Reconstituted skin models

Viktorie Hepová

Bachelor Thesis
2023

Tomas Bata University in Zlín
Faculty of Technology

Univerzita Tomáše Bati ve Zlíně
Fakulta technologická
Ústav technologie tuků, tenzidů a kosmetiky

Akademický rok: 2022/2023

ZADÁNÍ BAKALÁŘSKÉ PRÁCE

(projektu, uměleckého díla, uměleckého výkonu)

Jméno a příjmení:	**Viktorie Hepová**
Osobní číslo:	**T19624**
Studijní program:	**B0711A130009 Materiály a technologie**
Specializace:	**Biomateriály a kosmetika**
Forma studia:	**Prezenční**
Téma práce:	**Rekonstituovaný model kůže**

Zásady pro vypracování

Studentka zpracuje literární rešereši zaměřenou na metody testování bezpečnosti kosmetiky, in vitro techniky a přípravu rekonstituovaných tkání, včetně problematiky lab-on-chip.

Forma zpracování bakalářské práce: **tištěná/elektronická**
Jazyk zpracování: **Angličtina**

Seznam doporučené literatury:

[1] SNUSTAD, D.P., SIMMONS, M.J., RELICHOVÁ, J. et al. Genetika. Brno: Masarykova univerzita, 2009.
[2] ALBERTS B. et al. Molecular Biology of the Cell 5th ed. Garland Science.
[3] DAVID A.P. BIZIOS R. Biological Interactions on Material Surfaces. ISBN 978-0-387-98160-4.
[4] DRAELOS, Zoe Kececioglu a Peter T. PUGLIESE. *Physiology of the skin*. 3rd ed. Carol Stream: Allured Books, c2011, ix, 674 s. ISBN 9781932633771.

Vedoucí bakalářské práce: **prof. Ing. Petr Humpolíček, Ph.D.**
Ústav technologie tuků, tenzidů a kosmetiky

Oponent bakalářské práce: **doc. Ing. Zdenka Víchová, Ph.D.**
Centrum polymerních systémů

Datum zadání bakalářské práce: **1. února 2023**
Termín odevzdání bakalářské práce: **19. května 2023**

L.S.

prof. Ing. Roman Čermák, Ph.D.
děkan

doc. Ing. Marián Lehocký, Ph.D.
ředitel ústavu

PROHLÁŠENÍ AUTORA
BAKALÁŘSKÉ PRÁCE

Beru na vědomí, že:

- bakalářská práce bude uložena v elektronické podobě v univerzitním informačním systému a dostupná k nahlédnutí;
- na moji bakalářskou práci se plně vztahuje zákon č. 121/2000 Sb. o právu autorském, o právech souvisejících s právem autorským a o změně některých zákonů (autorský zákon) ve znění pozdějších právních předpisů, zejm. § 35 odst. 3;
- podle § 60 odst. 1 autorského zákona má Univerzita Tomáše Bati ve Zlíně právo na uzavření licenční smlouvy o užití školního díla v rozsahu § 12 odst. 4 autorského zákona;
- podle § 60 odst. 2 a 3 autorského zákona mohu užít své dílo – bakalářskou práci nebo poskytnout licenci k jejímu využití jen s předchozím písemným souhlasem Univerzity Tomáše Bati ve Zlíně, která je oprávněna v takovém případě ode mne požadovat přiměřený příspěvek na úhradu nákladů, které byly Univerzitou Tomáše Bati ve Zlíně na vytvoření díla vynaloženy (až do jejich skutečné výše);
- pokud bylo k vypracování bakalářské práce využito softwaru poskytnutého Univerzitou Tomáše Bati ve Zlíně nebo jinými subjekty pouze ke studijním a výzkumným účelům (tj. k nekomerčnímu využití), nelze výsledky bakalářské práce využít ke komerčním účelům;
- pokud je výstupem bakalářské práce jakýkoliv softwarový produkt, považují se za součást práce rovněž i zdrojové kódy, popř. soubory, ze kterých se projekt skládá. Neodevzdání této součásti může být důvodem k neobhájení práce.

Prohlašuji,

- že jsem na bakalářské práci pracoval samostatně a použitou literaturu jsem citoval. V případě publikace výsledků budu uveden jako spoluautor.
- že odevzdaná verze bakalářské práce a verze elektronická nahraná do IS/STAG jsou obsahově totožné.

Ve Zlíně, dne:

Jméno a příjmení studenta:

.....
podpis studenta

ABSTRAKT

Porozumění komplexitě anatomie a fyziologie lidské kůže je důležité pro vývoj in vitro rekonstituovaných kožních ekvivalentů modelujících funkce kůže. Rekonstituované kožní modely se obvykle skládají ze scaffoldů, poskytujících strukturu, a keratinocytů, tvořících stratifikovanou diferenciovanou vrstvu napodobující epidermis, pomocí modulování signálních drah. Běžně používanými a komerčně dostupnými rekonstituovanými modely napodobujícími kůži jsou epidermální a dermo-epidermální modely. Je možné je použít k hodnocení bezpečnosti a účinnosti kosmetiky, léčiv a dalších látek, nebo k léčení pacientů s rozsáhlým poraněním kůže. Tyto modely nabízejí alternativu k testování na zvířatech, které bylo zakázáno pro testování kosmetických ingrediencí a produktů Nařízením o kosmetických přípravcích (ES) č. 1223/2009.

Klíčová slova: kůže, pokožka, škára, rekonstituované modely, testování kosmetiky, in vitro metody, bezpečnost a účinnost, skin-on-a-chip

ABSTRACT

Understanding the complexity of human skin anatomy and physiology is important to develop in vitro reconstituted skin equivalents modelling skin functions. The reconstituted skin models are usually composed of a scaffold providing structure and keratinocytes forming a stratified differentiated epidermis-like layer by modulating signalling pathways. Commonly used and commercially available reconstructed models mimicking the skin are epidermal (RHE) or dermo-epidermal (HSE) models. They can be used to evaluate the safety and efficacy of cosmetics, drugs, or other substances or to treat patients with large skin wounds. These models offer an alternative to animal testing, which has been banned for testing of cosmetic ingredients and products by the Cosmetics Regulation (EC) No 1223/2009.

Keywords: skin, epidermis, dermis, reconstructed models, cosmetic testing, in vitro methods, safety and efficacy, skin-on-a-chip

I hereby declare that the print version of my Bachelor's/Master's thesis and the electronic version of my thesis deposited in the IS/STAG system are identical.

CONTENTS

INTRODUCTION

The subject of reconstituted skin models has been researched a lot in recent decades and every year brings new findings or procedures. I would like to get oriented with the topic, and therefore I will try to summarize the basic information on this issue so far.

A reconstituted skin model is a tissue-engineered skin construct showing great promise in research and the treatment of burns and wounds. It is an interdisciplinary matter, which involves knowledge and cooperation of several fields like biology, medicine, and engineering.

The initial section is dedicated to the (detailed) characterization of skin. Anatomy of the skin and physiology are described, for they are like two sides of the same coin. Understanding the structure and properties of the skin is crucial to construe the problematics of skin models, which are described in the second part along with methods of evaluating of cosmetics.

The skin is a very complex system featuring various processes and performing a wide range of functions. The models cannot reach the full characteristics of native skin but dispose of some of the properties for specific utilization. There are different kinds of skin models, that vary in the degree of sophistication in which they substitute or mimic the hierarchy of skin. This thesis introduces the main platforms, which are: artificial membranes, excised samples, reconstructed tissues, and microfluidic devices, with a focus on the reconstructed tissues and microfluidic devices, also called lab-on-chip, a perspective technology for research imitating the dynamics of a real environment in a body.

Investigating the effects of drugs and chemicals on animals before scientists deem them safe for pharmaceuticals or cosmetics is nowadays being criticized. Alternatives to animal testing include tests performed *in vitro*, using cells and skin models, or *in silico*, using advanced computer-modelling techniques. Utilization of the skin constructs is in sundry toxicological, cosmetological, or dermatological studies on processes like diffusion, efficacy, wound healing, inflammation, ageing, and shear stress. Diseases can be induced in skin equivalents and studied. This and other methods for approving cosmetic compounds and products are listed and explained.

Besides research, skin models find application in healthcare. They show great potential in the treatment (replacement and repair) of extensive burns and deep wounds.

The thesis then moves on to the description of biological and artificial components making up the structure of the skin constructs.

In conclusion, different approaches to the preparation of reconstituted skin models and the suitability of different models for different purposes are assessed by comparison of different studies.

1 THE INTEGUMENTARY SYSTEM

The integumentary system involves the skin and its various derivatives like hair, nails, nerve endings and receptors, and glands. The skin envelops the entire surface of the body, which is on average about 1.6 m^2. [1] Its epithelium is continuous with the epithelia of the digestive, respiratory, and urogenital systems. [2]

The skin is the primary body part that comes into contact with the surroundings, [1] as well as it is an organ involved in a wide range of intrinsic body functions. [3] As such, the skin creates an essential interface providing a balance between the external and internal environment. Exchanges between the outside and the inside of the body are controlled. [1]

There are various processes taking place in the skin, mechanical, thermal, biological, chemical, and electromagnetic. It is a very complex and dynamic system performing a wide range of functions, most importantly acting as a barrier. [1]

It is an organ continuously renewing itself through the growth and differentiation of its cells which leads to the production of a dead horny layer on the surface. The integumentum commune can be thought of as a huge, mostly holocrine, glandular system. All living epidermal cells metabolize fibrous proteins or lipids. The cells then die with the holocrine secretion. [2] Both protein and lipid substances (keratin and sebum), accumulated in and between the dead cells of the horny layer, are crucial for the barrier function. Antimicrobial peptides are also present. [4] In addition to the complexity, skin is naturally inhabited by microbiota, which contributes to skin barrier function. [5]

The protection of an organism from the exterior against assaults and from the interior against water loss is provided. [6] Thus, the integument maintains homeostasis. [4]

Many characteristics of the skin vary according to ethnicity, to sex, from individual to individual, in different regions of the body, and in the same individual within ageing. [1] Yet there are basic common features. [2]

The skin is made up of cutaneous and subcutaneous tissue. There are different opinions on what layers are included under the definition of the skin as such, and whether the subcutaneous tissue should be included. The word cutaneous is derived from the Latin cutis, meaning the skin. There are two of the main four kinds of tissues comprised in the skin, epithelial and connective. The skin is defined as two or three main distinct layers: epidermis, formed of the epithelium, and dermis (corium), formed of dense connective tissue. The third,

innermost, layer is termed hypodermis, which is the subcutaneous tissue, formed of looser connective tissue. The difference in the definitions gives rise to confusing discrepancies in skin properties data, like its weight and thickness. [6] The skin is composed in this way, although it may vary in different sites of the body, e.g. the hypodermis is markedly lacking in the eyelid skin. [7]

1.1 Epidermis

1.1.1 Epidermal epithelium and cells

The epidermal epithelium is stratified, which means its cells are arranged in layers. [3] Epithelial cells are closely packed with little intercellular material. Based on their shape, epithelial cells can be categorized as columnar, cuboidal, and squamous. [8] They exhibit polarity in the form of distinct membrane domains, apical and basolateral, which differ in their lipid and protein composition. The apical domain faces the "outside" of the body, while the basolateral faces the "inside" of the body. [9]

The cells of the epidermis are called keratinocytes, because of the abundance of keratin filaments in their cytoplasm. Keratinocytes are rich in protein, especially keratin. [6] There are two types of keratin, type I is acidic and type II is basic. These assemble through disulfide bonds into heterodimers to form a fibrous cytoskeleton. The keratins expression is specific for different layers of the epidermis and different regions of the body. [10]

There are also other cells than epithelial in the epidermis. Overall, there are four kinds. Keratinocytes take up most of the cell population of the epidermis. Melanocytes, Merkel cells, and Langerhans cells are interspersed between them in some layers. [6]

Most of the cells are attached to each other by desmosomes, which are complex anchoring structures, connected to the cytoskeleton. Desmosomes include cadherin proteins of two types, desmocollins, and desmogleins. Their isoforms are differentially expressed in different epidermal layers. [10]

1.1.2 Extracellular matrix

Except for tightly packed cells, the epidermis contains an extracellular matrix (ECM), occurring in the form of a basement membrane. It is a sheet-like structure separating epidermal and dermal compartments. [11] As the epidermis does not contain any vessels, it needs to be nourished otherwise. [2] The epidermis is nourished through the basement

membrane by substances diffusion from the dermal blood vessels. [3] This epidermal-dermal junction undulates and creates a pattern of the tissues projecting into each other. The epidermal extensions into the underlying tissue are termed rete ridges. Between them, highly vascular dermal papillae are enclosed. [2]

The ECM influences the maintenance of keratinocytes and homeostasis. It plays a significant role in the regulation of keratinocytes´ behaviour like cell division, mobilization, and differentiation. Thus, the basement membrane regulates self-renewal and determines the fate of the epidermal cells. [11]

1.1.3 Renewal

The epidermis continuously renews itself by proliferation and maturation of its cells (keratinocytes), as implied previously. [10] This process from the generation of new cells to the endpoint, when they slough off the skin surface, takes about a month. [3]

The process begins in the innermost layer of the epidermis, which contains stem cells. With each division of a stem cell, one daughter cell remains in this basal layer, as the other withdraws the cell cycle and commits to differentiate terminally. The keratinocyte moves up through the upper layers of the epidermis to the skin surface, where dead cells are shed into the environment. As they move up, keratinocytes gradually and continuously mature, differentiate, flatten parallel to the skin surface; they loosen their desmosomal connections, become enucleated and die. The final product is a fully cornified keratinocyte. [3], [10]

1.1.4 Layers of epidermis

From the basal layer (stratum basale), where they are generated, keratinocytes subsequently go through the spinous layer (stratum spinosum), granular layer (stratum granulosum), stratum lucidum and finally cornified layer (stratum corneum). Each layer is different in the function and morphology of the keratinocytes, some contain other specific cells. [6]

Homeostasis is enabled by constant proliferation. The adult epidermis is in continual flux. The rapidly dividing progeny of basal stem cells ensures the repair of wounds and yields a hydrophobic barrier. [10]

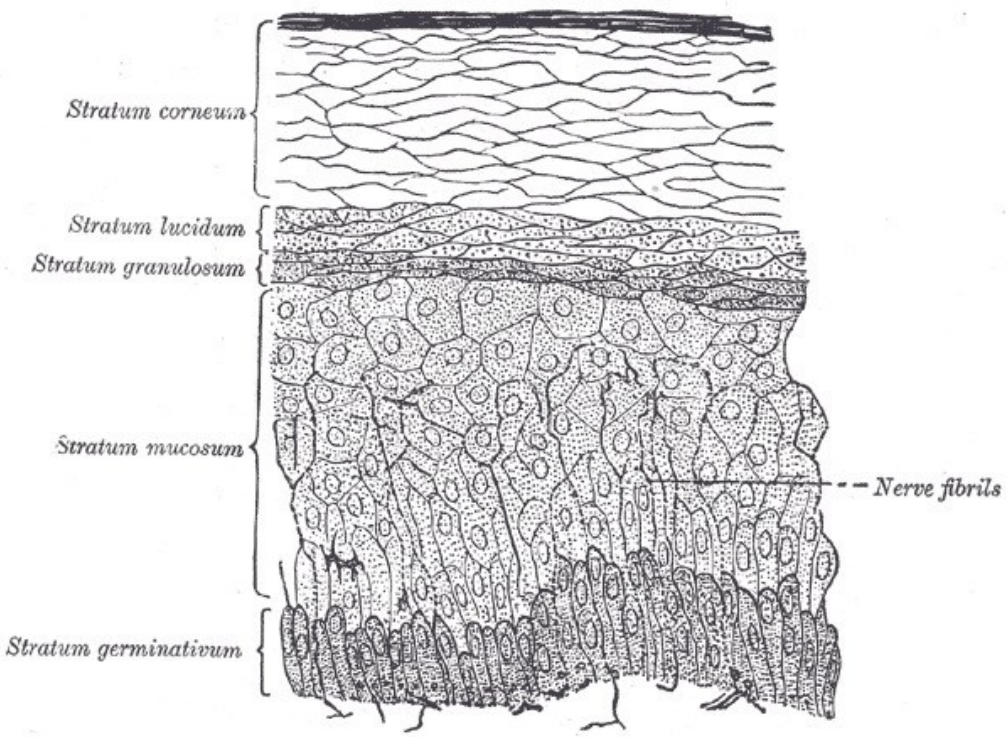

Figure 1 A schematic representation of epidermal sublayers, from down to top: Stratum basale, Str. spinosum, Str. granulosum, Str. lucidum, Str. corneum [12]

1.1.5 Thickness

Based on skin constitution, epidermal thickness, and epithelial layers, skin can be characterized as thick or thin (also hairy). The skin thickness depends a lot on the epidermis. It varies at different body locations. Epidermal thickness ranges from 0.05 to 1 mm, dermal from 1 to 2 mm. The thinnest skin is found on eyelids, whilst the thickest is on the palms and soles of the feet, where the epidermis can reach a thickness of 1.6 mm. [6]

Thin skin, also called hairy skin, extends over most of the body area. Its epithelium is constituted of 4 strata – basale, spinosum, granulosum, and corneum. The thickness is usually about 1-2 mm.

Thick skin is located in areas where abrasion occurs frequently, which are the palms of the hands, soles of the feet, and fingers and toes. It does not contain any hair follicles, nor does it contain sebaceous glands. However, there are more sweat glands and sensory receptors, accordingly to the function of the skin in these locations. The epidermis of thick skin contains a fifth distinct layer between stratum granulosum and stratum corneum, stratum lucidum. Each epidermal layer is thickened, especially the horny layer. The dermis, on the

other hand, is thinner. The dermal papillae of thick skin intervene more profoundly and regularly than on other parts of the body. The thickness of thick skin can reach 6 mm. [6]

1.1.6 Innervation

There are free endings of nerves extending into the epidermis from the dermis. They are responsible for sensing pain, heat, and cold. They are most abundant in the granular layer and surround hair follicles. Nerve endings connected to Merkel cells form Merkel discs, which register light touch and inform about shape, texture, and sustained pressure. [12], [13]

1.1.7 Sublayers

1.1.7.1 Stratum basale

The basal layer, which supplies the epidermis with new keratinocytes continuously, is also called stratum basale or a germinative layer, as it sits at the base of the epidermis and contains stem cells, from which new keratinocytes generate. Stratum basale is a single row of cuboidal to columnar keratinocytes, among which immigrated and reside two kinds of non-epithelial cells – Merkel cells and melanocytes. Keratinocytes are bound to each other by desmosomes, as well as are Merkel cells, whereas melanocytes are not attached to any of the surrounding cells by desmosomes. [3], [6], [10]

In this layer, a small population of stem cells interspersed between the others ensures slow-cycling renewal. The stem cells undergo proliferation into transiently amplifying cells and generate all the keratinocytes. The stem cells are, too, mutually attached by desmosomes to adjacent and overlying cells. They are bound to the underlying basement membrane by hemidesmosome connections. [10]

The basal keratinocytes contain a relatively big nucleus, endoplasmic reticulum, Golgi complex, mitochondria, melanin granules, and an abundance of free ribosomes. Their cytoplasm is rich in keratin filaments. The keratin filaments attach to desmosomes. K5/K14 filaments are settled perpendicular to the skin surface and attached to hemidesmosomes. The keratin filaments thus enable the attachment and anchoring of the keratinocytes. [3], [6]

Merkel cells´ origin is unclear, they possibly originate from epidermal or neural stem cells. Besides the stratum basale, they can also be found in hair follicles. Myelinated nerve endings coming from the dermis connect to the Merkel cells to form complexes functioning as mechanoreceptors. They are more concentrated in sensory areas like fingertips. They

extend cell processes into the intercellular spaces. [3], [6] The Merkel cells possess dense-core granules. Specific immunohistochemical markers comprise cytokeratins, neurofilaments, neuron-specific enolase, and desmosomal proteins. [10]

Melanocytes originate from the neural crest and migrate to the epidermis during the early fetal stage. [10] Two groups of melanocytes can be distinguished according to where they migrate and reside: epidermal and hair. Some migrate to the hair follicle, specifically the external root sheath, and are responsible for hair pigmentation. The other melanocytes migrate to the stratum basale, where they take up 3 % of the cell population. [6], [10]

The melanocytes are stellate-shaped. Although they lack desmosomes and hemidesmosomes and thus a connection to local cells, they are connected to keratinocytes in the stratum spinosum via their long dendrites. [3] In this way, they transport melanin pigment. One melanocyte usually reaches 10-20 keratinocytes with which it forms an epidermal melanin unit. [10] The main function of melanocytes is to generate melanin pigment, which essential purpose is photoprotection. The process of melanin synthesis takes place in melanosomes. Melanosomes are membrane-bound organelles originating from the Golgi apparatus. [10] The tyrosinase enzyme first catalyzes the conversion of tyrosine to 3,4-dihydroxyphenylalanine, which is then transformed into melanin. [3] Most of the melanin pigment in the skin can be found in the stratum basale. [10] Melanin, packed in vesicular melanosomes, is transferred to the dendrites and into intercellular space to be picked up by adjacent keratinocytes by phagocytosis. Keratinocytes distribute the melanin so it would overlay the surface of their nuclei, especially the apical side, creating a UV shield. [3]

Skin pigmentation

Human skin contains the same number of melanocytes across races with different skin colours. The colour difference is caused by the amount of tyrosinase activity, which is higher in dark-skinned races. In addition, the rate of the degradation of melanin is lower. [3] Genetics also plays a role in the amount of produced melanin. The tyrosinase activity is increased by sun exposure. [6] Exposure to UV light increases melanogenesis which leads to forming a cap of melanin granules over the nucleus. Another factor, which may affect the pigmentation, is local inflammation. Two possible responses to inflammatory mediators occur. Either the melanogenesis is increased or decreased or the melanin transfer is altered. [10]

1.1.7.2 Stratum spinosum

Stratum spinosum is composed of the largest number of sublayers in the epidermis, it is 8-10 cells thick. It is also called the suprabasal layer as it sits on the basal layer. [3], [6] The keratinocytes in the stratum spinosum form strong prominent desmosomal interconnections, which leads to a spiny appearance of the cells in stained specimens. [3] The desmosomal prickle-like artefacts gave this layer another name, the prickle cell layer. [10] Except for keratinocytes, another kind of cell constitute this layer – dendritic antigen-presenting Langerhans cells. [6] Stratum spinosum adds to the water barrier function of the epidermis, contributes to skin strength and flexibility, and participates in immunosurveillance. [3], [6]

The keratinocytes of the stratum spinosum exhibit a polyhedral to slightly flattened shape. [10] The desmosomal bonds between them are even stronger and more abundant than in the basal layer. [3] The suprabasal keratinocytes express specific K1/K10 proteins. These dimers form intermediate filaments which are spanned radially to insert into desmosomes. [6] During tissue processing for observation, the cell membranes shrink due to dyeing. The desmosomes remain intact, though, generating protrusion artifacts. [3], [6] In their cytoplasm, the keratinocytes exhibit numerous endoplasmic reticula, free ribosomes, melanin granules, and lamellar granules. [3] The lamellar granules are also called Odland bodies or keratinosomes. They are membrane-bound vesicles derived from the Golgi apparatus with a water-repelling content. The lamellar granules contain membrane-like stacks of lipids, mainly glucosylceramides, glycerophospholipids, sphingomyelin, and cholesterol sulfate. Along with the lipid content, many modifying enzymes are present. The lamellar granules first appear in the stratum spinosum, then they are found in the layers above to compose a water seal. [14]

About 5 % of the epidermal cells make up Langerhans cells, which are derived from bone marrow. [3] These cells constitute up to 25 % of the skin area. [6] Exposure to UVB radiation lessens their amount. [10] Langerhans cells are not attached to adjacent cells by desmosomes. [3] They appear clear on hematoxylin and eosin sections. Their cytoplasm comprises rod- or rocket-shaped Birbeck granules of unknown function. [3], [10] The Langerhans cells form flatten dendrites, which give them a stellate shape. The dendrites run parallel to the skin surface. [3], [6] Through the dendrites, the cells extend some processes into the intercellular spaces, specifically to the stratum granulosum. [3], [10] The Langerhans cells are functionally related to macrophage cells. [10] Their immunological function is possible thanks to the receptor for IgE, which allows them to recognize and capture allergens

or antigens they contact in the skin. After the uptake of an antigen, the Langerhans cells migrate through afferent lymphatics to regional lymph nodes, where they present the antigen to T cells, which are sensitized and start to proliferate. The Langerhans cells thus induce an immunological response. [3], [10]

1.1.7.3 Stratum granulosum

The keratinocytes in the granular layer are even more flattened, their nuclei are shrunken and their cytoplasmic membranes thickened. This layer is composed of 1-2 sublayers of keratinocytes in thin (haired) skin and 4-8 sublayers in thick (non-haired) skin. Tight junctions, partaking in the skin barrier, are formed. The process of keratinization begins in this layer. [6], [10]

The keratinocytes exhibit two kinds of characteristic granules, lipid-rich lamellar granules, and deeply basophilic keratohyalin granules. The granules give the keratinocytes a grainy appearance, which led to the term stratum granulosum – granular layer. [6], [10], [15]

The abundant lamellar bodies from the spinous layer accumulate in the granular layer aligned along the plasma membrane. When the cells move to the stratum corneum, excretion occurs. The lamellar granules fuse with the plasma membrane in order to release their lipid content and the enzymes into the intercellular space. [3], [14], [15]

The keratohyalin granules are bigger than the lamellar bodies. The keratohyalin granules contain histidine and cysteine-rich binding protein, profilaggrin, which is a precursor of filaggrin. Profilaggrin has a mass of approx. 400-500 kDa, as the keratinocytes move to the upper layer, profilaggrin is cleaved into approx. 26-48 kDa filaggrins. The term for filaggrin comes from three words describing its function: filament aggregating protein. Filaggrin works as a biological glue, it binds keratin filaments together. The filaments aggregate and align. [3], [6], [10], [14], [15]

Cells at the outer edge of stratum granulosum lose their nuclei and cytoplasmic organelles as they move upwards to further differentiate. [3]

1.1.7.4 Stratum lucidum

The stratum lucidum is a thin translucent layer laying at the transitional zone between the stratum granulosum and stratum corneum. This layer exhibits a clear (i.e. lucid), seemingly translucent appearance, which is why it is termed this way. The stratum lucidum is found

only in thick skin – on the palms of the hands and the soles of the feet. This layer is composed of dead keratinocytes, which have further flattened. The keratinocytes are packed densely with eleidin, which is a protein enriched with lipids. Eleidin is derived from keratohyalin and will be changed again in the process of the maturation of keratinocytes as it is an important part of the water barrier. [3], [6]

1.1.7.5 Stratum corneum

The outermost layer of the skin, the stratum corneum, is the most fundamental layer for the skin in terms of defence. [6] It is not easy to infiltrate a healthy stratum corneum for common external substances that the skin can come into contact with. Regarding the changes in the keratinocytes, this layer is also called the cornified layer. About 15-30 overlapping layers of keratinocytes constitute the stratum corneum, so the thickness is about 10-20 μm. [6] The thickness may vary in different sites of the body, as the skin is able to adapt in accordance to the level of external friction or trauma by forming a more compact stratum corneum, as seen on the soles of the feet and palms of the hands. [10] It may even result in forming a hard callus when exposed to harsher conditions. Whereas in most body parts, a thinner and looser layer of the stratum corneum is present. [3], [16]

The keratinocytes move continuously outward to slough off the skin surface. While doing so, they are under constant change, so finally they could slough off the skin surface, either singly or in clusters termed squamae. This process of keratinocytes sloughing off is termed desquamation. From the granular layer to the end, the process called cornification or keratinization takes place. The keratinocytes entering the stratum corneum are called corneocytes. The corneocytes are filled mainly with keratin filaments. These cells are bland polyhedral flattened discs with a thickened compressed plasma membrane. Because they have undergone a terminal differentiation and are enucleated, they are technically considered to be dead. Apoptosis, programmed cell death, takes place so the keratinocytes could differentiate into highly specialized corneocytes with a protective function. Within the keratinocytes, biochemical and morphological pathways are followed resulting in their death without causing harm to the surrounding cells. [3], [10], [16], [17]

The stratum corneum is arranged in a specific way, which is essential for its barrier function. The corneocytes form a seal-like pattern, the so-called “brick and mortar” structure. This analogy depictures that multiple layers of corneocytes, represented by the bricks, are

sandwiched in orderly, vertical stacks between layers of lipid matrix, which is represented by the mortar that cements the cells together. [10], [16]

While the cells move to the stratum corneum, they need to lose a great volume of water content, from 70 % in nucleated layers to 15 % in the nonviable layer. Keratohyalin or eleidin is transformed into keratin. The extracellular spaces are filled with lipid material excreted from the lamellar granules along with the enzymes that modify the polar "pre-barrier" lipids to nonpolar "barrier" lipids. The final product of this modification is a more or less equimolar ratio of ceramides, cholesterol, and free fatty acids. The lipid matrix contains a small percentage of other lipids, although phospholipids are absent. [6], [10]

Certain processes must occur in the stratum corneum to remain a healthy balance in the skin and ensure that an effective barrier structure is formed. These encompass:

- an establishment of a firm corneocytes core
- developing a thick cornified corneocytes envelope
- construction of an extracellular lipid matrix
- active desquamation [10]

The keratohyalin granules of the stratum granulosum contain profilaggrin, which is processed by proteolysis and dephosphorylation to take the form of an active filaggrin in the stratum corneum. Filaggrin cross-links the cytoplasmic keratin filaments. These bundled intracellular filament assemblies are crucial for the structural integrity of the stratum corneum. They attach to the interior surface of the cornified envelope. [6], [10]

The cornified envelope is established under the cytoplasmic membrane. Assembled keratins, filaggrin, and other small protein molecules like involucrin, loricrin, and cystatin A form a 10-15 nm thick structure, which eventually replaces the cytoplasmic membrane. The assembly is catalyzed by the enzymes transglutaminases, which contain calcium, and sulfhydryl oxidases. The cornified envelope surrounds the protein core and secures a mechanical barrier as both intracellular keratins and extracellular lipids bind to it. It also serves as a scaffold and organizes the extracellular lipids into lamellar bilayers/membranes. [6], [10]

The lipids thus constitute a multilamellar pattern. The lipid matrix helps prevent the cells from drying out. It is not all the same within the stratum corneum, though. The deep and the

intermediate zone exhibit more compact consistency, functioning as the main diffusion barrier. It gets gradually looser while moving to the upper zone. [6], [16]

Also, the bonds between the corneocytes get looser as they move upward. In the deeper zone of the stratum corneum, the cells hold through retained desmosomes – corneodesmosomes. The corneodesmosomes are made of desmoplakins, desmogleins, desmocollins, plakoglobins, and desmocalmins. They help maintain the mechanical and chemical barrier, but during the route to the surface, these strong intercellular bridges get enzymatically broken down. Thus, lacunar spaces between the cells are created, which means a barrier disruption, but it allows the corneocytes to be desquamated. [6], [10]

The keratin´s properties protect the underlying tissue from chemicals, microorganisms, and chemicals. The overall design of the epidermis, the stratum corneum especially, presents an active sturdy barrier regulating inward and outward water movement. This is why even minor superficial injuries lead to increased transepidermal water loss. The stratum corneum is not fully impermeable, though. It may be considered a semipermeable membrane. The lipid bilayers allow a slow passage to external substances. The permeation is possible by passive diffusion. According to the substances´ nature, they can go either via the inter- (or para-) cellular pathway or penetrate the corneocytes (transcellular way). The solubility of the matter determines which way it goes. Lipophilic substances are more likely to diffuse past the cells, whilst polar or hydrophilic substances prefer the transcellular route. If the molecule passes through the horny layer, the native cells of the layers below show little resistance to further permeation. When they enter the dermis, the substances get absorbed into the circulatory system through the plexuses underneath the basal membrane. [6], [10], [16]

1.1.8 Balance in the epidermis

To remain a healthy balance, continuous self-renewal happens. Beyond standard homeostasis, the skin regulatory system also reacts to wounding and regenerates the lost tissue. During the physiological state, the desquamation rate matches the proliferation rate of the basal stem cells. Dermis and the basal membrane play a key role in the regulation of the epidermal processes. The number of keratinocytes should be kept relatively constant. The interactions between both the keratinocytes and the immigrant cells should be managed as they mature. Signalling molecules such as hormones, growth factors, and cytokines are used as regulatory messengers. When the basal stem cells are activated, they divide. The

newly created cells push the overlying cells further up generating the movement. A newly added cell spends about 14 days until it reaches the stratum corneum. Apoptosis is also a crucial part of the epidermal regulatory mechanisms. Apoptosis is involved in the epidermal tissue remodelling during its development, regulating the cell numbers, and disposal of mutated, infected, or damaged cells. Apoptosis facilitates the terminal differentiation, thereby creating the stratum corneum, where it takes about another 14 days before the cells are desquamated. [6], [17]

1.2 The epidermal-dermal juncture

The epidermis and the dermis fold into each other, creating undulation called dermal papillae. Thus, the surface area between these two layers is increased which favours the nutrition of the epidermis because of the enhanced diffusion. [6]

There is a physical barrier between the epidermis and dermis, the basement membrane. The basement membrane gives mechanical support as well as mediates nutrient transport to the epidermis. [6]

1.3 Dermis

The dermis lays below the epidermis. The dermis is an example of a dense irregular connective tissue. It is a bilayer composed of the superficial papillary and the deep reticular parts. The papillary layer is a looser tissue than the reticular layer. [3] The thickness of the skin depends greatly on the thickness of the dermis. The dermis imparts elasticity, softness, and tensile strength to the skin. The dermis regulates the skin's salt-water balance and facilitates sensory reception and defence against foreign substances. The dermis is vascularized, innervated, and participates in the immune system. Epidermis creases into the epidermis and participates in forming the skin major appendages: hair follicles, sebaceous glands, eccrine (sweat) glands, and apocrine glands. Besides the main connective tissue cells, fibroblasts, other cells are also present in the dermis, mainly the immune cells. Although, most of the volume of the dermis is acellular. The extracellular matrix, comprising of ground substance and fibres, is mostly synthesized by the fibroblasts. [3], [6], [10] These dermal components are held together by adhesion proteins such as fibronectin, laminin, and some others. [3]

1.3.1 Dermal sublayers

The dermis exhibits different features in its different depth levels. It can be divided into two layers. [3] Within the upper layer, we can distinguish the part which creases into the epidermis, which would be called the papillary layer. The underlying bed, which contains incidental content as the papillary layer, would be called the subpapillary layer. The superficial and deep dermis differ in some features like density, fibrous content (distribution, the ratio of different kinds, the thickness, their orientation), cellular content (kinds of cells, their amount), distribution, thickness, and percolation of lymphatic and blood vessels, branching, distribution and myelination of nerves, hosting of different appendages. [18]

1.3.2 Appendages

The skin appendages open to the skin surface, so they are a way for secretion, but they also present a passage for foreign substances from the outer environment to get to the deeper layers of the skin. [6]

The whole body is covered with hairs, except for the palms, soles, lips, and the back of the ears. The hair follicle appendage is divided into two segments by the skin surface. Hidden underneath the skin is the hair follicle (or root), and the exposed segment is the hair. The hair follicle is built of seven concentrically arranged tubular layers of epithelial cells. The outermost tubular layer, called the outer root sheath, is continuous with the basal layer of the epidermis. The inner-most tubular layer is called the inner root sheath. A group of fibroblasts from the dermis aggregates at the base of the follicle forming a dermal papilla. Actively proliferating hair progenitor epithelial cells form the hair bulb, which is the deepest hair follicle portion. The hair follicle is supplied by the blood vessels and receives signals from nerves. The arrector pili muscle is attached to the hair follicle. By contracting, this muscle brings about the straightening of the follicle perpendicular to the skin surface. The arrector pili muscle attaches to the part of the follicle under the sebaceous gland. [6]

The sebaceous gland located in the upper level of the hair follicles is made of polyhedral cells with small nuclei and plasma filled with lipids. [6] The maturation of sebocytes leads to their rapture. Their content is released by holocrine secretion. [18] The lipid content, sebum, empties through a duct into the hair follicle, through which it secretes to the skin surface. The sebum lubricates the hair and the epidermis creating an oily coating. When the sebum is mixed with water substances like sweat, an emulsion is created and free fatty acids are formed protecting the skin against bacteria. [18] The sebaceous gland associated with

the hair follicle together form a structure termed the pilosebaceous unit. [6] So-called free sebaceous glands do not attach to hair follicles but open directly to the surface of the skin. They are located at hairless sites. [18]

The eccrine sweat glands are distributed in most sites of the skin, they can be found in large numbers in the thick skin and axillae, but are lacking in lips and foreskin. The eccrine gland is made of one layer of cuboidal cells surrounded by myoepithelial cells. The eccrine gland produces water and salt, which are secreted as sweat to the skin surface through a duct. The duct is coiled when deep in the dermis, but as it goes up, it straightens. Sweat evaporation is one of the mechanisms of body thermoregulation leading to cooling. [6]

Apocrine glands are present in the skin of the axillae and areoles and nipples. They can be considered scent glands. The secretory part of the apocrine gland is actually located in the subcutis. It produces milky viscous odorless substances that are secreted through a single duct with an opening to the hair follicle. By secerning its product, the cells of the apocrine gland die. The product later gets broken down by bacteria, generating smelly short-chained fatty acids. [6]

1.3.3 Blood vessels

The dermal blood vessels compose an extensive network of plexuses, that extends immediately beneath the epidermis. The dermal vasculature is formed of three connected plexuses: The deep (subcutaneous) plexus is located at the interface with the hypodermis. Vessels arising from the deep plexus reach to the middle (subpapillary) plexus, present at the level of sebaceous glands. The middle plexus sends branches further upward to the dermal papillaries to form superficial plexus from which the capillary loops, paralleling the skin surface right beneath the epidermis, arise. [6], [10], [18] In the capillary loops, the arterioles connect to venules through which the blood flows back to the deeper dermal layers, where the veins also form two kinds of plexuses. [18] The rich papillary vasculature helps to supply the avascular epidermis. [3]

The capillary loops mediate an important two-direction transit. From them to the dermis, nutrients are diffused. Also, the capillary loops and surrounding vessel network allow access to the blood system to the external substances applied topically onto the skin that managed to pass through the epidermis and the basement membrane. [6], [10]

1.3.4 Lymphatic vessels

The dermis is richly supplied with lymphatic vessels. [19] The lymphatic vessels are inconspicuous in the normal dermis. [10] Angiogenesis and lymphangiogenesis in the skin are controlled molecularly by vascular endothelial growth factor and vascular endothelial growth factor-C. [19] Blood and lymphatic vessel network in the dermis are closely intertwined with each other. [20] The blood and the lymphatic vessels are alike in the way that they are a system of tubes with endothelial lining, but they differ in some aspects. [19] The structure of the lymphatic vessels is less regular than the one of the blood vessels. [18] They are collapsed under normal conditions. The lymphatic vessels are attached to the fibres of the connective tissue. When the interstitial fluid volume increases, it stretches the dermal fibres which leads to an increase in the diameter of the vessel lumen, even though the interstitial pressure increased. [19]

The lymphatic vessels drain excess fluid, low-molecular-weight solutes, proteins, fragments of cells and inflammatory cells from the interstitial spaces. [20] Reducing the interstitial fluid amount decreases the local pressure and keeps the dermal components like cells and capillaries at a proper distance for nutrition. For instance, Langerhans cells leave the skin through the lymphatic vessels. Directing the antigen-presenting cells to the lymph nodes is crucial in the development of cellular immunity. [19]

The content collected with the capillaries flows through the lower lymphatic vessels, then to the regional lymph nodes and ultimately to the blood vessels. [18] The network of cutaneous lymphatic vessels is somewhat similar to the venous network. The lymphatic vessels form a superficial plexus at the level of the superficial plexus of the blood vessels. [20] The underlying tissue contains pre-collectors, larger post-capillary vessels, which form a subcutaneous plexus under the one of blood vessels. [19], [20] Except for the lymphatic capillaries, the valves help the flow of fluid in the lymphatic vessels. [19]

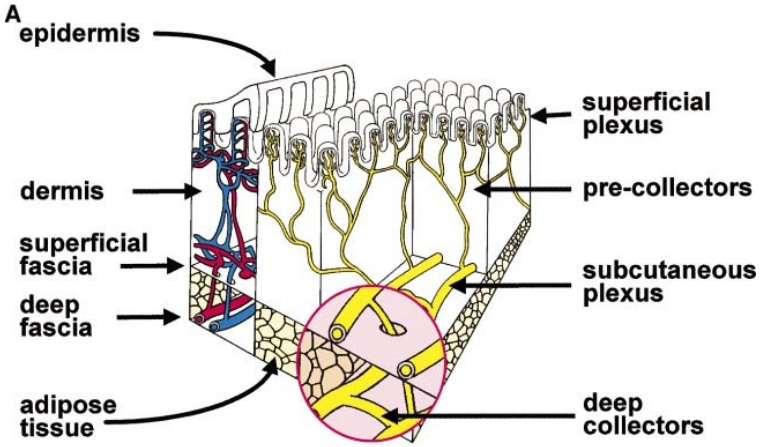

Figure 2 A schematic representation of blood and lymphatic vessels in the skin [20]

1.3.5 Cells inhabiting dermis

The cells take only about 10 % of the dermal content. However, this percentage doesn´t apply for both its layers, as the reticular layer is less cellular that the papillary. The imperative resident cells of the dermis as a connective tissue are fibroblasts. [3] Also, non-fibroblast cells, either resident or immigrant, can be found in the dermis. These encompass adipocytes, mast cells and other immune cells. [6], [10] Mast cells and dermal adipocytes are also considered resident cells. [3]

Stem cells capable of multilineage differentiation are found in various niches in the dermis. Some of the niches are hair follicles, sweat glands, and perivascular areas. [21]

The most abundant cell type in the dermis is **fibroblasts**. Fibroblasts originate from the mesoderm. They are spindle-like shaped. They generate the majority of the extracellular dermal content. [6], [10]

Adipocytes in the dermis are a distinct cell population from the adipocytes residing in the hypodermis. Dermal adipocytes provide insulation, store energy, and support hair follicle regeneration and wound healing. Every adipose depot contains stem cells, which undergo differentiation during wound healing. [22], [23]

Immigrant cells are white blood cells, leukocytes, and their derivates, executing the immunity function. [3] Some immune cells dwell in the dermis normally, some are recruited when a threat occurs. [24], [25], [26] **Leukocytes**, just like other blood cells, differentiate from progenitors produced by hematopoietic stem cells. [3], [26] From the bone marrow, they migrate via the bloodstream. [3] Two main progenitors give rise to immune cell subsets:

common myeloid progenitor and common lymphoid progenitor. [26] Leukocytes enter the dermis when they are needed to promote defence against pathogens, tissue repair, tissue remodelling, disposal of genetically defective tissue (TNF), induction of tolerance towards self-antigens and commensal bacteria. [24], [27]

A variety of leukocyte subsets cooperate with each other and other tissue-resident cells in order to orchestrate an immune response and preserve homeostasis. [27] Dysregulation of the immune responses can lead to cancer, chronic inflammatory diseases like psoriasis, or metabolic diseases, although not all of the mechanisms are known yet. [24], [27], [28] Leukocytes exit the dermis by blood or lymphatic vessels. [24]

Mast cells are typically found in the interfaces between the inner and outer environment such as the skin. In the dermis, they are located close to the blood vessels. Mast cells are able to both support and suppress inflammation. They participate in pathogen defence and wound healing, but also in allergic diseases. [24] Upon stimuli of their IgE receptor, pro-inflammatory and vasodilatory molecules like histamine, heparin, prostaglandins, leukotrienes, and several enzymes are released from their granules. [18], [24]

A common myeloid progenitor can differentiate into several types of cells, among which granulocytes, monocytes, macrophages, and dendritic cells can be found in the dermis. [29]

Although mast cells also contain granules, the term granulocytes regards neutrophils, eosinophils, and basophils. [26] Granular cells are recruited from the blood to tissues invaded by pathogens. Granular cells´ cytoplasm contains granules with enzymes and other substances. [3] These substances get released from stimulated cells and play a role in different immune responses by fighting pathogen invasion. However, the granular substances are also involved in processes leading to allergies or other not-homeostatic states. [30], [31]

Neutrophils are the most abundant leukocytes. Neutrophils respond to chemical signals produced by tissue and tissue-resident immune cells upon stress caused by foreign microorganisms. Neutrophils immigrate to the acutely impacted areas to represent the first line of defence and destroy the pathogens by phagocytosis or release of their antimicrobial granular content. [32]

Eosinophils can release specific toxic proteins, enzymes such as lysozyme and histaminase, cytokines, and chemokines. They help to fight mainly against parasites but can

also harm healthy tissue. [3], [30] Histaminase dampens allergic reactions by the neutralization of a common inflammatory substance histamine. [3]

Basophils represent a very small group of circulating leukocytes. They contribute to immunity by the release of leukotrienes, cytokines, histamine, heparin, and heparin sulfate from their granules. They are the only group of circulating leukocytes that contain histamine, which makes them similar to tissue-resident mast cells. [3], [31]

Monocytes are immune cells that terminally differentiate in tissues into phagocytic macrophages. [3]

Macrophages vary according to the place that they inhabit. [33] Macrophages in the skin represent the most numerous population of immune cells. [24]

Tissue-resident macrophage populations were established in the skin prenatally. Some populations are derived from monocytes immigrating via the bloodstream when the skin is injured or infected. [24]

Macrophages detect and ingest damaged cells and foreign substances. [3], [24], [34] They present the antigen of the digested pathogen on the cell surface to T-lymphocytes and thus continuing the chain of immune responses. [3], [34] Another way macrophages use to inform other immune cells of the pathogen is through the release of cytokines. [34]

In the skin, a macrophage called histiocyte is present. Histiocytes release collagenase, elastase, and other enzymes to digest interstitium. They are involved in tissue repair processes. [18]

Dermal dendrocytes, just like epidermal dendritic Langerhans cells, capture antigens, travel through the lymphatics to lymph nodes to present the antigen to naive T cells. However, their origin and behaviour are not the same. [24] Dermal dendrocytes are characterized by clotting factor VIIIa. [18] Dermal dendrocytes inhabit and leave the skin under both steady-state and threatening conditions. [24] They shape the immune response to microorganisms, vaccines, and tumours, participate in the response to tissue damage, but also generate tolerance to host tissue and commensal bacteria. [24], [25]

A common lymphoid progenitor gives rise to T-lymphocytes and B-lymphocytes, exerting adaptive immunity, and innate lymphoid cells. [27] Lymphocytes travel through the blood stream and lymphatic system. They are released into the dermis when needed and can re-enter these pathways repeatedly. [3]

T cells reside within the epidermis and dermis. [35] T cells have a T-cell receptor, through which they interact with antigen-presenting cells. T cells can be classified according to the variation of the receptor they express. Some express receptors with α and β chains, which are further classified as helper or cytotoxic T cells. The T cells expressing γ and δ chains are referred to as γδ T cells. [36], [37] Besides the antimicrobial function, T cells target tumours and participate in tissue homeostasis and wound healing. [28], [35]

Subtypes of helper T cells (Th cells) need to be in balance otherwise some autoimmune diseases can occur. The Th cells respond to cytokines from other immune cells and produce cytokines to help fight infection. Some Th cells are involved in the control of intracellular pathogens (viruses, some bacteria), and some in the control of extracellular organisms like helminths. However, they do not act directly but rather help other immune cells. [38]

Activated cytotoxic T lymphocytes which recognize the antigen destroy infected or malignant cells while avoiding harm to healthy cells. They help by secretion of cytokines with anti-microbial and anti-tumour effects, by the release of cytotoxic granules, or by binding to a receptor of affected cells which leads to the apoptosis of the targeted cell. [39]

γδ T cells are a heterogeneous population with a variety of functions and memory cell properties. [28] γδ T cells help other immune cells and interact with epithelial cells. They can serve as antigen-presenting cells. They are also capable of releasing cytokines, chemokines, or cytotoxic proteins. They are able to target diverse cancer cells. [37] However, some of the cytokines induce and aggravate psoriasis, although a bigger source of the pathogenic cytokines is a subtype of Th cells. [28], [37]

There are several subtypes of B cells within the dermis with functions in adaptive and innate immunity. [40] B cells respond to pathogens by producing antibodies and produce pro-inflammatory cytokines, but some secrete anti-inflammatory cytokines. [41] With the help of T cells, B cells can differentiate into plasma cells, which also produce antibodies. [18], [40]

Innate lymphoid cells (ILCs) are concentrated at barrier surfaces like the skin. Innate lymphoid cells are involved in innate immunity against all kinds of pathogenic organisms by quickly responding to host-derived signalling molecules. They also have a role in states of chronic inflammation, cancer, and metabolic diseases. ILCs can either be cytotoxic or non-cytotoxic and these groups further involve other subtypes. [27]

Cytotoxic ILCs are represented by natural killer cells. Natural killer cells can fight especially intracellular infections and tumors without previous activation. [27], [42]

Non-cytotoxic ILCs express three subgroups labeled as ILC1, ILC2, and ILC3, from which the second and the third group are found in the dermis. [27]

ILC2s interact with and activate other immune cells. ILC2s produce several cytokines and amphiregulin, a kind of epidermal growth factor. ILC2s with ILC3s promote tissue remodeling and repair to support homeostasis. [27] On the other hand, they are involved in the pathology of eczema and psoriasis. [24], [27] ILC3s produce an interleukin contributing to acanthosis, i.e., skin thickening. [27]

1.3.6 Nerves

Nerve fibers enter the dermis in bundles covered with a membrane and branch into many unmyelinated fibers which are distributed in the upper dermal layers and around appendages. The neural control in the dermis is managed by sensory and autonomic nerves. Tactile, pain, pressure and temperature sensation are transmitted via sensory nerves. The sensory nerves in the dermis are terminated either with a corpuscle of different functions or with free endings. Meissner corpuscles are nerve endings spiraling through Schwann cells and perceive tactile and pressure sensation. Pacinian corpuscles are responsible for vibration perception. The autonomic nerves govern the blood vessels and appendages such as the sweat glands or arrector pili muscles. [18]

1.3.7 Acellular dermal content

The extracellular matrix in the dermis comprises of an amorphous ground substance, in which fibers and cells, vessels and nerves included, are embedded. [2]

1.3.7.1 Fibers

The dermal fibers are arranged in an interwoven network, mostly oriented roughly parallel to the skin surface. [2], [16] They provide the skin with the ability to stretch and contract back to its original shape. [16] The fibers can be distinguished with a light microscopy as three kinds – collagen, reticular and elastic. [43]

The major component of the dermis, constituting about 70 % of its dry weight, is collagen. [18] Collagen is a family of fibrous α-chained proteins of 20 subtypes according to molecular structure, from which at least 15 subtypes are a part of the skin. [17], [18] In the dermis,

mostly type I and type III collagen fibers are found. [10] 80 % of dermal collagen is type I. [18] Collagen is a structural protein conferring tensile strength to the dermis as the fibers are tough and cannot be extended much. [17], [18] Thus they resist tension parallel to their direction which prevents the skin from tearing when stretched. [16], [18]

Collagen fibers are formed by aggregation of several consequentially smaller fibrillar collagen sub-units. The bundle-like structures are stabilized with glycoproteins. [2], [18]

The process of the creation of a collagen fiber begins in the rough endoplasmic reticulum of a fibroblast, which produces procollagen chains. Procollagen, a triple helix molecule, is transformed into tropocollagen. [18] Three to five tropocollagen molecules aggregate into ultrathin filaments. Bundles of these filaments constitute a microfibril. Parallelly bundled microfibrils form long fibrils. [2] Numerous fibrils form the final bundle, which is observed as a white fiber with the naked eye. [2], [18] The fibers can vary in thickness according to the amount of aggregated subunits. With increasing thickness, they get stronger. [18] In the papillary layer, the fibers are thinner and loosely positioned with greater spaces between them. [2], [17] The fibers in the reticular layer are thicker and more densely distributed. [2] The collagen meshwork exhibits remarkable structural integrity and flexibility. [2]

The collagen fibers are continuous with another kind of fiber, called reticular fibers, through an exchange of collagen fibrils. [43] The reticular fibers are composed of a special thin type of collagen III. [10] The reticular fibers can be distinguished from the collagen fibers by the silver staining method. The fibrils of the reticular fibers are quite heavily surrounded with glycoproteins, which most likely allow the silver to attach to them. They are observed as fine meshwork of fibrils. Their fibrils are uniform in diameter and can be observed individually or forming thin bundles. [43] They can be located in perivascular regions, covering the surface of some cells, and underlying the epithelium. [18], [43] The reticular fibers make up about 15 % of all dermal fibers. [18]

Elastic fibers are less tough than collagen fibers, however, they are extremely elastic. [18] The elastic fibers comprise two parts. A core made of elastin is surrounded by microfibrils made of a complex of proteins. The elastic fiber is assembled in the following manner: Secreted tropoelastin coacervates or aggregates via self-assembly. The assembly is facilitated by cell surface components, e.g. by heparan sulfate proteoglycans, on the cell surface. Elastin forms an amorphous hydrophobic core that is enzymatically cross-linked.

The aggregates transfer to microfibrils which compose of glycoproteins like fibrillins and which are up to 15 nm in diameter. The formed elastic fiber is 1-3 μm thick. [18], [44]

The elastic fibers are sparsed between the collagen fibers in the reticular layer in a way that they run more or less parallel to the skin surface. The closer to the epidermis the elastic fibers are, the more perpendicular to the skin surface they are directed and connect to lamina densa perpendicularly. [18]

Another property of the elastic fibers changes according to the depth of the dermis, which is the diameter. The closer to the superior layer, the thinner they are. [18]

1.3.7.2 Ground substance

It is a gel-sol that fills the space between the components in the dermis. The ground substance confers structural support to other dermal components but allows plasma proteins, electrolytes, growth factors, nutrients, and cells to pass through it. It contains water, proteoglycans, and glycoproteins. The proteoglycans are high in molecular weight. They are complexes of a protein core with covalently attached glycosaminoglycans, like hyaluronan and chondroitin sulfate. The proteoglycans, especially their glycosylated component, constitute most of the dermal volume, although they comprise only about 0,2 % of the dry dermal weight. The proteoglycans can bind some chemical mediators, which gives them lubrication and structural properties, and the ability to store substances. Glycoproteins are molecules containing protein and saccharide chains. For instance, fibronectins are glycoproteins mediating interactions between cells and between cells and the ECM. These interactions are crucial for adhesion, migration, and phagocytosis. [6], [10], [45]

1.4 Hypodermis

Hypodermis lies under the dermis and connects the skin to underlying bone and muscles via periostea and fascia. As such, hypodermis is also referred to as subcutaneous tissue. [16], [18] Hypodermis is constituted of a looser connective tissue. Hypodermis contains abundant adipocytes, also called fat cells, and is well vascularized. [3], [6] This layer stores fat which functions as an energy reserve, insulation, heat generator, cushion protecting from external physical pressure, and it also retains moisture. [16], [18] Hypodermal adipocytes take up, store, synthesize, and release fat. Most of their cytoplasm is formed of fatty content. They also affect metabolism by producing hormones, growth factors, and cytokines. [3] Adipocytes assemble into lobules separated by the connective fibroid fat septum.

Hypodermis is connected to the underlying tissues with fiber bundles produced in the hypodermis. [18] Hypodermis, just like the two overlying layers, contains stem cells. [46]

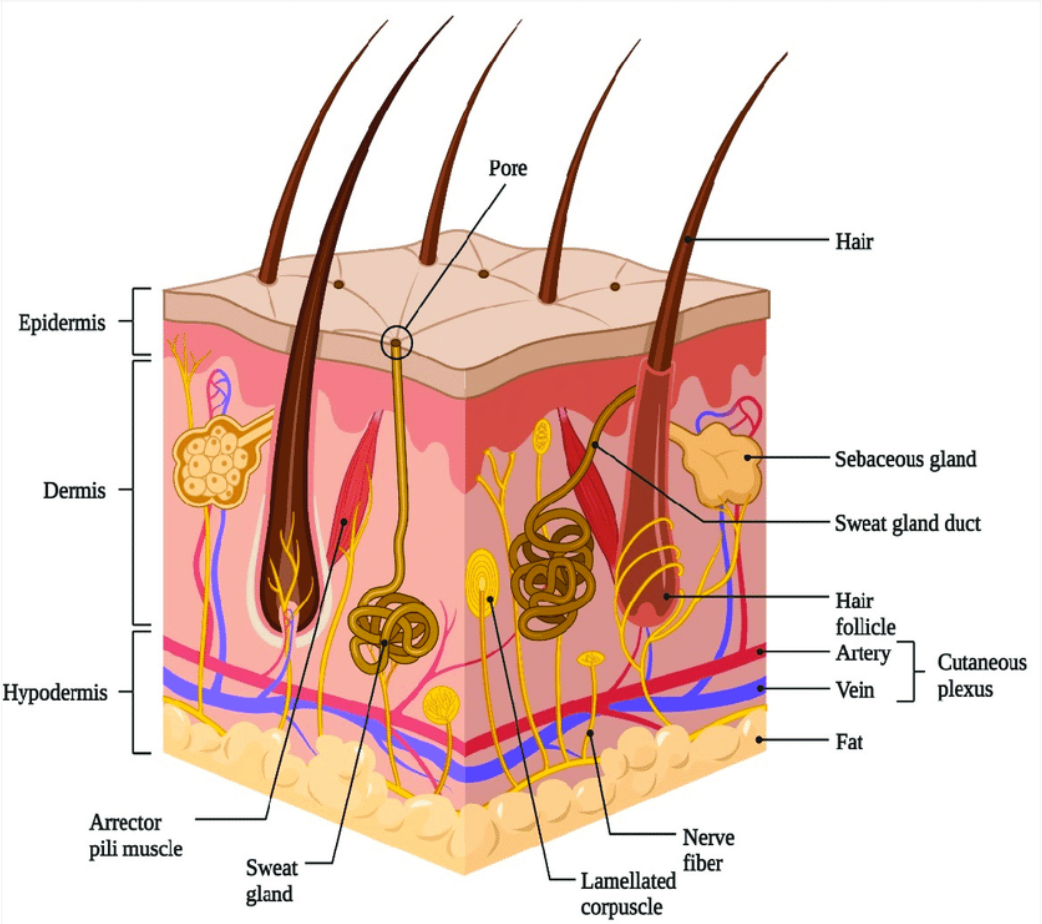

Figure 3 A schematic representation of full-depth skin structure [47]

2 SKIN MODELS

2.1 Introduction

For decades, animal models have been a crucial source of obtaining information on the development of human organism, functions of human body, diseases, and the effect of various factors on the body including toxicology tests. The most common animal models used have been the nematode, the fruit fly, the zebrafish, the common house mouse, and non-human primates. [48] However, animal use in science has several disadvantages, namely the biological differences between other species and humans. Furthermore, ethical problems lead to the prohibition of obtaining toxicology data for cosmetic compounds via animal testing by the European Union (EU) (beginning in 2009), although animals are still being used in related areas for various purposes. [48], [49], [50] For instance, animal models are still crucial in the evaluation of chemicals, systemic toxicity effects, and in preclinical drug development. [50], [51]

Skin models are systems undergoing development as a great interest has been shown to this topic in the last decades and is preserving. The first attempts to prepare a skin substituent in a modern manner in the 1960s resulted from the progress in tissue culturing. [46] Several skin models already exist that are utilized in various fields. The models mimic the functions and structure of real skin up to a certain level of complexity as the skin is a multi-functional organ with many features important to organism´s overall homeostasis. So far, it is usually constructed to model specific function or process rather than fulfilling all the functions of a native in vivo skin, which would be an ideal towards the research can move. It is crucial to choose materials and methods that enable the skin substituents to perform the specific functions as closely as possible to the in vivo skin and exhibit biocompatible properties, especially in the case of those substituents used as implants for surgery patients. The exploitation of skin models has become a popular research direction as the models often provide more accurate results. In addition, it has reduced ethical concerns regarding animal use in dermatology, cosmetic, and preclinical studies. [46] [52], [53]

2.2 Skin models for different utilizations

Skin models encompass animal-derived materials, ex vivo skin explants [54], skin-humanized mouse models [55], human bioengineered skin substituents and equivalents [56], in vitro models like 2D or 3D models, organoids, 3D bioprinted models, skin-on-a-chip

platforms, and artificial skin models [52]. Also, in-silico models are being developed. Mathematical and computational modelling present promising tolls in skin research. [1] In addition, some aspects of the skin reactions towards tested items can be modeled (predicted) using in chemico assays. [57]

The main motivation for skin models/substitutes establishment and expansion has been skin regeneration and testing of different substances, especially drugs and cosmetics. [46] The skin models have been developed and are being developed in ongoing research in order to treat large skin injuries caused by burns, traumas, or chronic inflammation, for skin grafting [56], [52], [58], to study tissue regeneration [55], to research the effect of different external stressors and stimuli (mechanical, electro-magnetic, chemical) on the skin, to develop and validate drugs and therapeutic solutions, to understand pathological skin conditions [52], to assess skin corrosion and skin barrier formation, to test topical irritation substances [59], to perform absorption and permeation studies, to evaluate the safety of chemicals, xenobiotics, pharmaceutical substances, and cosmetics [60], to study inflammatory or cancer skin diseases like psoriasis or melanoma and the safety and efficacy of potential therapeutics, [52], [61], [62], and to study other processes regarding the skin like aging. [63]

In general, skin models should mimic the barrier function of the skin and react to various (hazardous) factors in the same way as living human skin. [49]

In vitro skin models allow experiments to be performed in a controlled and repeatable environment. [52] On the other hand, their lack of complexity often impedes their use alone, and so usually for an adequate evaluation, it is required to perform a combination of different in vitro tests. [57], [64]

2.3 Testing of cosmetics

Two general properties of cosmetic products are crucial. A cosmetic product should be safe to use in regard to both human health and the environment, as well as it should be efficient. To ensure that cosmetic ingredients and products are safe and to support the claims, toxicological and other assessments need to be done. [64]

2.3.1 Regulations and guidelines on product safety

This chapter mentions some of the EU regulations regarding testing of chemicals and cosmetic substances and the intention for reduction, replacement, and refinement of animal

testing, although recently the effort to restrict animal use in research and the pursuit of alternative approaches has been initiated worldwide. [50]

The Organization for Economic Co-operation and Development (OECD) offers guidelines for the assessment of the safety of chemicals for its 38 member countries. The guidelines are being amended according to up-to-date research results so that member governments can include them in their regulations. The potential effect of chemicals on human health and the environment can be explored using these guidelines. Out of the five sections regarding chemicals assessment, Section 4 regards health effects. [65]

The tests for the cosmetic industry used to be done on animals, however, alternative methods have been developed and validated. For evaluations that can be done differently than using animals, animal testing has been banned by the 7th Amendment of the Cosmetics Regulation (EC) No 1223/2009 of the European Parliament and of the Council. [64], [66], [67] The cosmetic regulation has regards to the proposals from the European Commission. This regulation also gives information and sets requirements for cosmetic ingredients and finished products. Some ingredients must be listed due to the risk for human health (colorants, preservatives, UV filters), some are restricted, and some are prohibited. These ingredients are published in different annexes of the cosmetic regulation. Safety assessments of the substances with a potential health risk are done by the Scientific Committee on Consumer Safety (SCCS) prior to ingredients being listed in an annex. [64], [67] The SCCS is an independent body and should assist the European Commission. [66] The SCCS regularly publishes revised Notes of guidance, which rely on scientific opinions and focus on methods that can replace former animal tests. The SCCS have implemented the cosmetic legislation that had undergone changes in the past two decades in their Notes of guidance to help public authorities and cosmetic industry with the methodology of cosmetic substances testing and evaluation with emphasis on ingredients rather than the final products. [64], [67]

According to the cosmetic regulation, for each cosmetic product placed on the market must be designated a responsible person. The responsible person should ensure that the product fulfils the set-out obligations, from which the safety assessment of the product is particularly relevant to the topic of this paper. [66] The safety of the ingredients that are not evaluated by SCCS and annexed must be ensured by the responsible person that is putting them on market. [67] The safety assessment should be carried out by a qualified person and approached using an appropriate weight-of-evidence, considering intended use of the product and systemic exposure to each ingredient, and the safety report should be kept up to

date with new information. Annex I of the regulation states what information and assessment the safety report should contain. [66] Furthermore, Commission Implementing Decision 1213/647/EU provides guidelines on the aforementioned Annex I [68], and Commission Regulation (EU) No 655/2013 specifies criteria for claims justification. [64], [69]

2.3.2 Methods for safety evaluation

The safety assessor can use existing toxicological data and identify data gaps. Any manufactured and marketed substance in the EU must be registered. [64] According to Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), it is required to assess safety of exposure to workers and for the environment for every chemical, including chemicals solely used as a cosmetic ingredient. The requirements should be fulfilled using existing information and alternative test to vertebrate animal models. Animal models should only serve as a last resort. [50]

It might be required or beneficial to provide data on the following toxicological endpoints: skin and eye irritation/corrosion and damage (respectively); skin sensitization; genotoxicity/mutagenicity; carcinogenicity; photo-induced toxicity; and acute systemic, short-term, sub-chronic, long-term, prenatal developmental, one-generation, repeat dose, and reproductive toxicity. [50], [64] Eventually, the understanding of ADME (absorption, distribution, metabolism, excretion) and toxicokinetics may also be of great help to assess cosmetic substances. [50]

Several in chemico, in vivo, in vitro, in silico and mathematical test methods are at the researcher´s disposal to acquire information about the listed endpoints. They are defined in Regulation (EC) No 440/2008 and are largely based on the Test Guidelines from OECD. [50]

Tested health endpoints and suitable methods prescribed by Regulation (EC) No 440/2008 are further enumerated:

Skin corrosion and irritation can be tested in vitro with reconstructed human epidermis (RhE) test method, transcutaneous electrical resistance test, human skin model test, and membrane barrier method, and acute dermal irritation/corrosion can be tested in vivo. [50]

Serious eye damage and irritation can be tested with these in vitro test methods: bovine cornea opacity permeability, isolated chicken eye, fluorescein leakage, short time exposure,

reconstructed human cornea-like epithelium (RhCE), vitrigel-eye irritancy, and macromolecular. Acute eye irritation and corrosion might be tested in vivo. [50]

Allergic reactions are frequent undesirable effects of cosmetics and thus the assessment of **skin sensitization** is of high importance. The in vivo methods include guinea pig tests and local lymph node assays. The in vitro methods include: direct peptide reactivity assay, amino acid derivative reactivity assay, ARE-Nrf2 luciferase tests (evaluating keratinocyte activation), and skin sensitization assays addressing the key event on activation of dendritic cells on the adverse outcome pathway. [50]

In vitro 3T3 NRU **phototoxicity** test serves for assessment of photo-induced toxicity [50], where the cell viability of immortalized mouse fibroblasts BALB/c 3T3 is determined by the uptake of Neutral Red. [70]

Mutagenicity tests encompass in vitro tests such as mammalian chromosome aberration, reverse mutation using bacteria (Ames test), and mammalian cell gene mutation, and in vivo mammalian bone marrow chromosome aberration and mammalian erythrocyte micronucleus tests. Furthermore, **mutagenicity and genotoxicity** can be investigated using in vitro mammalian cell micronucleus test and other in vivo tests: rodent dominant lethal, mammalian spermatogonial chromosome aberration, and mouse heritable translocation test, unscheduled DNA synthesis test with mammalian liver cells, transgenic rodent somatic and germ cell gene mutation assay, and alkaline single-cell gel electrophoresis assay for DNA strand breaks also known as comet assay. [50] The combination of the Ames test and the in vitro micronucleus test is especially recommended for the purpose of genotoxic carcinogen detection. When both tests are positive, the tested chemical is regarded as a mutagen, the negativity of both tests can exclude the mutagenic potential, while if the tests differ in results, further testing is necessary to determine the mutagenicity. The solution in the case of equivocal results might be the comet assay in mammalian cells or on reconstructed human skin. Stem cell-based models, organoids, in silico models, artificial intelligence and other models will become increasingly pertinent. [64]

Besides genotoxic, non-genotoxic substances can have a carcinogenic potential. [64] Tests for **carcinogens** are called: Carcinogenicity test, Combined chronic toxicity/Carcinogenicity test, which are performed in vivo, and In Vitro Mammalian cell transformation test. [50]

Acute systemic toxicity plays a minor role in regards of safety of cosmetics for consumers, as cosmetics usually do not present a risk, and so data on this endpoint are not required. Alternative methods to animal models cannot portray the acute systemic toxicity well enough, thus all validated methods for testing acute systemic toxicity are performed in vivo. If needed, data from studies performed prior to the animal testing ban are often available. However, it is desirable to evaluate nanoparticles, impurities, raw materials, and interactions of ingredients using validated non-animal models. [50]

Repeated dose toxicity is a crucial endpoint to take into account when evaluating the safety of cosmetics because they are repeatedly applied to skin and mucosa. In general, toxicity depends on the route of administration and the duration of exposure. For repeated dose toxicity, data from studies lasting 90 days (subchronic toxicity) or more are preferable, even though 28-day-lasting studies are common and present an asset that can be transformed into subchronic estimation mathematically. The most common routes of administration of the cosmetic test ingredient are oral. Sometimes dermal and rarely inhalation routes are explored. Some studies especially focus on immunotoxicity or neurotoxicity. For repeated dose toxicity, only in vivo methods on rodents are validated. [50]

SCCS mentions in its 12th revision of Notes of Guidance that **reproductive/developmental toxicity** assessment cannot be fully replaced by alternative methods and that a battery of tests should be used for no single alternative assay covers all required information on reproductive and developmental toxicity. Therefore, the data need to be from tests performed prior to animal testing ban or with compliance to legislation regarding tests of compound for other than cosmetic use. Three so called “new approach methods” (NAMs) have been developed for embryotoxicity assessment: the Whole Embryo Culture test, the MicroMass test, and the Embryonic Stem Cell Test. These three tests might be used for qualitative research to categorise non-, weak/moderate-, and strong-embryotoxic substances, but are not suitable for quantitative risk evaluation. The two most common in vivo methods are Two-generation reproductive toxicity study and Prenatal developmental toxicity study performed on rodents or other species. Also some other validated tests are available. [50], [67]

Toxicokinetics, describing the processes of ADME, should be considered when assessing risks to human health. Animal tests for this purpose are restricted, nevertheless necessary in special cases. [67] Testing methods of ADME described in the Regulation 440/2008 are

called: Toxicokinetics, Skin absorption: in vivo method, and Skin absorption: in vitro method. The first two methods are in vivo. [50]

2.4 In-vitro skin models

2.4.1 Brief evolution

Since the 1960s, many attempts to bioengineer skin substituents happened. A skin graft from rabbit skin epithelial cells was proposed. In the 1970s, Rheinwald and Green cultured an epidermis from the host´s own – autologous – keratinocytes. It was made possible to serially cultivate and produce large quantities of keratinocytes. Cultured epidermal autografts emerged in the 1980s and soon became commercially available, to cover skin wounds. Dermal substituents were developed along the epidermal grafts and appeared on the market. With the use of epidermal substituents, the need for a vascularized dermal bed was recognized. Bi-layered substituents comprising of both epidermis and dermis were developed in the 1990s. The establishment of tissue engineering, combining engineering and biological knowledge and approaches, was attributed to Langer and Vacanti. Since then, many in vitro reconstructed skin model products have been offered on the market by various brands including big cosmetic companies such as L´Oréal and used commonly instead of animal models for testing and to treat extensive skin injuries. [46]

2.4.2 Human skin explants

Human skin explants may be obtained from cadavers, cosmetic surgeries, or biopsies. [49] They have been successfully used to answer basic questions about organ development and disease, and in irritancy and efficacy studies of cosmetics and drugs. However, their use is limited largely by being short in supply, life span, and due to ethical reasons and law. [48], [49] Thus, a different approach needs to be taken. Construing models by culturing cell lines and seeding them on scaffolds presents a good alternative, as well as even more complex models combining cell culturing with engineered microdevices end microfluidic systems. [48]

2.4.3 Reconstituted skin models

Skin substituents prepared in vitro by seeding corresponding cell types on scaffolds mimicking the extracellular matrix are called reconstituted or reconstructed skin models or equivalents. [46] There are several types of skin models based on different criteria. The

models can be cellular or acellular. They can be constituted of just one layer (epidermal or dermal) or epidermis and dermis or all three of the layers including the hypodermis (composite models). [56], [71] Based on the origin, the cells are called autologous if they come from the body of the latter recipient or allogeneic if they come from another source. [56] Also, the material, source, and preparation technique of the scaffold differ and determine the use of the whole model. [52]

Scaffolds, serving as ECM, should induce an environment for the cells that is analogous to the conditions in the body – encourage the micronutrient distribution, provide support for attachment and enable proliferation in multiple directions. Scaffolds can be made of natural or synthetic materials or a combination. They are often made of decellularized ECM, which can be derived from animal tissues. [52] Hydrogels, chitosan, collagen, fibrin, hyaluronic acid, alginate are common natural materials, also synthetic polymers can be used. [46], [52] Scaffolds can be fabricated with numerous techniques, for example electrospinning, freeze-drying, photo crosslinking, melt moulding, solvent casting, and 3D printing. [52]

The following sub-chapters describe different kinds of skin culture models and their utilities and limitations.

2.4.3.1 2D cell culture models

Quite simple models grown on a flat – 2D – surface have been valuable systems broadly used for research for over a century. 2D models helped in understanding of cell behaviour and reactions to biochemical and biophysical factors. On the other hand, cells in the environment of 2D cultures can show different morphology and behaviour than in in vivo tissues, as 2D cultures provide rigid conditions in comparison to more advanced models simulating natural conditions more closely. The cells are cultured attached on a polystyrene or glass dish. Cells in a monolayer can grow homogenously thanks to the equal access to nutrients. The dead cells detach into the medium. [46]

A common system is a keratinocyte monolayer used as an epidermal model utilized in permeation studies and screening of substances. In an air-liquid interface (ALI), keratinocytes can form a stratified structure. [46] For this purpose, early passage keratinocytes should be used. The stratification is controlled by using high-calcium medium. [72] 2D models provide acceptable approximation for many epithelial tissues. Binding the cells to the surface is often aided by seeding them on a collagen I or fibronectin coating substituting ECM, however this only allows ventral adhesion. Further approximation of

these system to in vivo conditions is using a sandwich model where the cell layer is overlayed with another coated substrate and thus both ventral and dorsal cell receptors are activated. Good receptor engagement enables more accurate modulation of cell morphology, adhesion, migration, and intracellular signalling pathways. [46]

Overall disadvantage of 2D models is that the ECM deposition, the secretion of growth factors and the gene expression differs from those in vivo, therefore 2D systems insufficiently perform the natural processes like cell-cell and cell-matrix interaction and signalling pathways. [46]

2.4.3.2 3D cell culture models

Generally, 3D skin models are used in therapeutic and cosmetic studies and clinical applications. [46]

2.4.3.2.1 Organoids

One of the most recent in vitro human organ models are organoids. Organoids are formed by the self-assembly of cells. They can mimic development and architecture and function (even disease) of different organs, including skin. [48]

Stem cells seem to be a good choice for organoids. Pluripotent stem cells (PSC) exhibit organization and form complex structures in organoids modelling development of embryonic and fetal stages of a particular organ. After the PSCs undergo an expansion phase and aggregate, they are exposed to different culture conditions in a stepwise manner to induce subsequent developmental stages. Adult stem cells (ASC) are suitable for organoids modelling function of adult tissues. ASC organoids often model only an epithelial part of the represented organ. Organoids composed of ASC take little time to grow, which makes them great tools for personalised medicine. [48]

It is possible to prepare organoids comprising of multiple skin types including skin appendages. [48] Lee et al. cultured cells for 4-5 months, modulating signaling pathways, before they developed an inside-out cyst-like skin structure comprising of cells that self-assembled to form stratified epidermis, fat-rich dermis with fibroblasts, cartilage, melanocytes, neuron network and Schwann cells targeting Merkel cells in pigmented hair follicles equipped with sebaceous glands. This construct almost completely mimics skin and promises an improvement in disease modelling, drug development, and skin grafting. [48], [73]

An issue for organoids nor including vasculature is the formation of necrotic tissue due to insufficient oxygen and nutrient supply, especially in the most inner parts. [48]

The ECM used in many organoids is animal derived. That limits their potential for clinical application. Besides, the production method, involving inducing tumours in mice, is not exactly ethical and human sources are restricted. Their advantage, on the other hand, is mechanical support and the preservation of native signalling. Single and multiple components of the ECM or synthetic hydrogels might suffice as well. [48]

Some organoids do not rely on an externally provided ECM component since the fibroblasts produce the ECM themselves. These scaffold-free organoids are also referred to as spheroids or microtissues. [46], [48] Fibroblasts produce the ECM core, which gets surrounded with keratinocytes, eventually other cells like melanoma cells. Spheroids are non-adherent aggregates, they can be prepared on low-adhesion, round-bottom surface, but are usually produced by the hanging drop method where the cells self-assemble and form the structure. Spheroids are fully immersed in the culture medium. Even though this allows efficient fabrication and reproducibility of tests, the situation in vivo differs in the presence of a gaseous environment on one side. Spheroids find applications mainly in toxicological assessment and development of anticancer drugs. They can adequately imitate the ECM deposition, intercellular interactions, and generate gradients of nutrients, waste, and gases. [46]

Organoids offer option to study organogenesis and organ function and even the processes that were difficult to study in animals, and allow high through-put screening of chemicals and assessment of their effect on healthy or diseased human organs in different developmental stages. [48]

2.4.3.2.2 Organotypic co-cultures

Another kind of scaffold-free skin equivalent are cell sheet-based constructs of organotypic co-cultures. They can be prepared on transwell plates. First, dermal fibroblasts form a dermal layer; ascorbic acid can support the ECM protein deposition. Keratinocytes are seeded on the dermal layer and co-cultured with the fibroblasts. The stratification and keratinization of the epidermal layer is aided by lifting the well to an air-liquid interface (ALI). Hear-bearing model can be prepared by embedding pilosebaceous units in the dermal layer. Moreover, co-culturing of endothelial cells with fibroblasts enables the formation of a vascularized dermal equivalent, over which keratinocytes can be again seeded. These

constructs are able to deposit ECM, exhibit high diffusion rates of nutrients, and allow interactions with cell proteins which makes them suitable for tissue repair applications. [46]

2.4.3.2.3 Reconstructed human epidermis

The basic version of reconstructed human epidermis (RHE) is composed of keratinocytes seeded on a polycarbonate membrane cultured at an ALI to form strata. [46] They have been broadly accepted for many applications, e.g. skin irritation, corrosion, phototoxicity, and absorption studies. [46], [49] Reconstructed epidermal models can be transformed using a set of cytokines into psoriasis-like models by altering gene-expression and phenotype. [72] Developed models should be proven to exhibit comparable features to in vivo skin to be accepted as a reliable research tool. Some of the important features assessed when evaluating different epidermal models are morphology (macro- and microscopic appearance and structure), lipid composition (the ratio of ceramides – normally mainly in the stratum corneum – to phospholipids – normally mainly in the viable strata; crucial for permeability, flexibility, and other aspects), and biochemical markers (models should contain similar amounts of proteins, e.g. keratin 1 and 10, loricrin, involucrin, which are specific for the degree of differentiation in corresponding sub-layers). Their suitability for different usage can be compared by testing of phototoxicity (application of phototoxic substances irradiated at different wavelengths), irritancy (application of irritating substances to evaluate the biochemical and histological reaction), and transport of different substances through the model (permeated amount measured as a function of time). [49]

Some of the commercially available RHE models are EpiSkin, SkinEthic, and EpiDerm. SkinEthic by L´Oréal company comprises 3 layers. Second-passage keratinocytes are cultured for 13 days to form a stratified epidermal layer which is then placed on type IV human collagen film covering type I bovine collagen matrix serving as a dermal layer in the base. SkinEthic by SkinEthic Laboratoties is composed of normal human keratinocytes on polycarbonate filters cultured for 17 days on an ALI. EpiDerm by MatTek Corporation is composed of normal human epidermal keratinocytes (NHEK), which differentiate into epidermal sublayers. All these models find application in toxicity, including phototoxicity testing. [49]

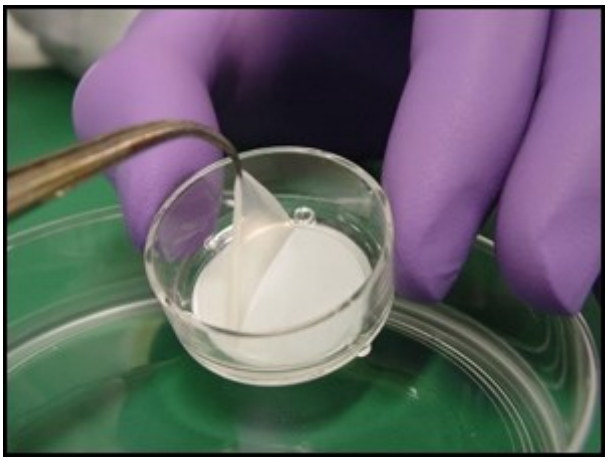

Figure 4 A photograph of EpiSkin model
detaching from the filter [74]

On one hand, the models exhibit lower barrier function in comparison to human skin, which is also their greatest weakness, on the other hand, they seem to show more consistent results then human skin, as the human skin can vary in some factors. [49]

Important reason for low barrier function of the RHE models is the absence of dermis, which in in vivo conditions acts as a barrier against lipophilic chemicals. Thus, transport studies might suggest higher permeability than it would be in in vivo. Furthermore, the dermal capillaries would remove the substances which would pass through the epidermis, while in vitro, the dermal aqueous environment hinders the permeation of lipophilic substances. The absence of dermal vasculature is thus another aspect that differentiates these models from in vivo skin. [49]

Their other shortcoming is the absence of the other components constituting epidermis such as the ECM, melanocytes, and immune cells. This limits the immunological response to the one that keratinocytes can perform. [46]

Still, RHEs are quite easy to reproduce and have a high throughput capacity. [46]

2.4.3.2.4 Human skin equivalent (dermo-epidermal model)

8 (HSE), also referred to as full-thickness models or reconstructed skin, were developed by combining a dermal and an epidermal layer in one construct. The epidermal equivalent is put on top of the dermal equivalent and the cells from both parts participate in creating a dermal-epidermal juncture, which usually lacks the waving pattern except for some cases of use of “de-epidermized” dermis. General common fabrication of HSE proceeds in following order: An acellular collagen layer is prepared on an insert membrane. The plain collagen

prevents the latter applied layer from contracting and detaching. Collagen matrix containing human fibroblasts is cultured for a week, as the fibroblasts remodel and secret new ECM, after that the cellular collagen is put on top of the acellular collagen. Keratinocytes are seeded on the collagen layers, after the layers stabilize, to form a confluent monolayer. Then, lifted to an ALI, the keratinocytes form a differentiated, organized, stratified epidermis. The HSE is superior to THE in the possible crosstalk between epidermis and dermis, which is crucial for homeostasis. [46]

Some of the commercially available HSE models are PhenionFT and EpidermFT. These models can be utilized in skin ageing, penetration, metabolism, genotoxicity, wound healing, disease processes, and sensitization studies and environmental effects studies. [46]

2.4.3.2.5 Possible improvements of skin equivalents

To prepare a skin model that would encompass skin appendages remains a challenge. [73] However, it is important to include skin appendages, as well as other parts of skin like hypodermis and vessels to mimic the functions of the native skin more closely. [46] The method of bioprinting and 3D cell printing seem to offer technological advances and solutions for these issues. [71], [75] Other aspect could be considered in modelling the complexity of the skin, which is the presence and interaction of microbiota, since the microbiota contributes to the barrier ability of the native skin. For instance, a model composed of agar layer and callus suspension was developed to grow and research skin bacteria. As more advanced 3D models have been developed, they can be used to investigate the skin-microbiota interplay. [5] Furthermore, integrating immune cells in the models would include the crosstalk between keratinocytes and immune cells and allow to study the inflammatory processes in the skin. Such models have also been introduced. [5]

2.4.4 Skin-on-a-chip

Various microfluidic systems for research and industry have been developed during the last decade. The interest in organ-on-a-chip systems, developed of skin equivalents on microfluidic platforms, is rising. It is possible to test organs like the liver, kidney, and heart using these minimized platforms and even connect these organ equivalents together micro-fluidically. Skin-on-a-chip can facilitate dermatological, pharmacological, cosmetic, or toxicological research. These devices could potentially replace animal testing and, thanks to micronization, lower material and tissue expenses. With skin-on-a-chip, it is possible to study various processes such as diffusion, wound healing, ageing, repair, disease pathology,

shear stress and other. The results obtained with skin-on-a-chip can be more reliable than animal studies. The flow of the fluid – medium – can be controlled. The flow enables communication between different parts of the skin, allows long-term viability due to nutrient supply better mimicking physiological conditions and induces shear stress supporting the formation of skin layers. Mathematical models are useful in predicting physical phenomena in the chips like fluid movement, drug diffusion, and heat transfer. [76]

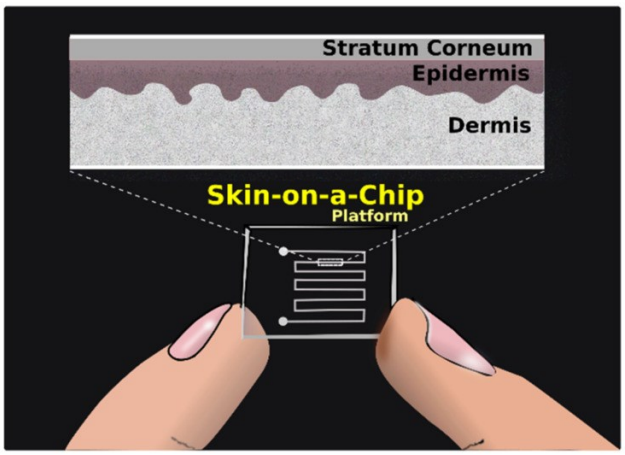

Figure 5 A sketch of skin-on-a-chip [76]

Different structures according to its complexity can be used to as diffusion materials for skin on a chip. Probably the least sophisticated structure is a membrane, which consists of specific macromolecular substances that can further be layered. It is important that the membrane is porous, compatible with a solvent, does not chemically react with other materials or chemicals, and available for purchase. Their availability, the possibility to store them, zero ethical concern regarding animal use, and high reproducibility and low variability of results makes them desirable for many research applications. They are frequently used in diffusion studies. Various membranes can differ in material, thickness, porosity, or tortuosity, which are all properties to consider when choosing a membrane. Most of the membranes are based on silicon, many on cellulose-acetate, some on synthetic polymers or their combination with chitosan and a membrane called StratM of multi-layered polyester sulfone became popular in the last few years. The biggest limitation of membranes presents the inability to imitate the barrier function. Many membranes are unacceptable to study metabolism, distribution, and excretion. [76]

Other than membrane, excised human or animal skins are an option as a diffusion material. Human ex vivo samples vary in thickness according to the source body location.

Skin excised from pig resembles human skin in structure and barrier function, contrary to rat skin, which is more permeable. [76]

Reconstructed tissues, either commercial or in-house produced, might be chosen as well. RHEs and HSEs are commonly used. Their advantage is stratified epidermis, but they might lack dermal layer, adipose tissue, immune cells, vasculature, or appendages. [76]

CONCLUSION

Many industry fields and health sector use various chemical substances for various reasons. The chemicals might affect the workers who come into contact with them, the consumers of commercial and medical products, and the environment. Therefore, it is necessary to investigate the effect of the substances on human health and the environment to prove their safety and efficacy. A different approach to the testing of chemicals can be taken, however legislation gives the frame of what is mandatory, possible, and forbidden. Although animals have been used for testing for a long time and provide certain advantages, it is unethical to use them, therefore many attempts to eliminate their exportation have been made, suggesting alternatives for testing and validating "new approach methods". Animal models might also exhibit inaccurate characteristics when assessing the effects on humans due to inter-species differences. In the European Union, Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) demands every marketed chemical to be registered, which includes cosmetic ingredients. The main legislative demands on cosmetics are given by the Cosmetics Regulation (EC) No 1223/2009 of the European Parliament and of the Council. Regulation (EC) No 440/2008 prescribes validated testing methods. In correlation with these regulations, the Scientific Committee on Consumer Safety provides guidelines on testing methods. More general guidelines are also available from OECD.

Except for in vivo animal models, it is possible to assess many features of tested ingredients and formulations by in chemico, ex vivo, in vitro, and in in silico modelling.

Since the beginning of cell culturing, and tissue engineering, many cellular and tissue models to test ingredients have been developed. Some of them were validated and adapted by the laws and guidelines. Most common in vitro models include reconstructed human epidermis (RHE) and human skin equivalts (HSE) comprising epidermal and dermal layer.

The general requirement for the models is to accurately model skin functions and reaction to various factors. It is still not possible to use one model which would encompass all functions of the skin, and thus, a battery of tests needs to be used for a complex evaluation. The limitations of RHEs are mainly their lower barrier function than the in vivo skin and the lack of other cutaneous components. However, thy are widely used for good reproducibility and low variability in results. Many models can be transformed to mimic pathological processes, diseases. HSEs present a step forward towards the native skin complexity,

however, further improvement would be beneficial. Advances in in vitro skin models would be incorporation of the hypodermis, appendages, vasculature, nerves, immune cells, and skin microbiota. The listed components of skin interact with each other, that is a crucial factor. It is important to realize that the skin is a complex organ interacting with the inner and outer environment, exhibiting hierarchical and interconnected structure and various vital functions for the homeostasis of human organism.

Furthermore, transferring the in vitro reconstituted models from the static conditions, provided solely by cell culturing techniques, on microfluidic platforms would greatly improve the reliability in mimicking the dynamic conditions. Skin-on-a-chip could be used in many fields for testing substances for pharmacology, toxicology, and cosmetic science. It brings promises for novel methods and in combination with computational and mathematical modelling could move the research regarding human health much further.

BIBLIOGRAPHY

[1] G. Limbert, 'Mathematical and computational modelling of skin biophysics: a review', *Proceedings of the Royal Society A: Mathematical, Physical and Engineering Sciences*, vol. 473, no. 2203, p. 20170257, ervenec 2017, doi: 10.1098/rspa.2017.0257.

[2] W. Montagna, *The structure and function of skin*. New York: Academic Press, 1956.

[3] F. E. Hossler, *Ultrastructure Atlas of Human Tissues*, 1st ed. Hoboken, New Jersey: John Wiley & Sons, Ltd, 2014. Accessed: Jan. 20, 2022. [Online]. Available: https://onlinelibrary.wiley.com/doi/book/10.1002/9781118284551

[4] L. P. da Silva, M. T. Cerqueira, and A. P. Marques, 'Regeneration Using Tissue Engineered Skin Strategies', in *Therapeutic Dressings and Wound Healing Applications*, John Wiley & Sons, Ltd, 2020, pp. 255–289. doi: 10.1002/9781119433316.ch12.

[5] F. Rademacher, M. Simanski, R. Gläser, and J. Harder, 'Skin microbiota and human 3D skin models', *Experimental Dermatology*, vol. 27, no. 5, pp. 489–494, 2018, doi: 10.1111/exd.13517.

[6] W.-M. Woo, 'Skin Structure and Biology', in *Imaging Technologies and Transdermal Delivery in Skin Disorders*, John Wiley & Sons, Ltd, 2019, pp. 1–14. doi: 10.1002/9783527814633.ch1.

[7] L. Glass, '1 Anatomy of the Eyelids, Orbit, and Lacrimal System', *Ento Key*, May 07, 2020. https://entokey.com/1-anatomy-of-the-eyelids-orbit-and-lacrimal-system/ (accessed Jan. 20, 2022).

[8] J. Daintith and E. Martin, 'Dictionary of Science (6th Edition)', [Online]. Available: https://app.knovel.com/hotlink/toc/id:kpDSE00001/dictionary-science-6th/dictionary-science-6th

[9] R. W. Putnam, '17 - Polarity of Cell Membranes', in *Cell Physiology Source Book*, N. Sperelakis, Ed., Academic Press, 1995, pp. 230–242. doi: 10.1016/B978-0-12-656970-4.50023-3.

[10] E. A. Mauldin and J. Peters-Kennedy, 'Chapter 6 - Integumentary System', in *Jubb, Kennedy & Palmer's Pathology of Domestic Animals: Volume 1 (Sixth Edition)*, M. G. Maxie, Ed., W.B. Saunders, 2016, pp. 509-736.e1. doi: 10.1016/B978-0-7020-5317-7.00006-0.

[11] E. Chermnykh, E. Kalabusheva, and E. Vorotelyak, 'Extracellular Matrix as a Regulator of Epidermal Stem Cell Fate', *International Journal of Molecular Sciences*, vol. 19, p. 1003, březen 2018, doi: 10.3390/ijms19041003.

[12] H. Yousef, M. Alhajj, and S. Sharma, 'Anatomy, Skin (Integument), Epidermis', in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2022. Accessed: Jan. 20, 2022. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK470464/

[13] E. Martin and R. Hine, *A Dictionary of Biology*. Oxford University Press, 2008. Accessed: Jan. 21, 2022. [Online]. Available: https://www.oxfordreference.com/view/10.1093/acref/9780199204625.001.0001/acref-9780199204625

[14] P. Wertz, 'Epidermal Lamellar Granules', *SPP*, vol. 31, no. 5, pp. 262–268, 2018, doi: 10.1159/000491757.

[15] 'Lamellar Bodies and Keratohyalin Granules in Normal Human Epidermal Keratinocytes » Nanolive - A complete solution for your label-free live cell imaging', *Nanolive - A complete solution for your label-free live cell imaging*, Sep. 06, 2019. https://www.nanolive.ch/keratinocytes-promocell/ (accessed May 11, 2022).

[16] E. McLafferty, C. Hendry, and A. Farley, 'The integumentary system: anatomy, physiology and function of skin', *Nursing Standard (through 2013)*, vol. 27, no. 3, pp.

35–42, Sep. 2012, Accessed: May 11, 2022. [Online]. Available: https://www.proquest.com/docview/1081981576/abstract/99C2FD3F67454APQ/1

[17] P. A. J. Kolarsick, M. A. Kolarsick, and C. Goodwin, 'Anatomy and Physiology of the Skin', *Journal of the Dermatology Nurses' Association*, vol. 3, no. 4, pp. 203–213, 2011, doi: 10.1097/jdn.0b013e3182274a98.

[18] Hiroshi Shimizu, *Shimizu's Textbook of Dermatology*. Hokkaido University Press/Nakayama Shoten, 2007.

[19] M. Skobe and M. Detmar, 'Structure, Function, and Molecular Control of the Skin Lymphatic System', *Journal of Investigative Dermatology Symposium Proceedings*, vol. 5, no. 1, pp. 14–19, Dec. 2000, doi: 10.1046/j.1087-0024.2000.00001.x.

[20] G. Mariani *et al.*, 'Radioguided Sentinel Lymph Node Biopsy in Breast Cancer Surgery*', *Journal of Nuclear Medicine*, vol. 42, no. 8, pp. 1198–1215, Aug. 2001, Accessed: May 07, 2023. [Online]. Available: https://jnm.snmjournals.org/content/42/8/1198

[21] N. Vapniarsky, B. Arzi, J. C. Hu, J. A. Nolta, and K. A. Athanasiou, 'Concise Review: Human Dermis as an Autologous Source of Stem Cells for Tissue Engineering and Regenerative Medicine', *Stem Cells Transl Med*, vol. 4, no. 10, pp. 1187–1198, Oct. 2015, doi: 10.5966/sctm.2015-0084.

[22] T. M. Brown and K. Krishnamurthy, 'Histology, Dermis', in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2023. Accessed: May 07, 2023. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK535346/

[23] I. L. Kruglikov and P. E. Scherer, 'DERMAL ADIPOCYTES: FROM IRRELEVANCE TO METABOLIC TARGETS?', *Trends Endocrinol Metab*, vol. 27, no. 1, pp. 1–10, Jan. 2016, doi: 10.1016/j.tem.2015.11.002.

[24] S. S. Tay, B. Roediger, P. L. Tong, S. Tikoo, and W. Weninger, 'The Skin-Resident Immune Network', *Curr Dermatol Rep*, vol. 3, no. 1, pp. 13–22, 2014, doi: 10.1007/s13671-013-0063-9.

[25] M. Haniffa, M. Gunawan, and L. Jardine, 'Human skin dendritic cells in health and disease', *J Dermatol Sci*, vol. 77, no. 2, pp. 85–92, Feb. 2015, doi: 10.1016/j.jdermsci.2014.08.012.

[26] G. A. Parker, Ed., *Immunopathology in Toxicology and Drug Development: Volume 1, Immunobiology, Investigative Techniques, and Special Studies*. in Molecular and Integrative Toxicology. Cham: Springer International Publishing, 2017. doi: 10.1007/978-3-319-47377-2.

[27] D. Artis and H. Spits, 'The biology of innate lymphoid cells', *Nature*, vol. 517, no. 7534, Art. no. 7534, Jan. 2015, doi: 10.1038/nature14189.

[28] C. Qi, Y. Wang, P. Li, and J. Zhao, 'Gamma Delta T Cells and Their Pathogenic Role in Psoriasis', *Frontiers in Immunology*, vol. 12, 2021, Accessed: May 07, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fimmu.2021.627139

[29] E. Lebegge, S. M. Arnouk, P. M. R. Bardet, M. Kiss, G. Raes, and J. A. Van Ginderachter, 'Innate Immune Defense Mechanisms by Myeloid Cells That Hamper Cancer Immunotherapy', *Frontiers in Immunology*, vol. 11, 2020, Accessed: May 07, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01395

[30] 'Eosinophils | British Society for Immunology'. https://www.immunology.org/public-information/bitesized-immunology/cells/eosinophils (accessed May 07, 2023).

[31] 'Basophils | British Society for Immunology'. https://www.immunology.org/public-information/bitesized-immunology/cells/basophils (accessed May 07, 2023).

[32] 'Neutrophils | British Society for Immunology'. https://www.immunology.org/public-information/bitesized-immunology/cells/neutrophils (accessed May 07, 2023).
[33] P. J. Murray and T. A. Wynn, 'Protective and pathogenic functions of macrophage subsets', *Nat Rev Immunol*, vol. 11, no. 11, pp. 723–737, Oct. 2011, doi: 10.1038/nri3073.
[34] 'Macrophages | British Society for Immunology'. https://www.immunology.org/public-information/bitesized-immunology/cells/macrophages (accessed May 07, 2023).
[35] L. D. Munoz, M. J. Sweeney, and J. M. Jameson, 'Skin Resident γδ T Cell Function and Regulation in Wound Repair', *Int J Mol Sci*, vol. 21, no. 23, p. 9286, Dec. 2020, doi: 10.3390/ijms21239286.
[36] 'Helper and Cytotoxic T Cells | British Society for Immunology'. https://www.immunology.org/public-information/bitesized-immunology/cells/helper-and-cytotoxic-t-cells (accessed May 07, 2023).
[37] 'Gamma Delta (γδ) T Cells | British Society for Immunology'. https://www.immunology.org/public-information/bitesized-immunology/cells/gamma-delta-gd-t-cells (accessed May 07, 2023).
[38] 'Cells T CD4+ | British Society for Immunology'. https://www.immunology.org/public-information/bitesized-immunology/cells/cells-t-cd4 (accessed May 07, 2023).
[39] 'Cells T CD8+ | British Society for Immunology'. https://www.immunology.org/public-information/bitesized-immunology/cells/cells-t-cd8 (accessed May 07, 2023).
[40] 'B Cells | British Society for Immunology'. https://www.immunology.org/public-information/bitesized-immunology/cells/b-cells (accessed May 07, 2023).
[41] G. F. Debes and S. E. McGettigan, 'Skin-associated B cells in health and inflammation', *J Immunol*, vol. 202, no. 6, pp. 1659–1666, Mar. 2019, doi: 10.4049/jimmunol.1801211.
[42] 'Natural Killer Cells | British Society for Immunology'. https://www.immunology.org/public-information/bitesized-immunology/cells/natural-killer-cells (accessed May 07, 2023).
[43] T. Ushiki, 'Collagen fibers, reticular fibers and elastic fibers. A comprehensive understanding from a morphological viewpoint', *Arch Histol Cytol*, vol. 65, no. 2, pp. 109–126, Jun. 2002, doi: 10.1679/aohc.65.109.
[44] A. D. Bradshaw, 'The Extracellular Matrix', in *Encyclopedia of Cell Biology (Second Edition)*, R. A. Bradshaw, G. W. Hart, and P. D. Stahl, Eds., Oxford: Academic Press, 2023, pp. 211–221. doi: 10.1016/B978-0-12-821618-7.00088-2.
[45] 'What is a Glycoprotein?', *News-Medical.net*, Jun. 15, 2020. https://www.news-medical.net/health/What-is-a-Glycoprotein.aspx (accessed May 17, 2022).
[46] T. Zidarič, K. S. Kleinschek, U. Maver, and T. Maver, *Function-Oriented Bioengineered Skin Equivalents: Continuous Development Towards Complete Skin Replication*. Springer Nature, 2023.
[47] 'Figure 1. Schematic representation of basic human skin anatomy...', *ResearchGate*. https://www.researchgate.net/figure/Schematic-representation-of-basic-human-skin-anatomy-depicting-the-different-skin-layers_fig1_362551152 (accessed May 19, 2023).
[48] N. S. Corsini and J. A. Knoblich, 'Human organoids: New strategies and methods for analyzing human development and disease', *Cell*, vol. 185, no. 15, pp. 2756–2769, Jul. 2022, doi: 10.1016/j.cell.2022.06.051.

[49] F. Netzlaff, C.-M. Lehr, P. W. Wertz, and U. F. Schaefer, 'The human epidermis models EpiSkin®, SkinEthic® and EpiDerm®: An evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport', *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 60, no. 2, pp. 167–178, Jul. 2005, doi: 10.1016/j.ejpb.2005.03.004.

[50] F. Pistollato *et al.*, 'Current EU regulatory requirements for the assessment of chemicals and cosmetic products: challenges and opportunities for introducing new approach methodologies', *Arch Toxicol*, vol. 95, no. 6, pp. 1867–1897, Jun. 2021, doi: 10.1007/s00204-021-03034-y.

[51] M. A. Wallig, W. M. Haschek, C. G. Rousseaux, and B. Bolon, Eds., 'Chapter 22 - Special Senses', in *Fundamentals of Toxicologic Pathology (Third Edition)*, Academic Press, 2018, pp. 673–747. doi: 10.1016/B978-0-12-809841-7.00022-8.

[52] E. Sutterby, P. Thurgood, S. Baratchi, K. Khoshmanesh, and E. Pirogova, 'Evaluation of in vitro human skin models for studying effects of external stressors and stimuli and developing treatment modalities', *VIEW*, vol. 3, no. 2, p. 20210012, 2022, doi: 10.1002/VIW.20210012.

[53] 'Innovations in Human Skin Models | Frontiers Research Topic'. https://www.frontiersin.org/research-topics/51357/innovations-in-human-skin-models (accessed May 09, 2023).

[54] E. Rousi *et al.*, 'An innervated skin 3D in vitro model for dermatological research', *In vitro models*, Jun. 2022, doi: 10.1007/s44164-022-00021-0.

[55] M. A. Nilforoushzadeh *et al.*, 'Tissue engineering in dermatology - from lab to market', *Tissue and Cell*, vol. 74, p. 101717, Feb. 2022, doi: 10.1016/j.tice.2021.101717.

[56] S. Tavakoli and A. S. Klar, 'Bioengineered Skin Substitutes: Advances and Future Trends', *Applied Sciences*, vol. 11, no. 4, Art. no. 4, Jan. 2021, doi: 10.3390/app11041493.

[57] R. Kreiling *et al.*, 'In chemico, in vitro and in vivo comparison of the skin sensitizing potential of eight unsaturated and one saturated lipid compounds', *Regulatory Toxicology and Pharmacology*, vol. 90, pp. 262–276, Nov. 2017, doi: 10.1016/j.yrtph.2017.09.023.

[58] 'Skin substitutes - UpToDate'. https://www.uptodate.com/contents/skin-substitutes#!# (accessed May 08, 2023).

[59] F. F. Schmidt, S. Nowakowski, and P. J. Kluger, 'Improvement of a Three-Layered in vitro Skin Model for Topical Application of Irritating Substances', *Frontiers in Bioengineering and Biotechnology*, vol. 8, 2020, Accessed: May 08, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fbioe.2020.00388

[60] B. Sarmento, '3.8.1 Introduction', in *Concepts and Models for Drug Permeability Studies - Cell and Tissue Based in vitro Culture Models*, Elsevier. [Online]. Available: https://app.knovel.com/hotlink/pdf/id:kt00URYJU2/concepts-models-drug/concepts-m-introduction-5

[61] 'Psoriasis Skin Model | Creative Bioarray'. https://www.creative-bioarray.com/psoriasis-skin-model.htm (accessed May 08, 2023).

[62] V. W. Rebecca, R. Somasundaram, and M. Herlyn, 'Pre-clinical modeling of cutaneous melanoma', *Nat Commun*, vol. 11, no. 1, Art. no. 1, Jun. 2020, doi: 10.1038/s41467-020-15546-9.

[63] M. Sarkiri, S. C. Fox, L. E. Fratila-Apachitei, and A. A. Zadpoor, 'Bioengineered Skin Intended for Skin Disease Modeling', *Int J Mol Sci*, vol. 20, no. 6, p. 1407, Mar. 2019, doi: 10.3390/ijms20061407.

[64] M. Barthe *et al.*, 'Safety Testing of Cosmetic Products: Overview of Established Methods and New Approach Methodologies (NAMs)', *Cosmetics*, vol. 8, no. 2, Art. no. 2, Jun. 2021, doi: 10.3390/cosmetics8020050.

[65] 'OECD Guidelines for the Testing of Chemicals'. https://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals_72d77764-en (accessed May 16, 2023).

[66] *Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products (recast) (Text with EEA relevance)*, vol. 342. 2009. Accessed: May 16, 2023. [Online]. Available: http://data.europa.eu/eli/reg/2009/1223/oj/eng

[67] 'SCCS Notes of guidance for the testing of cosmetic ingredients and their safety evaluation - 12th revision'. https://health.ec.europa.eu/publications/sccs-notes-guidance-testing-cosmetic-ingredients-and-their-safety-evaluation-12th-revision_en (accessed May 16, 2023).

[68] *2013/674/EU: Commission Implementing Decision of 25 November 2013 on Guidelines on Annex I to Regulation (EC) No 1223/2009 of the European Parliament and of the Council on cosmetic products Text with EEA relevance*, vol. 315. 2013. Accessed: May 16, 2023. [Online]. Available: http://data.europa.eu/eli/dec_impl/2013/674/oj/eng

[69] *Commission Regulation (EU) No 655/2013 of 10 July 2013 laying down common criteria for the justification of claims used in relation to cosmetic products Text with EEA relevance*, vol. 190. 2013. Accessed: May 16, 2023. [Online]. Available: http://data.europa.eu/eli/reg/2013/655/oj/eng

[70] OECD, *Test No. 432: In Vitro 3T3 NRU Phototoxicity Test*. Paris: Organisation for Economic Co-operation and Development, 2019. Accessed: May 17, 2023. [Online]. Available: https://www.oecd-ilibrary.org/environment/test-no-432-in-vitro-3t3-nru-phototoxicity-test_9789264071162-en

[71] B. S. Kim, G. Gao, J. Y. Kim, and D.-W. Cho, '3D Cell Printing of Perfusable Vascularized Human Skin Equivalent Composed of Epidermis, Dermis, and Hypodermis for Better Structural Recapitulation of Native Skin', *Adv Healthc Mater*, vol. 8, no. 7, p. e1801019, Apr. 2019, doi: 10.1002/adhm.201801019.

[72] E. Desmet, A. Ramadhas, J. Lambert, and M. Van Gele, 'In vitro psoriasis models with focus on reconstructed skin models as promising tools in psoriasis research', *Exp Biol Med (Maywood)*, vol. 242, no. 11, pp. 1158–1169, Jun. 2017, doi: 10.1177/1535370217710637.

[73] J. Lee *et al.*, 'Hair-bearing human skin generated entirely from pluripotent stem cells', *Nature*, vol. 582, no. 7812, Art. no. 7812, Jun. 2020, doi: 10.1038/s41586-020-2352-3.

[74] 'EPISKIN FAQ: Frequently asked questions'. https://www.episkin.com/frequently-asked-questions (accessed May 19, 2023).

[75] R. Cai, N. Gimenez-Camino, M. Xiao, S. Bi, and K. A. DiVito, 'Technological advances in three-dimensional skin tissue engineering', *REVIEWS ON ADVANCED MATERIALS SCIENCE*, vol. 62, no. 1, Jan. 2023, doi: 10.1515/rams-2022-0289.

[76] J. Ponmozhi *et al.*, 'Development of Skin-On-A-Chip Platforms for Different Utilizations: Factors to Be Considered', *Micromachines*, vol. 12, no. 3, Art. no. 3, Mar. 2021, doi: 10.3390/mi12030294.

LIST OF ABBREVIATIONS

ECM	Extracellular matrix
OECD	Organization for Economic Co-operation and Development
UV	Ultra-violet
SCCS	Scientific Committee on Consumer Safety
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
ADME	Absorption, distribution, metabolism, excretion
DNA	Deoxyribonucleic acid
ALI	Air-liquid interface
RhE	Reconstructed human epidermis
RHE	Reconstructed human epidermis
HSE	Human skin equivalent
PSC	Pluripotent stem cells
ASC	Adult stem cells

LIST OF FIGURES