, rather than a linear or preferential alignment. In the superficial reticular dermis, a small number of fiber bundles intersected the major fiber orientation, a pattern similarly observed in the deeper layers. Elastic fibers were found to closely align with collagen fibers in the superficial and mid-reticular dermis, while a looser alignment was noted in deeper regions. The study also observed a high degree of local similarities in the direction of collagen and elastin fibers across the dermal layers (61).

1.3 Hypodermis

The hypodermis, also known as subcutaneous tissue or superficial fascia, is the deepest layer of the skin, lying beneath the dermis and above muscles or bones (62). It is primarily composed of adipocytes (or fat cells), which are organized into lobules separated by fibrous septa containing collagen and reticulin fibers (63). These septa are continuous with dermal collagen and provide structural support. Moreover, they anchor the skin to underlying tissues, facilitating vascular and nervous supply to the overlying dermis (10). Each fat lobule is supplied by a muscular artery branching into arterioles and capillaries, with venules draining blood through the septa, ensuring oxygenation and nutrient exchange (8, 63).

Adipocytes are derived from mesenchymal stem cells (MSCs). They are large, reaching up to 100 μm in diameter. The principal role of adipocytes is to store neutral lipids and triglycerides in a single intracytoplasmic vacuole, giving them a signet-ring morphology in histological sections stained with H&E (8), as can be noticed in **Figure 6**. In frozen sections, their full lipid content is visible, and immunohistochemical staining reveals expression of S-100 protein and vimentin (63).

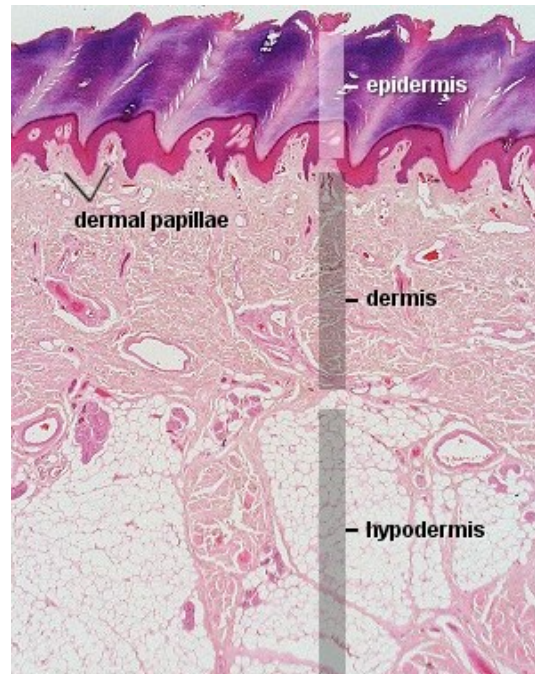


Figure 6: Thick adult skin with visible layers of epidermis, dermis and hypodermis, H&E stain.

(source: (46))

Approximately 80% of body fat resides in the hypodermis, with non-obese males having 10–12% of body weight as fat and females 15–20%. The hypodermis includes white and brown adipose tissue, the latter being more prevalent in infants and children, characterized by distinct mitochondrial properties and increased heat production (11). The thickness of the hypodermis varies by anatomical region. It is influenced by nutrition, age, sex, and body weight, with thicker deposits forming the panniculus adiposus in areas like the abdomen, hips, and thighs (45).

Functionally, the hypodermis serves as a reservoir of energy, provides insulation for thermoregulation, and cushions deeper tissues against mechanical and thermal damage (8). Another function of this skin layer is its ability to act as an endocrine organ, producing hormones such as leptin, which regulates body weight through the hypothalamus, and facilitating hormone conversion, such as androstenedione to estrone by aromatase (45, 62). Adipocytes also play signaling roles in osteogenesis, angiogenesis, and phagocytosis, with multipotent stem cells in the hypodermis capable of differentiating into adipocytes, osteoblasts, myoblasts, and chondroblasts (11). The loose connective tissue of the hypodermis allows the skin to slide over underlying structures, enhancing mobility (62).

1.4 Skin Appendages

A characteristic feature of the skin is the presence of skin appendages, or the adnexa of the skin. These appendages are derived from the skin and are usually adjacent to it. They are anatomical skin-associated structures that serve a particular function. In humans, common skin appendages include hair follicles and arrector pili muscles, sebaceous glands, sweat glands that encompass two types, apocrine and eccrine glands, and finally nails (8).

1.4.1 Hair Follicles

The pilosebaceous unit, a critical integumentary structure, integrates the hair follicles, the arrector pili muscles, and sebaceous glands distributed across the dermis on hair-bearing skin. Hair follicles vary in density across different anatomical locations. Moreover, they also exhibit a variation in size, shape, number, and distribution of across the body that depends the on anatomic location, race, and sex (62, 63). Hairs can be divided into vellus hair, which are short, fine and light-colored, or terminal hair, which are thicker, darker and also hormonally regulated post-puberty (8, 10). The hair follicle is closely connected to the

function of several other organs, specifically sweat and sebaceous glands, and is thus regulated by related hormonal activity (45).

The hair follicle is composed of three regions. The *infundibulum*, extending from the epidermis to the sebaceous duct, serves as the follicular opening for sebum release, aiding lubrication. The *isthmus*, from the sebaceous duct to the arrector pili insertion, is a narrower segment housing the bulge region with stem cells essential for hair regeneration. The bulb encases the dermal papilla and germinative matrix cells with high proliferation rates, forming the hair shaft (11, 63). The hair shaft consists of a central medulla with condensed cells, a cortex of keratin-filled cortical cells with a dense protein matrix cells analogous to the SC, and a cuticle forming an outer protective layer that interdigitates with the cortex to anchor the hair (8, 10). The inner root sheath supports the shaft and degenerates above the sebaceous gland. The outer root sheath is continuous with the epidermis and expresses epithelial keratins (11). A collagen-rich fibrous sheath from the corium ligament encases the follicle. The arrector pili muscle is anchored between the sebaceous gland outlet and dermal papillae ligament, elevates hair for thermoregulation but is absent in beard, pubic, and axillary hair (8).



Figure 7: Hair follicle of terminal hair, H&E stain.

(source: (46))

The hair matrix harbors germ cells and keratinocytes, resembling epidermal spinous layer cells, which divide rapidly during anagen (8, 62). Germ cells are undifferentiated progenitors from basal epidermal cells. They differentiate into matrix cells forming the hair shaft and sheaths and express hard keratins distinct from the epidermal keratins (8, 11, 63). The matrix keratinocytes, similarly to the cells in the *stratum spinosum*, undergo keratinization and melanin accumulation (62).

The hair follicles go through three cycles. The first is the anagen, an active growth phase that last for 2–6 years, making it the longest. There is a high mitotic activity of the cells of the follicular matrix. The next phase is the catagen, a transitory growth phase during which the cells of the matrix undergo apoptosis to form a club hair. This phase lasts for 2–3 weeks. The last phase is the telogen, resting phase that lasts for approximately 3 months. The keratinization of the hair bulb is completed and the hair growth stops (8, 45, 63).

1.4.2 Sebaceous Glands

Sebaceous glands, much like the sweat glands, are another structure in the skin specialized in excretion. Their main product is the sebum, which is an oily secretion that hydrates and protects skin and hair. These glands are primarily associated with hair follicles, forming part of the pilosebaceous unit. They are distributed across most of the body, with higher density in specific regions. Their activity is regulated by hormones, particularly androgens (8, 45).

Histologically, they are holocrine alveolar glands, formed by one or more lobules, as can be seen in **Figure 8**. These glands are most abundant on the face, scalp, and upper chest, but absent in areas like the palms, soles, or the oral mucosa. Sebaceous glands sometimes show an anomaly and may not be bound to follicles – one such example are sebaceous glands on the edges of the lips and the buccal mucosa, referred to as Fordyce's glands (8, 63).



Figure 8: Sebaceous glands of the pilosebaceous unit of hairy adult skin, H&E stain.

(source: (46))

Sebaceous glands are acinar, meaning the glands consist of multiple sac-like lobules, or acini. Acinar glands consist of a base layer of epithelial cells on the basal layer. These cells proliferate and differentiate and fill the acini with round cells with ample lipid droplets in their cytoplasm. The nuclei of these cells gradually degenerate, with the cytoplasm loaded with fat droplets, and burst. The acini connect to an excretory duct that opens into the hair follicle's *infundibulum* (62, 63). Sebaceous glands produce sebum, a substance composed of triglycerides, wax esters, squalene, and fatty acids. It is formed through holocrine secretion, where entire acinar cells disintegrate and release lipid-rich contents (10, 45).

Sebaceous glands are under hormonal control. They are mature at birth due to maternal hormones but atrophy in childhood. However, during puberty, their growth and activity is stimulated by androgens and sebum production rapidly. With age, however, the production drops, leading to skin dullness in older individuals (8, 63). The produced sebum functions in a way that forms a protective layer on the skin surface, helping to maintain skin moisture, supports the acidic mantle, and blocks microbial and chemical penetration. It also aids sweat evaporation and enhances skin flexibility (10, 45).

1.4.3 Sweat Glands

Sweat glands are elements of the excretory and thermoregulatory systems of the skin. The two main types of sweat glands are eccrine glands and apocrine glands. Each type has distinct structures, distributions, and functions that support skin homeostasis (45).

The more abundant are the eccrine glands as they cover nearly all body surfaces. Their density is the highest on palms, soles, forehead, and axillae, however, they are absent in the genital area (10, 11). Within the dermis, the eccrine glands are surrounded by intervertebral cells, which contract to help empty the gland contents. Structurally, these glands are tubular and spiral (62), consisting of three parts. They have a secretory coil that is located in the lower dermis or the subcutis, measuring 30–50 μm in diameter (11). The secretory area has clear and dark myoepithelial cells that have the morphology of a stratified cubic epithelium. The clear cells are located at the basal layer and are responsible for producing sweat. Additionally, they have many microvilli to increase their surface area (62).

Another part of the eccrine glands is made up the intradermal duct, which is straight and lined with one to two layers of cuboidal cells. The last part is the intraepidermal duct, or acrosyringium, is spiral and opens directly onto the skin surface (63). The sweat produced by eccrine glands is odourless and consists of water, sodium chloride, and urea. Its components are released by exocytosis to support thermoregulation through evaporative cooling. Cholinergic sympathetic nerves control eccrine glands. They respond to thermal and emotional stimuli (11, 62).

Apocrine glands are found in areas abundant in hair follicles. They are larger when compared to the eccrine glands, measuring 3–5 mm in diameter (63). Specialized forms of the apocrine glands are ceruminous glands found in the ear canal, where they produce earwax, and Moll's glands in the eyelids (8). Structurally they are also composed of three parts. Their secretory portion is located in the subcutaneous tissue. It consists of columnar cells with an apical cap and myoepithelial cells. The columnar cells release lipid-rich viscous fluid through "decapitation secretion", which means the free luminal end of the cell is lost with the secretory products into the follicular *infundibulum* above the sebaceous duct (8, 63). The secretions are initially odourless; however, a characteristic scent is produced upon bacterial decomposition on the skin surface. Therefore, these secretions may function as pheromones. Another distinctive feature is that the apocrine glands become active at puberty under hormonal control. They play a minimal role in thermoregulation but contribute to the skin's chemical barrier (11, 45, 62).

1.4.4 Nails

The nails are specialized keratinized epidermal structures that only form on the fingers and toes to protect digit tips. They are located on the distal phalanges (62). The main function of the nail is to protect the digit tips through their robust structure (10).

The nail unit consists of these main parts: nail plate, nail matrix, nail bed, proximal nail fold, lateral nail folds, hyponychium, and lunula. The nail plate is a hard keratin structure. Its cells undergo keratinization to form sulfur-rich, calcium-containing onychocytes. The nail matrix is a germinative layer with cuboidal basal keratinocytes that divide and differentiate into flat onychocytes for the nail plate. The nail bed is a thin epidermal layer with keratinocytes that adhere to the nail plate's onychocytes through ridges. The proximal nail fold is an extension of the skin, it has ventral epithelial cells that form the eponychium, a horny layer sealing the matrix. The lateral nail folds are also skin extensions, formed of cells that align with the nail plate's edges. The hyponychium is an epidermal layer under the free nail edge. Its cells transition to normal skin keratinocytes. And lastly, the lunula is a white crescent at the matrix's edge, with cells that are less keratinized, appearing opaque (8, 11, 62).

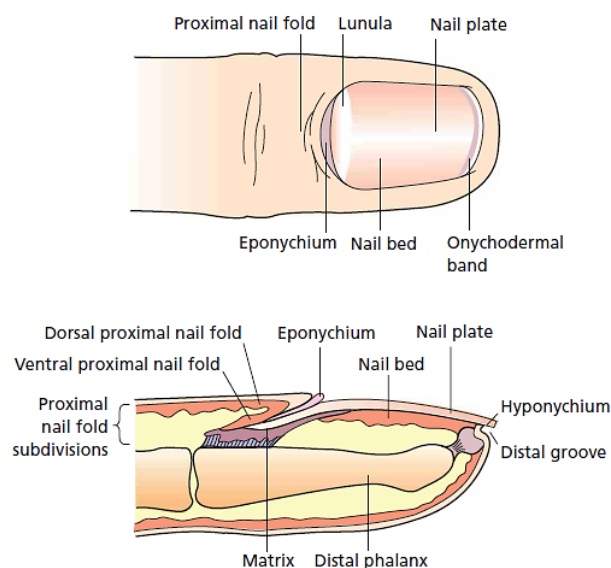


Figure 9: Anatomy and structure of the human nail.

(source: (11))

2 THE PHYSIOLOGY OF WOUND HEALING

As explored in the previous chapter, the skin is a large and multifunctional organ that serves as a vital protective barrier against water loss and environmental insults. When this barrier is disrupted, a wound forms, triggering a complex and dynamic biological process known as wound healing. This process comprises multiple overlapping phases that are rightly regulated by a variety of cellular and molecular mechanisms. Moreover, it is essential for restoring the skin's structural and functional integrity (1, 24). A simplified overview of this process is illustrated in **Figure 10**.

Wound healing is generally divided into four sequential yet overlapping phases: hemostasis, inflammation, proliferation, and remodeling, each regulated by intricate cellular and molecular interactions (11). Hemostasis is the initial phase and begins immediately after tissue injury. Platelets rapidly aggregate to form a clot, preventing blood loss and releasing growth factors that initiate the healing cascade (64, 65).

The inflammatory phase follows, marked by the infiltration of neutrophils and macrophages into the wound site to clear pathogens and cellular debris. These immune cells also secrete cytokines and additional growth factors to support the transition to the next phase (66). In the proliferative phase, granulation tissue forms, consisting of fibroblasts, endothelial cells, and a newly synthesized ECM rich in collagen and proteoglycans (11). During this phase, re-epithelialization is initiated as keratinocytes migrate to cover the wound, and angiogenesis ensures an adequate blood supply (24). Finally, the remodeling phase sets in, and the provisional ECM is replaced with new components (65, 66).

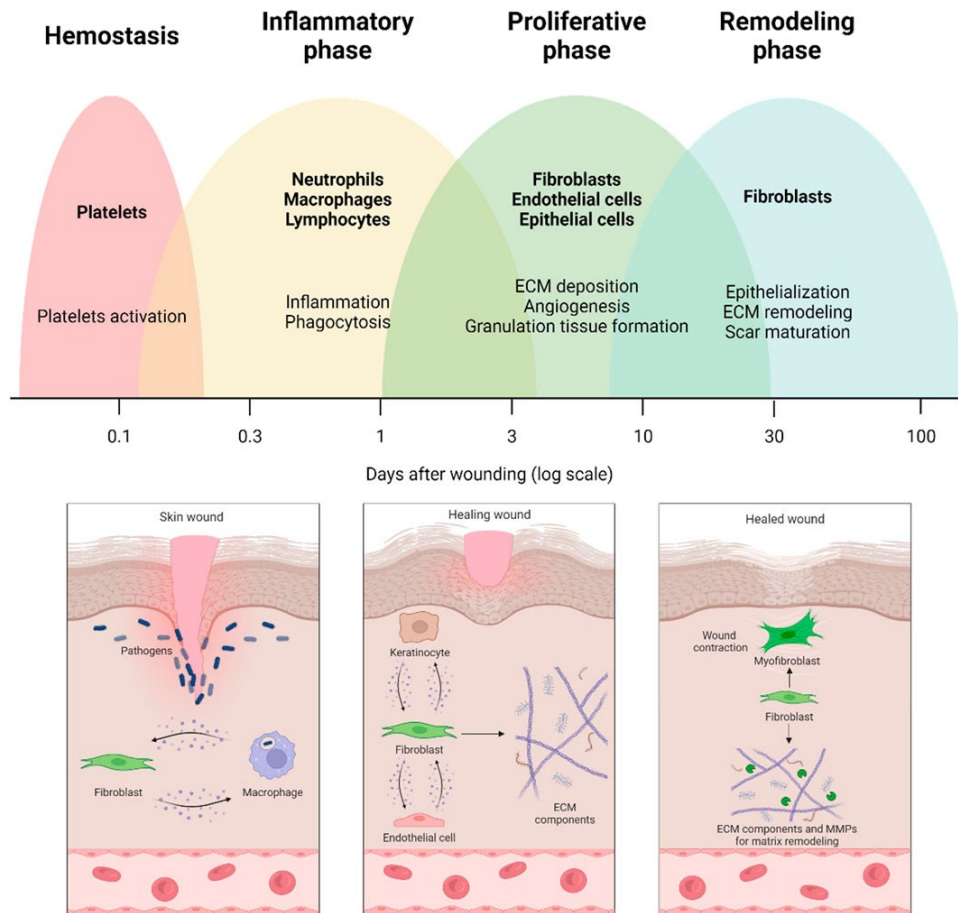


Figure 10: The overlapping stages of skin tissue wound healing.

(source: (67))

The duration and efficiency of each phase can vary depending on factors such as wound type, severity, and systemic conditions, including age, nutritional status, and underlying diseases (68). For example, acute wounds, like surgical incisions, generally heal within weeks, while chronic wounds, including diabetic ulcers, may persist for months due to impaired cellular function and prolonged inflammation (24).

Successful wound healing requires the precise coordination of various cell types – such as platelets, immune cells, and fibroblasts – and signaling molecules. Key molecules include epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF- β) and vascular endothelial growth factor (VEGF). These regulate critical cellular activities such as migration, proliferation, and differentiation (64).

In adult mammals, the healing of full-thickness skin injuries typically results in scar formation rather than complete regeneration (1). While scar tissue restores structural support and basic function, it lacks the original dermal architecture and often exhibits reduced tensile strength and elasticity compared to uninjured skin (11, 24).

2.1 Hemostasis

Hemostasis is the immediate physiological response to skin injury, primarily aimed at halting bleeding and initiating wound healing. Upon injury, the epidermal barrier is disrupted, and blood components are released into the wound site, triggering the activation of the clotting cascade. This results in a formation of a fibrin clot that not only stops bleeding but also serves as a provisional matrix for the subsequent influx of inflammatory cells (11, 64).

The process begins with rapid vasoconstriction of injured blood and lymphatic vessels, lasting only a few minutes. This action is mediated by endothelin released from damaged endothelial cells and circulating catecholamines released from injured cells (69). Simultaneously, platelets aggregate at the site of injury to form a thrombus, preventing excessive blood loss. Upon exposure to collagen in the subendothelial matrix, platelets adhere to the damaged vessel walls by G-protein-coupled receptors undergo morphological changes. They lose their disc shape, form pseudopodia – an arm-like projection from the cell membrane in the movement direction – and begin to cluster into larger aggregates (24, 69, 70).

Platelet membrane receptors also bind to collagen, activating intracellular signaling pathways that result in the production of thrombin. Thrombin, in turn, catalyzes the conversion of fibrinogen into insoluble fibrin (66). The clotting cascade ultimately culminates in the thrombin-mediated polymerization of fibrin monomers into long fibrin chains, forming a dense 3D fibrin network that incorporates platelets, erythrocytes, neutrophils, and monocytes (24).

The fibrin monomers assemble into protofibrils that become anchored to native type I collagen fibers through integrins. This creates a provisional ECM composed of fibrin, fibronectin, vitronectin, and thrombospondin. As the wound surface dries, this matrix solidifies into a protective scab (24, 66). Platelets further stabilize the thrombus by releasing adhesive glycoproteins and sphingosine-1-phosphate, enhancing their aggregation and adhesion to the extracellular matrix (69).

2.2 Inflammation and Immune response

The inflammatory phase of wound healing begins immediately after injury and plays a critical role in preventing infection, clearing debris, and preparing the wound bed for subsequent tissue repair (71). It is initiated by the disruption of the skin barrier and occurs

in parallel with hemostasis (72). This phase is tightly regulated to prevent excessive tissue damage, and its timely resolution, typically resolving within a few days, is essential for the progression to the proliferative and remodeling phases (66).

Following hemostasis, inflammation is marked by vasodilation and increased vascular permeability. Locally released mediators such as prostaglandins, histamine from mast cells, and serotonin from platelets initiate this vascular response, which peaks around 10 minutes after injury and lasts for approximately one hour, leading to characteristic signs such as redness, heat, and edema (24). The platelets degranulate, releasing granules rich in signalling molecules including EGF, PDGF, TGF- β , insulin-like growth factor (IGF), and interleukin-1 (IL-1). These mediators are most intensely released within the first hour, but the secretion can continue for up to 7 days. They attract neutrophils and macrophages while activating subsequent wound healing phases (64, 69). TGF- β , in particular, acts as a chemoattractant for immune cells and also modulates their responses. Other factors, such as fibrinopeptides, which are generated during fibrinogen cleavage, further draw inflammatory cells to the wound site (24, 66).

Because of the vascular damage, there is oxygen depletion, leading to hypoxia. This stimulates the migration of keratinocytes, angiogenesis and proliferation of fibroblasts. Moreover, the production of PDGF, VEGF and TGF- β further supports tissue repair (24).

Neurogenic inflammation, driven by substance P and calcitonin gene-related peptide (CGRP) from sensory neurons, further amplifies the inflammatory response. Substance P promotes vascular permeability and induces degranulation in mast cells, while CGRP induces vasodilation to aid in the recruitment of leukocytes (66). Mast cells contribute to this response by releasing histamine, VEGF, IL-6, and tumor necrosis factor- α (TNF- α), promoting endothelial permeability and immune cell migration (73).

Chemokines (e.g., IL-8) and growth factors (e.g., PDGF) from the fibrin clot that formed during hemostasis promote leukocyte recruitment by diapedesis, mediated by selectins and integrins on endothelial and immune cells (24). Concurrently, mediators such as damage-associated molecular patterns from necrotic cells and pathogen-associated molecular patterns activate pattern recognition receptors, which amplifies cytokine production and immune cell activation (66, 74). Calcium ions (Ca^{2+}) signals and hydrogen peroxide rapidly guide the migration of neutrophils, supported by PDGF and neutrophil-activating peptide from platelets (69, 75, 76).

Neutrophils arrive within an hour and peaking at day 2 phagocytose bacteria, release reactive oxygen species, and form neutrophil extracellular traps to eliminate pathogens (76, 77). Additionally, they secrete TNF- α , IL-1 β , IL-8, and VEGF, stimulating fibroblasts and epithelial cells (24). Neutrophils undergo apoptosis after 24–36 hours, triggered by monocytes attracted primarily by IL-8 to the wound site (76).

The number of monocytes reaches its peak around days 4–5. In the wound, monocytes bind to fragments of collagen and fibronectin through integrins and eventually differentiate into macrophages. Initially, macrophages adopt a pro-inflammatory phenotype, referred to as “M1”, eliminate apoptotic cells as well as debris and microorganisms (24, 78). As the tissue begins to repair, the overall macrophage population transitions to “M2” phenotype, which promotes anti-inflammatory effects. They facilitate the migration and proliferation of fibroblasts, keratinocytes and as well as endothelial cell (78). Macrophages also attract additional monocytes and lymphocytes, and their depletion delays healing (76).

Mast cells peak around 3 hours post-injury and decline after 6 hours or more. However, there is an increase in mast cell numbers over the following 10 days, with a decline to baseline by day 21. These cells contribute to clot stabilization through the release of TNF- α . Additionally, they support angiogenesis and fibroblasts activity by producing VEGF, chymase, and tryptase (73). Dendritic cells (DCs), including LCs, begin to accumulate at the wound site within an hour and remain for up to two weeks. These cells interact with mast cells, leading to the secretion of TGF- β and TNF- α to drive differentiation of fresh precursors into DCs (76). Plasmacytoid DCs, absent in normal skin, are recruited rapidly and support healing through interaction with regulatory T cells (TREGs) (79).

Lymphocytes, including T cells, B cells, and innate lymphoid cells (ILC), infiltrate the wound site approximately 72 hours after injury, largely attracted by macrophage activity (76). ILCs contribute to re-epithelialization (66), while T cells secrete keratinocyte growth factor (KGF)-1, KGF-2, and IGF-1, driving keratinocyte proliferation. Through EGF receptor expression, TREGs facilitate healing, with their depletion delaying closure (69, 76).

2.3 Proliferation

The proliferative phase of wound healing spans for approximately 2–10 days post-injury and further supports the restoration of tissue integrity through granulation tissue formation, angiogenesis, re-epithelialization, and wound contraction (72). This phase is orchestrated by macrophages, fibroblasts, keratinocytes, and endothelial cells, which coordinate the

replacement of the fibrin clot with a provisional connective tissue and epithelium, transitioning to the last phase of wound healing – tissue remodeling (24, 66).

Granulation tissue typically begins to form around 4–5 days after injury. Comprising fibroblasts, capillaries, and ECM, it gradually replaces the fibrin clot over the course of 2–3 weeks (24, 67). Fibroblasts are activated by PDGF, TGF- β , fibroblast growth factor (FGF), and other inflammatory mediators. They migrate into the wound, guided by integrin receptors that bind collagen, fibronectin, and vitronectin (24, 67, 80). Their proliferation is stimulated by TGF- β , IL-1, and TNF- α . Additionally, they secrete collagen type III, hyaluronic acid, fibronectin, and proteoglycans, thereby forming a provisional matrix in place of the fibrin clot, which is being simultaneously degraded by matrix metalloproteinases (MMPs). The activity of MMPs is regulated by tissue inhibitors of metalloproteinases (TIMPs) to prevent excess ECM breakdown and chronic wound formation (80). By day 4 post-injury, the fibroblasts become the predominant cell type in the wound site. Mechanical tension and cytokines, such as TGF- β , promote their differentiation into contractile myofibroblasts (81).

Another product of fibroblast activity are pro-angiogenic molecules, such as VEGF, FGF, angiopoietin-1, and thrombospondin, which stimulate neovascularization (67). Angiogenesis initiates as early as day 2 and is strongly driven by hypoxic conditions within the wound environment (11). Hypoxia induces the expression of hypoxia-inducible factor-1 in macrophages, endothelial cells, fibroblasts and keratinocytes. Endothelial cells, primarily responding to VEGF, differentiate into tip cells which guide sprouting vessels, proliferative stalk cells that elongate the new vessel, and phalanx cells that form the blood vessel lining (66). Another cell type that participates in neovascularization is the pericyte, which stabilizes the newly forming capillaries (82).

Keratinocyte migration marks the initiation of re-epithelialization and begins within hours after injury, originating from wound margins and adnexal structures such as pilosebaceous units (24). They adopt a migratory phenotype through an activation process lead by various cytokines and growth factors, such as EGF, TGF- β , TNF- α and IL-1, IL-6. This activated phenotype is marked by changes in the cytoskeleton network and modulation of surface adhesion receptors. A key marker of this activation is the upregulation of K6 and K16, which contribute to enhanced cellular flexibility. Prior to migration, desmosomal and hemidesmosomal adhesions are weakened by protein kinase C- α , enabling detachment from neighboring cells. A proposed mechanism involves a switch in integrin expression from

$\alpha 6\beta 4$ to $\alpha 3\beta 1$, promoting interaction with laminin-5 and facilitating movement across the ECM (80).

In the basal layer, keratinocytes guided by growth factors begin to proliferate, peaking at 1–2 days and decline to normal levels by day 14 (11). These growth factors include EGF or IGF-1. Moreover, ECM components modulate growth factor signaling through integrin-mediated pathways, enhancing receptor sensitivity (80). In about 7 days after injury, wound contraction guided by myofibroblast activity reduces the size of the wound. These cells develop pseudopodia, which enables them to connect to the surrounding collagen and fibronectin via actin-myosin interactions and exert their contractile force (71).

2.4 Tissue Remodeling

The final phase of wound healing, tissue remodeling, begins approximately 2–3 weeks post-injury and can persist for several months to years, depending on the wound type and individual healing capacity (83). During this phase, the connective tissue in the wound site is slowly remodeled into a mature scar, acquiring its final strength and appearance (24). Due to the fibroblast activity, type III collagen, which was synthesized during the earlier stages of wound healing, is being replaced with type I collagen (84).

Initially, the type III collagen fibers are degraded by MMPs secreted by several cell types, such as myofibroblasts, endothelial cells and macrophages. Simultaneously, myofibroblasts synthesise type I collagen and form cross-linked, aligned bundles, boosting the tensile strength of the scar tissue to approach the original, unwounded tissue (85). Compared to the normal dermis, the scar tissue is characterized by collagen that, unlike in the normal dermis, forms smaller and more densely packed fibers in parallel bundles. Additionally, there are often higher proportions of type III collagen fibronectin (11).

As the remodeling progresses, the number of cells in the scar tissue declines as myofibroblasts, macrophages, endothelial cells and other granulation tissue cells undergo apoptosis (66, 69). Endothelial cells undergo apoptosis, which halts the growth of new blood vessels and reduces the capillary density (11). Apoptotic cells are cleared by macrophages through phagocytosis (69). The components of the ECM also undergo changes. Notably, the levels of proteoglycans and GAGs, which provide hydration in granulation tissue, diminish. Elastin reappears in the scar tissue, but forms fragmented, chaotic structures. Another distinguishing feature of scar tissue is thickened epidermis, flattened dermal-epidermal junction and the absence of skin appendages (11, 66).

3 TISSUE ENGINEERING OF THE SKIN *IN VITRO*

The principles of tissue engineering emerged in the mid-1970s, notably with the work of Langer and Folkman, who utilized polymers for the sustained release of drugs to prevent tumor growth. This research was followed by studies in the 1980s when researchers around the world began seeding cells onto substrates to investigate cell-material interactions and tissue development. Tissue engineering has since evolved into an interdisciplinary field that integrates engineering, material and life sciences to develop biological substitutes that are capable of restoring, maintaining, or improving the function of tissues and organs (1).

Nowadays, tissue engineering has rapidly developed. Advancements have led to successful establishment of tissue-engineered substitutes of part of the human body, aiding in regenerative medicine and in research as a substitute to help eliminate the need for animal models. One such body part is the skin tissue, which has been well developed to closely mimic the structure of the native skin (86).

Despite the ongoing reliance on animal models to investigate skin-related pathologies or toxicity, notable anatomical and physiological differences between species often hinder the translatability of animal-based findings to human clinical settings (87). These differences include variations in metabolism, immune responses, and skin architecture (88). Consequently, the predictive power of animal models remains limited, and ethical concerns regarding animal use have further fueled the pursuit of alternative approaches.

This shift is reinforced by legislative and regulatory changes, such as the EU ban on animal testing for cosmetic products (Regulation 2009/1223/EC), the REACH regulation for chemical safety (2006/1907/EC), and Directive 2010/63/EU, which mandates adherence to the 3Rs principle (Replacement, Reduction, and Refinement) in scientific research (3). These principles advocate for the replacement of animals with alternative methods, the reduction in the use of animals, and lastly, the refinement of animal use practices to minimize suffering. In this context, tissue-engineered skin models have emerged as valuable *in vitro* tools for studying human skin biology and testing the safety and efficacy of compounds under physiologically relevant conditions (89).

3.1 A Brief Historic Overview

The earliest *in vitro* skin models consisted of 2D cell monolayer cultures, which remain widely used in initial testing due to their simplicity, cost-effectiveness, and rapid turnaround.

These models offer valuable insights into cytotoxicity and molecular mechanisms (86). The foundation for *in vitro* skin modeling was laid in the 1950s with the development of techniques to separate the epidermis from the dermis and culture keratinocytes. A pivotal advancement occurred in 1975 when Rheinwald and Green successfully cultured human keratinocyte colonies from single cells using irradiated 3T3 fibroblast feeder layers. This breakthrough enabled large-scale keratinocyte expansion for burn treatments and dermatological research and established keratinocyte monolayers as a cornerstone method for studying skin biology, including their capacity to form multilayered structures (2).

However, monolayer cultures fall short in replicating the full stratification and architecture of the native skin. Cells grown on flat plastic surfaces in submerged conditions adapt to the artificial environment, often resulting in altered gene expression profiles (90). Compared to 3D environments, 2D-cultured cells display distinct morphology, a more uniform life cell cycle, faster proliferation, and heightened sensitivity to active substances (91).

The development of 3D skin models began in 1976 with the introduction of an explant model, which used inverted dead pig skin to support keratinocyte outgrowth. This approach was significantly refined in the 1980s by culturing keratinocytes on collagen-based matrices at the air-liquid interface (ALI), which facilitated the keratinocyte differentiation and the creation of an epidermal layer on human de-epidermized dermis (DED) while preserving essential basement membrane proteins. From the 1980s onward, optimized culture protocols led to the development of diverse array of HSEs that recapitulate both dermal and epidermal compartments, and human epidermal equivalents (HEEs) that feature only the epidermal layer. These constructs are collectively known as organotypic cultures or living skin equivalents and utilize various dermal substrates, including DED and collagen matrices (2).

A basic protocol for HSE construction consists of fibroblasts embedded in an ECM gel followed by seeding keratinocytes seeded on top. These constructs are cultured in cell culture inserts with porous membranes to allow for epidermal stratification at the ALI. Today, such models are well-established and commonly applied *in vitro* for purposes such as cosmetic product testing, drug screening and dermatological research (7). Several commercial 3D skin models have been validated by the Organisation for Economic Co-operation and Development (OECD) and the European Centre for the Validation of Alternative Methods (ECVAM). Examples of HEEs include SkinEthic™ (EpiSkin, L'oréal, Lyon, France) and StrataTest® (Stratatech, Madison, WI, USA), and notable HSEs include EpiDerm™

(Mattek, Ashland, USA) or Phenion® Full-Thickness skin model (Henkel, Düsseldorf, Germany) (92).

Recent advancements in tissue engineering have led towards the development of more physiologically relevant skin equivalents by incorporating additional human cell types such as adipocytes, dermal papilla cells for hair follicle modelling, endothelial cells for vascularization, immune cells, and neurons for peripheral nerves. These innovations improve the models' functionality and relevance, particularly for studying irritation, immune responses, and toxicity. Nonetheless, a major limitation of current HSEs remains the absence of a functional vascular network, which, along with issues like limited lifespan, excessive cell contraction, and matrix degradation, constrains their utility (93).

To address these challenges, emerging technologies such as 3D bioprinting and microfluidic systems, including skin-on-a-chip (SOC) platforms, are being explored (94). These approaches enable the incorporation of multiple cell types, dynamic fluid flow, and improved vascularization. While promising, these novel models must undergo rigorous validation to ensure that their morphology and barrier function are comparable to those of well-established HSEs and HEEs (2, 94).

3.2 Human Skin Equivalents

As mentioned in the previous subchapter, HSEs are 3D skin models that combine dermal and epidermal components to mimic native skin. This structure enables them to replicate key histological and functional properties of human tissue. HSEs are also referred to as dermo-epidermal substitutes or reconstructed human skin (1, 87, 92).

A general protocol for constructing an HSE is illustrated in **Figure 11**. Typically, the process begins with creating a dermal layer comprising a scaffold populated with fibroblasts, which is cultured for up to one week under submerged conditions. Next, keratinocytes are seeded on top of this layer and the construct is incubated for another week, still submerged. Finally, the model is raised to an ALI to promote keratinocyte differentiation and stratification, resulting in the formation of a multi-layered epidermis (5).

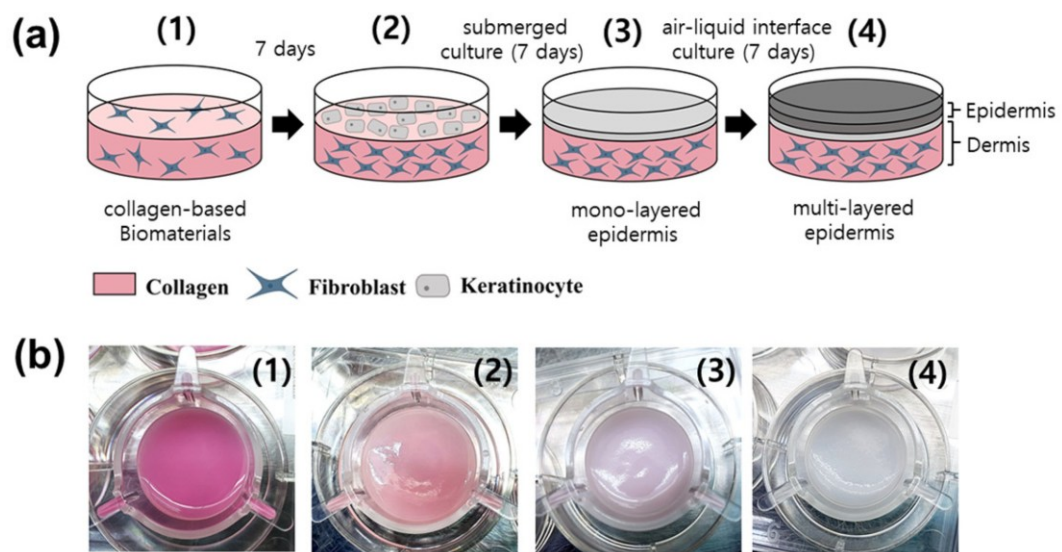


Figure 11: The process scheme of constructing HSEs.

(source: (95))

Various scaffold materials have been employed in the development of the dermal layer, including fibronectin, gelatin, and collagen (96). Collagen type I is the most commonly used, as it is the predominant component of the native dermal ECM and can be readily extracted from natural sources such as calf skin or rat tail tendon (87, 97). However, one downside of collagen and other natural polymer-based gels is that they exhibit weak mechanical properties due to high water content and are susceptible to contraction exerted by fibroblasts (98, 99). To improve scaffold stability, techniques such as chemical cross-linking (e.g., using glutaraldehyde), non-enzymatic glycation, or blending collagen with other polymers have been applied (87, 100).

The key cellular components of HSEs are fibroblasts and keratinocytes. Fibroblasts support keratinocyte proliferation and differentiation through paracrine signaling, essential for forming a well-structured basement membrane (87). The fibroblasts are incorporated into the dermal matrix, where they become synthetically active instead, leading to the reorganization of the scaffold through endogenous secretion of ECM components. This construct can be referred to as a dermal equivalent (DE). The fibroblasts contract the dermal matrix, which allows for the next step in the preparation of HSEs (1, 97). Epidermal keratinocytes are subsequently seeded onto the surface of fibroblast laden dermal matrix. A crucial step in preparing the HSE is exposing the epidermal cells to the air, which triggers epidermal stratification (87). At this point, nutrients are only by diffusion through the porous

membrane of the cell culture insert (97). After about two weeks at the ALI, a fully stratified 3D structure has formed with a similar architecture to the native human skin (101).

In addition to scaffold-based methods, scaffold-free approaches have also been described. For instance, Michel et al. reported a technique using only cultured fibroblasts and keratinocytes. In this system, the presence of ascorbic acid enhances fibroblast-mediated ECM secretion, leading to a self-assembled dermal layer. Keratinocyte stratification is likewise promoted under ALI conditions, yielding a multi-layered epithelium (102).

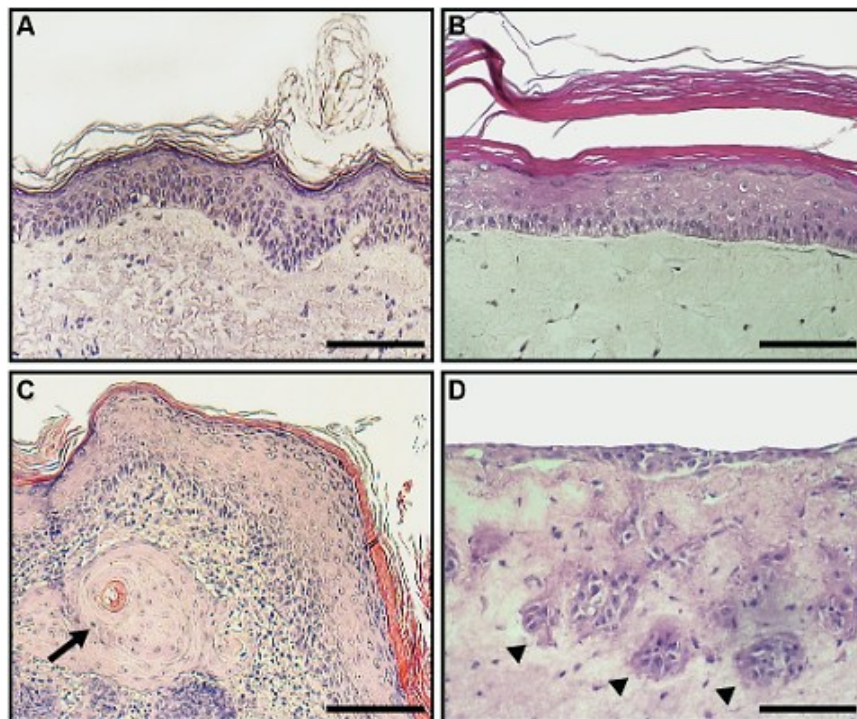


Figure 12: Histological staining of human skin specimen (A) and squamous-cell carcinoma (SCC) sample (C), and *in vitro* skin equivalents generated by seeding normal human keratinocytes (B) or SCC keratinocytes (D) onto fibroblast-containing collagen gel. Arrow indicates a “horny pearl” in the skin dermis. Arrowheads indicate clusters of tumor cells in the dermal compartment of the skin equivalents. Bars: 200 μ m

(source: (3))

3.3 3D Bioprinting

3D bioprinting offers a promising approach to fabricate HSEs by precisely depositing living cells and biomaterials to replicate the structure and function of native skin (103). Over the past decade, this technology has developed rapidly from custom-built laboratory-specific printers to commercially available off-the-shelf systems. Typically, 3D bioprinting utilizes scaffold-based models, where cells grow within a supportive matrix composed of natural, synthetic, or hybrid polymers (99). The complete process includes three stages: pre-

processing (e.g. imagining, model design, bioink preparation), processing (tissue printing), and post-processing (tissue maturation) (104).

As an additive manufacturing technique, 3D bioprinting employs computer-aided design to deposit bioinks, which are mixtures of biomaterials and living cells, in a layer-by-layer fashion to construct tissue-like structures (105). For a bioink to be suitable for printing, it must possess appropriate rheological properties and crosslinking potential. Collagen and gelatin are commonly used for their favorable biological compatibility and support for cell adhesion; however, their mechanical properties are relatively weak. Therefore, bioinks often incorporate multiple materials to improve both structural integrity and printability (106).

HSEs can be constructed using bioinks containing fibroblasts and keratinocytes to form the dermal and epidermal layers, respectively (103). Among the available bioprinting techniques, extrusion-based bioprinting is the most widely employed. This method uses pneumatic, piston, or screw-driven systems to continuously extrude high-viscosity bioinks such as collagen or fibrin. It is particularly well-suited for generating robust dermal layers with high cell densities and for printing large-scale skin constructs. However, it offers lower resolution and poses a risk of mechanical stress on the cells during extrusion (104, 105).

Inkjet bioprinting, another common method, dispenses low-viscosity bioinks via thermal or piezoelectric mechanisms in a drop-on-demand manner. This technique is ideal for high-resolution patterning of epidermal layers, allowing for precise cell placement. Nonetheless, it is limited by nozzle clogging and is compatible only with low-viscosity materials, which can restrict material selection (87).

Laser-assisted bioprinting employs laser-induced forward transfer to position bioinks without the need for a nozzle. This method enables high cell viability, single-cell resolution, and the formation of intricate tissue structures. It is particularly advantageous for printing complex spatial arrangements, such as vasculature or skin appendages, although it remains costly and technically demanding (104, 105).

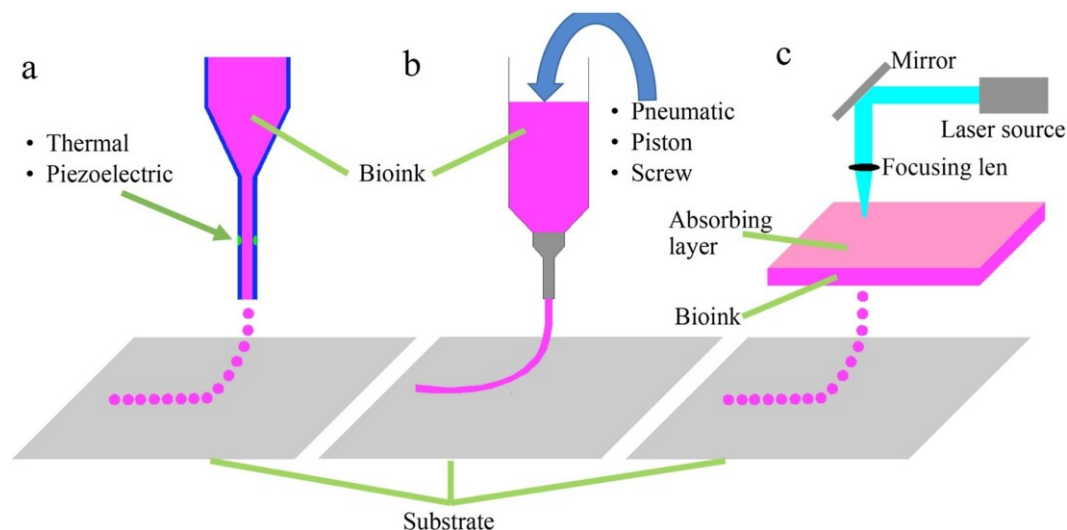


Figure 13: Three main bioprinting strategies: Inkjet printers (a), extrusion-based printers (b) and laser-assisted printers (c).

(source: (104))

The primary cell types used in 3D bioprinted HSEs are fibroblasts and keratinocytes, though increasing efforts are being made to incorporate other skin-relevant cells such as melanocytes and endothelial cells (103). Recent advancements have also enabled the partial integration of skin appendages, such as hair follicles and sweat glands, into 3D printed models (105). These developments underscore the growing potential of 3D bioprinting to produce anatomically precise, multi-cellular skin constructs suitable for both research and regenerative medicine. The ability to spatially arrange various cell types, including vasculature-like structures, is one of the most significant advantages of this technology (87).

Overall, the main advantages of 3D skin bioprinting is high resolution, flexible operation and repeatable printing. However, some limitations and current challenges include key technical problems with bioinks. For instance, selected biological materials need to correspond to the native human skin tissue characteristics, such as physical and mechanical properties. On top of that, the selected materials must be also compatible with the 3D printing system, and they need to possess sufficient mechanical strength after printing for proper structural stability. The research status of 3D bioprinting for skin tissue engineering indicates that an ideal bioink remains to be identified (105).

3.4 Skin-on-a-chip

The SOC is a specialized subset of organ-on-a-chip (OOC) technology, which comprises microfabricated devices that mimic the physiological functions and structural properties of

specific human tissues or organs *in vitro*. OOC platforms, also known as tissue-chips or micro-physiological systems, use microfluidic systems seeded with human cells to simulate dynamic fluid environments, offering more accurate models than traditional 2D or 3D cultures (107). These specialized platforms allow for the control of the microenvironment, including mechanical forces and chemical gradients. They are designed to replicate the structure and function of human skin *in vitro*, addressing the demand for reliable models in drug and cosmetic testing (108). An illustration of a SOC design is shown in **Figure 14**.

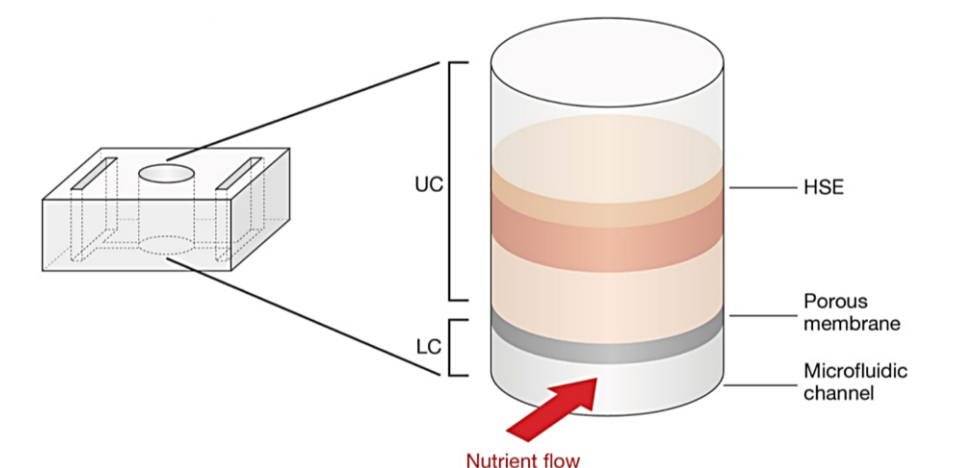


Figure 14: Schematic diagram of the interaction of three layers in the SOC. LC means lower chamber, which encompasses a microfluidic channel, UC means upper chamber.

(source: (109))

SOCs employ two main strategies for developing skin equivalents. The first approach, named transferred SOC, incorporates either patient-derived skin fragments from biopsies or post-mortem skin samples. The second approach, called *in situ* SOC, focuses on generating the tissue directly on the chip by utilizing immortalized human skin cell lines (109).

Transferred SOC offer a more accurate in representing the cellular population of the skin and their interactions at a microscopic level. The process of generating HSE through this approach is simpler than cultivating skin cells and creating an optimized environment within the device for the tissue to sustain itself over an extended period of time. There are, however, concerns with variability of the skin samples, which may in turn affect the reproducibility and consistency of research finding. This problem arises due to the nature of the skin tissue, because the skin physiological profile may vary in terms of thickness and cell types among individuals (109). These models allow for the study of different factors, such as molecules diffusion or drug sensitivity and toxicity (93).

In situ SOC models offer greater control over tissue formation and microenvironmental conditions. On the other hand, *in situ* SOC face challenges in fully replicating the 3D skin structure within channels, often relying on simplified constructs or manual tissue formation, and some models show suboptimal performance under dynamic conditions compared to static cultures such as traditionally prepared HSEs (93). The biomimicry is also limited by the absence of vasculature, skin appendages and various cell types within the skin (110). To enhance the physiological relevance of these *in situ* SOC, several research entities have integrated LCs, macrophages and T cells into these models, which are reported to be sustained for up to 50 days, making them ideal for long-term studies (109).

Overall, the SOC field is currently experiencing growing global. The leveraging of SOC technology has a potential to support more efficient drug development and reduce reliance on animal testing, aligning with broader regulatory and industrial trends (111).

3.5 Multi-Cellular 3D Skin Models

Despite the ability of HSEs to replicate key histological features, basic fibroblast-keratinocyte HSEs remain limited in complexity. They lack skin appendages such as hair follicles and sweat glands and typically rely on simple scaffold compositions (92). Moreover, the absence of a vascular system restricts nutrient delivery and waste removal, thereby shortening the models' lifespan and limiting physiological relevance (87). To address these issues, recent research has focused on the integration of additional cell types into HSEs.

The inclusion of immune cells such as T cells, dendritic cells, and macrophages enables the study of inflammation, infection, and allergic reactions. However, full immune system integration remains rare. The inclusion of multiple cell types at once presents challenges, particularly in developing a compatible culture medium that supports all cells without altering phenotype, viability or function (112).

The addition of melanocytes is crucial for modeling pigmentation-related processes. Although these cells have been successfully cultured for decades, replicating their full range of functions *in vitro* remains challenging. Their complex behavior is difficult to replicate in the current 3D models. While melanocyte-keratinocyte co-cultures and 3D epidermal models have improved our understanding of melanogenesis and melanin transfer, they still fail to fully mimic the cellular and tissue polarity seen *in vivo*. Because of these limitations, many researchers continue to use short-term *ex vivo* skin explants to study pigmentation, allowing

for more natural interactions between melanocytes, keratinocytes, and fibroblasts. These explants preserve melanogenesis and melanin transfer for up to 48 hours without disrupting skin structure (7).

The physiological relevance may be additionally elevated by the addition of a hypodermal layer, thereby yielding a tri-layered 3D skin model. Incorporating the hypodermis could enable research on hormonal regulation, lipid metabolism, and structural integrity (87). The literature reports that the most common cell types used are mature adipocytes. However, isolated mature adipocytes are fragile and display inconsistent viability when introduced into 3D matrices *in vitro* (113). Additionally, there are concerns with de-differentiation under suboptimal culture conditions. Adipose-derived stem cells represent an alternative, though they require differentiation *in vitro*, which increases time and cost. Some tri-layered models also incorporate native adipose tissue, which introduces other cell types such as endothelial cells and macrophages, further enhancing complexity (87).

The incorporation of hair follicles and sebaceous glands remains challenging due to the complexity of the pilosebaceous unit. It encapsulates all important physiologic processes found in the human body: controlled cell growth/death, interactions between cells of different histologic types, cell differentiation and migration, and hormone responsivity, to name a few. Thus, the value of the hair follicle as a model for biological scientific research goes way beyond its scope for cutaneous biology or dermatology alone. Hair follicle morphogenesis is a very complex process involving many different signaling pathways that is restricted to embryogenesis and cannot be induced during adulthood (7).

Many efforts have been driven by the scientific community during last decades to reconstruct human hair follicle using different approaches. The most promising approach involves using reprogrammed dermal papilla cells and 3D printed scaffolds to produce a fully human HSE with intact hair (114). Recent breakthroughs have even shown the possibility of creating hair-bearing human skin from pluripotent stem cells, opening new paths for regenerative medicine (115).

As for sebaceous glands, their regeneration is still in early stages. Most current methods rely on isolating glandular cells from human skin and cultivating them. A newer method involves forming spheroids or organoids from these cells, which can mimic gland behavior. However, challenges remain, especially regarding cell viability and organoid formation efficiency (116). Materials like Matrigel and growth factors have been shown to support

sebaceous gland formation, but these methods are mostly applied *in vivo*. So far, no reliable method exists for fully integrating sebaceous glands into *in vitro* skin models (7).

Approaches to simulate vascularization use cell-based methods by incorporating endothelial cells within the dermal compartment. It has been demonstrated that stem cells or endothelial cells seeded and exposed to VEGF. While this leads to capillary-like structures, these vessels cannot yet be externally perfused (93). Another approach lies in the structural modification of the scaffold by including pore size and their interconnectivity (117). However, challenges remain in achieving reproducibility, appropriate vessel diameter, and efficient perfusion. Emerging technologies such as 3D bioprinting offer promising solutions, enabling precise spatial placement of vascular structures and reproducible fabrication of complex, physiologically relevant models (99).

4 OVERVIEW OF *IN VITRO* WOUND HEALING RESEARCH

As established in previous chapters, skin wound healing is a critical physiological process essential for health and survival. However, it is highly complex, involving multiple, dynamically interacting cell types. Consequently, substantial research has been devoted to understanding the cellular and molecular mechanisms underlying wound healing. Despite these efforts, the full complexity of this process cannot yet be fully replicated *in vitro*. As a result, various animal models continue to be used to investigate normal and impaired wound healing and to test for possible therapeutic interventions (3).

Animal experiments have greatly advanced our understanding of wound healing, skin diseases, and treatment strategies. They remain a common approach for studying both physiological healing and pathological conditions. However, there are significant concerns about their translational value due to anatomical and physiological differences across species (118).

Rodents, for example, are frequently used in wound healing research. Yet, rodent skin differs from human skin in several key aspects: it contains more appendages, has fewer epidermal layers, and is loosely attached to the underlying musculature. Moreover, rodent wound healing predominantly occurs by contraction, whereas in humans, it proceeds mainly through new tissue formation (3).

Among animal models, the pig is considered the most comparable to humans in terms of dermal structure and healing mechanisms. Accordingly, many wound healing studies have been conducted in pigs. Nevertheless, differences still exist – for instance, pig skin exhibits reduced dermal vascularity and lacks eccrine sweat glands. It also remains unclear how these distinctions affect wound healing outcomes, and many molecular aspects of pig cutaneous healing remain insufficiently characterized (119).

In light of the translational limitations of animal models and increasing pressure from regulatory and ethical frameworks, as discussed in Chapter 3, the development of non-animal alternatives for studying wound healing has gained momentum. These range from simple 2D monocultures of key skin cell types, such as keratinocytes and dermal fibroblasts, to more complex systems including co-cultures and 3D tissue-engineered constructs. Among these, HEEs and HSEs are particularly valuable for modeling wound healing under various experimental conditions (2). Nevertheless, the complexity of the wound healing process requires the adoption of both *in vitro* and *in vivo* models, despite their dissimilarities (120).

4.1 2D Wound Healing Assays

In vitro 2D wound healing models are widely used for studying cell migration due to their simplicity and cost-effectiveness. The models allow researchers to directly examine how environment changes or therapeutic compounds influence cellular behaviour in the absence of the tissue complexity (120, 121). The fundamental aim of any 2D wound healing assay is to intentionally disrupt a confluent cell monolayer, creating a cell-free area into which cells migrate and proliferate. Various wounding techniques are used, including mechanical, thermal, electrical, and optical methods. Across these models, three key steps are consistently observed: wound creation, monitoring of the healing process, and data analysis (6, 118).

A general overview of the 2D wound healing assay workflow is presented in **Figure 15**. The wound area is typically monitored using time-lapse photomicrographs. Quantitative analysis is most commonly performed by calculating wound closure rates through changes in wound width or area. While this method is inexpensive, it can be time-consuming, requiring manual measurement at multiple time points. More advanced systems use real-time optical monitoring with automated software that tracks cell migration and proliferation (122, 123).

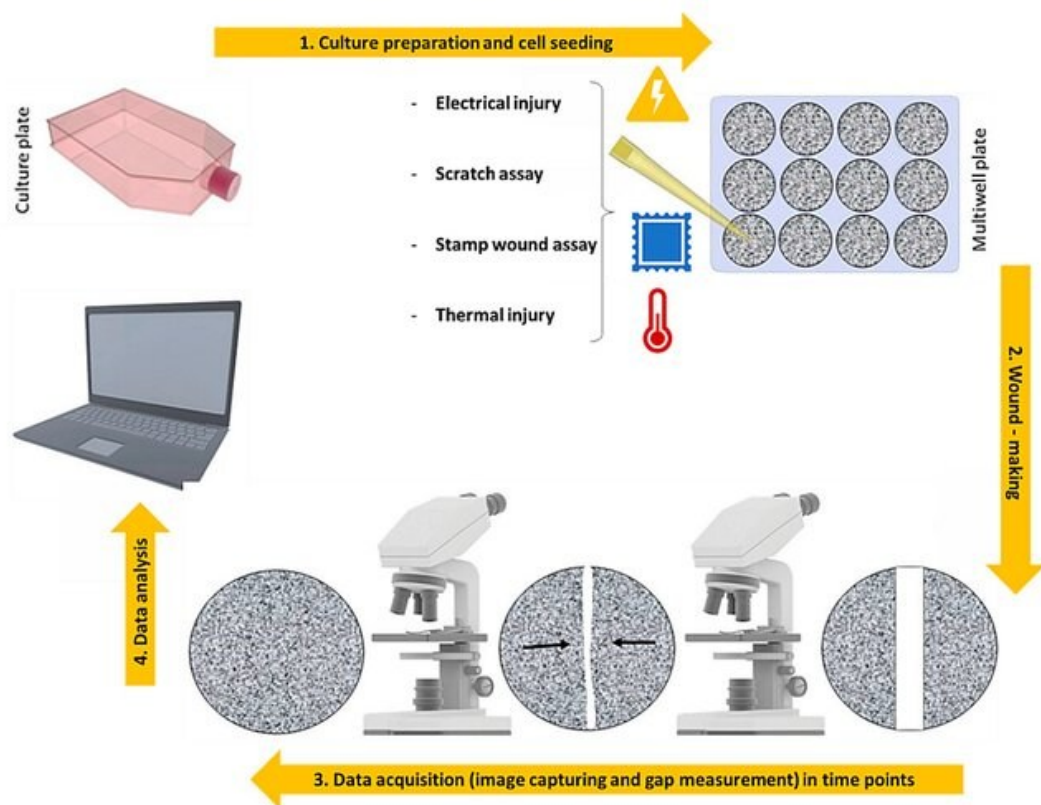


Figure 15: Summarized workflow of the 2D *in vitro* wound healing assay.

(source: (4))

Among the simplest and most widely used methods is the scratch assay, which involves mechanically creating a linear “wound” in a confluent monolayer (usually of keratinocytes or fibroblasts) using a pipette tip, cell scraper, metallic indenter, or even a toothpick. Cells are typically cultured to 80–90% confluency on plastic dishes, which may be coated with ECM proteins such as collagen or fibronectin to assess migration on different substrates (120). Despite its simplicity, the manual nature of this method often leads to variability in wound width and geometry, reducing reproducibility. Mechanical injury can also disrupt the ECM coating, potentially confounding the results (4). In addition, dislodged cells may accumulate at the wound edge, influencing subsequent migration (124).

To address these limitations, various automated or alternative methods have been developed. For example, devices such as the WoundMaker™, can generate consistent linear wounds approximately 700–800 μm wide in a cell monolayer (125). Another method, stamping, uses the application of pressure on a defined area. Stamping assays often use rubber or polydimethylsiloxane moulds to create wounds of varied shapes, preserving ECM coatings but suffering from inconsistent manual pressure application (6).

Wound can be also induced thermally. Heated aluminum stamps pressed into the monolayer culture mimics a burn wound. However, the method lacks consistency due to uneven heat transfer. Hence, this method requires a control group, which is a stamp at room temperature, to determine mechanical damage (120).

A more standardized method is electrical wounding, implemented through Electric Cell-substrate Impedance Sensing (ECIS®). Wounding is achieved by a high-voltage pulse, creating a defined wound. The samples are probed with small alternating current over a range of frequencies, enabling real-time monitoring of wound closure on the electrode. Capacitance increases due to the cells' insulating properties as they migrate and proliferate to form a monolayer. ECIS® also informs about barrier function through impedance values (126). Overall, this method offers high reproducibility for medium- to high-throughput screening, but requires specialized equipment and localized heat generation can damage surrounding cells, affecting their viability (3, 118).

Cell ablation using a laser beam can also be used to introduce a defined wound in a confluent cell monolayer. The instrument used for the purpose of wounding is the Laser Enabled Analysis and Processing instrument (LEAP™) (127). Like ECIS®, this method requires additional instrumentation and may cause collateral thermal damage to adjacent cells (4, 6).

Despite their limitations, 2D wound models remain widely used, particularly in early-stage drug and biomaterial development. They are inexpensive, easy to standardize, and amenable to automation. For example, they are a valuable tool for assessing keratinocyte and fibroblast responses to therapeutic agents or hydrogel coatings intended for cutaneous application (118, 120).

However, 2D assays oversimplify *in vivo* conditions, as they lack the complex multicellular architecture and ECM that is essential for replicating physiological wound healing (4). *In vivo*, dermal fibroblasts are embedded in the ECM with few direct cell-cell contacts, and keratinocytes in 2D culture often lack differentiation and barrier formation (3). Furthermore, when testing the effects of chemical compounds, cells cultured under 2D conditions commonly show downregulation of drug-metabolizing genes, limiting the predictive power of organ-specific toxicity (128). For these reasons, 3D cell culture systems are increasingly favored for providing a more physiologically relevant environment that better mimics native tissue architecture and cell-cell interactions (129).

4.2 3D Wound Healing Assays

3D wound healing assays are experimental systems that simulate the 3D architecture of human skin, typically utilizing HSEs. These models offer more accurate insights into skin repair processes compared to traditional 2D cultures, capturing complex interactions among cell types and extracellular matrix components (6). These models serve as a vital bridge between *in vitro* experiments and clinical applications, offering insights into therapeutic efficacy and pathological healing that 2D systems cannot provide. They provide a platform to enable researchers to investigate critical wound healing mechanisms, most importantly re-epithelialization and inflammation with greater fidelity (3, 4).

Wounds in HSEs are typically induced mechanically using scalpels for incisions or punch biopsies for excisional wounds (6). Burn and cold injuries can be simulated with heated or cooled metal rods (130). These injuries range from superficial epidermal damage to full-thickness wounds. A recurring issue with both mechanical and thermal methods is poor reproducibility or unintentional detachment of the epidermal layers. To address this, automated wounding techniques, such as rotating drills or dermatological lasers, have been explored to enhance consistency and reproducibility (6).

In models with full-thickness mechanical injuries, some studies describe transferring the wounded HSE onto a new dermal compartment. This supports keratinocyte migration and proliferation, facilitating re-epithelialization (131, 132). Alternatively, Geer et al. demonstrated that filling the wound gap with fibrin as a scaffold promotes keratinocyte growth and migration, accelerating the healing process and increasing response uniformity. This approach closely mimics *in vivo* conditions by reproducing the kinetics of re-epithelialization, the spatial distribution of proliferating cells, cellular phenotypes in the newly formed epidermis, and upregulation of key migration-related markers, including basement membrane formation during closure (133).

The healing process in 3D constructs is typically assessed via histological analysis, which often provides qualitative data. However, additional insights can be gained through immunological techniques and molecular assays such as reverse transcription polymerase chain reaction (RT-PCR), which help to characterize gene expression and signaling pathways involved in healing (131). Techniques like flow cytometry and macrophage polarization assays are used to investigate cellular dynamics (120), while confocal and time-lapse microscopy allow real-time tracking of cell migration in 3D. Nevertheless,

standardized and automated methods for calculating migration rates are still lacking, limiting high-throughput applications (6, 134).

The wound healing process in 3D models is typically assessed using histological techniques such as H&E staining, which reveals tissue architecture. Hematoxylin stains nuclei, while eosin highlights ECM and cytoplasmic proteins. This approach enables evaluation of re-epithelialization, epidermal thickness, and tissue organization (135). An example is provided in **Figure 16**, which illustrates the re-epithelialization of a commercially available HSE, Apligraf® (Organogenesis, Canon, MA), following mechanical injury and transfer onto a new dermal compartment (a), after 12 h (b), 48 h (c) and 96 h (d).

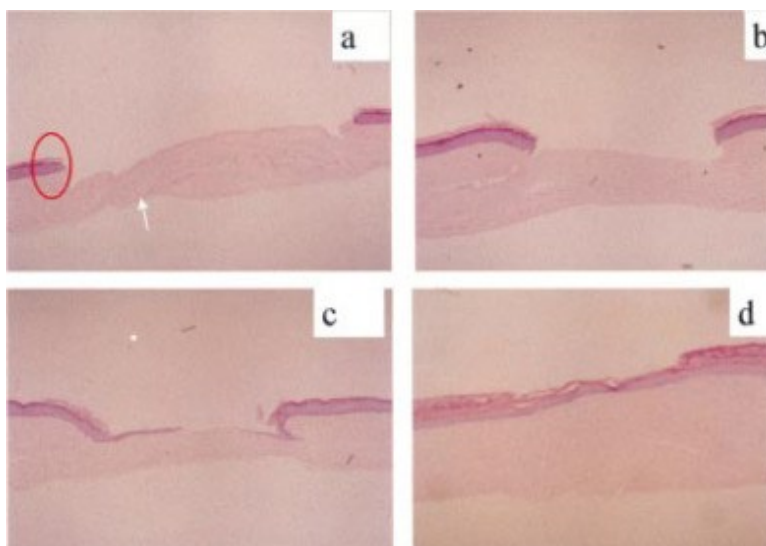


Figure 16: Photomicrographs showing the process of re-epithelialization of Apligraf® after mechanical injury and transfer to a DE.

(source: (131))

Wound closure rates are commonly measured from bright-field micrographs by manually tracking wound area or the distance between wound edges over time. Stamm et al. highlight this as a bottleneck due to the time-consuming and subjective nature of manual analysis. Additionally, a lack of standardized interpretation methods and insufficient methodological reporting in literature further complicate reproducibility (6). Some examples of softwares employed to measure the wound closure include AxioVision (136) or ImageJ (133).

Immunohistochemistry (IHC) is used for protein localization within tissue sections by utilizing their marking with monoclonal antibodies. It enables visualization of key healing markers, such as collagens type I and III, IL-10, TGF- β , VEGF, keratins (K10, K14), and

laminin (137). Enzyme-linked immunosorbent assay (ELISA) is another widely used technique to quantify inflammatory mediators, cytokines, and growth factors in tissue homogenates or cell culture supernatants (120). These include TNF- α , IL-6, EGF, and FGF – key mediators of wound healing (137).

HSEs are also applied in the investigation of scar pathogenesis. Scar-like conditions can be modeled by incorporating keloid fibroblasts with keratinocytes from normal skin. Similarly, hypertrophic scar-derived keratinocytes seeded on fibroblast-populated dermal matrices have recapitulated abnormal features, such as increased dermal and epidermal thickness and altered collagen I deposition, thus highlighting keratinocyte involvement in scar formation. However, their use in therapy development remains limited due to a lack of validated biomarkers and reliance on excised scar tissue (121). To develop effective treatments, deeper insight into the molecular pathways driving hypertrophic and keloid scar formation is essential. Validated, human *in vitro* preclinical models are therefore critical for both mechanistic studies and therapeutic screening (118).

4.3 Limitations and Future Perspectives

Despite their utility, *in vitro* 3D wound healing assays face several challenges, as discussed in Chapter 3. For recapitulation, these include the absence of vasculature, immune cells, and skin appendages, all of which limit the models' ability to replicate the full complexity of *in vivo* healing (3). Another major limitation is the low reproducibility of wound induction methods. Manual tools such as scalpels introduce operator-dependent variability, while automated tools like lasers or drills, although more consistent, require costly equipment (6). Moreover, certain concerns are raised due to the use of serum in culture media as it introduces undefined variables, such as growth factors and cytokines, due to its origins. It is theorized that the unstandardized nature of the components may affect experiments, prompting interest in defined, serum-free systems (6, 121, 138).

Advancements in 3D wound healing assays aim to overcome these limitations through integration with novel technologies. Among the most promising are SOC platforms and 3D bioprinting. A modified SOC model, a so-called wound-on-a-chip, developed by Bilgari et al., has been used to model the inflammatory phase of healing. This system integrates fibroblasts, endothelial cells, and polarized M1/M2 macrophages in collagen or Matrigel matrices using microfluidic platforms. It has shown potential in modeling cytokine

production, vascular formation, and the paracrine signaling crucial to early-stage inflammation, making it suitable for anti-inflammatory compound screening (139).

3D bioprinted, vascularized skin models are also gaining traction for simulating angiogenesis more accurately. This rapidly advancing technology allows for layer-by-layer construction of complex tissue structures using bioinks, resulting in customizable, reproducible constructs (118). Endothelial networks can be printed and seeded with endothelial cells, including those derived from induced pluripotent stem cells (iPSCs), to form perfused skin equivalents (140).

Marquardt et al. developed a standardized human skin wound model by introducing three types of injuries – mechanical, thermal, and irradiation – using tissue from healthy donors. Upon treatment with dexpanthenol, a commonly used wound healing agent, significant and reproducible changes in the proliferation marker Ki-67 were observed (141). This novel, standardized model enabled characterization of genes involved in ECM remodeling, including matrix metalloproteinases and keratin-associated proteins. While this study did not use an SOC platform, Cho et al. suggested that such a model could be integrated into a 3D wound-on-a-chip system, enabling deeper investigations into wound healing mechanisms and drug efficacy (109).

Although a universally standardized 3D wound healing model has yet to be established, defining and optimizing a reproducible model within individual laboratories remains highly beneficial. While high-throughput screening of artificial skin constructs is currently hindered by labor-intensive and costly analytical methods, reproducibility can be enhanced by automating key assay stages such as wounding. Ultimately, 3D wound healing assays offer a cost-effective and ethically favorable alternative to animal models. They more accurately represent human skin anatomy and metabolism, and are therefore expected to significantly advance our understanding of wound healing while serving as a valuable platform for evaluating bioactive compounds (6).

II. PRACTICAL PART

5 MATERIALS AND EQUIPMENT

5.1 Biological Material and Culture Medium Composition

The Human Neonatal Dermal Fibroblast (HDF) (Cat. N.: 106-05n, Cell Sigma-Aldrich) cell line originated from the human skin responsible for producing the components of the extracellular matrix in the dermis. Cells were grown routinely in T75 flasks (TPP, Trasadingen, Switzerland). The Fibroblast culture medium consisted of Dulbecco's Modified Eagle's Media (DMEM) (BioSera, France) and Ham's F12 (Life Technologies Limited, UK) in a 1:1 ratio supplemented with 10% Fetal Bovine Serum (FBS) (BioSera, France), and 1% Penicillin/Streptomycin (GE Healthcare HyClone, UK) as an antibiotic. The cells were maintained at 37°C in humidified air and 5% CO₂. The cell culture medium was changed every 2–3 days. For routine passaging, the cells were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS), trypsinized with trypsin/ethylenediaminetetraacetic acid (EDTA) (Biosera, France), centrifuged at 37 °C, 1,200 RPM for 3 minutes, resuspended in a new cell culture medium and seeded at a 1:3 ratio. Cells were not allowed to reach >90% confluence. The HDF cells were used in passages 11–16.

The spontaneously immortalized HaCaT cell line (Cat. N.: 300493, CLS cell lines service, Germany) is derived from adult human epidermal cells. The cells retain the capacity to proliferate and undergo differentiation, much like basal keratinocytes of the human epidermis. Cells were grown routinely in T75 flasks (TPP, Trasadingen, Switzerland). The keratinocyte culture medium consisted of DMEM (BioSera, France) supplemented with 10% Fetal Bovine Serum (BioSera, France), 1.0 mM Sodium Pyruvate (Life Technologies Limited, UK) and 1% Penicillin/Streptomycin (GE Healthcare HyClone, United Kingdom) as an antibiotic. The cells were maintained at 37°C in humidified air and 5% CO₂. The cell culture medium was changed every 2–3 days. The passaging procedure was the same as for the HDF cell line. The HaCaT cells were used in passages 11–23.

5.2 Laboratory Equipment

Airstream® Gen 3 Class II Biological Safety Cabinet AC2-4E8 (Esco Lifesciences, Singapore)

Centrifuge 5702 R cooled model for temperature-dependent samples (Eppendorf, Czech Republic)

CLASSIC vortex mixer (VELP Scientifica Srl, Italy)

Culture-inserts 2 well for self-insertion (Cat. N.: 80209; ibidi GmbH, Germany)

Extractor hood MERCI G NextGen (MERCI s. r. o., Czech Republic)

Histology bath (Falc Instruments, Italy)

Incubator with accessories Heracell™ 150i (Thermo Scientific, USA)

Inverted microscope IX51 (Olympus Corporation, Japan)

Laminar flow hood Bio130 A2 with accessories (Alpina, Poland)

Magnetic stirrer Arex digital (VELP Scientifica Srl, Italy)

Manual Microtome Ls-2065A (Shenyang Longshou Electronic Instrument Co., Ltd., China)

Millicell® 12 well hanging cell culture inserts (Cat. N.: PTSP12H48; Merck KGaA, Germany)

Millicell® 6 well hanging cell culture inserts (Cat. N.: PTHT06H48, Merck KGaA, Germany)

Photometer Infinite 200 Pro M Nano (Tecan group Ltd., Switzerland)

Ultrasound Elmasonic S 70 H with heating (Elma Schmidbauer GmbH, Germany)

5.3 Chemicals

Adenine (Cat. N.: A2786-5G; Sigma-Aldrich, USA)

Calcium chloride (Cat. N.: C7902-500G; Sigma-Aldrich, USA)

Collagen type I, High Concentration, Rat Tail (Cat. N.: 354249; Corning, USA)

Dimethylsulfoxide (DMSO) (Cat. N.: D1370.1000; Duchefa Biochemie, Netherlands)

Dulbecco's Phosphate Buffered Saline (DPBS) (Cat. N.: LM-S2041/500; BioSera, France)

Dulbecco's Modified Eagle's Media (DMEM) powder (Cat. N.: PM-D1115/50L; BioSera, France)

Eosin Y solution (Cat. N.: 318906-500ML; Sigma-Aldrich, USA)

Fetal Bovine Serum (FBS) (Cat. N.: FB-1280; Biosera, France)

Ham's F12 – F12 Nut Mix (Cat. N.: 2170-018; Life Technologies Limited, UK)

HEPES solution (Cat. N.: H0887-100ML; Sigma-Aldrich, USA)

Hydrocortisone (Cat. N.: H0888-10G; Sigma-Aldrich, USA)

ibidi Mounting Medium with DAPI (Cat. N.: 50011; ibidi GmbH, Germany)

Insulin-Transferin-Selenium (Cat. N.: 414000-045; Life Technologies Corporation, USA)

L-Glutamine 100X (Cat. N.: XC-T1715/100; Biosera, France)

Mayer's hematoxylin solution (Cat. N.: MHS1-100ML; Sigma-Aldrich, USA)

NaHCO₃ (Cat. N.: 28000-31000; Penta Chemicals, Czech Republic)

NaOH (Cat. N.: 28000-31000; Penta Chemicals, Czech Republic)

O-phosphorylethanolamine (Cat. N.: P0503-5G; Sigma-Aldrich, USA)

Paraffin (Cat. N.: P3558-1KG; Sigma-Aldrich, USA)

Paraformaldehyde (CAS N.: 30525-89-4; Sigma-Aldrich, USA)

Penicillin-Streptomycin Solution 100X (Cat. N.: XC-A4122/100; Biosera, France)

Progesterone (Cat. N.: P8783-5G; Sigma-Aldrich, USA)

Sodium dodecyl sulfate (SDS) (Cat. N.: 71725-100G; Sigma-Aldrich, USA)

Sodium Pyruvate 100mM (Cat. N.: 11360070; Life Technologies Limited, UK)

Thiazolyl blue tetrazolium bromide (MTT) (Cat. N.: M1415.025; Duchefa Biochemie, Netherlands)

Triiodothyronine (Cat. N.: T2877-100MG; Sigma-Aldrich, USA)

Trypsin/EDTA 10X (Cat. N.: XC-T1717/100; Biosera, France)

Xylene (isomeric mixture) (Cat. N.: 1082984007; Merck, Germany)

6 METHODS

This chapter describes experimental methods to construct a HSEs and investigate its potential in wound healing. The methods are divided into five parts, and each experiment is detailed in the following subchapters. All statistical operations were carried out in Excel version 2502.

6.1 Keratinocyte Migration Assay

To evaluate the keratinocyte migration, a simple scratch assay was carried out with HaCaT cell line, passage 23, in a 24-well cell culture plate using a two-well cell culture insert (ibidi GmbH, Germany). The inserts were placed approximately into the middle of the wells with sterile pliers to prepare the plates. Next, the keratinocytes were passaged at ~90% confluency. The resulting cell suspension was then diluted with keratinocyte culture medium to reach a final concentration of approximately 100,000 cells per well, and 500 µl of this solution was pipetted into each well of the cell culture insert. The plates were then incubated until a confluent monolayer had formed. After the cells reached about 90%–100% confluency, the inserts were removed using sterile pliers, creating a wound approximately 500 µm wide. The remaining medium was aspirated, the wells were gently rinsed twice with DPBS to remove cell debris, and 1 ml of keratinocyte culture medium was added to each well. For positive control (PC), a keratinocyte culture medium without serum was used. The wound was then monitored in phase contrast using a microscope equipped with a camera. The wound was photographed immediately after creation, then at hours 2, 4, 6, 8, and 24. The migration assay was done in a triplicate.

The wound area and width were measured in the software Olympus cellSens Standard version 4.1. Two evaluation methods were employed to quantify wound closure. The first method is described by **Equation 1**. It compares the initial (A_i) and final (A_f) wound area, expressing the migration rate as a percentage reduction in the cell-free space. The second method is described by **Equation 2** and compares the initial (W_i) and final (W_f) wound width over time (t) to evaluate the cell migration.

$$\text{Wound closure [\%]} = \frac{A_i - A_f}{A_i} \cdot 100 \% \quad (1)$$

$$\text{Migration rate } [\mu\text{m} \cdot \text{h}^{-1}] = \frac{W_i - W_f}{t} \quad (2)$$

6.2 Fabrication of a 3D Human Skin Equivalent

This subchapter is divided into several sections due to the nature of the experiment. Briefly, the HSE was fabricated by first constructing a DE out of Collagen type I and HDF cells. This structure comprised an acellular layer of only collagen hydrogel and a cellular layer of collagen hydrogel with fibroblast cells. After 7 days of submerged cultivation, the HaCaT keratinocytes were introduced, and the whole structure was in a submerged co-cultivation. After another 7 days, the structure was co-cultivated at the ALI for at least 14 days, but no more than 21 before further manipulation.

6.2.1 Collagen Hydrogel Preparation

A neutralization buffer was prepared to neutralize an acidic solution of collagen type I. The reagents used in this mixture are shown in **Table 1**, along with their respective volumes. The neutralization buffer is constructed so that the neutralized collagen solution has a pH of about 7.4. After preparation, the neutralization buffer was sterile-filtered and stored in a fridge until use. To construct the collagen matrix, the acidic solution of collagen was diluted by the neutralization buffer so that the collagen concentration was $1 \text{ mg} \cdot \text{ml}^{-1}$.

Table 1: Neutralization buffer reagents and their respective volumes.

Components	Volume [μl]
10x PBS	10,000
HEPES	2,000
NaOH	6,000
NaHCO_3	575
Ultra-pure water	56,400

(source: own elaboration)

To prepare 1 ml of neutralized collagen at a concentration of $1 \text{ mg} \cdot \text{ml}^{-1}$, 250 μl of the acidic collagen solution was added to 750 μl of the neutralization buffer in a small plastic tube. The content of the tube was homogenized by slow, repeated pipetting to avoid bubble formation. All the steps were performed in an ice tub placed in a laminar flow hood to prevent the collagen from cross-linking at room temperature.

Next, collagen hydrogel was prepared by pipetting 250 μl of the neutralized collagen mixture per 12-well plate cell culture insert. The plate with the cell culture inserts can be

gently moved or circled to allow the collagen mixture to cover the inserts fully. It was then placed in an incubator for 45 minutes.

6.2.2 Assembly of a Dermal Equivalent with Fibroblast Cells

To construct a DE, HDF cell line were passaged one day prior at a 9:10 split ratio. The next day, during the incubation to generate the collagen hydrogel, the cells were passaged, resuspended in 1 ml of Fibroblast culture medium, and counted using a Bürker counting chamber. The cells were then resuspended to a final concentration of $3 \cdot 10^5$ cells per ml. A mixture of collagen and HDF cells was prepared to construct a dermal equivalent according to **Table 2** below. The reagents were added gradually from top to bottom except for the HDF cells, which were added last after mixing the components thoroughly by repeated pipetting to ensure the used collagen was neutralized. As with the preparation of the clear collagen hydrogel, this mixture was made ready in an ice tub and placed in a laminar flow hood.

Table 2: Mixture of collagen and HDF cells to fabricate a DE.

Components	Volume [μ l]
10x DMEM	170
L-Glutamine	15
FBS	192
7.5% NaHCO ₃	53
Collagen	1,423
HDF cell suspension	157

(source: own elaboration)

After adding the cells, the mixture was gently mixed by repeated pipetting. Then, 400 μ l of this mixture was added into each cell culture insert on top of the collagen hydrogel. The 12-well plate with the inserts was transferred into an incubator for about 45 minutes. To support the fibroblasts, 2 ml of fibroblast culture medium in total was added: 1.6 ml into the bottom well of the 12-well plate and 0.4 ml into the cell culture insert. For the next 7 days, the DE was incubated with the culture medium changed every 2–3 days.

6.2.3 Addition of Keratinocytes

To generate HSEs, keratinocytes were added to the DE constructed of collagen and fibroblast cells. The HaCaT cells were passaged, and the cells were counted using a Bürker counting

chamber. The cells were resuspended in Epidermalization I culture medium, so the number of cells was $5 \cdot 10^6$ per 50 μl of the cell suspension.

During the passaging process of HaCaT cells, the 12-well plates with the inserts containing the DEs were aspirated of all fibroblast culture medium. They were then left in the laminar flow hood for 20 minutes, allowing the remaining medium to evaporate slightly. To ensure the HaCaT cell's adhesion to the DEs, glass cylinders were placed on top of them. After that, 50 μl of the HaCaT cell suspension was pipetted to the cylinders on top of the moist DE. To allow the keratinocytes to attach, the plate was left for 15 minutes in the laminar flow hood and subsequently moved to an incubator for 45 minutes to ensure the cells fully adhered to the collagen gel with fibroblasts. Afterward, the cylinders were removed, 1.6 ml of Epidermalization I (EpI) culture medium was added into the bottom well of the 12-well plate, and 0.4 ml of the medium was gently added on top of the inserts. The plates were then incubated. The medium was changed every 2–3 days until the next step. The EpI medium was switched with Epidermalization II (EpII) medium after 2 days.

The Epidermalization mediums were prepared and sterile-filtered at least one day prior and stored in the fridge at 4 °C before each use. The composition of both mediums is summarized in **Table 3** below.

Table 3: The Epidermalization mediums components along with their respective volumes.

Components	EpI [ml]	EpII [ml]
DMEM medium	363	363
Ham's F12	120	120
L-glutamine	10	10
Adenine	11	1
Hydrocortisone	1	1
Triiodothyronine	1	1
Transferrin	1	1
Insulin	1	1
Progesterone	0.5	0.5
O-phosphorylethanolamine	1	1
Calcium chloride	-	1.8
FBS	0.5	0.5

(source: own elaboration)

6.2.4 Co-culture at an Air-Liquid Interface

The HSEs were co-cultured at an ALI 7 days after adding the keratinocytes to facilitate the development of a proper epidermal layer. To do so, the remaining medium was aspirated from both the wells and the inserts, and the plates with the HSEs were in the laminar flow hood for about 20 minutes to allow them to dry. Subsequently, 580 µl of Cornification medium was added to each bottom well. Such a volume was enough to keep the medium slightly above the level of the membrane of the insert. The tissue models were incubated for at least 14 days to ensure a thick epidermal layer before being used further for subsequent experiments. The medium was changed every 2–3 days.

The Cornification medium was prepared and sterile-filtered at least one day prior and stored in the fridge at 4 °C before each use. The composition of the medium is summarized in **Table 4** below.

Table 4: The Cornification medium components and their respective volumes.

Components	Volume [ml]
DMEM medium	237
Ham's F12	237
L-glutamine	10
Adenine	1
Hydrocortisone	1
Triiodothyronine	1
Transferrin	1
Insulin	1
Progesterone	-
O-phosphorylethanolamine	1
Calcium chloride	1.8
FBS	10

(source: own elaboration)

6.3 Viability Assessment

The viability of the tissue models was assessed by a 3D skin model irritation test on HSEs 13 days after the start of ALI co-cultivation. The viability of the tissue models was evaluated in a fibroblast culture medium. Sterile DPBS was used for negative control, and a 5% SDS

solution was used for positive control. MTT solution at a concentration of $5 \text{ mg} \cdot \text{ml}^{-1}$ was prepared from powdered MTT one day prior and stored in a plastic tube wrapped in aluminium foil to protect the solution from light and stored in a fridge at 4°C .

The MTT assay consisted of several steps that took place over two days. Briefly, the tissue models were pre-incubated before applying the test substances. After the application, they were incubated for half an hour, washed with sterile DPBS, and placed in an incubator overnight for post-incubation. The MTT solution was applied the following day, the formazan crystals were dissolved in DMSO, and the absorbance was read. This viability assay was performed in a triplicate. Photographs taken during these steps are compiled in **Figures 17 and 18**.

The pre-incubation was carried out by pipetting 0.9 ml of the pre-warmed fibroblast culture medium into the wells of a 6-well plate. The inserts with the HSEs were carefully removed from the 12-well plate and placed into the wells of a 6-well plate. Any bubbles that were trapped under the inserts were removed. The 6-well plates containing the tissue models were put into an incubator for 30 ± 1 minutes. Afterward, $40 \mu\text{l}$ of the pre-warmed test substances were taken with a micropipette and slowly dispensed near the center of the tissue models. The inserts were tilted and rotated to ensure the substance would be applied uniformly to the whole surface. Subsequently, the plates with the treated tissue models were placed in an incubator for 30 ± 1 minutes.

For washing, the tissue models were removed from the incubator, and the insert was grabbed with sterile forceps and filled with DPBS by gently spraying it from a wash bottle to the side walls of the insert. To dispose of the DPBS, the insert was flipped over a beaker. Each tissue was washed a total amount of ten times. While washing, care was taken to prevent the insert from hitting the beaker to avoid damaging the HSEs. The washed tissue models were placed in a 6-well plate pre-filled with 0.9 ml of pre-warmed culture medium. The plates were then incubated for 24 ± 2 hours as post-incubation.

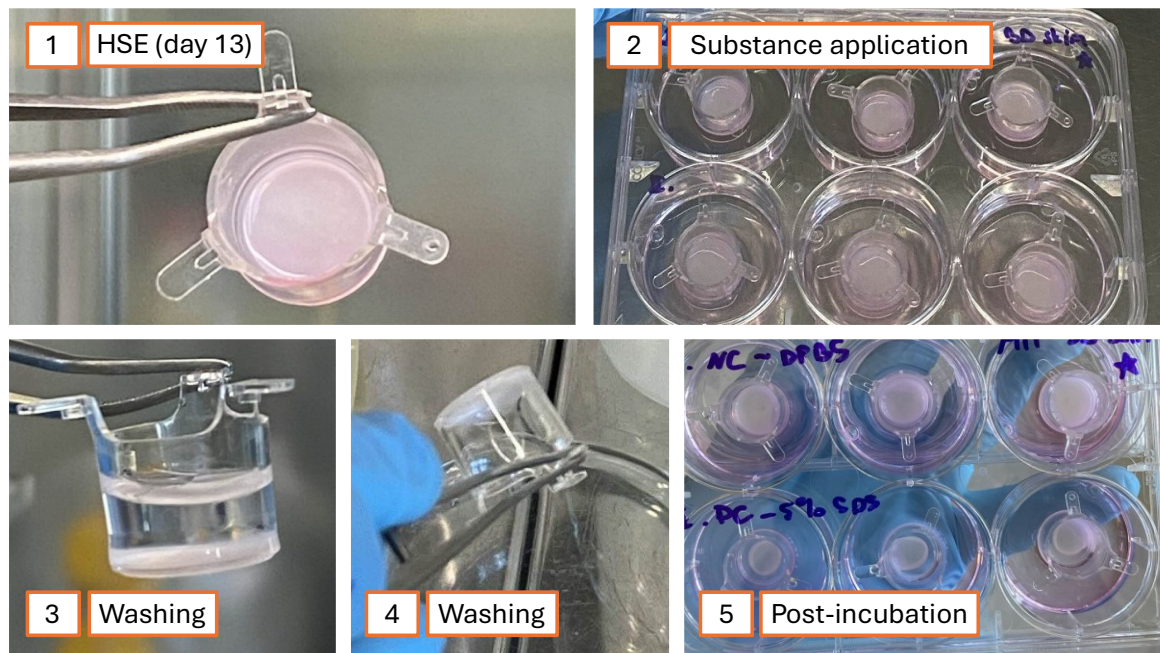


Figure 17: Day one of the MTT assay.

(source: own elaboration)

The next day, after the post-incubation period, the inserts were transferred from the 6-well plates into a 24-well plate pre-filled with 200 μl of MTT solution per well, and 100 μl of MTT solution was added inside per insert. In this step, the MTT solution was diluted with fibroblast culture medium to a $0.4 \text{ mg}\cdot\text{ml}^{-1}$ concentration. The plate was covered with aluminium foil to protect it from light and placed in the incubator for 3 hours $\pm$ 5 minutes. Afterward, using a pipette, MTT residuals were removed from the inside and outside of the inserts. The inserts were transferred to a new 6-well plate with 1.9 ml per well of DMSO, and then 100 μl of DMSO was pipetted inside the insert. The plates were covered with aluminium foil and placed on a shaker for 3 hours $\pm$ 5 minutes to extract formazan.

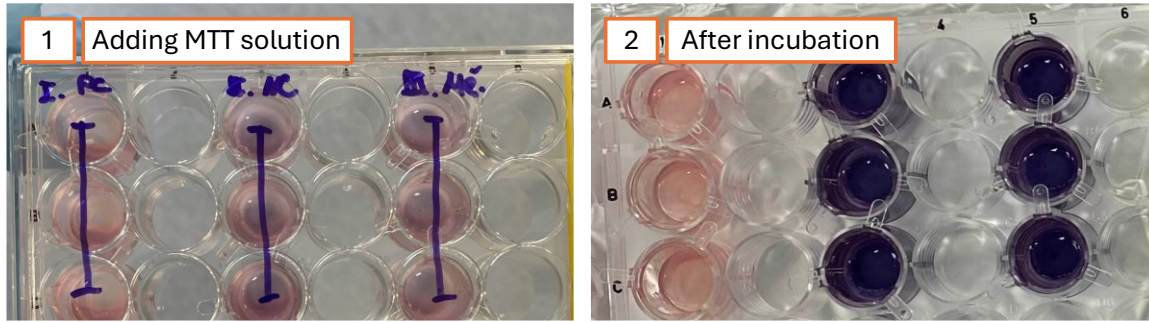


Figure 18: Day two of the viability assay.

(source: own elaboration)

To measure the absorbance, for each tissue model, the extract inside the insert was combined with the external extract from the well, and the mixture was pipetted well to ensure a uniform color. The combined mixture from each HSE was transferred into a 96-well plate, each well containing 200 μ l, and absorbance was measured using a spectrophotometer at a wavelength of 570 nm. DMSO was used as a blank. The relative viability of the HSEs was calculated from the ratio of the DMEM/F12 sample absorbance (A_{sample}) to the negative control absorbance (A_{DPBS}) multiplied by 100, as described by **Equation 3**.

$$Relative\ viability\ [\%] = \frac{A_{sample}}{A_{DPBS}} \cdot 100\ \% \quad (3)$$

6.4 Fabrication of a 3D Wound Healing Model of Human Skin

The HSEs to be wounded were generated as described above in Subchapter 6.2. Before wounding, another batch of DEs was prepared at least 7 days prior according to the procedure in sections 6.2.1 and 6.2.2. The wounded HSEs will be placed directly on top of the DEs, providing the keratinocytes with a surface to migrate to. Before harvesting the tissue models for wounding, a stainless steel dissecting needle, scalpel, spatula, and tweezers with sharp tips were disinfected by washing them with 70% ethanol solution and putting them under UV light for 30 minutes.

The following steps are pictured in **Figure 19** below this paragraph. First, the medium was aspirated from the HSE to be harvested. Then, the tissue model was removed from the insert and an incisional wound was created in the middle of the tissue using a scalpel. Subsequently, the wounded tissue model was moistened with medium and transferred onto a DE and adjusted to smooth out any wrinkles.

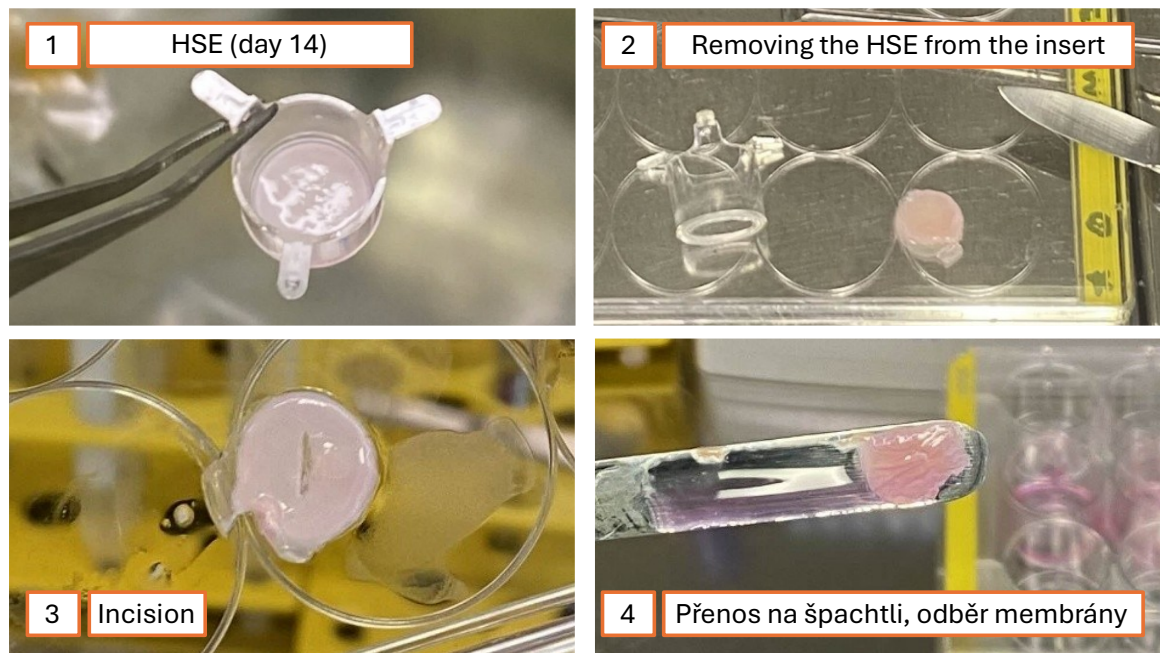


Figure 19: Preparation to establish the wound healing model (WHM) on day 14 of ALI co-culture.

(source: own elaboration)

Next, another drop of warm medium was added, and the wounded HSE was transferred onto the DE and smoothed out. Its position was adjusted if necessary, so the wound sat roughly in the middle. Finally, the wound's edges were pushed away to create an approximately 2–3 mm wide elliptical space. With this being done, the WHM was established. It was left to dry for about 20 minutes, and then 580 μ l of EpI medium was added to the bottom well. The plate with the WHMs was kept in the incubator, and the medium was changed every 2–3 days. The tissues were harvested for histological analysis on days 0, 2, 5, and 14.

6.5 Tissue Model Harvesting, Processing for Histology and Staining

The tissue models were gently washed with DPBS twice before being harvested. They were then removed from the insert as described above in subchapter 6.3. The transfer from the cut insert membrane was the same, too, except that the tissue models were moved to a tissue processing cassette, and DPBS was used to moisten the spatula and the models to facilitate the transfer. The cassettes were immediately immersed in 4% paraformaldehyde for 1 hour. They were then gradually moved to ethanol for dehydration, xylene for clearing, and lastly, paraffin for wax infiltration at 60 °C. The whole procedure involving each step is listed in **Table 5** below.

Table 5: The procedure of processing the tissue models for histology.

Step	Time [min]
4% paraformaldehyde	60
30% Ethanol	30
50% Ethanol	30
70% Ethanol	30 or overnight at 4°C
80% Ethanol	30
90% Ethanol	30
≥96 % Ethanol	30
Xylene I	30
Xylene II	30
Xylene III	30
Paraffin I	60
Paraffin II	60
Paraffin III	60

(source: own elaboration)

After wax infiltration, the tissue models were transferred into a metal mould and embedded in paraffin. They were oriented parallel to the knife. Subsequently, the processing cassette was placed on top of the mould and topped with more wax. The embedded tissue models were stored in a fridge at 4 °C. For sectioning, thickness of 5–7 µm was chosen.

The H&E staining procedure was performed according to **Table 6** below. The nuclei were stained with Meyer's hematoxylin and the fibers were stained with eosin. The slides were gently tapped on cellulose cotton wool after each step. Before microscopy, the stained tissue model sections were mounted with mounting media and covered with a glass coverslip.

Table 6: The procedure for H&E staining.

Step	Time [min]
Xylene	10
Xylene	10
96% Ethanol	10
96% Ethanol	10
Rinse in tap water	
Meyer's hematoxylin	5
Rinse in warm tap water	
Blueing in warm tap water	5
Eosin	3
Winse in cold tap water	
96% Ethanol	10
96% Ethanol	10
Xylene	10
Xylene	10

(source: own elaboration)

7 RESULTS

This section presents the results of the experiments described in Chapter 6. It is divided into four subchapters, each detailing the acquired outcomes of the experiments. Subchapters 7.2 and 7.4 also compile the results collected via the methods from Subchapter 6.5.

7.1 Keratinocyte Migration Assay

The photos captured with the microscope camera are shown in **Figure 20**. Each compares growth between the representative samples of each group – HaCaT cells cultured in DMEM and a serum-free DMEM (PC). After 24 hours, the DMEM-cultured replicates exhibited near complete closure of the wound area.

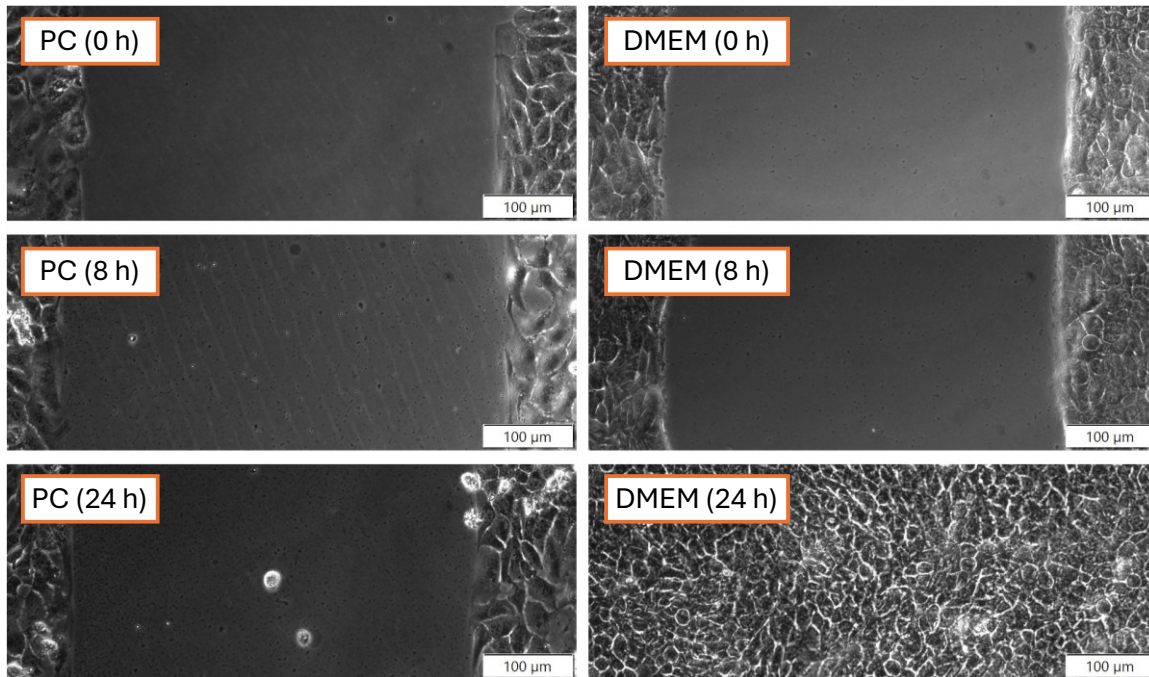


Figure 20: Comparison of HaCaT cell migration between the cells grown in keratinocyte culture medium (DMEM) (right side) and PC (left side). Magnified 100x.

(source: own elaboration)

The mean values of area of each replicate were calculated and used to determine the wound closure in % according to **Equation 1**. A representative calculation for one sample in DMEM at the 24th hour is shown below this paragraph, along with **Table 7**, which includes the mean area measurements and wound closure calculations.

$$\text{Wound closure}_{DMEM\ 24h} = \frac{355.75\ mm^2 - 1.86\ mm^2}{355.75\ mm^2} \cdot 100 = 99.48\ \%$$

Table 7: The mean area in mm² and calculated wound closure in % for each timestamp over the duration of the migration assay.

t [h]	PC		DMEM	
	Area [mm ²]	Wound closure [%]	Area [mm ²]	Wound closure [%]
0	362.44	0.00	355.75	0.00
2	357.29	1.42	353.20	0.72
4	359.68	0.76	349.79	1.67
6	363.27	-0.23	340.76	4.21
8	366.65	-1.16	330.49	7.10
24	363.04	-0.16	1.86	99.48

(source: own elaboration)

The mean values of each replicate's width were then calculated and used to determine the migration rate in $\mu\text{m}\cdot\text{h}^{-1}$ according to **Equation 2**. A representative calculation for one sample in DMEM at the 24th hour is shown below this paragraph, along with **Table 8**, which includes the mean width measurements and migration rate calculations.

$$\text{Migration rate} = \frac{483.82 \mu\text{m} - 30.13 \mu\text{m}}{24 \text{ h}} = 18,90 \mu\text{m} \cdot \text{h}^{-1}$$

Table 8: The mean width in μm and calculated migration rate in $\mu\text{m}\cdot\text{h}^{-1}$ for each timestamp over the duration of the migration assay.

t [h]	PC		DMEM	
	Width [μm]	Migration rate [$\mu\text{m}\cdot\text{h}^{-1}$]	Width [μm]	Migration rate [$\mu\text{m}\cdot\text{h}^{-1}$]
0	489.90	0.00	483.82	0.00
2	481.62	4.14	478.89	2.47
4	481.62	2.07	470.91	3.23
6	486.22	0.61	468.42	2.57
8	494.27	-0.55	444.55	4.91
24	487.37	0.11	30.13	18.90

(source: own elaboration)

7.2 Fabrication of a 3D Human Skin Equivalent

Photographs were taken during the subsequent steps during the fabrication of the 3D tissue models. **Figure 21** below shows the initial steps of establishing the DE. The mechanical integrity of both layers was verified by inverting the cell culture insert.

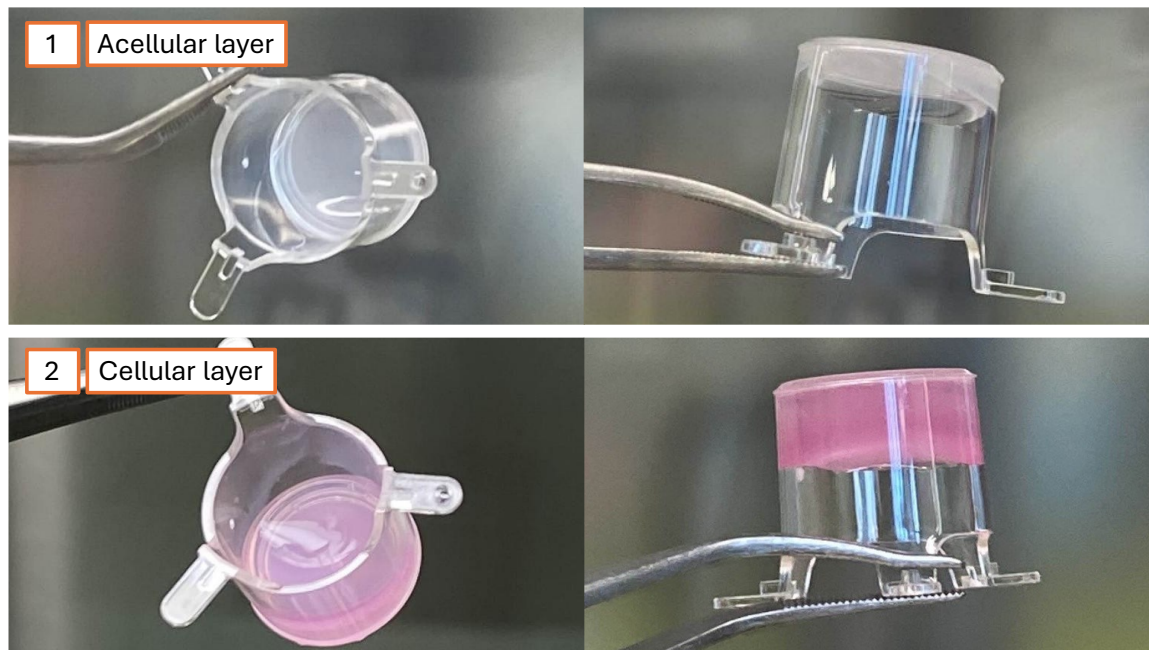


Figure 21: The preparation of a DE.

(source: own elaboration)

Over the next 7 days, the DEs exhibited noticeable contraction, and thus reduction in size. A week later, HaCaT cells were seeded onto the DEs, and following an additional 7 days of submerged culture, the tissue models were transferred to the ALI. Throughout the co-cultivation period, the tissue models developed an increasingly opalescent appearance, as depicted in **Figure 22**.

The HSEs were harvested for histological evaluation on the second week, following the procedure described in Subchapter 6.5. Prior to microscopy, the sectioned tissue models were mounted in a liquid mounting medium. A representative photomicrograph is presented in **Figure 23**. Although an artefact, visible as a circular inclusion in the upper left corner, partially obscures the tissue model, the majority of the structure remains clearly observable. Nonetheless, a well-developed epidermal layer (indicated by the white arrow) positioned above a fibrous dermal layer (black arrow) can be distinguished in the section.

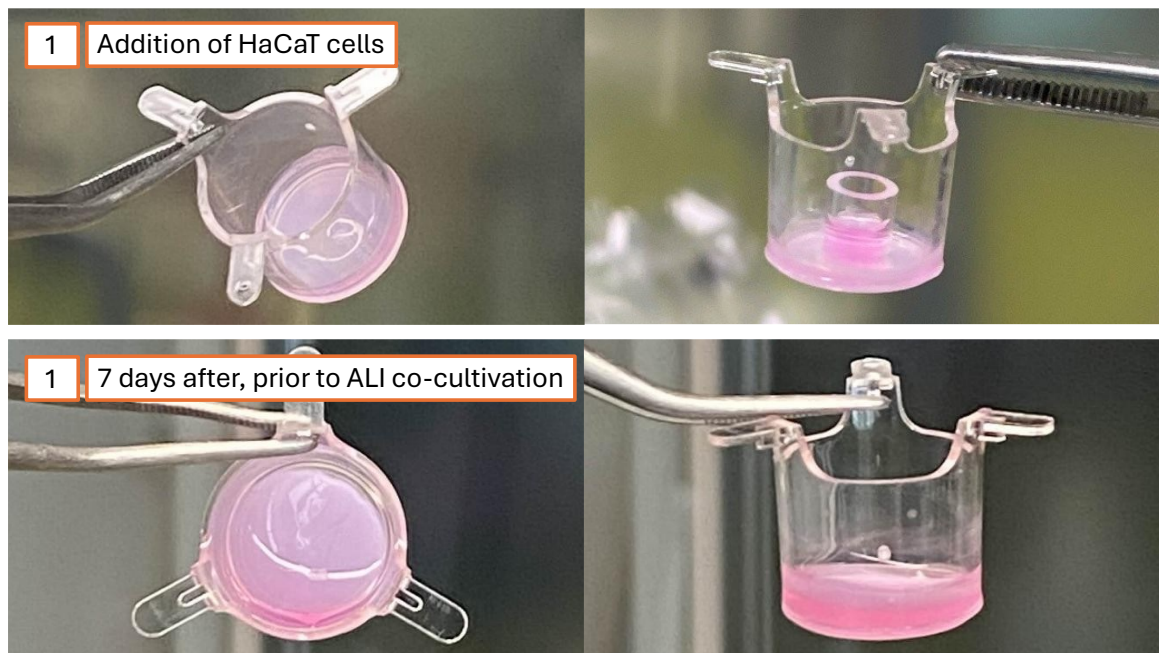


Figure 22: Addition of HaCaT cells to the DE (top) and beginning of ALI co-cultivation (bottom).

(source: own elaboration)



Figure 23: H&E-stained section of the HSE, 16 days of ALI co-cultivation. Magnified 100x.

(source: own elaboration)

7.3 Viability Assessment

The viability of the HSEs was evaluated by measuring their metabolic activity using a modified MTT assay. Testing was performed during the second week of ALI co-cultivation. The negative control (DPBS) was used as a reference value, while 5% SDS was a positive control. The experimental HSE samples were cultured in a fibroblast culture medium (DMEM/F12). The calculated results of the relative tissue model viability are summarized in **Figure 24**.

The HSEs demonstrated high relative viability, averaging $141.7\% \pm 32.3\%$ ($n = 3$) compared to the negative control. The positive control exhibited the expected cytotoxic effect, with relative viability averaging $11.9\% \pm 0.9\%$ ($n = 3$). Although the standard deviation (SD) is relatively high, possibly reflecting variability between the HSEs, the overall results suggest active cell metabolism and overall tissue vitality at day 13 of ALI co-cultivation.

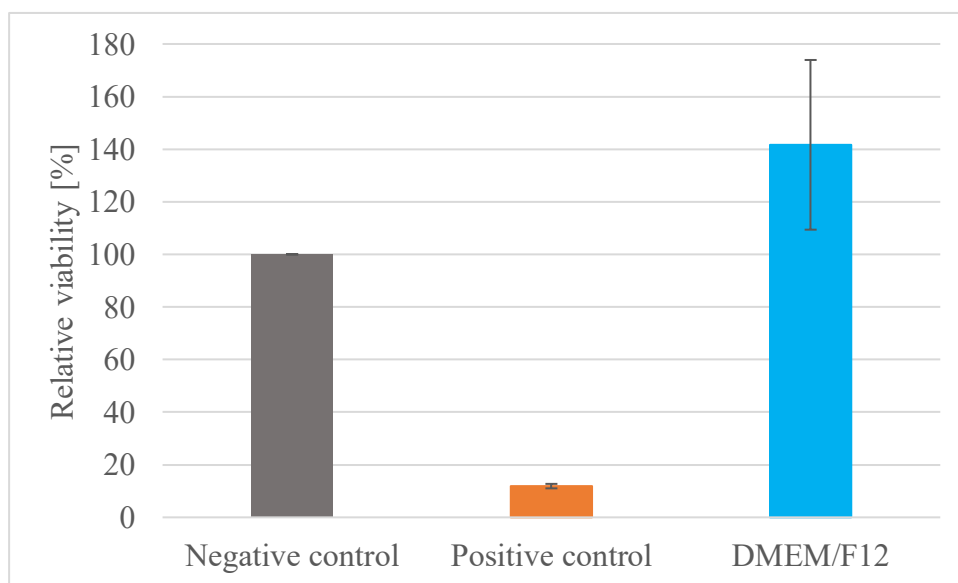


Figure 24: The viability of the HSEs (%).

(source: own elaboration)

7.4 Fabrication of a 3D Wound Healing Model of Human Skin

To assess the potential of 3D skin models for *in vitro* wound closure, WHMs were established after 14 days of ALI co-cultivation. **Figure 25** shows a representative WHM in co-culture. The photograph was taken immediately after preparation. Additionally, an

unwounded HSE was also prepared to compare the tissue morphology to the wounded HSEs. Fixed and H&E-stained sections are shown in **Figure 26**.

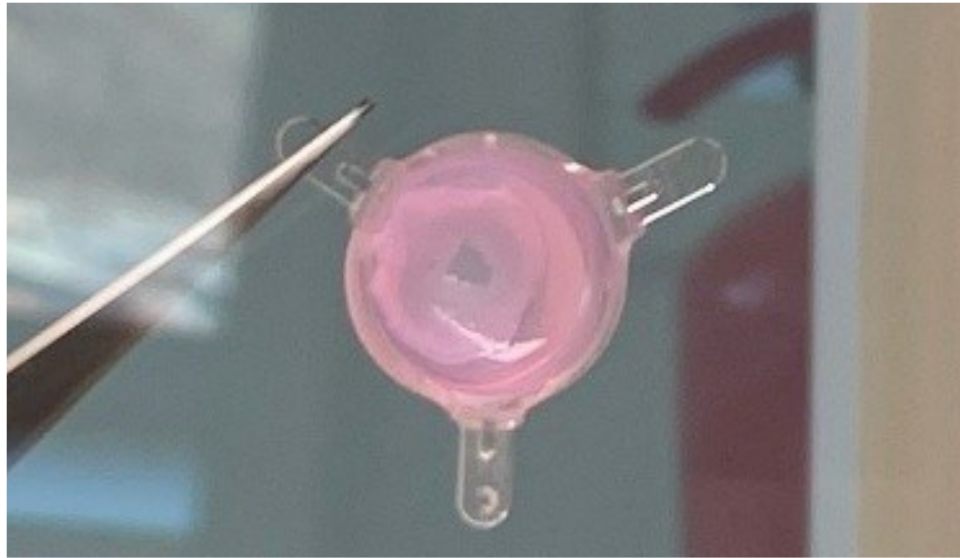


Figure 25: The established WHM on day 0.

(source: own elaboration)



Figure 26: HSE harvested on day 14 after ALI co-cultivation, H&E stain. Magnified 200x.

(source: own elaboration)

The wound closure was assessed by imaging the tissue models at days 0, 2, 5, 7 and 14. By day 2, the wound gap appeared significantly reduced, suggesting early signs of closure. However, the WHM deviated from the expected outcome, which should feature a wounded HSE sitting directly on top of a DE, as evidenced by **Figure 27**. This DE was, however, present even after dehydration step of the histology processing procedure, which is visible, though faintly, on photographs featured in **Figure 28**.

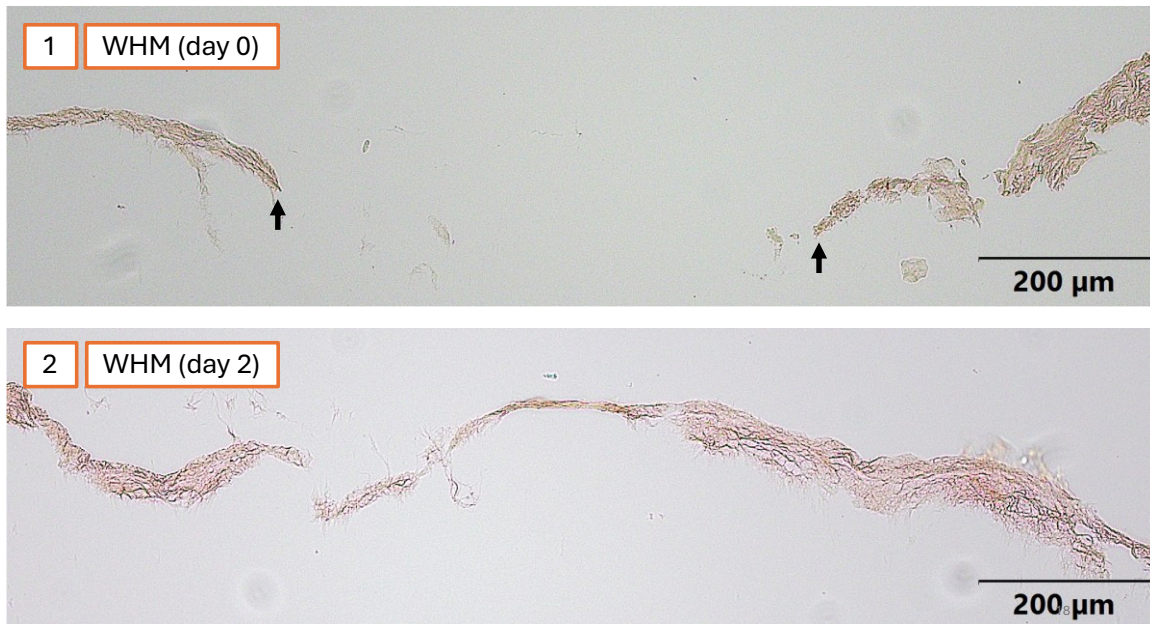


Figure 27: The WHM on day 0 (top) and day 2 (bottom), H&E stain. Black arrows mark the initial wound edges. Magnified 100x.

(source: own elaboration)

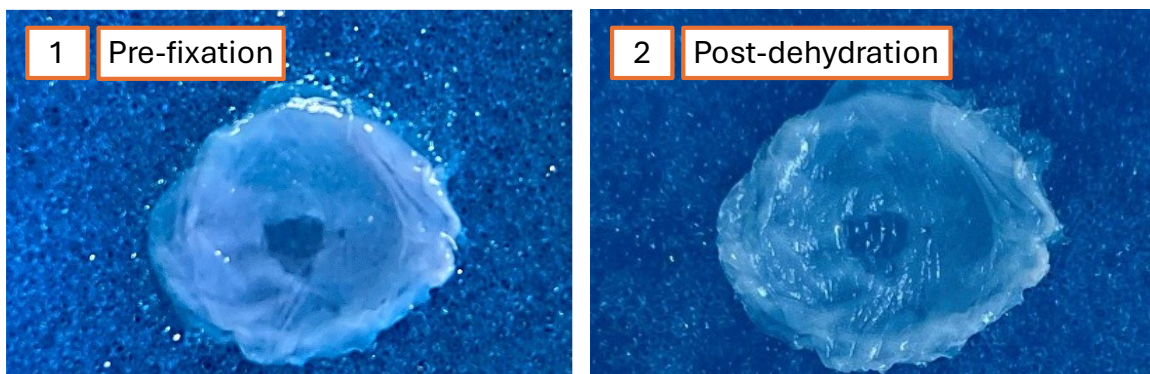


Figure 28: Morphology of a harvested WHM on day 14 before fixation (left), after dehydration (right).

(source: own elaboration)

8 DISCUSSION

The scratch assay performed using HaCaT cells served as a preliminary *in vitro* model to assess keratinocyte migration and re-epithelialization. As explored in Chapter 2, these processes are critical in the early stages of wound healing. The results obtained in a 2D environment are essential for confirming the behavior of keratinocytes before exploring their ability to migrate in a more complex 3D system.

The HaCaT cells cultured in a complete DMEM medium supplemented with 10% FBS exhibited a clear, time-dependent migration into the scratch area in a sheet-like manner. Photographs acquired at regular intervals (0, 2, 4, 6, 8, and 24 hours) demonstrated that the gap was 99.48 % closed within 24 hours, indicating high migratory capacity under serum-rich conditions. Conversely, the positive control group, cultured in serum-free DMEM medium, exhibited a visibly impaired closure. Notably, several rounded and detached cells were observed at the 24-hour mark. It is possible that serum withdrawal contributed to reduced cell-cell adhesion and stress-induced detachment. Such observation further highlights the importance of a bioactive environment provided by the serum-derived components in supporting proper cellular function (138).

It is also important to consider that various chemical and biological modulators can influence the outcome of a scratch assay, which opens an opportunity to study their effects on cell migration. For example, the exposure to growth factors, such as TGF- α , TGF- β , EGF, FGF and KGF can be employed to study how they promote keratinocyte motility, providing valuable insights into wound healing mechanisms (142, 143).

The scratch assay of keratinocytes may be elevated by employing a co-culture system with dermal fibroblasts to explore factors that influence cutaneous wound healing. One study employed such model with medium conditioned with MSCs to test the influence of factors secreted by the MSCs on both skin cell types in wound healing. According to the authors, the conditioned medium is enhanced with several wound healing mediators, such as TGF- β or chemokines IL-6 or IL-8 and collagen type I, among others (144).

The scratch assay represents a simple and rapid method for evaluating the cytotoxicity of various compounds. However, 2D cell cultures are unable to replicate the complex architecture and microenvironment of biologically active 3D tissues. For example, in classic scratch assays performed on 2D monolayers, keratinocyte cultures demonstrate very limited stratification, partial differentiation, and hyperproliferative growth (132).

Therefore, a 3D model of human skin, composed of collagen type I matrix with HDF seeded with HaCa keratinocytes was established. The tissue model, cultured for 14 days at the ALI, showed a developed epidermal and dermal layer. As such, the overall morphology of the developed HSE resembles previously reported tissue-engineered skin models. For instance, Schoop et al. successfully established a differentiated epidermis using HaCaT cells seeded on a fibroblast-populated collagen type I matrix. Their model exhibited visible stratification within 14–21 days of ALI co-culture along with the formation of epidermal layers. Furthermore, they demonstrated that HaCaT cell differentiation into a squamous stratified epithelium was depended on fibroblast density, with optimal epidermal development observed at a fibroblast concentration of $5 \cdot 10^5$ cells per ml (145).

To assess the viability of the 3D skin models after 13 days of ALI co-culture, a skin irritation test using MTT was performed in biological triplicates. The assay allowed for the quantification of metabolically active cells based on mitochondrial reduction of MTT to formazan. The negative control, consisting of 3D skin models treated with DPBS, served as the reference standard for determining the relative viability. The positive control group, treated with 5% SDS solution, a known cytotoxic agent, exhibited significantly reduced viability per expectations. The experimental group, consisting of models maintained in a DMEM/F12 (1:1) medium (fibroblast culture medium), demonstrated higher relative viability as opposed to the relative viability of the negative control group, indicating that the models retained metabolic activity during culture in this cell culture medium.

Importantly, the viability assessment of the HSEs cultured in fibroblast culture medium revealed a relatively high SD. Only one replicate presented a significantly lower relative viability compared to the other two replicates, which may reflect a degree of biological variability. This variability can arise from several factors including differences in cell seeding density, matrix composition or tissue model thickness. Despite the observed variability, all samples consistently exhibited high relative viability compared to the negative control, indicating successful model establishment further supported by the histological evidence. These findings confirm the overall presence of metabolically active, viable cells in the prepared HSEs.

Although this modified MTT assay is a simple method for evaluating the tissue model irritability, or in the case of this thesis, viability, the drawback of MTT assay lies in the fact that it is a destructive method and is only suitable if there are no other points of interest to be measured. An alternative to this modified MTT assay is a modified lactate dehydrogenase

(LDH) release assay (146). LDH is a cytosolic enzyme that is released after damage or lysis of cell membrane, therefore serving as a reliable cytotoxicity marker. The cells are subjected to a potentially toxic agent, the LDH released in culture media is measured and the percentage of cytotoxicity is calculated. It allows to monitor cytotoxicity from the same sample over time, therefore providing more data per tissue model, making it very useful and cost-effective (147).

To further validate the structural and functional properties of the fabricated HSE, additional characterization techniques should be employed. PCR methods can be used to assess the expression of key epidermal markers, such as K14 for basal cells and K10 or involucrin for differentiated suprabasal cells (148). Additionally, immunohistochemical staining would enable the spatial visualization of these proteins within the tissue context, providing deeper insight into the morphology and physiology of the 3D skin tissue models (149).

The HSEs were employed for an *in vitro* 3D wound healing assay after positive viability assessment. To better mimic *in vivo* conditions, the wounded HSEs were transferred onto a DE. By day 2, the WHM exhibited a reduced gap, indicating early signs of re-epithelialization. This finding is consistent with the initial phases of wound healing described in the literature. The cellular migration observed in this wounded tissue model at this point likely involves keratinocytes migrating from the wound edges to cover the wound bed, which is a key step in cutaneous repair (80).

These findings are similar to the results reported by Egles et al. in a comparable study. Their team demonstrated that fibroblasts accelerate the rate of re-epithelialization of wounded HSEs, achieving near-complete wound closure by migrating keratinocytes within 2 days of the wound healing assay (132).

However, a notable limitation of the WHMs prepared in this thesis was the absence of DEs on photomicrographs after H&E staining. As shown in **Figure 28**, the wounded tissue model remained attached to the DE during histological processing, suggesting that the loss of the DE likely occurred later. This structural limitation suggests that the WHMs may require further optimization to ensure the integrity of all components throughout the experimental and processing stages.

Despite this technical challenge, the observed wound closure by day 2 in the presented tissue model aligns with the early migratory responses seen in other similar systems within

the first 24–48 hours post-wounding. While the rate of closure in our model appears consistent with these initial stages, complete wound closure *in vivo* and *in vitro* typically involves a more extended period encompassing proliferation, matrix deposition, and tissue remodeling (150, 151).

Nevertheless, the high viability of HSEs in Subchapter 7.3 suggests potential for applications such as cytotoxicity evaluation. Properly constructed and validated 3D skin models remain a powerful tool in drug and cosmetics development, offering a reliable alternative to animal models for evaluating cytotoxicity and drug safety (2). Such models can be employed to assess the safety of consumer products intended for topical application (152) or to study the pathophysiology of wound healing under various experimental conditions (153, 154). Recently, an interesting study by Reddersen et al. evaluated the biocompatibility of tattoo inks and studied the wound healing progression after tattooing commercially available HSEs (155). This highlights the growing scope of applications for 3D skin equivalents beyond conventional toxicological studies.

On the other hand, one of the critical challenges employing 3D skin tissue models for wound healing research is their complexity compared to 2D cell cultures. In addition to the longer maturation time (approximately four weeks), evaluation of re-epithelialization requires labor-intensive histological processing and analysis (156).

The results obtained in this thesis are an early step towards developing a wound healing model that mimics the *in vivo* microenvironment. However, the wound healing process in native skin is much more complex. It involves various cell types, including platelets, endothelial cells, macrophages, neutrophils and myofibroblasts along with the many chemokines, cytokines and growth factors secreted by these cells (69). These cell types and signaling molecules could be added to these tissue models to further mimic the complex wound healing process in the native skin. Additionally, the space of the wound can be filled with fibrin to allow for the control over mechanical and physiological environment and can be used to investigate cell behaviour for each cell type (157).

CONCLUSION

This thesis examined the potential of *in vitro* skin models for studying wound healing processes, combining both theoretical background and experimental research. The theoretical part provided a comprehensive overview of the human skin structure, wound healing physiology, principles of engineering 3D skin substitutes for *in vitro* applications, and current approaches for the modelling and analysis of wound repair *in vitro*. Together, these chapters laid the foundation for understanding how engineered skin constructs can mimic key aspects of native skin and its capacity for application in research and cosmetics and drug development.

The experimental part of this thesis successfully established and characterized a HSE based on HaCaT keratinocytes and human dermal fibroblasts embedded within a collagen type I matrix. A combination of photographic documentation, histological analysis, and viability assay confirmed the formation of epidermal layers atop a dermal-like structure and demonstrated the metabolic activity of the tissue models. The fabricated HSEs showed morphological and functional features similar with those described in the literature.

The prepared HSEs were used to evaluate keratinocyte migration in a 3D environment. Despite the structural limitations of the wounded tissue model, early signs of re-epithelialization were observed, suggesting that keratinocytes initiated migration into the wound area. This cellular behavior parallels the migration patterns typically seen in 2D scratch assays, but within a more physiologically relevant 3D context. Overall, these findings represent a promising step toward the development of a functional *in vitro* platform for assessing the effects of potential therapeutic compounds on wound healing under conditions that more closely mimic native human skin.

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LIST OF ABBREVIATIONS

2D	Dvourozměrné/Two-dimensional
3D	Trojrozměrné/Tree-dimensional
Ala	Alanine
ALI	Air-liquid interface
Asp	Aspartic acid
Ca ²⁺	Calcium ions
CGRP	Calcitonin gene-related peptide
DCs	Dendritic cells
DE	Dermal equivalent
DED	De-epidermized dermis
DMEM	Dulbecco's modified eagle's media
DMSO	Dimethylsulfoxide
DPBS	Dulbecco's phosphate buffered saline
dSEARCH	Dendrite surveillance extension and retraction cycling habitude
ECIS®	Electric Cell-substrate Impedance Sensing®
ECM	Extracellular matrix
ECVAM	European Centre for the Validation of Alternative Methods
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-Linked Immunosorbent Assay
EPB	Elastin binding protein
EpI	Epidermalization I
EpII	Epidermalization II
FBS	Fetal bovine serum
FGF	Fibroblast growth factor

GAGs	Glycosaminoglycans
Glu	Glutamic acid
Gly	Glycine
H&E	Hematoxylin and Eosin
HDF	Human neonatal dermal fibroblast
HEEs	Human epidermal equivalents
HSEs	Human skin equivalents
Hyl	Hydroxylysine
Hyp	Hydroxyproline
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL	Interleukin-1
ILC	Innate lymphoid cells
KGF	Keratinocyte growth factor
LCs	Langerhans cells
LDH	Lactate dehydrogenase
LEAP TM	Laser Enabled Analysis and Processing TM
LOX	Lysyl oxidase
Lys	Lysine
MCs	Merkel cells
MMPs	Matrix metalloproteinases
MSCs	Mesenchymal stem cells
MTT	Thiazolyl blue tetrazolium bromide
OECD	Organisation for Economic Co-operation and Development
OOC	Organ-on-a-chip
PDGF	Platelet-derived growth factor

Pro	Proline
RT-PCR	Reverse-transcription polymerase chain reaction
SC	<i>Stratum corneum</i>
SDS	Sodium dodecyl sulfate
SOC	Skin-on-a-chip
TGF- β	Transforming growth factor-beta
TIMPs	Tissue inhibitors metalloproteinases
TNF- α	Tumor necrosis factor-alpha
TREGs	Regulatory T cells
UV	Ultraviolet
Val	Valine
VEGF	Vascular endothelial growth factor
WHM	Wound healing model