

Department of Plastic Surgery  
and  
Department of Pharmacology

Faculty of Medicine  
University of Helsinki  
Finland

**Outcome of split thickness skin grafts on excised burns  
with different dermal compositions**

Heli Lagus

Academic dissertation

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination Porthania lecture hall P674, Helsinki, on June 26<sup>th</sup> 2020, at 12 o'clock noon.

Helsinki 2020

**Supervised by**

Professor Jyrki Vuola, M.D., Ph.D.  
Department of Plastic Surgery  
Helsinki Burn Centre  
Helsinki University Hospital and University of Helsinki  
Finland

Docent Esko Kankuri, M.D., Ph.D.  
Department of Pharmacology  
University of Helsinki  
Finland

**Reviewed by**

Julian Dye, MA (Oxon), Ph.D. (Lon) CBIol, FRSB  
Institute of Biomedical Engineering  
University of Oxford  
United Kingdom

Docent Ilkka Kaartinen, M.D., Ph.D.  
Department of Plastic Surgery  
Tampere University Hospital  
Finland

**Opponent**

Professor Esther Middelkoop, Ph.D. in biochemistry  
Plastic, Reconstructive and Hand Surgery  
Amsterdam UMC - Vrije Universiteit Amsterdam  
The Netherlands

The Faculty of Medicine uses the Urkund system (plagiarism recognition) to examine all doctoral dissertations.

ISBN: 978-951-51-6066-9 (print)  
ISBN: 978-951-51-6067-6 (on-line)  
University Printing House  
Helsinki 2020

To my family

# Table of Contents

List of original publications.....	6
Abbreviations .....	7
Abstract .....	8
1. Introduction .....	9
2. Review of the literature .....	10
2.1 Function and structure of skin.....	10
2.1.1 Epidermis.....	10
2.1.2 Basement membrane .....	12
2.1.3 Dermis .....	12
2.1.4 Subcutis or hypodermis .....	15
2.1.5 Skin homeostasis and maintenance by ECM and dermal fibroblasts .....	15
2.2 Cell structure and function.....	15
2.2.1 Cell cycle.....	16
2.2.2 Gene expression.....	16
2.2.3 Cell signaling.....	17
2.3 Wound healing.....	25
2.3.1 Wound healing phases.....	26
2.3.2 Factors affecting wound healing.....	34
2.4 Temporary and permanent wound covers.....	35
2.4.1 Temporary wound covers .....	35
2.4.2 Permanent wound covers/ skin replacements .....	35
2.4.3 Dermo-epidermal substitutes /composite skin substitutes.....	37
2.4.4 Bioengineered wound covers.....	37
2.5 Scar assessment methods .....	41
2.5.1 Scar assessment scales currently available.....	41
2.5.2 Objective scar measuring devices.....	42
2.5.3 Protein profiling methods .....	43
2.6 Psoriasis .....	44
3. Aims of the study .....	46
4. Materials and methods.....	47
4.1 Patients.....	47
4.1.1 Burn patients.....	47
4.1.2 Psoriasis patients .....	47
4.1.3 Healthy controls.....	47
4.2 Wounds and wound cover materials .....	47
4.2.1 Burn wound type and location.....	47
4.2.2 Timeline of the study.....	48
4.2.3 Skin samples (I, II, and III).....	49
Assessment and analysis methods.....	51
4.2.4 Vancouver Scar Scale.....	51
4.2.5 Proteomics .....	51
4.2.6 Imaging and image analysis .....	52
4.2.7 Statistical and biostatistical analyses, tools and databases .....	53
4.2.8 Cell stimulation experiments .....	54
5. Results .....	57
5.1 Histology and immunohistochemistry .....	57
5.1.1 Inflammation .....	57
5.1.2 Proliferation.....	57
5.1.3 Remodeling phase 3 weeks to 12 months.....	58
5.2 Clinical findings-VSS .....	60
5.3 Proteomics .....	62
5.3.1 Gene expression analyses .....	65

5.3.2	Cell stimulation experiments .....	65
6.	Discussion .....	67
6.1	The effects of different treatment materials on wound healing and on STSGs, and long-term outcome one year after surgery, studies I & II .....	67
6.1.1	Biocompatibility and biodegradability .....	68
6.1.2	Inflammation .....	68
6.1.3	Proliferation phase.....	69
6.2	The long-term effects of different early wound-bed treatment materials detected in proteomics and reflected to psoriasis, study III .....	75
6.2.1	Proteomic findings in skin samples of burn patients .....	75
6.2.2	Validations.....	76
6.2.3	IPA and TRANSFAC® analyses .....	77
6.2.4	DNAH10 mRNA splice variants .....	77
6.2.5	DNAH10 expression in inflammatory skin pathology psoriasis .....	78
6.2.6	DNAH10 in cell stimulation experiments .....	78
7.	Conclusions .....	81
8.	Future prospects .....	82
9.	Acknowledgements .....	84
10.	References .....	86
11.	Original publications .....	107

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

- I Lagus H, Sarlomo-Rikala M, Böhling T, Vuola J. Prospective study on burns treated with Integra®, a cellulose sponge and split thickness skin graft: comparative clinical and histological study-randomized controlled trial. *Burns* 39:1577-87, 2013. doi: 10.1016/j.burns.2013.04.023.
- II Lagus H, Kankuri E, Nuutila K, Juteau S, Sarlomo-Rikala M, Vuola J. Induced Granulation Tissue but not Artificial Dermis Enhances Early Host-Graft Interactions in Full-Thickness Burn Wounds. *World J Surg* 42:981-991, 2018 doi: 10.1007/s00268-017-4270-6.
- III Lagus H, Klaas M, Juteau S, Elomaa O, Kere J, Vuola J, Jaks V, Kankuri E. Discovery of increased epidermal DNAH10 expression after regeneration of dermis in a randomized with-in person trial - reflections on psoriatic inflammation. *Sci Rep* 9:19136, 2019. doi:10.1038/s41598-019-53874-z.

The publications are referred to in the text by their roman numerals.

The original publications are reproduced with the permission of the copyright holders.

## ABBREVIATIONS

STSG	split thickness skin graft
ADM	acellular dermal matrix
IGT	induced granulation tissue
DNAH10	dynein axonemal heavy chain 10
KRT	keratin
UV	ultraviolet
BM	basement membrane
LC	Langerhans cell
DC	dendritic cell
DETC	denritic epidermal T cell
ECM	extracellular matrix
MMP	matrix metalloprotease
TIMP	tissue inhibitor of matrix metalloproteases
GAG	glycosaminoglycan
DCN	decorin
TGF- $\beta$	transforming growth factor-beta
CTGF	connective tissue growth factor
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
dWAT	dermal white adipose tissue
KGf	keratinocyte growth factor
GM-CSF	granulocyte macrophage colony-stimulating factor
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
FGF	fibroblast growth factor
VEGF	vascular endothelial growth factor
PDGF	platelet-derived growth factor
RTK	receptor tyrosine kinase
MAPK	mitogen-activated protein kinase
IL-1	interleukin-1
TNF- $\alpha$	tumor necrosis factor-alpha
TRM	T memory cell
Treg	T regulatory cell
Th2	T-helper 2 cell
MSC	mesenchymal stem cell
VSS	Vancouver scar scale
HE	hematoxylin and eosin
IHC	immunohistochemistry
AP-2	Activating enhancer binding protein 2
KLF-6	Krüppel-like factor 6
ZF-5	zink finger 5 protein
C6S	chondroitin-6-sulfate
PHK	primary human keratinocytes

## ABSTRACT

Although the use of split thickness skin grafts (STSGs) is the gold standard to cover excised deep burns, relatively little is known about how regenerating dermal connective tissue underneath the STSGs affects wound healing and ultimately the outcome from grafting.

We compared three ways to treat a wound bed of a deep burn after excision onto fascia. The recipient sites before transplanting the STSGs were: a plain excised wound as a control, a site treated with permanent artificial dermal template (Integra®), and a site with granulation tissue promoted by cellulose sponge (Cellonex™).

In study I ten adult burn patients with deep burns covering 22-45 % of total body surface-area were included. Test areas were divided into three equal sections (5 x 10 cm each) that were covered in random order with one of the aforementioned materials. After two weeks, the artificial dermal template and the wound bed under the viscose cellulose sponge were also covered with STSGs. At each site the wound healing process was followed-up for up to one year. Histological analysis from punch biopsy samples and clinical scar assessments were performed using the Vancouver Scar Scale at 3 and 12 months.

Study II concentrated on the healing process of STSGs one week and 3 months after grafting from the material retrieved from study I. Healing was assessed using immunohistochemistry of late inflammation, angiogenesis/vascularity, and proliferation .

In study III, samples from study I were analysed using proteomics. The differentially expressed proteins (DEPs) were further analysed using Ingenuity Pathway Analysis. Based on the results of pathway analysis the potential role and function of DNAH10 (a motor protein usually found in cilia and flagella) was assessed in a group of patients with psoriasis as well as healthy controls. This thesis has five main findings: 1) A valuable experimental model was designed for objective comparative studies of different treatment modalities with-in a patient-controlled study protocol. 2) In study I, inflammation was more distinctive in the granulation tissue promoted by cellulose sponge. In contrast to expectations, more pronounced inflammation combined with granulation tissue formation did not jeopardize the outcome in terms of scarring problems when the wound bed was covered in a timely fashion. 3) After one year, the clinical outcome and the histological findings of the artificial template site did not differ from the two other test sites. 4) In study III, at one-year follow-up, approximately 12% of all proteins detected demonstrated differential expression across comparisons between the different wound bed treatments, and 5) the biggest differences were found in the expression of the axonemal heavy chain dynein DNAH10. STSGs on pre-treated wound beds demonstrated higher expression of DNAH10 than STSGs of control sites. The expression of DNAH10 was also higher in intra-lesional psoriasis skin samples than non-lesional or healthy control skin samples.

The dermal composition of the wound bed has an impact on cellular behaviour during wound healing, and it affects functions such as proliferation and angiogenesis. Surprisingly by the end of the follow-up period, healthy granulation tissue resulted in as little scarring as dermal substitute in the STSGs. Despite the lack of clinical and histological differences with those assessment methods used, clear differences were found in protein levels at the long-term follow-up.

# 1. INTRODUCTION

Large deep burns are devastating life-threatening injuries, however patient survival has increased during the past decades. Early excision combined with emerging new technologies in tissue engineering show promising results, also reducing the morbidity of the patients. Yet, a substantial deficiency remains regarding the basic knowledge of wound healing of burns and the effect of different types of wound covers at the cellular, protein, and molecular levels. One of the most challenging issues in the future will be to improve the quality of remaining scars to closely resemble healthy skin.

Patients can suffer from disfiguring scars, limited range of motion of the affected area, pain, and itching. In addition to physical symptoms the scars cause also psychosocial problems. Improving the quality of the scars has a tremendous impact on the quality of life and on daily life activities of the patients.

Skin substitutes may improve the outcome both functionally and cosmetically. Severe burns with a limited amount of healthy skin for grafting have also prompted a need to search for other alternatives to cover the wounds either temporarily or permanently.

The clinical use of skin substitutes is continuously increasing. Even though there is a substantial amount data on the products from laboratory testing, with both cell-culture and animal testing, and an increasing number of case reports and descriptive studies, there is an obvious deficit in comparative systematic human studies due to difficulties in finding similar wounds with similar etiologies and similar circumstances. Therefore, a large deep burn wound offers a good model for research with a possibility to divide the wound bed into comparative areas.

The primary goal of temporary wound covers is to protect the wound from microbial invasion and from further injury, whereas permanent skin replacements also restore the barrier function from inside-out. Autologous options to cover the wound are not always possible. When the condition of the wound bed or the general condition of the patient do not allow definitive wound closure, temporary and/or less invasive options are considered. Commercially available wound care products and dressings range from passive dressings to interactive products, even with cells. As the technology in tissue engineering progresses it becomes more difficult to differentiate between wound dressings and skin substitutes. Even when the products are integrated to the wound bed they are usually replaced by autologous tissue in the long run. These innovations benefit also wound and skin-cancer patients with larger defects (after operation), since dermal substitutes can be used in both patient groups.

Wound healing is a complex long-standing process with overlapping different phases from injury of the tissue to mature scar. Many factors affect the healing process and contribute to the final outcome. This thesis addresses some clinical, cellular, and even molecular-level questions on wound healing; the role of regenerating dermal connective tissue composition in intricate interaction between epidermal, dermal, and even bone marrow derived mesenchymal stem cells; and the long-term outcome of healed split thickness skin grafts. The results are reflected to cellular and molecular events in a skin disorder, psoriasis.

## 2. REVIEW OF THE LITERATURE

### 2.1 FUNCTION AND STRUCTURE OF SKIN

Skin is the outmost boundary to the external world that envelops the human body. It protects the body from various physical, chemical, and biological insults such as UV radiation; penetration of toxic substances and microbial invasion from the environment; and it controls fluid movement preventing dehydration. Skin also functions as a sensory organ and takes part in thermoregulation and it has metabolic functions, for example by producing vitamin D (1).

Skin consists of three layers: epidermis, dermis, and hypodermis or subcutis. Epidermis is mainly responsible for the barrier function, primarily in *stratum corneum* serving as a water-tight layer (2), whereas dermis provides tissue tensile strength, resilience, elasticity, blood vessels, and innervation. These two layers are connected to each other by a special extracellular matrix, basement membrane (BM) (3,4). Since epidermis is avascular, its' nurturing and oxygen supply are provided by diffusion from dermal vessel plexus. Underneath the dermis a subcutis containing adipose tissue and connective tissue attaches the skin to underlying structures like muscle fascias, functioning as cushion and insulation, and storing energy (5).

#### 2.1.1 EPIDERMIS

Epidermis consists of several layers of cells, mainly (approximately 90%) of keratinocytes (6). Other cells include melanin pigment producing melanocytes, Merkel cells as mechanoreceptors, and immune cells such as Langerhans cells (LC), antigen presenting dendritic cells (DC) (7), and epidermal T-cells (DETCs).

In the innermost layer, basal keratinocytes attach to the basement membrane. They form a population of interfollicular stem cells that proliferate and ensure the continuity and sufficiency of cells for barrier maintenance. They maintain their own population, but mostly remain in a relatively quiescent state and rarely dividing (6). In addition to interfollicular stem cells, three other epidermal stem cell niches serve as keratinocyte reservoirs: the epidermal appendages in the hair follicle bulge, in the base of sebaceous glands and in the eccrine sweat glands (8-10). These stem cells are the reserve of epidermal cells in case of injury and they become activated during wound-healing and repair. Epidermal stem cells regulate epidermal maintenance (11) and cellular homeostasis. During normal maintenance the cells are renewed by asymmetrical division of interfollicular stem cells. Upon division, stem cells produce transit-amplifying progenitors: one daughter cell remains on the basal layer, whereas the other daughter cell is destined to differentiate (12). Transit-amplifying cells divide rapidly a few times and then commit to terminal differentiation. When entering the terminal differentiation process, keratinocytes lose contact with the basement membrane and exit the cell cycle. Differentiation progresses from the suprabasal spinous cells to the lamellar granule-containing granular cells. In the late differentiation stage, a cornified cell envelope forms

and a bricks-and-mortar-like structure is generated from cross-linked corneocytes and lamellar body lipids secreted into the intercellular space generating a waterproof barrier (13). The outmost layer of epidermis forms of flat collapsed cornified dead keratinocytes, corneocytes, and extracellular lipids, cholesterol, free fatty acids, and ceramides (13).

### **Barrier function**

*Stratum corneum* prevents passive diffusion of water. Beneath the *stratum corneum* tight junctions seal the intercellular space between epidermal cells. Tight junctions are composed of transmembrane proteins that are linked to cytoskeletal actin (14). Crosslinks between molecules of involucrin, loricrin, filaggrin, and small protein proline-rich molecules of cornified envelopes of granular cells make this layer tear resistant. These two physical barriers, stratum corneum and tight junctions in stratum granulosum, together prevent the penetration of harmful substances from outside and escape of fluids from inside (15).

Epidermis also has immunological functions. Lipids, especially free fatty acids, antimicrobial peptides such as beta-defensins and cathelicidins, and antimicrobial proteins, create an antimicrobial barrier. Barrier breach provokes production of antimicrobial peptides (16,17). During wounding, stressed keratinocytes rapidly induce transcription of keratins KRT16/17-KRT6. Langerhans cells (LCs), members of the dendritic cell/macrophage family located under the tight junction barrier, take part in a variety of T-cell responses. They recognize and process soluble antigens (18).

Wounding causes acute breaches in barrier function, while chronic inflammatory conditions, such as psoriasis, lead to chronic impairment or decrease of barrier function. Deficiencies of different compounds of upper epidermal layers lead to barrier disturbances.

### **Keratins**

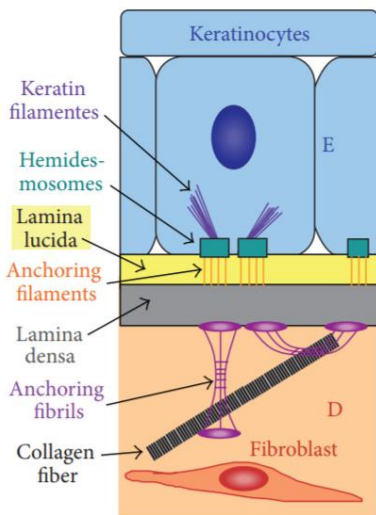
Keratins are cytoskeletal intermediate filaments of keratinocytes adding mechanical stability. Keratin links with desmosomes and hemidesmosomes create a network enhancing mechanical resilience in the tissue. Keratins in the upper epidermis are formed into the cornified epithelial barrier layer. Keratins form paired complexes of type I and type II keratins. In skin, keratin pairs KRT5 and KRT14 are expressed in the basal cell layer, whereas keratins KRT10 and KRT1 are expressed in differentiated keratinocytes in the suprabasal layers. Keratin pairs KRT16 and KRT6 are expressed in highly activated and proliferative suprabasal keratinocytes during wound healing and in stressed keratinocytes in skin pathologies, and KRT16 serves as a marker for hyperproliferation (19). Typically K6, K16, and K17 are rapidly induced as a result of various challenges to the interfollicular epidermis like wounding and infection, in response to oxidative or UV stress, as well as in chronic hyperproliferative diseases, such as psoriasis and cancer (20-22). Keratins are also involved in cell signaling. See chapter 2.2.3 on cell signaling.

### 2.1.2 BASEMENT MEMBRANE

Basement membrane (BM) is a special type of extracellular matrix attaching epidermis to dermis (3), but also functionally separating them. BM functions as a selective permeability barrier, acts as a gatekeeper for trafficking of the cells, and regulates cell growth. Together with BM-associated growth factors, structural proteins such as laminin and type IV collagen promote cell proliferation. BM takes part in cell signaling and thereby regulates cell survival and cell death (23,24). BMs create a biochemically and physically distinct environment (25) and modulate stem cell behavior by driving them to differentiate or to maintain their stemness (26).

Structurally, the BM of skin consists of two independent polymeric networks, one of laminin and the other of type IV collagen as major constituents, which are attached to each other by perlecan, a heparan sulfate proteoglycan (27). Basal keratinocytes and dermal fibroblasts produce BM components in co-operation. Most of the BM molecules are produced by both cell types, but laminin 5 is synthesized only by keratinocytes and nidogen only by fibroblasts (28).

Type IV collagen stabilizes and protects the BM from mechanical stress. In addition, laminins initiate BM formation, mediate early cell differentiation, and support migration by functioning as cell adhesion molecules (29). Laminin is also an important component of hemidesmosomes, and it interacts with integrins on cell surfaces as well as with integrins on type VII collagen of anchoring fibrils providing protection from shear forces.



**Figure 1.** Skin basement membrane (BM) has three layers: *lamina lucida*, *lamina densa*, and *sublamina densa*. In *lamina lucida*, hemidesmosomes attach basal keratinocytes to BM. Lamina densa contains mainly type IV collagen (23) but also laminins, as well as nidogen and perlecan. Sublamina densa connects lamina densa to underlying papillary dermis. Sublamina densa connects to anchoring plaques by anchoring fibrils and microfibrils of type VII collagen and heparan sulfate proteoglycan.

This figure is reproduced under the Creative Commons Attribution License CC BY 3.0 from Copyright 2013 Dirk Breitkreutz. <https://creativecommons.org/licenses/by/3.0/> <https://www.hindawi.com/journals/bmri/2013/179784/> (1)

### 2.1.3 DERMIS

Dermis provides pliability, elasticity, and tensile strength to skin. In addition to extracellular matrix (ECM), dermis contains fibroblasts, endothelial cells, smooth muscle cells, pericytes, neurons, immune cells such as different types of T cells, mast cells, B cells, dermal dendritic cells, innate lymphoid cells including NK cells, and macrophages (30,31).

### ***Connective tissue cells – Fibroblasts***

Fibroblasts are the main population of cells in dermis forming a poorly-defined group of cells. Fibroblasts are any stromal cells that express no markers for more specific mesenchymal lineage leading to heterogeneous and dynamic cell lineages that vary across tissue types. Different layers of human dermis exhibit different subpopulations of fibroblasts with unique properties (32). In dermis, fibroblasts express different quantities and ratios of collagen type I and type III mRNAs depending on their resident depth (32). Papillary dermis has more collagen type III than reticular dermis (33). Adult intact skin contains collagen I to collagen III in a ratio approximately 4:1 (34). Fibroblasts in the deeper dermis produce less collagenase enzyme (matrix metalloproteinase-1, MMP-1) mRNA than their “peers” in a more superficial location (35). Papillary and reticular fibroblasts also show a different profile in ECM synthesis, and growth factor and cytokine secretion. These different fibroblast populations possess distinct abilities to promote keratinocyte growth, migration, and differentiation, and in influencing both synthesis and remodeling of ECM in the dermis.

### ***Extracellular matrix (ECM) of dermis – an active scaffold***

ECM has been estimated to contain approximately 300 core proteins (36). Some selected proteins important in wound healing are addressed in this chapter.

ECM is the non-cellular component of tissue providing support for cells. It not only anchors cells, but also modifies cellular properties and regulates cell behaviour, and in turn cells build and (re)model ECM. There is a constant interaction between cells and ECM. ECM controls the majority of cell functions: cell proliferation, migration, differentiation, growth, survival and particularly the stemness of stem cells, cell adhesion and cell-cell communication contraction, transmission of forces into the cells, changes in shape, protein synthesis and expression of specific genes, degradation of proteins, and apoptosis (27,32,37,38). Skin ECM forms a dynamic interlocking network with a variety of macromolecules like proteolytic enzymes and their tissue inhibitors, growth factors and cytokines (39), serving as their reservoir and regulating their bioavailability. ECM also functions as physical barrier, as a track for cellular movement, and a binding site for cells.

The (bio)chemical and physical properties of ECM influence cellular behavior like cell migration, growth, and differentiation (40). The biochemical characteristics of ECM enable cells to sense and to interact with their microenvironment by different signal transducing pathways (36). Cells sense chemical signals like growth factors and different metabolic precursors. Signaling molecules bind to adhesive ECM proteins such as fibronectin, laminin, and collagens. These proteins in turn bind to cell surface integrins, which function as links between cells and ECM (36). Physical features of ECM like rigidity, elasticity, density, porosity, spatial arrangement, orientation, insolubility, and mechanical tension are sensed by the cells through integrins. Integrins are connected to cytoskeletal actin and mediate mechanical cues inside the cells (27). These interactions may affect migration, proliferation, differentiation, apoptosis, and stem-cell fate. Contraction of ECM may result in tensile stretch in cytoskeleton and nucleus. Compression of ECM may alter local ion concentrations thereby activating ion channels (40,41). The increased rigidity of ECM may increase proliferation and contractility. The stiffness of ECM also regulates the differentiation of mesenchymal stem cells: rigid ECM

leads to the osteogenic path whereas soft ECM favors the neurogenic path (36). The ECM laxity or rigidity is linked to fibroblast apoptosis signaling and MMP activity. Matrix stiffness is known to induce myofibroblast differentiation (32,42-44).

The main components of dermal ECM consist of collagens, elastin, proteoglycans, and glycoproteins as cell adhesion molecules (32,36). The different macromolecules in the dermis occupy distinct tasks: collagens (and laminins in BM) serve as structural proteins; elastins and fibronectins provide flexibility; proteoglycans and hyaluronan maintain the three-dimensional structure and bind growth factors. On the other hand glycoproteins on the cell surface like integrins regulate cell adhesion and mediate signaling between cells and the ECM (39).

Fiber-forming structural proteins create a three-dimensional structure. The most abundant fiber-forming structural proteins include collagens, fibrin, fibronectin, vitronectin, elastin, and fibrillin. Collagens provide integrity, rigidity, and elasticity to the tissue (45). Fibrin, fibronectin, and vitronectin are compounds of a healing wound. Fibronectin has an important role in guiding inflammatory cells and fibroblasts to the wound site (46). Elastin endows resilience to skin (47).

Non-fiber-forming structural proteins create a charged, dynamic, and osmotically active microenvironment. Proteoglycans and glycosaminoglycans (GAGs) fill the most interstitial space in the dermis (48). Proteoglycans are glycosylated proteins that provide mechanical support. They are able to regulate cell proliferation, migration, protein synthesis and degradation, and apoptosis (37). They consist of a core protein combined with GAG chain or chains. Together with other matrix proteins like collagens, proteoglycans (such as decorin, biglycan, fibromodulin, and versican) form a supramolecular structure to increase the stiffness of the tissue. Sulfated GAGs are often part of proteoglycans. The GAG chains are highly negatively charged, they enable space-filling, lubrication, hydration, and buffering functions of proteoglycans, and they distribute forces within the tissue (36). They maintain the mechanical strength of the tissue (49) and serve as storage for growth factors and other proteins. GAGs may function as a physical and biochemical barrier, creating a special microenvironment restricting the free movement of proteins (50). This specific microenvironment may be crucial for creating stem cell niches and to protect the stem cells from exposure to growth factors and receptor binding in order to maintain the stem cells in an undifferentiated state. The GAGs assist in interaction with other ECM components.

Four major classes of GAGs have been identified: heparan sulfate, chondroitin sulfate/dermatan sulfate, keratan sulfate, and hyaluronan, also called hyaluronic acid. Dermatan sulfate is also known as chondroitin sulfate B, and chondroitin-6-sulfate is known as chondroitin sulfate C (51). Dermatan sulfates take part in cell signaling through interactions with collagen and transforming growth factor- $\beta$  (TGF- $\beta$ ) (52).

Proteoglycan decorin (DCN) regulates collagen fibril and fiber bundle organization and the tensile strength of dermis (39). It binds to specific locations on the surface of type I collagen fibrils (27) and it interacts with fibronectin, thrombospondin, epidermal growth factor receptor (EGFR), and TGF- $\beta$  (53). It regulates the production of EMC molecules such as fibronectin and thrombospondin-1. In addition to blocking the action of TGF- $\beta$  by forming an inactive complex DCN exerts anti-fibrotic effects by inhibiting collagen I

maturation, and stimulating collagenases (53). DCN also regulates inflammation by recruiting mononuclear cells to the site of injury by inducing MCP-1 (54).

#### **2.1.4 SUBCUTIS OR HYPODERMIS**

Subcutis is a layer between dermis and fascia. This mainly adipose tissue functions as a cushion against physical pressure from outside. It also retains moisture and generates heat, provides thermal insulation and takes part in adaptive immunity (55). Between adipose cells, connective tissue fiber bundles reach from dermis to fascia and periost. Abundant mesenchymal stem cells also reside in adipose tissue and subcutis serves as reservoir for adipokines, which can exert pro- or anti-inflammatory functions (56).

A distinct adipose tissue type has recently been identified: dermal white adipose tissue (dWAT), a layer that comprises adipocytes in the reticular dermis, located primarily perifollicular, and superior hypodermis. Its functions differ from other adipose tissue; dWAT takes part in hair cycling, thermogenesis, wound healing, scarring and fibrosis, and immune defense producing cathelicidins and communicating directly with various types of cutaneous immune cells. (57)

#### **2.1.5 SKIN HOMEOSTASIS AND MAINTENANCE BY ECM AND DERMAL FIBROBLASTS**

ECM produced by dermal fibroblasts contributes to stem cell niche homeostasis by influencing local tissue architecture and the signaling microenvironment.

The epidermal stratification process depends on dermal fibroblasts. Complex interactions between epidermal keratinocytes and dermal fibroblasts are required in order for stratification to proceed properly. For example, IL-1 $\alpha$  from epidermal keratinocytes induces dermal fibroblasts to produce of keratinocyte growth factor (KGF) and granulocyte macrophage colony-stimulating factor (GM-CSF) that in turn stimulate keratinocyte proliferation and differentiation (58,59).

## **2.2 CELL STRUCTURE AND FUNCTION**

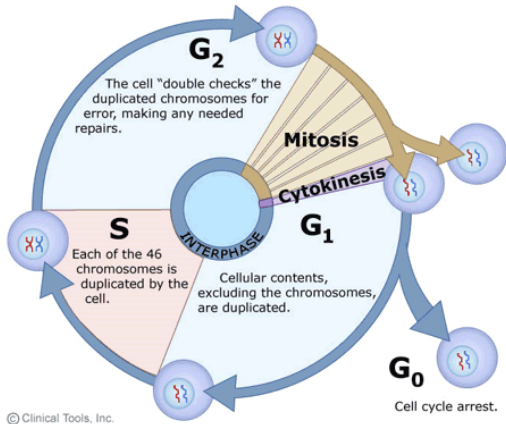
This chapter is provided to give a very brief overview of structure and function of keratinocytes and fibroblasts focusing on cell cycle, gene expression and cell signaling.

In general cells are surrounded by cell membrane, a barrier controlling the entry and exit of substances. The inside of cells contains cytoplasm, a nucleus with DNA, and cell organelles like Golgi, mitochondria, endoplasmic reticulum with or without ribosomes, and cytoskeleton.

Cytoskeleton includes microfilaments, microtubules, and intermediate filaments, and it is responsible for mechanical support, anchoring organelles, and cell movement. It is also involved in transportation macromolecules within the cell.

## 2.2.1 CELL CYCLE

The aim of the cell cycle is to duplicate, divide, and distribute chromosomal DNA into two genetically identical daughter cells. The cell cycle has two major phases, synthesis (S) and mitosis (M); and two minor or gap phases between the major phases, G<sub>1</sub> between M phase and S phase, and G<sub>2</sub> between S phase and M phase (60). See figure 2.



**Figure 2.** Phases of cell cycle.

Reproduced here under the Creative Commons Attribution License CC BY-NC-SA 2.0 UK from <https://www2.le.ac.uk/projects/vgec/highereducation/topics/cell-cycle-mitosis-meiosis>. Copyright 2019 Clinical Tools, Inc.

If the conditions are unfavourable, the cell either lengthens G<sub>1</sub> phase or may enter a resting phase, G<sub>0</sub>. In favourable conditions, the G<sub>0</sub> or the beginning of G<sub>1</sub> phase proceeds towards the end of G<sub>1</sub> phase to a commitment point, after which the replication of DNA is carried on even if the cell division stimulating extracellular signals are removed. During G<sub>2</sub> phase, cellular growth and protein synthesis takes place. The sequential phases from G<sub>1</sub> to S followed by G<sub>2</sub> together are called interphase (61).

A detailed description of gene replication is reviewed elsewhere (62,63).

## 2.2.2 GENE EXPRESSION

Made up of DNA, genes carry the instructions for proteins to be synthesized. When a gene is activated (e.g. through a signaling pathway) its instructions are decoded via transcription (of the nucleotide sequence), where the information is copied to create a complementary messenger RNA (mRNA). This copy is then translated to sequences of amino acids by ribosomes with the help of transfer RNAs (tRNAs) to build a protein or a protein precursor. See figure 3.

### **Transcription**

In gene transcription, the information of a gene is copied from the gene's DNA sequence by RNA polymerase to create a complementary RNA strand, a transcript. Transcription is regulated by transcription factors and coactivators and takes place in the nucleus. The next step, translation of the code to amino acid sequence, occurs by ribosomes in cytoplasm or across endoplasmic reticulum.

RNA polymerase enzyme "manufactures" the new messenger RNA molecule by attaching to DNA in a specific promoter region with the help of transcription factor. The transcription factor binds to DNA at a specific target sequence and thereby either assists or inhibits the binding of RNA polymerase.

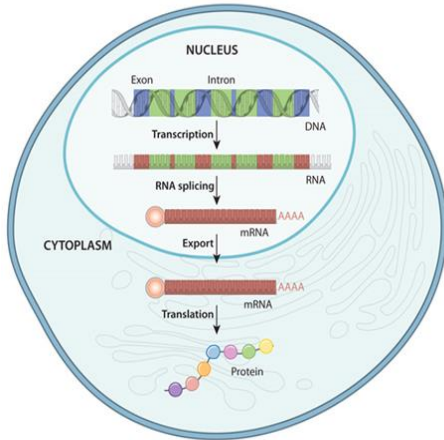
Gene promoters are regions of DNA, typically located upstream of the gene, in near proximity to the target gene that assists the RNA polymerase to attach to DNA and to begin the transcription/copying of the DNA.

Transcription factors are proteins that regulate transcription. They function as

activators that boost transcription of a gene whereas repressors decrease the transcription. Transcription factors bind to nearby DNA. These binding sites are called enhancers and silencers which can either turn a gene on or off.

In eukaryotic cells, the manufactured “primary transcript” mRNA needs further processing before leaving the nucleus. These post-transcriptional modifications of mRNA protect the transcript and assist in leaving the nucleus and for mRNA to become mature mRNA called an exon. After transcription, the transcript may also undergo an alternative splicing resulting in different (mRNAs and) proteins from the same RNA transcript.

MicroRNAs are small posttranscriptional regulator-RNAs that can cause silencing of the gene by degrading mRNA or by blocking mRNA translation.(64).



**Figure 3.** Gene expression.– from DNA to protein  
Transcription in nucleus. DNA strand is copied into a messenger RNA.  
Translation in cytoplasm. Messenger RNA is translated by ribosome. The code of mRNA is translated to sequences of amino acids forming a protein or a precursor of a protein.  
Figure reproduced here with permission from the copyright holder from <https://www.nature.com/scitable/topicpage/gene-expression-14121669>. Copyright 2010 Nature Education.

## Translation

Translation of the mature mRNA transcript takes place in a ribosome. (65,66).

After translation, the produced amino acid sequence form either a functional protein or they form a precursor of protein, an inactive pro-protein that requires further processing before becoming fully active. In order to become fully active, the protein may undergo proteolysis by proteasomes or post-translational modification and protein folding in the endoplasmic reticulum. Endoplasmic reticulum is responsible for the quality control of synthesized proteins. Unfolded or misfolded proteins are either degraded or they accumulate in the cell leading to cell dysfunction. (64). Depending on the severity of the dysfunction, this leads either to reduced general protein synthesis or to the apoptosis of the cell (21).

### 2.2.3 CELL SIGNALING

Cell signaling refers to processes that take part in endocrine, paracrine, juxtacrine, autocrine, and intracrine cellular communication and affect cellular behavior (67). Cells communicate with each other and their microenvironment by responding to and sending

chemical messages, although signals received may also be physical: mechanical, osmotic, thermal, electrical or light-related (68). Extracellular signaling involves the triggering physical parameter or the chemical signaling molecule, the mediator of the message. Intracellular signaling includes receptor activation that leads to a series of intracellular biochemical reactions or conformational changes called a signaling pathway. One signaling pathway may interact with another forming a network. Signaling pathways can be regulated by positive or negative feedback. Cellular responses to signals may include: increasing transcription of a certain gene; a metabolic response like enzyme activity; or cell growth, proliferation, migration, differentiation, or apoptosis. (68).

The effect of the signal may induce fast, slow, or even permanent changes in cell behaviour. Short-term cellular changes include changes in shape, secretion, contraction or relaxation, migration, and changes in metabolism. Long-term cellular changes may include gene expression, proliferation, differentiation, cell survival, and apoptosis. Based on mathematical modelling, it has been suggested that short-term signaling may determine long-term cell fate (69). More detailed information regarding the concept of cell signaling was reviewed recently (70).

### ***Extracellular signaling***

Cytokines and growth factors are signaling molecules that mediate the signals from ECM or from another cell into the target cell. Cytokines like interleukins, lymphokines, interferons, chemokines, and TNF- $\alpha$ , are small proteins that pre-dominantly have an effect on immune cells (71) whereas growth factors are heterogeneous secreted molecules that mainly regulate growth and differentiation of various cell types (72). Some cytokines and growth factors important in wound healing are presented in Table I.

#### ***ECM molecules as signaling molecules***

ECM molecules interact with cell surface receptors that forward signals from the cell membrane to molecules in the cytoplasm. ECM-cell interactions promote cell adhesion, migration, growth, differentiation, and apoptosis, and affect activities of growth factors and cytokines. The molecules of the ECM are able to bind either to growth factors or directly to their receptors to mediate signaling: fibronectin binds fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF). Tenascin-C and laminin-5 interact with epidermal growth factor receptor (EGFR) promoting cell proliferation. In contrast, decorin-induced activation of EGFR inhibits mitogenic signaling in fibroblasts and endothelial cells. Decorin takes part in multiple signaling pathways by binding to and activating growth factor receptors like insulin-like growth factor 1 receptor (IGF1R) and TGF- $\beta$ . Decorin interacts with macrophages and simultaneously activates transiently mitogen-activated protein kinase (MAPK) and NF- $\kappa$ B signal pathways and enhances the release of inflammatory factors like TNF- $\alpha$  and IL-10. Growth factors regulate ECM structure in co-operation with proteases: growth factors initiate deposition of ECM components, while proteases enable their degradation, ensuring the remodeling of ECM that is required for cell movement. (73).

**Table I.** Partial list of the cytokines and growth factors important in wound healing. The key growth factors and cytokines in wound healing are reviewed in detail in the articles of Werner and Grose (71), Barrientos et al. (74), Efron and Moldawer (75).

Cytokines or growth factors	Examples of target functions	Examples of secreting cells
<i>Pro-inflammatory cytokines and growth factors</i>		
TNF- $\alpha$	KGF secretion, synthesis of ECM proteins and TIMPs (-1, -2, -3, -9, and -13), re-epithelialization	Neutrophils, macrophages, keratinocytes
IL-1	K6 and K16 expression in keratinocytes, fibroblast activation, KGF secretion, neutrophil infiltration	Neutrophils, monocytes, macrophages, keratinocytes
IL-8	Neutrophil infiltration, keratinocyte migration and proliferation, MMP expression in leukocytes	Macrophages, epithelial cells, and endothelial cells
IL-6	Neutrophil infiltration, keratinocyte proliferation	Neutrophils and monocytes/macrophages
PDGF	Neutrophil, macrophage and fibroblast infiltration and proliferation, macrophage growth factor secretion, granulation tissue formation	Platelets, macrophages, endothelial cells, fibroblasts, and keratinocytes
GM-CSF	Neutrophil infiltration, keratinocyte proliferation, endothelial cell proliferation and migration	Keratinocytes upon wounding
MCP-1	Infiltration of monocytes/macrophages, T-cells, and mast cells	Keratinocytes upon wounding
<i>Angiogenic growth factors</i>		
VEGF	Angiogenesis, granulation tissue formation	Platelets, neutrophils, macrophages, endothelial cells, fibroblasts, keratinocytes
FGF-2	Granulation tissue formation, re-epithelialization, ECM formation and remodeling	Keratinocytes, mast cells, fibroblasts, and endothelial cells
<i>Pro-fibrotic growth factors</i>		
TGF- $\beta$	Inflammation, granulation tissue formation, re-epithelialization, ECM formation and remodeling	Platelets, keratinocytes, macrophages, lymphocytes, and fibroblasts
CTGF	Fibroblast infiltration and proliferation, endothelial cell endothelial proliferation, migration, survival, and adhesions in angiogenesis, keratinocyte migration	Fibroblasts
IL-4	Stimulation of fibroblast activation, M2 polarization	Mast cells, activated T-cells
<i>Growth factors promoting re-epithelialization</i>		
EGF	Re-epithelialization	Platelets, macrophages, and fibroblasts
KGF (FGF-7)	Keratinocyte proliferation, migration, and differentiation	Fibroblasts

## **Receptors**

Receptor proteins on cell membranes bind to signaling molecules, which leads to responses such as a conformational change of the receptor, which in turn transmits the signal to commence the signaling pathway. See Figure 4. Direct cell-cell contact can lead to stable interactions. Signal transduction into the cell may occur also through binding of the messenger to primary cilia. Primary cilia a cell organelle containing a certain subset of receptors and other proteins involved in signaling, detecting, and transmitting signals from the surrounding of the cell into the cell (74). Primary cilia are found on the surface of many growth-arrested or differentiated eukaryotic cells. See chapter Primary cilia for further details.

### **Cell surface receptors**

Most receptor tyrosine kinases (RTKs) take part in regulating complex functions like proliferation and differentiation (75).

Integrins are transmembrane receptors, proteins that cells use to respond to and attach to the ECM. They function as “dual receptors.” They transduce signals both from the external environment into the cell, and from inside to out, functioning as links between the ECM and the cytoskeleton of the cell. Integrins are coupled to intracellular cascades that can affect cell proliferation, differentiation, polarity, contractility, and gene expression (36). They are able to have crosstalk with receptor tyrosine kinases like EGFR (76). Some ECM molecules function as ligands to integrins leading to activation of the MAPK pathway.

### **Intracellular signaling**

Altogether 177 different signaling pathways have been identified (Panther classification system, Geneontology, Panther 14.1). This chapter focuses on the most important signaling pathways related to wound healing.

In wound healing many of the processes, such as cell proliferation, motility, and survival are regulated by multiple pathways. Epidermal cell differentiation, barrier function, and apoptosis are also affected by various signaling pathways. Several key signaling pathways (77) participate in mammalian cutaneous wound healing, such as Wnt/ $\beta$ -catenin, TGF- $\beta$ , Hedgehog, and Notch pathways. The NF- $\kappa$ B signaling pathway has an important role in inflammation.

The Wnt/ $\beta$ -catenin pathway is involved in reconstruction of dermis by affecting the numbers of fibroblasts, cellularity, and matrix production. In epidermis it may sometimes be involved in regeneration of hair follicles in large wounds. Several components of ECM act through the Wnt -pathway to regulate hair epidermal stem cell activity (78). TGF- $\beta$ 1 is one of the key regulators produced by keratinocytes. The TGF- $\beta$ 1 signaling pathway takes part in reconstitution of dermis by affecting fibroblast proliferation, matrix synthesis, fibroblast differentiation into myofibroblasts, and wound contraction. In epidermis, TGF- $\beta$ 1 has an inhibitory role of re-epithelialization.

Hedgehog pathway has been associated with accelerated wound healing, and Sonic hedgehog (Shh) participates in hair follicle regeneration. It has also been suggested to promote fibroblast proliferation and angiogenesis (77).

Notch signaling affects macrophage behaviour and angiogenesis. During skin development it affects epidermal differentiation and participates in asymmetric cell division during epidermal stratification (79).

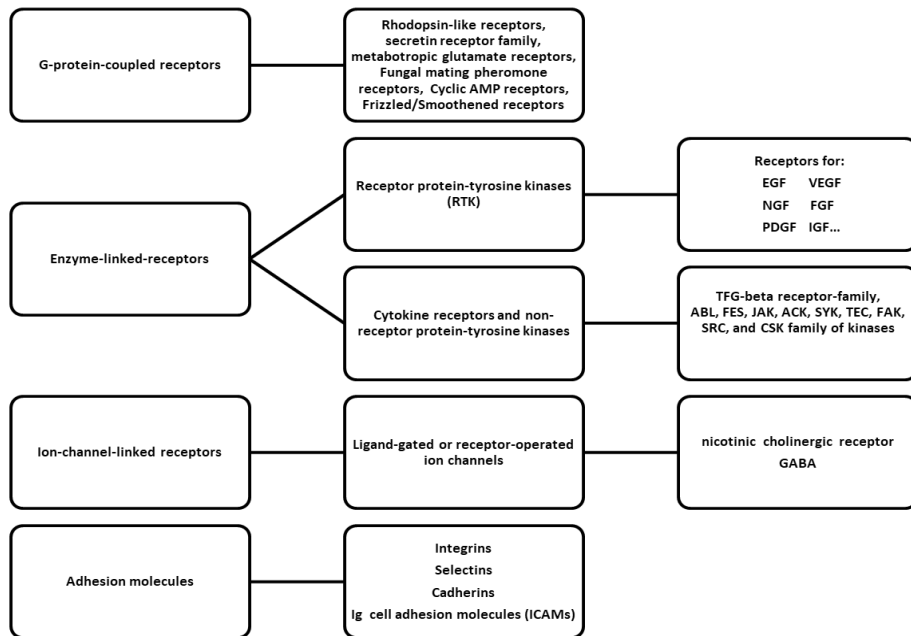


Figure 4. Cell surface receptor classification according to (66).

The NF- $\kappa$ B Signaling Pathway has been regarded to have a critical role in inflammation, the innate and adaptive immune response, and stress. Dysregulated signaling is seen to occur in inflammatory and autoimmune diseases (18,80).

The MAPK signaling pathway has been shown to have critical role in epidermal differentiation as well as in skin barrier function. The MAPK signaling pathway components can integrate and mediate various signals involved in keratinocyte differentiation.

The ERK1/2 signaling pathway has been shown to control keratinocyte differentiation; low ERK1/2 activity could induce keratinocyte differentiation and apoptosis (81). Under normal circumstances the signals for proliferation of keratinocytes are mediated through EGFR following the MAPK pathway. This mitogenic activity is limited to basal keratinocytes (76). Signaling pathways known to be crucial for epidermal stem cell homeostasis and skin morphogenesis include Wnt, TGF- $\beta$ /BMP, Notch, Hedgehog, and FGF. Wnt/ $\beta$ -catenin signaling is well recognized to play a role in controlling epidermal stem cell maintenance and fate decision. It participates in multiple processes in the skin, including cell-matrix interactions within the niche and outside (82).

**Table II.** Examples of molecules, signaling pathways, and cascades, and functions they regulate

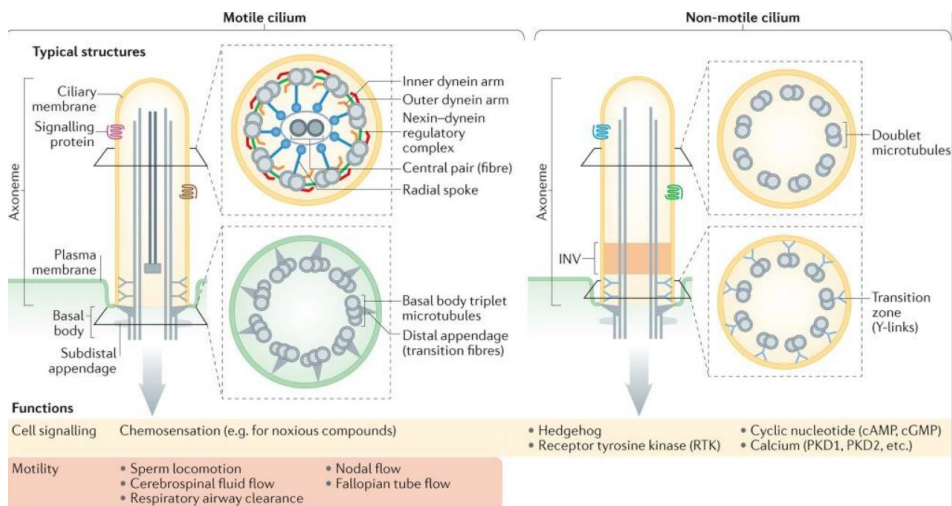
	<b>Example functions</b>
Wnt	Cellular differentiation and proliferation; disturbances may lead to the formation of tumors and promote metastasis.
TGF beta	Regulation of growth and proliferation of cells along with migration, differentiation, and apoptosis
Hedgehog	Required for proper cell differentiation, regulating adult stem cells involved in maintenance and regeneration of adult tissues
Notch	Determination of cell fate, regulation of pattern formation and other developmental settings. Disrupted signaling can cause developmental defects and a range of adult pathologies.
Akt/PKB	Mediation of protein synthesis, metabolism, proliferation and cell cycle progression, inhibition of apoptosis
AMPK	Takes part in cellular response to low levels of available ATP, often caused by stresses such as heat shock, ischemia, or hypoxia
Apoptosis	Programmed cell death
Insulin	Regulation of intracellular glucose levels
JAK-STAT	Control of cell proliferation and hematopoiesis, main signal transduction cascade from cytokine receptors
MAPK/ERK	Cellular response to extracellular stimuli such as heat and stress, can influence cell proliferation, metabolism and survival
mTOR	Regulates cell growth, proliferation and survival
NF- $\kappa$ B	Important role in inflammation, the innate and adaptive immune response, and stress
Nodal	Pattern formation and differentiation during embryo development, nodal family of proteins, a subset of the TGF- $\beta$ superfamily
Hippo	Controls organ size in animals through the regulation of cell proliferation (restraining) and apoptosis (promoting)
TLR	Cellular response to threatening molecules, results in an inflammatory and immunological response.
VEGF	Vasculogenesis and angiogenesis, also induces cell migration, proliferation, and survival
p53	Co-ordination of the cellular response different types of stress such as DNA damage and hypoxia. The downstream signals lead to apoptosis, senescence and cell cycle arrest.

## Cilia

Cilia are microtubule-based specialized cell organelles that interact with the extracellular environment and/or generate motion. Cilia are able to sense chemical, mechanical, and osmotic stimuli, as well as pH, oxygen concentration, and light (photoreceptors). They transduce chemical and mechanical signals into the cells controlling for example cell polarity, differentiation, and proliferation (83). Three major types of cilia have been identified: primary cilia, motile cilia, and nodal cilia (84). Primary and motile cilia share a common architecture consisting of an axoneme and a nine doublet-ring of microtubules as a central structure, as well as a ciliary membrane. Primary and motile cilia can be distinguished by the absence of a central pair of microtubules and a lack of dynein arms in primary cilia (84). See Figure 5. Nodal cilia share characteristics of both motile and primary cilia. Nodal cilia have a 9+0 axoneme, like primary cilia, but contain dynein arms like motile cilia.

### Primary cilia

Primary, also called sensory or non-motile, cilia are present on most vertebrate cells. They were found in fibroblasts in 1962 by Sorokin, and in keratinocytes 1963 by Wilson and McWhorter (85). They appear as cellular antennae-like protrusions of cell membrane that receive and transduce extracellular chemical or mechanical signals into the cell (86). Primary cilia are cell organelles that are assembled in cells during normal cell cycle progression in G1–S phases. They are assembled by centrioles of the mother centrosomes that have relocated from the nuclear membrane to the plasma membrane. In some cells, the disassembly of primary cilia is required by the end of mitosis to prevent a G1/S arrest, whereas in certain cell types, the ciliary membrane may persist through mitosis (87). The centrosomes can be seen as intracellular hubs, whereas primary cilia function as receivers of extracellular signals transmitting them into the cell. It has been suggested that there is a link between proliferation and ciliogenesis, but not all proliferating cells form primary cilia. Cilia take part in and act as key coordinators of signaling pathways during



**Figure 5.** Typical structures and function of motile and non-motile cilia.

Reproduced from Nat Rev Mol Cell Biol. 2017 Sep; 18(9): 533–547. Genes and molecular pathways underpinning ciliopathies, Jeremy F. Reiter with the permission of Springer Nature.

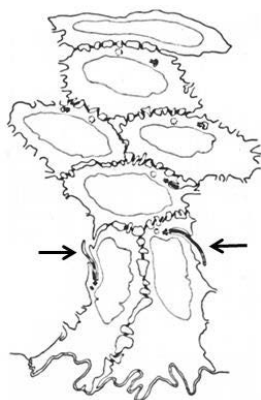
development and in maintaining tissue homeostasis (88). A single primary cilium can respond to several different kinds of signals and the same cilium may possess different receptors and different channels (88). Ciliary signaling coordinates cell migration, differentiation, transcription, cell division, cell polarity, and apoptosis (89). Misregulation or delay in primary cilia loss leads to defects in cell cycle progression, which cause aberrant development or imbalanced homeostasis seen in ciliopathies and many cancers (83). Some specialized primary cilia act as sensors for light, temperature, osmolality and gravity (90).

Various signaling pathways have been linked to the cilium, including Hedgehog, Wnt, Notch, Hippo, GPCR, PDGF (and other RTKs including FGFR, EGFR and IGF1R), mTOR, and TGF- $\beta$  (91) as well as NF-kappa B signaling and TGF $\beta$ /BMP signaling pathways (92).

Primary cilia and cell–cell junctions, including the adherens and tight junctions share several similar features: they are markers of cell polarity in epithelia; sites of mechanosensation; transducers of signals; and are regulated by the actin cytoskeleton (83).

During development, the composition of ciliary signal systems may change as part of the dynamic process that controls cell differentiation, allocating different signal systems to different cell types to determine cell fate and function.

In keratinocytes, the cilium is a typical feature of post-mitotic and differentiated cells which have exited the cell cycle (91). Figure 6. shows a cross-section of keratinocyte with cilia.



**Figure 6.** Keratinocytes. Arrows are pointing at cilia. Reproduced from *Journal of Ultrastructure Research*, 87, 212-220, (1984) Rolf Elofsson, *The Ciliated Human Keratinocyte*, with permission from Elsevier.

The fibroblast primary cilium, similarly to GPS, may coordinate cellular directional migration and PDGFR $\alpha$ -mediated chemotaxis. Primary cilia may also directly interact with extracellular matrix (ECM) proteins as the cell moves, transmitting mechanical information from the outside milieu to the cell. Recent work has further shown that stem cells possess primary cilia with signal transduction components that control maintenance of stem cell pluripotentiality and regulate early differentiation and proliferation.(93) Signaling through PDGFRs has been associated with resorption of cilia, an important step in cell cycle progression to permit the

centrioles to participate in mitosis (91).

### **Motile cilia**

Motile cilia are higher specialized organelles than primary cilia. They appear in differentiated specialized cells and generate bending waves to take part in cell motility or in transportation of fluids over the cell surface (94-96). Motile cilia differ from sensory primary cilia by the presence of motor proteins required to move fluid in the brain, respiratory, and reproductive tracts (97). An increasing amount of evidence suggests that

motile cilia can mediate sensory functions, as well as exhibit mechanoreceptive and chemoreceptive properties (98).

## **Dyneins**

Dyneins are a family of force generating motor proteins associated with microtubules. Dyneins are categorized into two main classes: axonemal and cytoplasmic. Motile primary cilia have also been recently discovered, like vertebrate nodal cilia that promote left-right asymmetry during development (99). Axonemal dyneins are expressed in cilia. Axonemal dyneins within motile axonemes cause sliding or bending along the axoneme resulting oscillation (100); outer-arm dyneins seem to be responsible for increase the frequency of axonemal beating, whereas inner-arm dyneins have distinct roles in shaping the ciliary waveform (95).

Cytoplasmic dyneins transport and position protein complexes and cell organelles inside the cells (101). Cytoplasmic dyneins may also translocate membrane-spanning proteins and signaling complexes in the plane of the lipid bilayer, for instance within the nuclear envelope (100). Cytoplasmic and axonemal dyneins show similarities in heavy chains across different species. Dyneins vary in their properties especially in velocities in translocation of microtubule (95). According to their location on the microtubule doublet, the axonemal dyneins are called either inner or outer arm dyneins. The inner-arm dyneins are further divided into two functionally different subgroups: two-headed inner-arm dyneins (DNAH10 belongs to this group) and one-headed inner-arm dyneins. Dynein arms of cilia are preassembled in the cytoplasm before transporting to into cilia (94). Dyneins “walk” towards the minus ends of microtubules.

## **Ciliopathies**

Ciliopathies vary from absence of cilia in specific tissue to malfunction/dysfunction of ciliary motion like reduction or lack/loss of motility. Primary ciliary dyskinesias are ciliopathies with known dysfunction of motile cilia caused by genetic recessive disorders. Out of these, the most well-known are dysfunction in respiratory and reproductive cilia/flagella and lateral asymmetry as situs inversus (nodal cilia disorder).

## **2.3 WOUND HEALING**

Wound healing is a complex process involving timely overlapping interaction between different types of cells and interplay between the cells and the ECM of the wound bed in a designated sequence. In man, wound healing is not a true regeneration of tissue after early gestation, 0–24 weeks (102), instead it results in an inferior quality of connective tissue, a scar.

During cutaneous wound healing, the barrier function and mechanical properties of skin are restored by the actions of numerous types of cells which undergo proliferation, migration, differentiation, and apoptosis to rebuild the skin.

### **2.3.1 WOUND HEALING PHASES**

Wound healing and scar formation involve epidermal, dermal, and subcutaneous mesenchymal cells, endothelial cells, and cells of the immune system and neurons. Wound healing is traditionally divided into four overlapping phases: hemostasis; inflammation; proliferation; and re-modeling.

#### ***Hemostasis***

To reduce bleeding, blood vessels contract and a coagulation cascade is activated to produce fibrin net, and together with adhering and aggregating thrombocytes to form a clot. Hemostasis is achieved from minutes to hours after injury. Platelets and the cells from the fibrin clot and surrounding wound bed release pro-inflammatory cytokines, and growth factors such as TGF- $\beta$ , PDGF, FGF, and EGF, attracting inflammatory cells as well as proliferating cells to the wound site.(103).

Upon wounding and breach of the epidermal barrier, keratinocytes also secrete pro-inflammatory cytokines IL-1, TNF - $\alpha$  and growth factor EGF (104).

#### ***Inflammation***

Inflammation phase can be separated into two distinct phases: early and late inflammation. Early inflammation serves to defend against microbial invasion and infection and late inflammation is a transition phase towards tissue repair.

Inflammatory cells migrate into the wound area. Leukocytes infiltrate the wound sequentially: neutrophils, macrophages, and lymphocytes (105). Neutrophils are the first leukocytes to arrive at the injured wound site within a few minutes, followed by monocytes/macrophages and lymphocytes (71). Mast cells release histamine and cytokines promoting the inflammatory phase, stimulating vasodilatation and increasing vascular permeability. Neutrophils combat invading microbes by secreting reactive oxygen species, antimicrobial peptides, and antimicrobial proteases (106). Cytokines released by neutrophils during apoptosis are chemotactic for monocytes. Monocytes converting into macrophages start to arrive 5 to 6 h after injury. Macrophages decrease the numbers of bacteria, secrete various cytokines and growth factors and phagocytose apoptotic neutrophils. Macrophages initiate granulation tissue formation (107). Other inflammatory cells also take part in initiating the proliferation phase by secreting growth factors and cytokines.

Classically activated M1 macrophages possess pro-inflammatory properties whereas M2, alternatively activated macrophages, are anti-inflammatory cells. Towards the transition from the inflammation to proliferation phase, macrophages change their phenotype from M1 to M2 leading to decrease of inflammatory cytokines and increase of anti-inflammatory cytokines (108). The removal of M1 macrophages at early stage has been shown to reduce granulation tissue formation, to impair epithelialization, and to reduce scar formation (106). M2 macrophages are a prominent source of cytokines and growth factors that promote matrix deposition like TGF- $\beta$ , PDGF, IGF1, TNF- $\alpha$ , and angiogenesis by releasing angiogenic factors including FGF-2, VEGF, placental growth factor and by releasing degrading enzymes (106). Cytokines and growth factors produced by macrophages activate keratinocytes, fibroblasts, and endothelial cells (108). During inflammation, macrophages also secrete chemokines that attract T cells to the wound site.

Epidermal and dermal T-cells produce pro-inflammatory cytokines and epithelial growth factors (109,110). Tissue-resident  $\gamma\delta$ T cells likely regulate keratinocyte proliferation and differentiation by secreting growth factors (106). T-cell receptor (TCR)-specific activation of  $\gamma\delta$ T cells accelerates the rate of early wound closure. Th2 cells and regulatory T cells (Tregs) also contribute to matrix formation by secreting TGF- $\beta$ 1, IL-4, -5, -13, and -21, which enhance anti-inflammatory macrophage polarization and suppress other inflammatory cell types. The resolution of inflammation is largely dependent upon macrophages, Tregs, and Th2 cells. Tregs seem to control Th2 responses (111).  $\alpha\beta$  T lymphocytes in the skin are long-lived resident memory T cells (TRM). Most are derived from antigen-specific effector T cells, which previously infiltrated the tissue as a result of an infection. TRM promote recruitment of other memory T cells from the periphery to sites of infection (112). Antigen-presenting cells with memory T cells are found around the hair follicles.

### ***Proliferation Phase***

In the proliferation phase, the wound bed is filled with new extracellular matrix produced mainly by fibroblasts and differentiated myofibroblasts with simultaneous (re)building of the vascular network by angiogenesis of endothelial cells and covering the wound with migrating and proliferating keratinocytes by re-epithelialization. Concomitantly, the myofibroblasts contract the wound to reduce its size to hasten wound closure.

#### **Fibroplasia**

The synthesis of new ECM into the wound bed driven by fibroblasts is called fibroplasia. The formation of granulation tissue formation begins 2–4 days after injury. It consists of blood clot, fibroblasts and their progenitors, as well as endothelial cells and inflammatory cells. Myofibroblasts in granulation tissue have similar functions as fibroblasts in terms of synthesizing ECM and contractile properties (34). TGF- $\beta$ 1 enhances granulation tissue formation by mediating the increase of fibronectin, the fibronectin receptor, and collagen and protease inhibitors (107). It also promotes ECM synthesis by stimulating mesenchymal cells to differentiate into myofibroblasts.

#### **Angiogenesis and lymphangiogenesis**

The formation of new blood vessels is critical to restore oxygen and nutrition supply to the wound site and to support the new building tissue. Angiogenesis takes place in response to pro-angiogenic factors like VEGF, FGF, angiogenin, angiotropin, and angiopoietin-1 released by wound site macrophages and keratinocytes (113). Angiogenesis is also stimulated by lactic acid, plasminogen activator, collagenases, and low oxygen tension (114). Beside a sufficient vascularization, lymphatic vessel formation is essential to re-establish skin function. Dermal lymphatic vessels are involved in the regulation of tissue fluid homeostasis and immune cell trafficking. Adipose tissue-derived stem cells have shown potential to support lymphangiogenesis.

#### **Re-epithelialization**

Upon wounding and release of inflammatory cytokines, basal keratinocytes become activated and migrate into the wound (115). The re-epithelialization of a wound requires coordinated interactions among inflammatory cells, keratinocytes, fibroblasts, vascular

endothelial cells, and immune cells, as well as the local microenvironment. Epithelial stem cells contribute to the re-epithelialization by differentiating into epithelial cells. The first signal from the injury is the release of IL-1 which activates endothelial cells and fibroblasts and attracts lymphocytes to the wound site (21). Keratinocytes and fibroblasts take part in reconstructing BM by both producing BM components. BM has an important function by regulating keratinocyte growth and terminal differentiation (116).

### ***Remodeling phase***

During the remodelling phase, the newly generated tissue is organized. It matures and simultaneously the cellularity of the scar decreases. The onset of the remodelling phase varies in the literature from 7–10 days to 2–3 weeks after injury and it can last for 1 year or more. In order for the actual matrix to form, the removal of granulation tissue is required. The granulation tissue is replaced by collagen and elastin fibres together with proteoglycans and glycoproteins. The formed matrix is then remodelled; TGF- $\beta$  induces synthesis of new collagen, whereas the breakdown of old collagen is induced by PDGF (107). Metalloproteinases released by fibroblasts and macrophages degrade collagen type III of the granulation tissue and replace it with collagen type I, which is further reorganized into parallel fibrils, forming a scar with low cellularity. In keloids the early excess of collagen type III is replaced by type I collagen, with a high ratio of type I to type III collagen (17:1) compared to normal scars (6:1) (117).

Apoptosis of myofibroblasts may be promoted by a decrease or absence of mechanical tension and by alterations in cell density or protease activity; changes in the pattern of secreted growth factors are also thought to contribute to cell death (46).

### ***Role of stem cell in wound healing***

Stem cells affect all phases of wound healing. Mesenchymal stem cells (MSC) may originate from different sources like bone marrow, adipose tissue, dermis and perivascular niches (118). MSC enhance wound healing by promoting proliferation of keratinocytes and endothelial cells; by decreasing apoptosis; by paracrine signaling; and by differentiating into keratinocytes and endothelial cells (108). They are also able to differentiate into pericytes and vice versa. MSCs may migrate to the wound site where they direct inflammation and antimicrobial activity, promote cell migration, proliferation, and differentiation, and recruit other host cells to assist in re-epithelialization and wound repair (119). MSCs can reduce inflammation by suppressing T-cells and activating macrophages. MSCs regulate macrophages through paracrine signaling supporting the transition of inflammatory type M1 macrophages to a type M2 anti-inflammatory phenotype which then decreases their expression of inflammatory cytokines and increases anti-inflammatory signaling. MSCs decrease TNF- $\alpha$  production and provide anti-scarring properties by secreting prostaglandin E2 (PGE2), which in turn induces expression of IL-10 by T cells and macrophages (120). MSCs also contribute to granulation tissue formation (108).

By differentiating into keratinocytes MSCs have been shown to enhance the speed of re-epithelialization, and increase the number of epidermal ridges, the thickness of the regenerated epidermis, and the number of appendage-like structures. In addition MSCs

release a variety of growth factors that enhance the proliferation of keratinocytes, fibroblasts, and ECs such as IGF-1, EGF, KGF, and other mitogens.(108).

Adipose-derived stem cells (ASC) have been shown to promote re-epithelialization by paracrine activation of keratinocytes by secreting of growth factors like PDGF, FGF-2, and TGF- $\beta$  (108). In a rodent model they also enhanced the granulation tissue formation (121). They exert their beneficial influence in wound healing and promote angiogenesis by secreting angiogenic factors like HGF, VEGF, GM-CSF, PDGF, SDF-1, FGF-2, TGF- $\alpha$ , IL-6, and IL-8. Like MSCs ASCs are able to differentiate into keratinocytes as well as into fibroblasts and endothelial cells. (122).

The role and function of dWAT and hair follicle stem cells has been described in the chapter of skin structure and function.

### ***Burn wound healing***

Burn toxins such as free radicals, lipoproteins, lipopolysaccharides, and lipid peroxides form as a result of thermal injury to skin. They are liberated during the early stages of burns and cause immunosuppression, mitochondrial destruction, energy metabolic disorders of cells and increase the permeability of the cell membrane (123). Oxidative stress may increase the development of necrosis. Free radicals are also associated with formation of pyridinoline cross-links, normally appearing in bone and cartilage, but also occurring in hypertrophic scars (46). Heat denatured proteins of the eschar may also result in an uncontrolled inflammatory response (123,124).

In large burns exceeding 20 % of total body surface area burn shock takes place in addition to local injury. Vasodilatation and the capillary permeability is increased and fluid escape accompanied with proteins leaking from blood vessels into interstitial space follows creating edema and on the other hand hypovolemia needing fluid resuscitation (125).

Even though neutrophils have been found in high numbers in burn wounds and for prolonged periods of time, their functions were impaired. In burns, mast cells have been shown to regulate both the inflammatory and the proliferative phases. Mast cells are among the first cells to react in burn injury. They release histamine and cytokines promoting the inflammatory phase. Mast cells may enhance tissue remodeling at later stage. On the other hand their uncontrolled activity may lead to scar formation.(126). Patients experiencing pain in burns secrete neuropeptide substance P increasing the release of histamine from mast cells possibly also contributing to formation of hypertrophic scar. (46). In burns the immune response is dysregulated. In addition to impairment of neutrophils it has been suggested that also T cells contribute deteriorating wound healing. The levels of IL-17 and IL-22 cytokines are significantly increased supporting Th17 responses. The skin-resident  $\gamma\delta$  T lymphocytes promote Th1, Th17 and regulatory T cell responses. (126). In burn wounds expression of the TGF $\beta$ -, FGF-, and VEGF families of cytokines are increased in keratinocytes which regulate re-epithelialization and regeneration of dermal tissue (127).

The patients fall into hypermetabolic state, chronic inflammation and their immune system is compromised leading to increased susceptibility to infection and sepsis and delayed re-epithelialization. The deeper and larger the burn is, the greater the level of circulating cytokine storm and the more difficult the hypermetabolic response. (125).

Regarding hypertrophic scar formation in children's scald burns, if an accurate prediction is made that the burns are expected to heal before 25 days, a better outcome is expected with conservative treatment than with skin grafting. In deep burns, the risk of pathological scar formation is increased even if the patients are grafted and healed within two weeks of injury. (126).

Early permanent wound closure warrants minimal or no scarring complications, whereas delayed treatment often leads to severe hypertrophic scarring which is directly proportional to the delay to wound closure. One study of 337 scalded children showed that if the wound closure was achieved within 21 days the risk for hypertrophic scar was low, and after this time frame a much higher incidence of hypertrophic scarring occurred (126). Earlier permanent wound closure is also associated with lower mortality and better functional long-term results (109,127,128). The thicker the STSG is, the less contraction there will be at the site of application, but the longer it will take to heal the donor site (129). Currently, split-thickness autologous skin grafting is the clinical "gold standard" in full-thickness injuries treatment (129,130).

### ***Factors promoting scar formation***

Scarring represents an outcome and "end-product" ECM resulting from wound healing. Problematic or pathologic scars evolve due to variety of triggering and/or predisposing factors. Most of these factors are related to chronic inflammation, myofibroblast activation, and/or mechanical stretch of the cells. Other factors may also contribute to formation of a problematic scar. Morphological characteristics of problematic scar are abundant fibroblast proliferation and differentiation into myofibroblasts; the excessive production of extracellular matrix (ECM) proteins, especially types I and III collagen that accumulate in the extracellular space (39,128); decreased apoptosis of myofibroblasts and fibroblasts; and decreased degradation of ECM by MMP.

Fibroblasts of hypertrophic scars resemble deep dermal (reticular) fibroblasts (35). Keloid scars do not progress beyond the early stages of wound healing and remain in this state for years.

### **Role of inflammation**

Pathological scarring can be seen as inflammatory disorders of reticular dermis. Chronic inflammation in reticular dermis has been suggested to lead to scarring problems such as hypertrophic scars and keloids (129). The sustained cross-talk between inflammatory macrophages and myofibroblasts results in prolonged myofibroblast activation, wound contraction and excess deposition of ECM especially collagen (34). ECM influences the pro-fibrotic signals between macrophages and myofibroblasts. In keloids and hypertrophic scars the reticular layer contains inflammatory cells, increased numbers of fibroblasts, newly formed blood vessels, and collagen. In keloids proinflammatory factors such as interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-6, and TNF- $\alpha$  have been shown to be upregulated. Systemic factors may also contribute to dermal inflammation. In adolescence and during pregnancy, estrogens and androgens intensify inflammation by vasodilatation. Systemic inflammation is also likely to be responsible for the tendency of patients with extensive burns to develop hypertrophic scars after surgery. In deep and large burn wounds, the duration of inflammation is prolonged. The severe burn itself is

associated with a cytokine storm that significantly increases the risk of developing keloids and hypertrophic scars for at least 1 year. Excess secretion of pro-inflammatory factors promotes degradation of both growth factors and ECM. Pro-inflammatory mediators also prohibit the conversion of M1 macrophages to M2 macrophages (106).

Most of the current therapies for scars target reducing inflammation like corticosteroid injection/tape/ointment, radiotherapy, cryotherapy, compression therapy, stabilization therapy, 5-fluorouracil therapy, and surgical methods that reduce skin tension (129).

### **Myofibroblast activation and origin of myofibroblasts**

One key factor in the formation of pathologic scars is the numerous myofibroblasts producing excess ECM. Myofibroblasts synthesize scar-type connective tissue. Many different terms are used for cells converting into myofibroblasts: e.g. fibroblast-to-myofibroblast transition; myofibroblast (trans)differentiation, activation, or transformation; or just fibroblast activation.

Myofibroblasts may originate from various precursor cells. They can be activated from tissue resident fibroblasts, from circulating fibrocytes, from pericytes, and they may be formed by transdifferentiation from epithelial-mesenchymal (EMT) or endothelio-mesenchymal transition (34). Dermal adipocytes may also undergo adipocyte-myofibroblast transition (AMT) (130). In addition to fibroblasts, other fibroblast-like cells take part in wound healing. Some fibroblasts or fibroblast-like cells may originate from bone marrow-derived mesenchymal stem cells, whereas fibrocytes originating from mononuclear cells also have similarities with mesenchymal cells. During wound healing even up to 30% of cells with a similar phenotype to fibroblast and myofibroblast are suggested to originate from circulating hematopoietic fibrocytes. (131,132) Myofibroblasts may form from cells of mesenchymal origin like adipocytes and pericytes by de-differentiation to fibroblasts (32). Decreased apoptosis also contributes to the number of wound site myofibroblasts (133).

TGF- $\beta$ 1 is the most well-known inducer of myofibroblast differentiation. It seems to necessitate the presence of a splice variant of fibronectin EDA. TGF- $\beta$ 1 is in the ECM in a latent form that needs to be activated by degrading enzymes. Profibrotic effects of TGF- $\beta$  are mediated via Smad-proteins and altered expression of the connective tissue growth factor (CTGF) gene. CTGF is a well characterized downstream target of TGF- $\beta$  that promotes fibrotic response and ECM production (133). TGF- $\beta$ 1 signals may also be mediated via Smad-independent pathways, including p38 MAPK, ERK, JNK, and c-Abl.

Fibrin degradation product FnE potentiates the myofibroblast activation and recruitment induced by TGF- $\beta$  (134). Proinflammatory factors promote degradation of growth factors and ECM and thus also activating latent TGF- $\beta$ 1. Another factor known to induce myofibroblast differentiation is mechanotransduction, stretching the cell and stiffness of the matrix. The signaling cascade involves integrin/FAK/Src via ERK and MAPK pathways. Matrix stiffness is increased with cross-linking by lysyl oxidase-like 2 (LOXL2) enzymes. Inhibition of these enzymes results in less mechanical stress. Many integrins (such as  $\alpha$ 3 $\beta$ 1,  $\alpha$ 11 $\beta$ 1,  $\alpha$ v $\beta$ 3,  $\alpha$ 4 $\beta$ 7, and  $\beta$ 1 integrin) contribute to fibrosis and myofibroblast differentiation through various pathways (135). Periostin is shown to contribute to myofibroblast activation. Hyaluronic acid (HA) and its receptor CD-44 are involved in myofibroblastic activation; high molecular weight HA inhibits fibrotic

pathways, but low molecular weight HA appears to induce them. Collagen III deficiency promotes myofibroblast activation and wound contraction (136).

Fibroblasts are the main executors of scar formation and driving myofibroblast differentiation in concert with macrophages, other inflammatory cells, epithelial cells, cytokines, and growth factors (137).

### **Role of re-epithelialization and basement membrane**

A burn wound that heals in less than 10 days has a 4% risk of developing into a hypertrophic scar (138,139). In case re-epithelialization is delayed up to 2 to 3 weeks, one third of the wounds develop hypertrophic scarring. If re-epithelialization is prolonged up to three weeks or more, the risk for hypertrophic scarring increases to 78% (140).

Scar tissue derived keratinocytes differ from normal keratinocytes, the thickness of epidermis is increased, and the basement membrane may be absent or aberrant in scar tissue (141). In hypertrophic scars the structural deviations in the BM trigger basal keratinocytes to transform to proliferative phenotype. An abnormal response to persistent or repetitive injury to epithelial cells is also suggested to result in fibrosis (128). The equilibrium between keratinocyte proliferation and differentiation is disturbed in scar tissue (115). Keloid-derived keratinocytes have been shown to be significantly more proliferative and resistant to apoptosis than normal skin derived keratinocytes. Keratinocytes also play an important role in the development of pathological fibrosis through paracrine regulation of fibroblasts like enhancing their proliferation and collagen production (115). Activated keratinocytes of hypertrophic scars produce growth factors that affect the inflammatory response and endothelial cells. The persistence of activated keratinocytes indicates abnormal keratinocyte migration and proliferation and deviant epidermal-mesenchymal interactions that delay re-epithelialization (140). Some studies imply that the abnormalities in proliferation and differentiation of the keratinocytes cause an increase in epidermal thickness and may lead to hypertrophic scar formation (142). The increased thickness of epidermis seems to correlate with the severity of the scar (143).

BM plays a significant role in regulating the cell fate decision of epidermal keratinocytes during skin wound healing. Alterations in the structure of the BM reduce attachment of basal keratinocytes to their microenvironment and promote them to differentiate and to adopt a proliferative phenotype during scar formation with possible involvement in pathogenesis of scarring similar to hypertrophic scars. In areas where BM is degraded, the differentiation of keratinocytes has been found to be decreased or absent.

Involucrin, a marker for early terminal differentiation, shows higher expression both in keloids and hypertrophic scars compared to normal skin (143). This abnormal early terminal differentiation has been found to be associated with disorganization of the stratum corneum in keloids. Also the turnover rate of stratum corneum has been found to be hastened. The stratum corneum of both keloids and hypertrophic scars shows increased transepidermal water loss and/or water-holding capacity compared to normal skin. (143). Decreased hydration levels have been shown to result in increased proinflammatory gene expression in epidermal keratinocytes. Dehydration of the stratum corneum induces keratinocytes to produce cytokines, which in turn activate dermal fibroblasts to produce collagen (46).

The depth and frequency of epidermal rete ridge formation has been shown be significantly reduced in all scar types compared to normal skin. In young hypertrophic

scars the proliferation of keratinocytes, thickness of epidermis and expression of keratinocyte activation markers keratins 6, 16, and 17 are increased (141,144). After 12 months, the increased keratinocyte proliferation and expression of keratin 16 normalize; not even abnormal mature scars exhibit increased rates of keratinocyte proliferation.

In addition to its role as a supportive structure and a barrier, BM also participates in signaling during angiogenesis. BM harbours various angiogenesis-modulating molecules, growth factors, and cytokines that are released and activated by the breach of BM. Laminin degradation exposes angiogenesis-inducing sites.

### **Role of angiogenesis**

Early-stage macrophages control induction of granulation tissue and myofibroblast differentiation. Mid-stage macrophages play a role in stabilization of vascular structures and transition of granulation tissue into scar tissue (145). Both keratinocytes and fibroblasts contribute to angiogenesis. Vascular endothelial growth factor (VEGF)-induced angiogenesis plays a major role in fibrogenesis and hypertrophic scar formation (146). VEGF is mainly secreted by macrophages, endothelial cells, keratinocytes, and fibroblasts. The expression of VEGF is positively correlated with vascular density and negatively with maturation of scar tissue. In the early post-injury scar, expression of VEGF in the basal layer of the epidermis is significantly increased. VEGF is able to promote inflammatory cell recruitment and increase vascular permeability. VEGF may also affect fibroblasts (147).

In hypertrophic scars, pathological angiogenesis is a crucial factor. Many angiogenesis-promoting factors have been shown to be abnormal in hypertrophic scars, like VEGF, TGF- $\beta$ 1, angiogenin, and thrombospondin (148). Mechanical stretch also promotes angiogenesis. In early scar formation, inflammatory factors contribute to angiogenesis. Conversely, the newly formed microvessels exhibit endothelial dysfunction leading to persistent inflammation. An excessive angiogenic response enhances scar formation. The hypoxia environment in hypertrophic scars can further induce angiogenesis, which promotes cell proliferation, thus creating greater demand for oxygen and promoting *circulus vitiosus* (148).

### **Mechanical stretch/ force**

Dermal fibroblasts are mechanosensitive to mechanical stimuli and stretch of fibroblasts leads to the secretion of Leucine-rich alpha-2-glycoprotein (LRG-1). In the presence of TGF- $\beta$ 1 LRG1 is mitogenic to endothelial cells and promotes angiogenesis (149). Stretch stimulation of dermal fibroblasts triggers signal transduction from ECM into cells mediated by  $\beta$ 1 integrin in the cell membrane and results in increased synthesis of ECM proteins, such as collagen I and III, and elastin, as well as increased production of protease inhibitors such as plasminogen activator inhibitor and tissue inhibitor of metalloproteinase (150).

In keratinocytes, stretch stimulation is also transmitted into cells involving interactions between  $\beta$ 1 integrin and EGFR and induces ERK phosphorylation as is the case in dermal fibroblasts. In epidermal keratinocytes, stretch application promotes cellular adhesiveness, cell proliferation, and protein synthesis (150).

### 2.3.2 FACTORS AFFECTING WOUND HEALING

Besides the normal contributors to the complex wound healing, the process is affected by multitude of different factors either concerning the local circumstances of the wound, or the general condition of the patient, or both. Many of these factors are related to impeding the tissue oxygen and nutrition supply.

Oxygen has a crucial role in energy production. During tissue maintenance and homeostasis, oxygen is essential in cell respiration in efficiently producing energy source adenosine triphosphate (ATP) in aerobic metabolism, 16 to 18 times more ATP per hexose sugar than anaerobic metabolism (151). In wound healing, oxygen facilitates especially inflammation and proliferation phases. In inflammation, oxygen is needed as a substrate in producing reactive oxygen species (ROS) by neutrophils. ROS help to destroy microbes during early inflammation in respiratory burst thereby reducing risk of infection (152). Oxygen also has enhancing effects with some antibiotics, such as beta-lactams, quinolones, and aminoglycosides (153).

Paradoxically, in early proliferation phase, hypoxia induces angiogenesis but in later stages oxygen facilitates angiogenesis. In ECM production, oxygen is essential in collagen synthesis for hydroxylation of proline and lysine. It promotes fibroblast proliferation and wound contraction (105). Oxygen also enhances epithelialisation secondary to ECM synthesis and increases keratinocyte differentiation (105,152).

Nutrition is essential in wound healing both as a substrate for energy production and as an important source of building blocks or cofactors in different phases of wound healing. Glucose is the main source of energy, but fat and proteins can also be consumed. Certain amino acids of proteins are particularly important like arginine, glutamine, glycine, and methionine for wound healing. Micronutrients, such as trace elements (like zinc, manganese, magnesium, copper, calcium, and iron) and vitamins (A, B, C, E, and K) influence immune system function, collagen synthesis, and reepithelialisation as cofactors in different enzymes (154). Some other important factors affecting wound healing are listed in table III.

**Table III.** Partial list of additional factors affecting wound healing not listed in the text (112,154,155)

Local	Systemic
Chemical factors: pH, toxic substances	Age, gender
Physical factors: Temperature, pressure, shear forces	Diseases: Diabetes, pulmonary diseases, cardiovascular diseases, kidney insufficiency, gout, rheumatoid arthritis
Moisture balance, excessive exudates, urine	Medications: steroids, non-steroidal anti-inflammatory drugs (NSAIDs), anti-rejection medications, chemotherapy
Foreign body	Lifestyle: Smoking, alcohol abuse, obesity, stress, nutrition
Necrosis	Immunocompromised conditions: Cancer, radiation therapy, AIDS
Repeated trauma, edema, tissue perfusion impairing factors	

## **2.4 TEMPORARY AND PERMANENT WOUND COVERS**

Literally temporary wound covers are intended for temporary use and to be removed and/or replaced with permanent covers integrating to the wound bed. Temporary covers of the wound inhibit the invasion of microbes, mechanically protect the wound and aid in wound healing. Permanent wound covers assist also in recovering the barrier function preventing the dehydration. In burns, traditional dressings are used to temporarily protect the wound or assist in treating the infection prior to surgery. After excision or debridement, the aim is to prepare the wound bed for skin grafting, and after transplantation of a skin graft, to protect the graft or to prevent the infection together with moisture balancing. More sophisticated dressings offer a wider range and more complex solutions temporarily mimicking skin functions. The latest era of nanotechnology in bioengineering has enabled adding various components to wound dressings or skin substitutes such as nanocomposites of metals, like silver and zinc, with antimicrobial properties (156). Other bioactive molecules can also be incorporated to nanofibers, like growth factors and cytokines, enzymes, or compounds of ECM to enhance wound healing (157). Immunomodulatory properties of the materials aim to reduce inflammatory response and avoid foreign body giant-cell formation, as well as rejection of an implant (157). One strategy is to modulate macrophage polarization towards the M2 type (158). Emerging 3D bioprinting allows tailored automated tissue engineering solutions with cells either in situ or in vitro printing in the future (159). Some of the different types of skin substitutes, both temporary and permanent, are listed in Table IV.

### **2.4.1 TEMPORARY WOUND COVERS**

Biological wound covers/skin substitutes include xenografts, amnion membrane, cadaver skin and cultured epidermal allografts. They offer naturally occurring biocompatible structures recognized by the host cells. They may elicit immune rejection even though they might become partially incorporated to the wound bed and leave elements behind assisting wound healing. Amniotic membranes are suggested to have anti-inflammatory, anti-microbial, anti-fibrotic, and anti-scarring effects. Cultured epidermal allografts provide the wound with a mechanical barrier and growth factors (56), and the cells also synthesize components of ECM.

### **2.4.2 PERMANENT WOUND COVERS/ SKIN REPLACEMENTS**

The use of permanent skin substitute should be safe and effective, preferably also cost-effective. The choice of the product should be tailored to the needs of the patient. The list of other properties desired is long and constantly lengthening. Even though the use of skin substitutes is slowly increasing they are not widely used. According to a survey in 2015 their use was considered essential or at least desirable in treating large burns (160).

Permanent skin substitutes replace either epidermis dermis or both. Permanent grafts need to be integrated to the wound bed even though they may be replaced later by

autologous tissue. In dermal substitutes, the vascular ingrowth into the material is essential to establish circulation to bring oxygen and nutrition for the cells.

Permanent epidermal grafts include cultured epidermal autografts, sprays, or sheets. Their take rate seems to depend on the type of wound bed: 45–75% when grafted onto dermal or neodermal wound bed, 28–47% when grafted onto early granulation tissue, and only 15% when grafted onto granulation tissue of chronic wound bed (161). Graft loss has been linked with infection, insufficient microcirculation of the wound bed, and poor adherence of the graft due to slow formation of anchoring fibrils (161).

Dermal substitutes or matrices with or without seeded cells are constituted either from natural or synthetic polymers.

Since collagen type I is a natural polymer and also the most abundant protein of human dermal ECM, it is a usual constituent of artificial dermal matrix (162). Collagen-based scaffolds are known to reduce wound contraction and scar formation and to promote epithelialization. Collagen has strong affinity to the soft tissue and blood vessels, and it can be replaced with host cells and vessels through acceleration of their penetration into the scaffold. The advantage of using collagen resides in its high biocompatibility, biodegradability, and weak antigenicity while offering support for cell attachment and growth compared with other natural biomaterials. Other natural biopolymers in currently commercially available skin substitutes contain gelatin or chitosan (163). Fast degradation and low mechanical stiffness limit their clinical use. Other naturally occurring polymers used in dermal substitutes include elastin, GAGs, fibronectin, fibrin, silk, and alginates (164). The benefit of natural polymers is that they exhibit low toxicity and elicit low inflammatory response (164).

One of the synthetic dermal substitutes is Integra®, the most widely used dermal substitute and the first FDA-approved artificial skin substitute (165). It is covered with 0.23 mm thick polysiloxane membrane mimicking epidermis. The silicone layer is stated to have vapor transmission characteristics similar to normal epithelium. The two-layer construct contains a 2-mm-thick dermal template layer of type I collagen from bovine hide and chondroitin-6-sulfate from shark's cartilage (166). This ADM has been shown to contain only modest inflammatory cell infiltrate—primarily macrophages and lymphocytes in the early phase after application (167). It has been explained that this would rely on the capability of chondroitin-6-sulfate to block aggregation of platelets and therefore to reduce pro-inflammatory signaling (166). Studies by Yannas et al. have strongly suggested that blocking of wound contraction follows extensive binding of myofibroblasts on the surface of the scaffold (168). Chondroitin-6-sulfate has also been suggested to bind TGF- $\beta$  (166).

The use of chondroitin-6-sulfate increased the resistance to collagenases of the material and decreased the need to use extensive cross-linking (165), even though the product contains only 8% of dry weight chondroitin-6-sulfate (169) thus delaying the degradation of the product.

Some of the synthetic polymers, such as polylactic-co-glycolic acid, polyhydroxybutyrate, and polyethylene glycol, are used as carrier scaffolds because of their good mechanical properties (162). Polyvinylpyrrolidone, polycaprolacton, polyethylene glycol, and poly lactic acid possess advantageous mechanical properties including elasticity and contractibility, similar to those of native skin with less

biocompatibility and in vitro and in vivo functionality and performance. There are some unsolved challenges, like wound contraction, impaired vascularization, scarring, and high cost associated with these products (170).

### **2.4.3 DERMO-EPIDERMAL SUBSTITUTES /COMPOSITE SKIN SUBSTITUTES**

These substitutes consist of two layers: epidermal cells and dermal substitute with fibroblasts. The cells can be either allogenic or autologous. If only allogenic cells are used the substitute may only be a temporary cover since the survival of allogenic cells limited to one to two months (171).

The cells of currently commercially available cellular skin substitutes contain only two types of cells, keratinocytes and fibroblasts. Regarding the quality of scarring one aspect is not to use just any kind of fibroblasts rather than using superficial dermal fibroblasts since they have been shown to be anti-fibrotic whereas deep dermal fibroblasts are shown to be pro-fibrotic (56). Keratinocytes and fibroblasts alone are nonetheless unable to supply sufficient pigmentation, pressure and temperature sensation, immune regulation, excretion, perspiration, thermoregulation, protection from ultraviolet radiation, synthetic function, and insulation (56). Current major differences between skin substitutes and the skin itself include the absence of stable vascular and lymphatic networks, skin appendages (such as hair follicles, sweat glands and sebaceous), and hypopigmentation (172). To resemble functional skin, melanocytes and Langerhans cells, adipose tissue and nerves, together with the vascular ingrowth, still need to be added together with the formation of skin appendices.

In burns the use of skin substitutes does not replace all skin elements and innervation. Their deficiency may cause the patient a variety of complications and discomfort, such as lack of sensation, perspiration, and motion flexibility (112). The pain in burn wounds may also play a role in controlling local inflammation.

### **2.4.4 BIOENGINEERED WOUND COVERS**

Bioengineered skin-replacement products may be either temporary or permanent. They can be either synthetic, or contain biological constructs, or a combination of both. Preference is given to products that are biodegradable containing natural polymers with binding sites for cells. The materials are expected to promote the ingrowth of surrounding cells or to serve as vehicle and scaffold for transplanted cells facilitating building of the new tissue (45). They may also contain growth factors, cytokines, other bioactive molecules, and ECM for host cells (173) or even allogeneic or autologous cells producing aforementioned. These high-tech products often induce cellular migration of local cells into the material or support attachment, proliferation, and differentiation of transplanted cells (45,156).

Artificial bioengineered scaffolds can be even further functionalized by adding signaling molecules regulating cell-cell and cell-matrix interactions to enhance matrix integration. The material can be cross-linked to resist wound contraction, add tensile strength, and reduce or delay degradation. On the other hand, cross-linking may decrease cellular infiltration, vascularization, and ECM deposition of the matrix (164).

**Table IV.** Skin substitutes, partial listing, P= permanent, T= temporary

<i>Epidermal substitutes</i>	Origin of material	Commercial product	P/T
Dehydrated Human Amnion/Chorion Membrane	Allogenic	Epifix	T
Cryopreserved Placental Membrane	Allogenic	Grafix	T
Lysate of cultured human keratinocyte	Allogenic	Lyphoderm	T
Cultured autologous keratinocytes fibrin substrate	Allogenic/Autologous	AcuDress	P
Cultured keratinocytes, multi-layer sheet	Autologous	EpiCel	P
Cultured epidermal autograft	Autologous	MySkin	P
Cultured keratinocytes (confluent cell sheet)	Autologous	EPIBASE	P
Cultured epidermal autograft suspension	Autologous	CellSpray	P
Uncultured suspension of auto hK, delivered as a spray	Autologous	ReCell	P
Autologus keratinocyte fibrin glue suspension	Autologous	Bioseed	P
Cultured keratinocytes from outer root sheath of scalp hair follicles (confluent cell sheet)	Autologous	EpiDex	P
Poly-dl-lactide	Biosynthetic	Suprathel	T
Benzyl ester of hyaluronic acid and a semipermeable silicone membrane	Biosynthetic	Jaloskin	T

**Table IV.** continues: Skin substitutes, partial listing, P= permanent, T= temporary

<i>Dermal substitutes</i>	Origin of material	Commercial product	P/T
Small intestine, acellular lyophilized	Porcine	OASIS Wound Matrix	P
Dermis, acellular lyophilized, coated with elastin hydrolysate	Bovine	Matriderm	P
Fetal bovine dermis	Bovine	Primatrix	P
Dermis, acellular diisocyanate cross-linked	Porcine	Permacol Surgical Implant	P
<i>Collagen based</i>			
Collagen, aldehyde cross-linked reconstituted	Porcine	EZ Derm	T
Multilayer bovine collagen matrix	Bovine	Collatamp	P
Collagen, cross-linked, Glycosaminoglycan, Polysiloxane	Bovine/synthetic	Integra	P
Collagen, lyophilized cross-linked sponge, heat-denatured, Silicone	Bovine/synthetic	Terudermis	P
Atelocollagen Silicone/silicone fortified with silicone gaze TREX	Porcine/synthetic	Pelnac Standard/ Pelnac Fortified	P
<i>Allogen dermis</i>			
Allogenic acellular dermis	Allogenic	Allopatch, DermaMatrix	P
Acellular lyophilized dermis	Allogenic	AlloDerm, Karoderm, SureDerm	P
A glycerol preserved acellular dermal collagen-elastin matrix obtained from human donorskin	Allogenic	Glyaderm	P
Cultured dermal allograft	Allogenic	ICX-SKN	T
Acellular pre-meshed	Allogenic	GraftJacket	P
Autologous dermal substitute	Autologous	DenovoDerm	P
Cultured dermal fibroblasts natural human collagen matrix	Allogenic/synthetic	ICX-SKN skin graft replacement	T
Polyglycolic acid/polylactic acid, extracellular matrix, derived from fibroblasts	Synthetic	Dermagraft	T
Polyethylene oxide terephthalate/Polybutylene terephthalate/Autologous keratinocytes and fibroblasts	Synthetic/autologous	PolyActive	T
Cultured allogeneic human dermal fibroblasts embedded in a human fibrin gel matrix	Allogenic	Cyzact (ICX-PRO)	T
Autologous cultured keratinocytes and fibroblasts in autologous fibrin	Autologous	MyDerm	P
Human fibrin and alginate matrix	Allogenic	Smart Matrix	T
Biodegradable polyurethane foam with a temporary non-biodegradable polyurethane seal	Synthetic	NovoSorb Biodegradable Temporing Matrix (BTM)	P

**Table IV.** continues: Skin substitutes, partial listing, P= permanent, T= temporary

<b>Composition substitutes and substitutes with cells</b>	<b>Origin of material</b>	<b>Commercial product</b>	<b>P/T</b>
Cultured composite autograft	Autologous	Cultured skin substitutes CSS	P
Autologous full thickness cultured skin	Autologous	Tiscover	P
Autologous full thickness substitute consisting of dermal and epidermal layers	Autologous	DenovoSkin	P
Cultured keratinocytes and fibroblasts in microperforated hyaluronic acid membrane (HAM)	Autologous	TissueTech Autograft System (Laserskin and Hyalograft 3D)	P
microperforated Hyaluronic acid membrane/Autologous fibroblasts	Allogenic	Hyalograft 3D	P
Cadaver skin	Allogenic		T
Native human cadaver skin with dermal and epidermal cells	Allogenic	Karoskin (Karocells)	T
Sheets of cells derived from neonatal (allogenic) foreskin	Allogenic	Celaderm	T
Sprayed suspension of allogenic keratinocytes and fibroblasts in fibrin substrate	Allogenic	Allox	T
Collagen/ Allogenic keratinocytes and fibroblasts	Bovine	Apligraf	T
Collagen/ Allogenic keratinocytes and fibroblasts	Bovine	Graftskin	T
Collagen, sponge/ Allogenic keratinocytes and fibroblasts	Bovine	OrCel	T
Collagen, Silicone film, Nylon mesh/Allogenic fibroblasts	Porcine/synthetic	Biobrane/Biobrane-L, TransCyte	T
Collagen/ Autologous keratinocytes and fibroblasts	Bovine	PermaDerm	P
Hyaluronic acid membrane (microperforated)/Autologous keratinocytes and fibroblasts	Recombinant rooster	TissueTech Autograft System, LaserSkin (Vivoderm)	P
Xenografts	Porcine	Mediskin	T

Data for the table was collected from following articles: (156,174-179).

## 2.5 SCAR ASSESSMENT METHODS

Scar assessments are needed to evaluate scar severity, make a treatment plan, follow up the efficacy of the treatments, and identify the need for modifying the treatments (180), but also to identify the risk factors for hypertrophic scars (181). Various tools have been used to assess scars but none of alone has yet been proven to be optimal. A gold standard is still lacking. In most cases, scars are assessed either with subjective scar assessment scales or with scar assessment devices measuring objectively one or more properties of the scar, and lately also with the combination of both.

The advantages of using scar scales are obvious: they are easy to use, inexpensive, and time-efficient (175), but their disadvantages are subjective assessments with low inter-rater reliability (168). The scar assessment devices instead are costly, and their use is often time-consuming. Even though devices give objective measures, they are prone to interpretation and measurement errors (176).

### 2.5.1 SCAR ASSESSMENT SCALES CURRENTLY AVAILABLE

The Vancouver Scar Scale (VSS), also called The Burn Scar Index, is the first validated scar scale and one of the most widely used scar assessment scales, both in clinical assessments and in research use (182-184). It has been developed for assessing burn scars. Four parameters are evaluated with separate scores ranging between 0 and 5: pigmentation, vascularity, pliability, and height. The higher the scar scores, the worse the scar is. The worst possible scar scores in the original version 14 points and in the amended version 13 points. The scale is subjective and the validity and reliability has been shown to be intermediate and it lacks the assessment of scar related symptoms. Most of the current scar scales and objective scar assessment devices have been compared to VSS in their validating process.

Only a few of the scar scales measure symptoms of the scars such as pain and itching. The Patient and Observer Scar Assessment Scale (POSAS), introduced in 2004, is one of the first, if not the first, validated scar scale that also takes into account the patient's perspective, and it also includes a patient rating for itching and pain (185). Parameters evaluated with the scale are the symptoms (e.g. pain and pruritus) and physical characteristics of a scar (e.g. vascularization, pigmentation, thickness, relief, pliability), ranked on a 1-10 ordinal scale. In a comparative study of VSS and POSAS, the latter has been found to be more reliable for single observers. Even this scale lacks the assessment of function or its deficits and the psychological and social aspects (182).

The Manchester Scar Scale (MSS) is based on three different types of evaluations: clinical assessment; rating of photographs; and evaluation of the histological properties of the scar. The ratings are combined with a visual analogue scale. Parameters evaluated include: color, contour, radiance (matte/shiny), texture, and distortion. This scale is best suited for assessing linear scars and it lacks the assessment of scar-related symptoms. (186).

The Stony Brook Scar Evaluation Scale (SBSES) is a photo-based scale and it was developed in the context of emergency medicine. The parameters assessed are: color;

elevation or depression; width; suture or staple marks; and overall appearance. Scores range from 0 to 5 for each parameter. This scale's inter-rater reliability is good, but it lacks the assessment of scar-related symptoms.(184).

The Hamilton Scale is based on evaluating photographs of the scars. The following parameters are assessed: color, vascularity, surface irregularity, and thickness. This scale has a good reliability, but it includes a possible disadvantage of distortion of observer's interpretation of the photograph. It also lacks the assessment of scar-related symptoms. (182).

The Seattle Scale is also based on evaluating photographs of the scars. The assessed parameters are: pigmentation, surface area, thickness, and height. The ratings also allow negative values for certain parameters, such as hypopigmentation or atrophy, to better differentiate between different scar types. It lacks the assessment of scar-related symptoms.(182).

The University of North Carolina 4P Scar Scale (UNC4P) was developed to widen the spectrum of the qualitative assessment of current scar scales. The "4Ps" of the UNC4P include pliability, pain, paresthesias, and pruritus, and the scale of the scar ranges from 0 to 12. It is designed to be used in combination with another scar scale like VSS (187).

Digital image capturing combined with in-person scar assessment has also been used to better classify scarring. A similar method is the Matching Assessment of Scars and Photographs (MAPS) that also combines in-person assessments with digital photos.

Visual Analog Scale (VAS) with scar ranking has been originally designed for assessing pain the patient is experiencing with a linear scale from 0 to 10, but has also been successfully used to assess patients' experience of their own scars. (182).

In addition to the above-mentioned scar-assessment tools, there are also patient-reported scales measuring health-related quality of life, such as the Patient-Reported Impact of Scars measure (PRISM) measuring also physical symptoms (188) and the Brisbane Burn Scar Impact Profile.

Since most of the original scar scales fail to address the symptoms caused by the scar, many have been modified to take symptoms into account. Not all the modifications have been validated (182). Itching and pain should be measured, but an objective measurement tool remains to be developed.

## **2.5.2 OBJECTIVE SCAR MEASURING DEVICES**

The following key characteristics of the scar can be measured objectively by various scar assessment devices: color in terms of pigmentation and vascularity; biomechanical characteristics such as pliability and elasticity, texture; and dimensions such as height/thickness, area, and volume of the scar. Pathophysiological disturbances in oxygen tension by transcutaneous oxygen tension (tcpO<sub>2</sub>) in water loss and moisture content could also be measured by transepidermal water loss (TEWL) in an open or closed chamber system, as well as tissue microstructure of the scar (189,190).

### ***Color measuring devices***

Color of the scar depends on vascularity of the scar, as well as the pigmentation of the skin involved. Inflammation and erythema increase the blood supply of the affected area.

Quantity of pigmentation can be measured by the intensity of a specific wave length of light reflecting back from the scar with the knowledge of light absorption patterns of haemoglobin, melanin, and collagen.

Devices based on reflectance spectroscopy utilize either tristimulus (three different wavelength illumination; regions for red, green and blue) or narrow-band scanning reflectance. Some examples of tristimulus-based devices include: Chromameter, Micro Color, and LabScan XE. Devices quantifying color with a narrow-band spectrophotometer utilize calculations of melanin and erythema index. Examples of such devices are DermaSpectrometer and Mexameter.

Laser imaging may be used for measuring microcirculation in the scar, for example, Laser Doppler Flowmetry (LDF), Laser Doppler Imaging (LDI), or Laser Speckle Imaging (LSI)/Laser Speckle Perfusion Imaging (LSPI) (190).

Cutaneous perfusion can also be measured light-based SIAScope or by in vivo confocal laser scanning microscopy (191). By intravital capillaroscopy it is possible to measure capillary density and the structure of vasculature by dermoscopy (190).

Digital photographs may also be analysed by quantifying color values in computerized analysis of two-dimensional (2D) or three-dimensional (3D) photographs (190).

### ***Scar dimensions measuring devices***

Surface area can be evaluated by planimetry or 3D imaging.

The height or the depth of the scar may be assessed by ultrasound, moulds, or dial caliper. Ultrasonography or ultrasound scanning may also include a tissue ultrasound palpation system (TULP or TUPS). Examples of TULP devices are PolyU, Aloka Echo Camera, and DermaScan-C and DermaScan-A (191). TUPS requires technical training and experience in image interpretation and is relatively expensive compared to other modalities though. Biomechanical characteristics, such elasticity, stiffness, and pliability can be measured by suction, tonometry, torsion, adherence, reviscometry, elastometry, ballistometry, dielectric measurements, and bio-impedance.

### ***Pliability measuring devices***

Devices suitable for measuring the pliability include pneumatometer, tissue tonometer, cutometer, dermal torque meter, and elastometer (191).

The firmness and/or hardness of the scar can be assessed by durometer, where vertically applied load causes indentation measured. Durometers have been used in assessing burn scars, but were originally developed for the assessment of scleroderma. Their disadvantage is high inter- and intraobserver variability (184).

Combination devices measuring multiple different properties of the scar are nowadays available, such as Dermalab combo.

## **2.5.3 PROTEIN PROFILING METHODS**

Different studies have found diverse amounts of proteins in skin using varying methods. In the Human Protein Atlas [www.proteinatlas.org](http://www.proteinatlas.org) (status 12.1.2020), based on transcriptomic analysis, 14857 proteins are expressed in human skin. This encompasses approximately 76% of all human proteins. The major problems when comparing different

proteomes is the differences in protein identification techniques (micro-array, immunohistochemistry, and mass spectrometry data), differences in anatomical sites and disease states.

The use of proteomic techniques in skin tissue has not proven to be easy due to an abundance cross-linking of proteins and high lipid content making isolation and digestion of proteins challenging for analyses (192). Proteomics may also be more sensitive and precise than previous or traditional methods, but translational and clinical significance of the findings often remain unclear.

The Manchester Proteome is one of the newer protein libraries of skin combining bioinformatics, systematic reviews, mass spectrometry, and immune-based techniques. It has 2948 identified proteins of healthy skin (193). Some proteomic studies have been conducted on scars (194,195).

## 2.6 PSORIASIS

This chapter provides a very brief overview of psoriasis characteristics and pathogenesis that can be related to the (patho)physiology of wound healing.

Psoriasis is a common chronic inflammatory (autoimmune) systemic disease affecting 2–3% of adults (196,197). It is characterized by abnormal keratinocyte proliferation, aberrant keratinocyte differentiation, and accelerated turnover of epidermal layers (196), as well as infiltration of inflammatory immune cells (197) and angiogenesis (198). Histopathologically typical changes include thickened stratum spinosum and stratum corneum, with retained nuclei as parakeratosis. In the suprabasal layer, keratinocytes proliferate excessively, whereas the granular layer is missing or abruptly (199). Psoriasis is also associated with other co-morbidities such as psoriasis arthritis and cardiovascular disease, together with risk factors such as dyslipidemia, higher blood pressure, and higher body mass index (197).

The following pathogenesis has been suggested: The innate immune system in psoriasis is dysregulated. Trauma or infection results in release of antimicrobial peptide LL37 by keratinocytes. LL37 binds to self-DNA and self-RNA fragments that are released from dying or stressed skin cells forming complexes. In turn, these complexes activate plasmacytoid dendritic cells (pDCs), which then produce type I interferons (IFN), for example IFN- $\alpha$ . Type I IFNs and immune complexes may then activate myeloid DCs. Activated mDCs release IL-23 and IL-12, which in turn activate Th17, Th1, and Th22 cells and their immune pathways. The latter produce cytokines, such as IL-17, IL-22, IFN- $\gamma$ , and tumor necrosis factor (TNF). These cytokines stimulate keratinocytes to intensify the inflammation characteristic of psoriatic lesion (200).

Keratinocyte hyperproliferation in psoriasis is possibly due to pro-inflammatory cytokines such as TNF, IL17A, and IL-23 produced by infiltrating immune cells like T cells, macrophages, and neutrophils (201,202). The cross-talk between epidermal keratinocytes and immune cells is disturbed (203).

Diseases like psoriasis exhibit aberrations in proliferation and differentiation through, in part, dysregulation in these epigenetic mechanisms (204). In psoriasis and other hyperproliferative skin diseases, integrins are also found in upper layers.

Psoriasis shares many immunological similarities with persistent wounding responses. Similar to wound-activated keratinocytes, suprabasal keratinocytes in psoriatic skin lesions also express high levels of KRT6, 16, and 17, which is considered to be a hallmark of psoriasis (19).

### 3. AIMS OF THE STUDY

The main goals of this thesis were to evaluate if the different early wound-bed treatments have effects on the wound healing process in the early phase, at three months, and at the final stage at the one-year time point.

The main hypothesis was that the use of an artificial dermal substitute would result in a better long-term outcome both functionally and cosmetically, whereas the induced granulation tissue was expected to result more hypertrophic scarring. The hypothesis in study II was that the additional neodermal ECM would have beneficial effects on STSG compared to fascially excised wound bed. The hypothesis in study III was that the treated sites differ in terms of their protein expression at the latest stage of follow-up, one year.

The objectives/specific aims of the individual studies were:

#### Study I

To assess how the use of different wound-cover materials during early wound healing affects the wound healing process within one year of an excised deep burn wound, using clinical, histological, and immunohistochemical analyses. This study focused on overall wound healing and scar assessment.

#### Study II

To evaluate how the pre-treatment of the excised wound bed, compared to no treatment, affects the vascularization, inflammation, and proliferation of the cells in the transplanted STSG one week after grafting and to evaluate the proliferation of the cells three months after grafting, focusing on computer-assisted image analysis of immunohistochemical findings.

#### Study III

To examine and compare the long-term effect of three different dermal compositions on transplanted STSG at the protein level one year after surgery of a deep burn wound. This study focused on one-year follow-up results.

To study the function and role of DNAH10, also with another group of patients (psoriasis) with similar features to wound healing, such as inflammation and epithelial hyperproliferation.

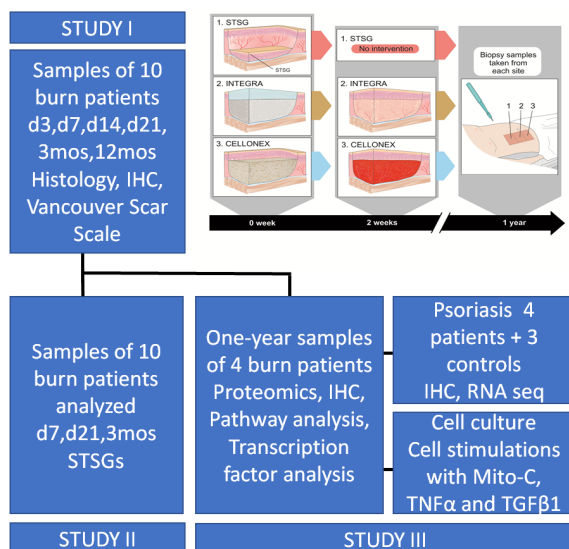


Figure 7. Experimental design

## **4. MATERIALS AND METHODS**

### **4.1 PATIENTS**

#### **4.1.1 BURN PATIENTS**

Ten patients with deep large burns admitted to Helsinki Burn Centre were recruited to participate in the study. The inclusion criteria were age between 17 and 70 years and deep burns over 20% of total body surface area on the anterior side of the body.

##### ***Burn patient demographics***

In studies I and II the age range of the patients was 19–58, mean  $36.8 \pm 4.0$  years at the time of the injury. Nine (90%) patients were men and one (10%) a woman. Total burned surface areas ranged from 22–45%, mean  $35.8 \pm 2.4\%$ .

The inclusion criteria were: age between 17 years and 70 years, the size of the deep burn over 20 % TBSA with possible test sites on the anterior side of the body to minimize shearing and maseration of the skin grafts. All burn patients received the same treatment, only the order of the materials used was randomized by simple randomization with no blinding.

Due to cost reasons in study III the samples only of four patients from study I at one year time point were used in assessments in study III. These four patients were selected due to technically best quality one year samples.

#### **4.1.2 PSORIASIS PATIENTS**

The age range of patients was 20- 65 years, mean 48,75 years. The samples of four patients were used for this study. Samples were originally collected for another study

#### **4.1.3 HEALTHY CONTROLS**

The age of control participants varied between 45 and 69 years with mean of 59,33 years, all female. Samples of the three healthy control participants were also obtained originally for another study. Ethics

All participants or their relatives in these studies provided a written informed consent adherent to Guidelines of Helsinki Declaration and the studies were approved by the Research Ethics Committee of Helsinki University Hospital (DNro 101/E6/2000).

## **4.2 WOUNDS AND WOUND COVER MATERIALS**

#### **4.2.1 BURN WOUND TYPE AND LOCATION**

Nine of the patients had flame burns and one had a burn from an electric arc. They all had deep third-degree burns needing fascial excision. In our burn-wound model the burned necrotic tissue from the anterior side of the body was removed tangentially on fascial level

within five days of the injury. The test areas selected for the study were located mainly on the chest area or chest/upper abdomen. For one patient, the test area was on the left medial thigh and for another patient on the right medial thigh. The test area was 10x15 cm, and was divided into three consecutive sections of 5x10 cm.

The three sections received a different cover material: one was covered with split thickness skin graft (STSG) right after excision; one with acellular dermal matrix (ADM); and one with a temporary wound cover of viscose cellulose sponge inducing granulation tissue (IGT) formation. The order of cover materials in each patient was randomized.

The STSGs were obtained with air driven Zimmer dermatome with setting of 8/1000 of an inch equalling around 0.2 mm of thickness. The STSGs in test areas were not meshed and they were attached with staples.

The artificial dermal substitute used in this thesis is Integra®, a two-layer construct. The upper layer is made of silicone and it functions as an artificial epidermis before it is peeled off or before it detaches from the underlying dermal part. The lower layer consists of bovine type I collagen and chondroitin-6-sulfate, a glycosaminoglycan from shark’s cartilage, which is chemically cross-linked. In these studies, the artificial dermal template was placed on an excised wound bed and attached with staples. After two weeks, the silicone layer was removed and replaced with an STSG.

Viscose cellulose sponge Cellonex™ (Vivoxid Ltd, Turku, Finland) contains 4–6 g of cellulose per 100g of viscose solution and it includes supportive cotton fibres about 20% of the weight of cellulose. The material has a porous structure with thin pore walls and the cells are able to migrate into the material. This temporary wound dressing was changed on days 3 and 7 and it was removed on day 14 and replaced with an STSG.

The surrounding areas were covered with meshed split thickness autografts. STSGs were covered with Surfisoft® monofilament polyamide woven sheet (Mediprof, Bleijswijk, the Netherlands) attached with staples. No local antibiotics or silver was used in test areas or their immediate proximity. In other areas, antimicrobial topical agents were used if needed.

#### 4.2.2 TIMELINE OF THE STUDY

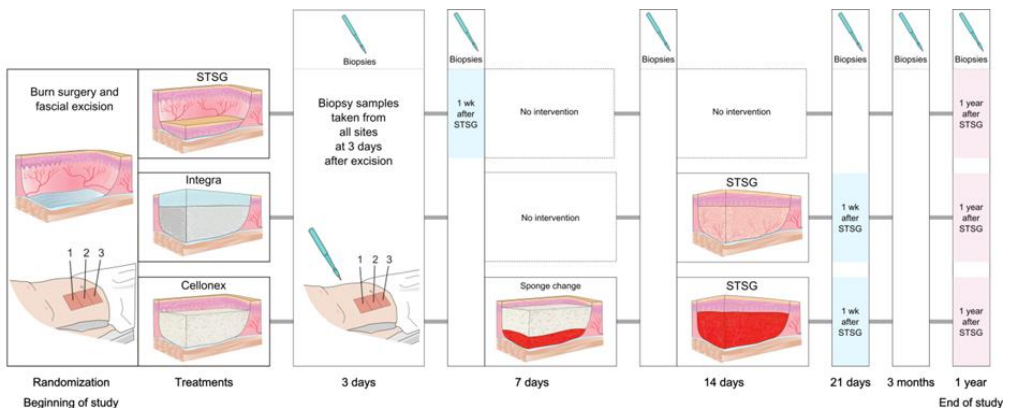


Figure 8. Timeline of the study. Illustration copyright Sole Lätti.

The burns were operated within five days of injury. During the initial debridement operation on day 0 of the study, a test area with three treatment sections measuring 5x10 cm, each adjacent to each other, was selected on the anterior side of the body within the deep third degree burn. After tangential excision on the fascial level, one of the treatment sections received a split thickness skin graft, another an artificial dermal substitute and the third was covered with a viscose cellulose sponge. At the times of changing the cellulose sponge, on days 3 and 7, punch biopsies and photographs of all treatment sections were also taken.

On day 14, the silicone layer of artificial dermal substitute was removed, the cellulose sponge was removed, and punch biopsies and photographs of all treatment sections were taken. Then, the two treatment sections without STSGs were covered with STSGs.

On day 21, as well as three and twelve months after primary operation, punch biopsies and photographs of all treatment sections were taken.

At three and twelve months after surgery, the Vancouver Scar Scale assessment was performed by an occupational therapist to evaluate the cosmetic and functional outcome of the treatment sections.

#### **4.2.3 SKIN SAMPLES (I, II, AND III)**

Skin samples of burn patients were obtained by 3–4 mm punch biopsies of the wound bed after tangential fascial excision of the burn and of all treatment areas 3, 7, 14, and 21 days, as well as 3 months and 12 months, after the primary operation.

The samples were fixed in 10% buffered formalin and after fixation embedded in paraffin.

For study I, all samples were used in analysis. In study II, samples from one week after transplantation of STSG (meaning day 7 for test areas of STSG alone and day 21 for test areas with ADM and IGT) and three months after primary operation were assessed. In study III, the samples of four patients at 12 months were analysed.

In study III, the skin samples of psoriasis patients were harvested with an air-driven dermatome with a setting of 4–6/ 1000 inches both from lesional and non-lesional sites.

Healthy control skin was obtained from reduction mammoplasties or microvascular free-flap surgeries. These samples were primarily collected for another study, and were fixed in 10% buffered formalin and after fixation embedded in paraffin. Then, 5µm-thick sections of these formalin-fixed paraffin-embedded samples were cut and stained immunohistochemically for evaluation of DNAH10 expression using the same staining protocol as detailed below for the burn-patient samples.

#### ***Stainings of the skin biopsy samples (I, II, and III)***

In study I, slides of all samples were stained with standard hematoxylin and eosin (HE).

In studies I, II, and III, samples were stained with various immunohistochemical (IHC) stainings using the following protocol:

After de-paraffination, the samples underwent four cycles of five and half minutes of heat-induced antigen retrieval with citrate-buffer (Citric acid monohydrate Merck and distilled aqua) pH 6.0 in a microwave oven. After cooling and washes with TBS, the

samples were blocked for endogenous peroxidase with 0.5% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min. Non-specific binding was blocked using 10% normal goat serum in TBS buffer at room temperature for 2h. The samples were then incubated in primary antibody solution overnight at +4°C. On the next day, after washes with TBS + 0.025% Triton, dilutions of secondary antibody in 0.5% BSA-TBS were added and the slides were incubated for 30 min at room temperature. The samples were then washed and incubated in ABC-complex followed by washes and incubation in aminoethyl carbazole (AEC) solution. After colour development, the slides were washed in lukewarm tap water and counterstained with Mayer's haematoxylin for 1 minute. Samples were then coated with Aquamount mounting medium.

Protocols varied according to the primary antibodies used. Instead of using citric acid in microwave treatment for IHC of collagen type IV, the deparaffinised slides were treated with trypsin digestion with 0.5% trypsin in PBS and then washed with PBS. In IHC of CD31, protease type VIII digestion was used instead of citric acid in microwave treatment. In MIB-1 antibody IHC, the endogenous peroxidase was not blocked. In alpha-SMA staining no previous citric acid in microwave was needed.

In study I, the staining of the samples was assessed with a semi-quantitative scale from 0 to 3 by two pathologists. In study I a 25-point Chalkey eyepiece graticule was used assess angiogenesis, particularly the vessel density. The calculations were made based on the average from assessment of three hot spots, where the number of points in the grid that hit stained vessels is counted. In studies II and III, the stained slides were scanned and the images were analysed as described in Chapter 8.

**Table V.** List of antibodies used in the studies.

Used in study	Staining/ antibody	Vendor	Dilution of primary antibody
I, II	CD31 as marker for endothelial cells	DakoCytomation, Glostrup, Denmark	1:100
II	CD163 a marker for type M2 macrophages, clone 10D6	Novocastra Laboratories Ltd, United Kingdom	1:200
I	Alpha SMA for smooth muscle cells and myofibroblasts, 6582	BioMakor, Israel	1:20,000
I, II, and III	MIB1 a Ki67 clone as a marker for proliferation, M7240	DakoCytomation	1:500
I, II	collagen type IV a marker for basement membrane, M0785	DakoCytomation	1:100
III	CASP14, a marker for keratinocyte differentiation, HPA027062	Sigma-Aldrich	1:1000
III	DNAH10 a marker for axonemal heavy chain dynein 10, HPA039065	Sigma-Aldrich	1:500

## ASSESSMENT AND ANALYSIS METHODS

### 4.2.4 VANCOUVER SCAR SCALE

Burn Scar Index/Vancouver Scar Scale is widely used clinical assessment for different kinds of scars. Four different qualities of scar were assessed by an occupational therapist. In study I, the assessment was performed by an occupational therapist treating scar problems. All the test areas were assessed twice, three months and twelve months after excision of the burn.

**Table VI.** Original Burn Scar Index assessment parameters and the criteria for values.

<b>Pigmentation M (0–2)</b>	Normal	0
	Hypopigmented	1
	Mixed	2
	Hyperpigmented	3
<b>Vascularity V (0–3)</b>	Normal	0
	Pink	1
	Red	2
	Purple	3
<b>Pliability V (0–5)</b>	Normal	0
	Supple	1
	Yielding	2
	Firm	3
	Banding	4
	Contracture	5
<b>Height H (0–3)</b>	Normal (flat)	0
	0–2 mm	1
	2–5 mm	2
	>5 mm	3

### 4.2.5 PROTEOMICS

#### *Tissue microdissection*

Sample preparation: paraffin-embedded three formalin-fixed 10- $\mu$ m-thick sections from each sample block were deparaffinized by incubation at room temperature in xylene for 10 min. After deparaffinization, tissue sections were rehydrated with a graded series of ethanol and briefly air-dried.

Microdissection: Skin epidermis and dermis including skin appendices were dissected with the PALM MicroBeam laser capture microdissection system (Zeiss, Göttingen, Germany) and collected into AdhesiveCap opaque tubes (Zeiss). The tissue was lysed in a buffer consisting of 0.3 M Tris-HCl, pH 8.0; 0.1 M DTT; 0.5% (w/v) polyethylene glycol 20 000; and 4% SDS. Then the samples were incubated at 99°C for 90 min.

## **Mass-spectrometry**

Sample purification was performed via methanol/chloroform protein precipitation. Protein reduction and alkylation was performed in 100 mM Tris (pH 8.0) containing 5% sodium deoxycholate by adding 5 mM DTT and 10 mM iodoacetamide for 20 min at room temperature. Protein digestion was carried out by diluting the solution 5 x with mQ-H<sub>2</sub>O, before adding 400 ng trypsin (Pierce, ThermoFischer Scientific, Waltham, MA) per reaction and incubating overnight at +37°C. Trifluoroacetic acid was added to a concentration of 0.5% to stop the digestion. The peptides were purified using C18 StageTips (3M Empore, Eagan, MN) and then analyzed on an Ultimate 3000 RSLCnano (Dionex, ThermoFisher Scientific) nano-LC system with a C18 cartridge column (Dionex) and an in-house packed (3 µm ReproSil-Pur C18AQ particles, Dr. Maisch HPLC GmbH, Ammerbuch, Germany) 50 cm 75 µm ID emitter-column (New Objective, Woburn, MA) using a 60 min 8–50% B gradient where buffer A was 0.1% formic acid in water and buffer B was 0.1% formic acid in 80% acetonitrile. Separated peptides were then eluted at 200 nl/min (spray voltage 2.4 kV) to a Q Exactive Plus (ThermoFisher Scientific) mass-spectrometer operating with a top-5 MS/MS strategy with a 0.6 s cycle time. Dynamic exclusion was set to 30 s. Only charge states +2 to +6 were analyzed. MaxQuant 1.4.0.8 software package was used to identify and quantify the raw data. Search was performed against the UniProt ([www.uniprot.org](http://www.uniprot.org)) human database using the tryptic digestion rule.

Analysis of proteomics data: Each sample of raw proteomic data was first normalized to the dissected tissue area, and then normalized to its detected proteins' average intensity. These sample tissue area- and intensity-normalized values were then used in subsequent analyses using a statistically significant cut-off of  $p < 0.05$  for each identified protein in any comparison between groups. Gene Cluster 3.0 software<sup>4</sup> was used for clustering of proteins and sample groups and heat map visualizations were produced by using Java TreeView 1.16r4 software.

## **4.2.6 IMAGING AND IMAGE ANALYSIS**

### **Scanning of slides**

For study III, the immunohistochemically stained slides from the samples were scanned with a 20x magnification microscope by the Digital microscopy and molecular pathology unit at the Finnish Institute for Molecular Medicine (FIMM, Helsinki, Finland).

Microscopy images were viewed using the Panoramic Viewer software (3DHISTEC Ltd, Budapest, Hungary). Snapshots of approximately 60–65x magnified views were captured.

### **Image analysis**

For studies II and III, the captured images of immunohistochemically stained slides were analysed by authors with FIJI ImageJ software (205). Macros for automated quantitative analysis of stainings were created and they are freely available from the authors.

#### **4.2.7 STATISTICAL AND BIostatistical ANALYSES, TOOLS AND DATABASES**

In study I, the nonparametric Friedman test was used for detecting differences between the effects of different wound-bed treatment materials in certain sectors of wound healing: in inflammatory cells, in angiogenesis, in numbers of epidermal cell layers, in type IV collagen of basement membrane, in myofibroblasts, and in VSS.

Spearman correlation test was used to analyse the correlation between fibroblasts and myofibroblasts. These analyses were performed by using Number Cruncher Statistical Systems-computer program (NCSS).

Page's trend test with R statistical software using package 'concord' was used for analyzing keratinocyte proliferation. For the rest of the analyses IBM SPSS version 19 computer program was used.

In study II, two-sided Student's t-tests were used to discover possible differences between different wound covers at one week and three months after skin grafting.

To calculate linear correlation (Pearson) between CD163+ macrophages and CD31+ endothelial cells GraphPad Prism 5.0 software was used.

In study III after normalization to their respective sample area, the identified proteins in the proteomics dataset that met the  $p < 0.05$  cutoff in normalized expression and were associated with biological functions and/or diseases in Ingenuity's Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone.

If not otherwise stated, statistical analyses were performed using Student's t-test for pairwise comparisons. P-values less than 0.05 were considered significant.

This was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. The proteins that met  $< 0.05$  cut off for p-value were included in the analysis.

Biostatistical tools such as Ingenuity Pathway Analysis (IPA, Qiagen Bioinformatics, Redwood City, CA) and TRANSFAC® were used in study III to analyse proteomic and sequencing transcriptomic data acquired from mass-spectrometry of the samples. Here both pathway analysis and upstream and downstream protein networks were generated using IPA.

TRANSFAC® is a database for eukaryotic transcription factors that was used in this study to predict potential transcription factor binding sites. The use of these bio-statistical pathway analyses is based on existing databases. The pathway here refers to an artificial simplified model of a process in cells or tissues. The model may involve signaling pathways, related proteins, identifying distinct cellular processes, or diseases with association to identified proteins or genes.

### ***RNA sequencing data comparison***

We collected and compared the fold-change values of differentially expressed dynein and kinesin genes from psoriatic patients' lesional, non-lesional areas and from the control samples of the 5'-end targeted RNA-sequencing data from Tervaniemi et al. (206) published data of psoriatic samples with other published studies.

## **4.2.8 CELL STIMULATION EXPERIMENTS**

### ***Cell cultures and cell stimulations (III)***

For study III, human primary keratinocytes for cell cultures were obtained from discarded full thickness skin of reduction mammoplasties and abdominoplasties. A written informed consent was obtained from all donors and the study was conducted according to the Helsinki Declaration. The primary keratinocytes were isolated from the epidermis.

### ***Keratinocyte isolation protocol of the collected skin samples***

The adipose tissue left on the skin samples was removed with a scalpel. The samples were minced into small pieces and rinsed carefully with phosphate-buffered saline (PBS). In order to separate epidermis from dermis using enzymatic digestion the minced pieces were placed into 6-well-plates filled with solution of 2 ml PBS and 2 ml of solution of lyophilised matrix metalloproteinase, dispase (Dispase, Roche Diagnostic Deutschland GmbH, Mannheim, Germany), 2 mg diluted to 10 ml of PBS and incubated overnight in the fridge + 4°C. After incubation the epidermis was peeled off from the dermis with needles. The isolated epidermal layer pieces were placed in a test tube containing 0.1% trypsin-PBS solution and incubated at 37°C for two minutes to detach the keratinocytes from the epidermis. The tube was vortexed cautiously until the clear trypsin became blurry. The gained single-cell suspension was transferred to another test tube using a cell strainer. To inhibit further trypsinization, a solution containing 10% of fetal bovine serum (FBS) – Dulbecco Modified Eagle Medium (DMEM) was added and then the new suspension was centrifuged for 5 minutes at 350 G. The supernatant was gently removed with a pipette and 5 ml of GIBCO's keratinocyte serum-free medium (KSFM) supplied with growth supplement and 1% penicillin-streptomycin was added and the suspension was centrifuged for 5 minutes at 350 G. Cells were counted and then the suspension was seeded on rat-tail collagen I coated cell culture flasks.

### ***The freezing and thawing protocols of the cell suspension***

Approximately 900 µl of cell suspension was transferred to each 1 ml cryovial containing 50 µl of dimethyl sulfoxide (DMSO) and 50 µl of 100% FBS. DMSO was used as a cryoprotectant to reduce ice formation and to prevent cell death during the freezing process. The cryovials were frozen in a cooler of -80°C for 24 hours and then stored in liquid nitrogen.

The cryovial containing the frozen cells from liquid nitrogen storage was thawed either in the incubator at 37°C or in the 37°C water bath. The thawed cells were transferred to a

Nunc centrifuge tube and pre-warmed complete growth medium was added. The cell suspension was gently centrifuged  $200 \times G$  for 3 minutes. After centrifugation, the supernatant was decanted without disturbing the cell pellet and replaced with new complete growth medium. A sample was taken for cell counting and the rest of the cells were re-suspended and transferred to a rat-tail collagen I coated cell culture flask for culturing.

### ***Cell culturing***

For study III both passages three and four were used. The isolated frozen primary human keratinocytes were cultured in defined keratinocyte serum-free growth medium (10744019, ThermoFisher Scientific, Gibco) for two passages and then passaged to glass slides coated with type I collagen for mitomycin C experiments. The keratinocytes were cultured for three passages and then passaged to 6-well plates for cell stimulation experiments.

### ***Protocol for mitomycin C treatment experiment for the fluorescence immunocytochemistry***

For cell cycle arrest,  $5\mu\text{g/ml}$  mitomycin C (Merck KGaA, Darmstadt, Germany) was used. The cultured keratinocytes of third passage (P3) on glass slides coated with type I collagen were incubated with  $5\mu\text{g/ml}$  mitomycin C for three hours. The control group was incubated with defined keratinocyte serum-free growth medium under the same circumstances. Cell cultures on glass slides were then washed with culture medium and incubated at  $37^\circ\text{C}$  for 72 hours.

### ***Protocol for mitomycin C treatment experiment for the Western Blotting***

The cultured keratinocytes (P3) on glass slides coated with type I collagen were incubated for three hours: one group with  $5\mu\text{g/ml}$  mitomycin C and the control group without mitomycin C. Then the wells of the glass slides of both groups were washed with culture medium and then incubated with fresh medium at  $37^\circ\text{C}$  for 24 hours. Western blotting was then performed to detect the differences in DNAH10 expression.

### ***Cell stimulation protocols***

For cell stimulation experiments, proinflammatory cytokines TNF- $\alpha$  and TGF- $\beta$ 1 were both from PeproTech PE Ltd, London, UK. The isolated frozen primary human keratinocytes were thawed and cultured as described above. After a third passage, the keratinocytes were passaged to 6-well plates (ThermoFischer Scientific) and then incubated. Two 6-well plates were used for four different groups, 3 wells for each group, approximately 3 million cells/ well. On the second day, the defined keratinocyte growth media was changed to Epilife. On the third day, the cells were kept in the incubator for growing. On day four, the media (Epilife ThermoFischer Scientific) of group one was changed. For stimulation of group two,  $10\text{ mg/ml}$  of TGF- $\beta$ 1 was added with the fresh media. For stimulation of group three,  $50\text{ ng/ml}$  of TNF- $\alpha$  was added with the fresh media. For group four,  $10\text{ mg/ml}$  of TGF- $\beta$ 1 and  $50\text{ ng/ml}$  of TNF- $\alpha$  was added with the fresh media. On day five, after 24 hours of incubation, the samples of Western blotting were harvested.

***Immunocytochemistry, immunofluorescence microscopy of treated cell cultures and Western blotting***

**Table VII. Antibodies**

Primary P, Secondary S	Staining/ antibody	Vendor	Dilution of antibody
Immunocytohistochemistry			
P	DNAH10 a marker for axonemal heavy chain dynein 10, rabbit IgG HPA039065	Sigma-Aldrich	1:500
S	Alexa Fluor 555-conjugated donkey anti-rabbit IgG A-31572	ThermoFisher Scientific	4 µg/ml
	ProLong™ Gold Antifade Mountant with DAPI for staining nuclei	ThermoFisher Scientific	
Western blotting			
P	anti-pan-actin for loading control	Thermo Fisher Scientific	1:500
S	goat anti-rabbit 800CW and goat anti-mouse 680LT	Li-Cor Biosciences, Lincoln, NE	1:10,000

To detect the fluorescence signals we used immunofluorescence microscope EVOS™ FL Imaging System (ThermoFisher Scientific).

For Western blotting to detect DNAH10 in mitomycin C experiments, the cell lysates of the two groups were collected into Laemmli Sample Buffer (Bio-Rad) and then separated by polyacrylamide gel electrophoresis (Bio-Rad). The proteins were then transferred onto Whatman nitrocellulose membranes (Sigma-Aldrich) and the non-specific binding to membranes was blocked in 5% BSA solution (Sigma-Aldrich). The fluorescence signals were detected using the Odyssey reader (Li-Cor Biosciences). Western blotting and fluorescence detection were also used in analysis of the effect of pro-inflammatory cytokines TNF $\alpha$ , TGF- $\beta$ 1, and their combination, on the expression of DNAH10 of P4 primary keratinocytes as described above.

## 5. RESULTS

### 5.1 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

In study I, hematoxylin and eosin staining of the first biopsy samples taken in the primary operation showed mainly muscle, some fascia, and some adipose cells. The samples were assessed by two pathologists with a semiquantitative scale from 0 to 3.

#### 5.1.1 INFLAMMATION

Inflammation, the amount of different types of leucocytes before covering the wound bed with split thickness skin graft, was higher in cellulose sponge treated granulating wound beds compared to ADM and control (C) groups. Neutrophils were highest in numbers on day 7 in cellulose-sponge treated areas of all test groups ( $p=0.0008$ , IGT mean  $1,45 \pm 1,12$  standard deviation (SD), C mean  $0,20 \pm 0,63$  SD, ADM mean  $0,00 \pm 0,00$  SD). The number of macrophages increased until day 14 in all test groups and the ADM group continued to rise until day 21. The same profile of increase was seen for lymphocytes. On days 7 and 14 IGT group showed highest numbers of lymphocytes of all test groups (on day 7  $p=0,03$ , IGT mean  $1,20 \pm 1,03$  SD, C mean  $0,38 \pm 0,46$  SD, ADM mean  $0,20 \pm 0,42$  SD, on day 14  $p=0,0029$ , IGT mean  $1,22 \pm 0,71$  SD, ADM mean  $0,350 \pm 0,41$  SD and C mean  $0,30 \pm 0,46$  SD). Similarly, the proliferation of inflammatory cells was highest for cellulose-sponge treated wound beds on day 14, whereas the other two treatment groups showed a minor increase in inflammatory cell proliferation on day 21 (on day 14  $p=0,009$ , IGT mean  $1,900 \pm 1,1972$  SD, C mean  $0,750 \pm 0,4249$  SD, ADM mean  $0,700 \pm 0,4830$  SD). Only occasional inflammatory cells were detected at later time points in any of the test areas. During the whole follow-up period, eosinophils and giant cells were only occasionally detected, so no allergic or foreign-body reactions as giant cell granulomas were seen.

In study II, the amount of anti-inflammatory repair M2 phenotype macrophages in STSGs was assessed one week after transplantation. The STSGs on IGT presented the most CD163 positive staining compared to the STSGs in the other two test areas ( $p=0,0002$  between IGT and ADM,  $p=0,0269$  between ADM and C, IGT mean  $0,5184 \pm 0,4032$  SD, C mean  $0,3901 \pm 0,4002$  SD, ADM mean  $0,2190 \pm 0,257$  SD).

#### 5.1.2 PROLIFERATION

In studies I and II, the proliferation phase from day 3 to day 21, overlapping with the remodelling phase, several measures were assessed, including: endothelial cells CD31, hotspots in Chalkley count, fibroblasts and myofibroblasts, pericytes/smooth muscle cells in blood vessels ( $\alpha$ -SMA), keratinocytes MIB-1 and fibroblasts MIB-1 and basement membrane collagen type IV.

## **Vascularization**

In study I, the single endothelial cells were detected from day three onwards. On days seven and fourteen the IGT group expressed the highest density of new capillaries of the test areas (on day 7  $p=0,007$ , IGT mean  $4,7996 \pm 1,2392$  SD, C mean  $4,3330 \pm 1,2668$  SD ADM mean  $3,2331 \pm 0,6673$  SD, on day 14  $p=0,01$ , IGT mean  $5,666 \pm 1,7213$  SD, C mean  $4,5996 \pm 1,7414$  SD, ADM mean  $4,4328 \pm 2,1548$  SD). The density of vessels was assessed by using Chalkley count. In this “hot spot” counting of new capillaries of the neodermis including also the dermal component of the STSG, the ADM group showed the highest density (of capillaries) by the third week ( $p=0,032$ , ADM mean  $6,4331 \pm 1,7429$  SD, C mean  $5,8297 \pm 2,1926$  SD, IGT mean  $5,0297 \pm 1,6174$ ). After STSG transplantation, the capillary density hot spots decreased in these areas.

In study I, the least smooth muscle cells were seen around vessels in the ADM group from day seven until day fourteen. The number of smooth muscle cells in the ADM group doubled thereafter and stayed that numerous until the end of the follow-up with comparable numbers to the other test areas.

In study II, one week after STSG transplantation, the STSGs in the IGT group showed the most CD-31 positive area ( $p=0.0453$ , IGT mean  $2,26 \pm 1.574$  SD, ADM mean  $1,697 \pm 1.095$  SD, C mean  $1,293 \pm 0.875$  SD) as well as largest average vessel size ( $p=0,0022$ , IGT mean  $83,4700 \pm 66.5100$  SD, ADM mean  $42.1800 \pm 42.1400$  SD, C mean  $51,8000 \pm 35.9500$  SD) and the highest vessel density (not significant) compared to the other groups.

Epithelialization results are shown combined in remodelling section.

## **Fibroplasia**

In the study I the numbers of fibroblasts were highest in IGT group on days 14 and 21 whereas the amount of myofibroblasts was the highest on same days in the control group with only significant difference in myofibroblasts on day fourteen ( $p=0,012$ , C mean  $1,500 \pm 1,1728$  SD, IGT mean  $0,778 \pm 0,833$ , ADM mean  $0,556 \pm 0,7265$ ).

In the study II the fibroblast proliferated at three months in control group the most, (almost five times more) compared to the other test groups. In study I in assessment of total fibroblast proliferation ADM group showed the most proliferating fibroblast compared to the other test groups with no significant difference. On days seven and fourteen the control group showed the most MIB-1 staining compared to the other test groups with significant difference (on day 7  $p=0,030$ , C mean  $0,667 \pm 0,7071$  SD, IGT mean  $0,500 \pm 0,500$  SD, ADM mean  $0 \pm 0,00$  SD, on day 14  $p=0,008$ , C mean  $0,700 \pm 0,6325$  SD, ADM mean  $0,050 \pm 0,1581$  SD and IGT mean  $0,200 \pm 0,4216$  SD). The highest MIB-1 staining was seen on day 21 with almost equal amounts both in ADM and IGT groups.

In general, the most fibroblast and myofibroblasts were detected on day 21, at the time the fibroblasts also proliferated the most. In a semi-quantitative scale there seemed to be more myofibroblasts present than fibroblasts on day 21.

### **5.1.3 REMODELING PHASE 3 WEEKS TO 12 MONTHS**

In study I and II the inflammatory cell proliferation was still detectable on day 21, also macrophages, also with phenotype M2 CD163 positive cells, were present in all test areas

together with a few lymphocytes. Thereafter there was a permanent reduction of inflammatory cells in all test areas.

The numbers of CD31 positively staining endothelial cells were reduced after day 21 in all test areas but the amount smooth muscle cells in vessels decreased only a little.

The amount of fibroblasts and myofibroblasts decreased gradually from three weeks towards 12 months with scarce fibroblasts and myofibroblasts in all test areas.

On day 21, a week after STSG transplantation, the dermal cell proliferation was higher in STSGs in both AMD and IGT groups compared to the control group ( $p= 0,0329$  between IGT and C, C mean  $1,112 \pm 1,077$  SD, ADM mean  $1,949 \pm 1,897$  SD, IGT mean  $1,869 \pm 1,212$  SD). By three months the proliferation of dermal cells in the control group increased whereas the in the other two groups it decreased with statistically significant differences ( $p= 0,0008$  between C and ADM,  $p= 0,0013$  between C and IGT, C mean  $2,345 \pm 3,988$  SD, ADM mean  $0,2569 \pm 0,2466$  SD, IGT mean  $0,2899 \pm 0,3345$  SD).

In study II the proliferation of keratinocytes in STSGs was assessed one week after grafting. The keratinocytes of STSGs on IGT group proliferated more compared to the two other groups ( $p= 0,0003$  between ADM and IGT,  $p < 0,0001$  between C and IGT, IGT mean  $5,725 \pm 3,956$  SD, C mean  $1,708 \pm 1,484$  SD, ADM mean  $2,028 \pm 2,59$  SD). The proliferation rate evened out and decreased by three months. In study III at twelve months follow-up the keratinocytes proliferated the most in IGT group and the least in ADM group with statistically significant differences ( $p=0,0025$  between ADM and IGT, IGT mean  $14,59 \pm 7,2759$  SD, C mean  $12,33 \pm 8,8377$  SD, ADM mean  $10,35 \pm 5,7116$  SD).

One week after grafting the STSGs on IGT group showed the most and the ADM group the least epidermal cell layers of the test areas but the difference was not significant.

The number on keratinocyte cell layers decreased from three months to twelve months.

In study I and III the BM component collagen type IV was assessed. The staining in STSGs was low or non-existing in most of the samples already from day three onwards; in control group it was only approximately 40 % of the maximum staining at one-year follow-up (unpublished data).

One week after grafting the staining of type IV collagen in STSGs on IGT group were lower compared to the other test groups, whereas on ADM group it stained the most (unpublished data). At three months the amount of collagen type IV was at the lowest. The increase in staining from three months to twelve months was substantial in all groups showing as almost a continuous line in most of the samples. The STSGs of ADM group showed the most staining of collagen type IV at twelve months compared to the other test areas ( $p=0,0168$  between ADM and C,  $p=0,0189$  between ADM and IGT, ADM mean  $0,1197 \pm 0,08018$  SD, IGT mean  $0,0801 \pm 0,07974$  SD, C mean  $0,08875 \pm 0,04161$  SD).

In study III the differentiation of keratinocytes in STSGs was assessed by staining with CASP14. STSGs on IGT group showed the least and control group the most staining at one-year follow-up ( $p=0,0395$  between C and IGT, IGT mean  $10,65 \pm 7,026$  SD, C mean  $15,65 \pm 10,51$  SD, ADM mean  $12,11 \pm 13,36$  SD).

DNAH10 staining at twelve months showed the least staining in epidermis of the control group with statistically significant differences compared to the other two test groups ( $p=0,00789$  between ADM and C,  $p=0,00024$  between C and IGT, ADM mean  $3,7713 \pm 4,1811$  SD, IGT mean  $2,2938 \pm 1,7273$  SD, C mean  $0,9855 \pm 0,5046$  SD).

## 5.2 CLINICAL FINDINGS-VSS

The original Vancouver Scar Scale (VSS) was used for scar assessment three months and twelve months after primary surgery. There were no significant differences between materials in either time point. See Table VII. None of the materials scored high in any of the parameters assessed in either time point.

Of the assessed parameters pigmentation and pliability improved the most between the follow-ups.

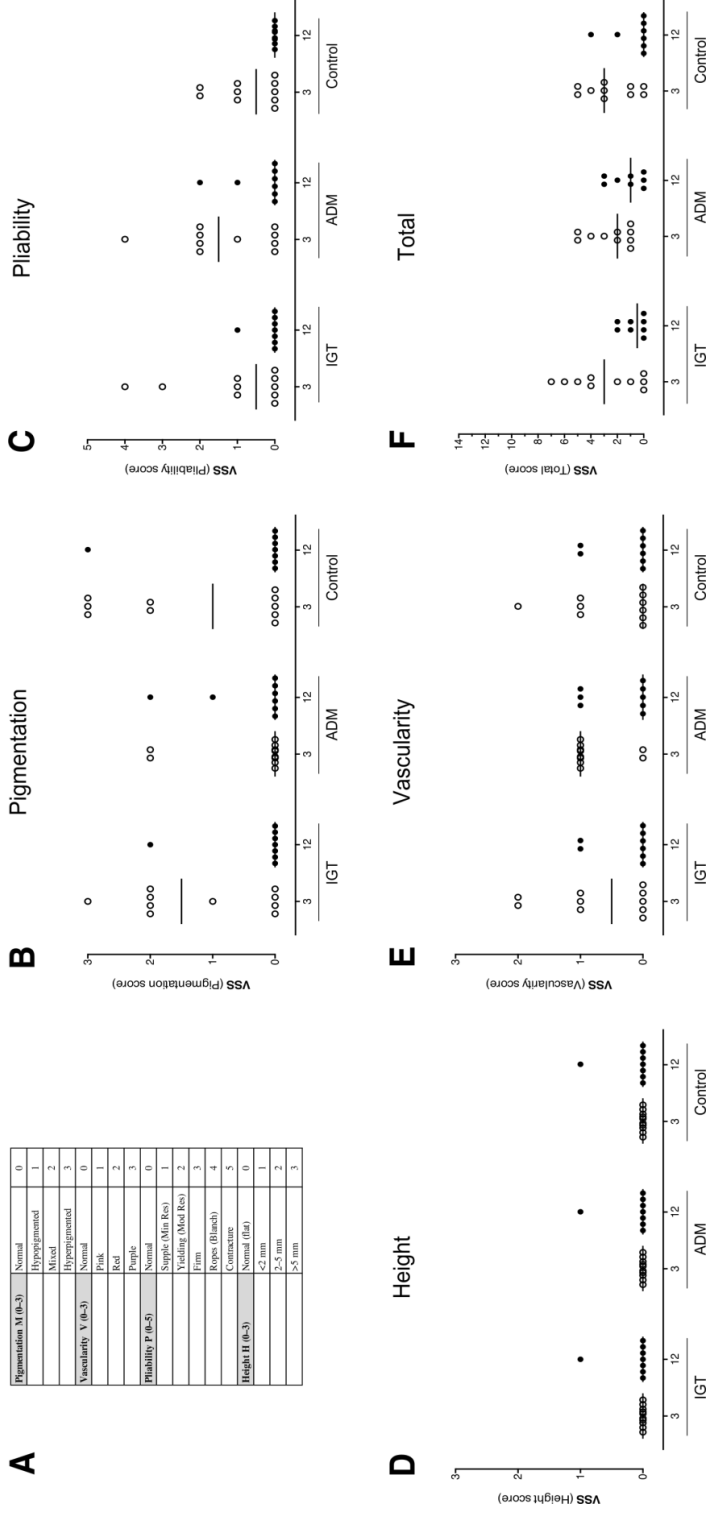
In pigmentation the ADM group scored very low and showed no changes, whereas IGT group and control group improved equally. The IGT group showed lower score in three month's follow-up though.

In pliability assessments the control group improved the most in mean values but the IGT group gained the lowest scores. ADM group had the highest scores at three months but also improved the most by twelve months. IGT group and control group performed very similarly and gained very low final scores.

In vascularity assessments the IGT group improved the most and gained the best scores. The control group improved the least, but the ADM group received the worst final scores.

The height of the scar was the only parameter that worsened during the course of time. All treatment sections scored the same. This occurred only in one patient of the series and equally in all materials.

The overall scores of VSS mean values improved for all materials from 3 to 12 months: in induced granulation tissue sections from 2.9 to 0.8, in STSG alone sections from 2.5 to 0.8 and in artificial dermal matrix sections from 2.5 to 1.3. In overall scores at 3 months  $p=0,971$  and at 12 months  $p=0,549$ . All scores are shown in Table VII.



**Figure 9.** Results of Vancouver Scar Scale. Horizontal lines represent median values. White dots represent three month's and black dots twelve month's assessment results.

### 5.3 PROTEOMICS

In study III protein content of one-year follow-up samples of four burn patients were analysed with mass-spectrometry. 481 proteins were identified out of which 131 proteins were epidermal and 181 were dermal proteins. The proteins not detected in all the samples were not counted.

The validation of the correct microdissection was performed by blind clustering analysis of all samples. As a result, the proteins divided into two main branches corresponding to epidermis and dermis with 46 proteins of epidermis and 90 mesenchymal proteins of dermis.

The proteins of different treatment sections were grouped accordingly. 12 percent of the proteins identified were statistically significantly differently expressed across the treatment sections. Of these differently expressed proteins (DEPs) of epidermis 2/3 were higher expressed in samples of control sections compared to treatment sections with additional dermal stimulus (either with dermal template or with cellulose sponge induced granulation tissue). Only one out of thirty, KRT34, of the DEPs of epidermis was higher expressed in the ADM group compared to other two groups.

The biggest differences were detected in expression of motor protein axonemal heavy chain dynein DNAH10 in epidermis with a 34-fold difference between the control sections and granulation tissue sections; here the control sections showing the least expression of DNAH10 protein.

The validation of proteomic results was performed by immunohistochemical stainings of same biopsy samples of most differently expressed protein DNAH10 together with CASP14. The results were similar even though not identical compared to results in proteomics confirming the validity of proteomics.

Of DEPs of dermis the biggest differences in expression were detected in four different keratin proteins: KRT1, KRT6C, KRT16 and KRT77. All these proteins showed higher expression in the IGT group compared to other treatment sections. The induced granulation tissue seemed to increase the expression of those four keratins in dermis.

Collagen 6A2 binds to extracellular matrix proteins, an interaction that explains the importance of this collagen in organizing matrix components. Here the highest expression was found in control group compared to other test groups.

COL12A1 is a member of the fibril-associated collagens with interrupted triple helices (FACIT) collagen family. Type XII collagen is found to be associated with type I collagen modifying interactions between collagen I fibrils and the surrounding matrix.

FN1, gene encoding fibronectin, is involved in cell adhesion and migration processes including embryogenesis, wound healing, blood coagulation, host defense, and metastasis. In both COL12A1 and FN1 the highest expression of the proteins were seen in ADM group compared to other test groups.

**Table VIII. Top 15 significantly differently expressed proteins**

EPIDERMIS																	
Protein	N (GT)	Average (GT)	SD (GT)	SEM (GT)	N (DM)	Average (DM)	SD (DM)	SEM (DM)	N (STSG)	Average (STSG)	SD (STSG)	SEM (STSG)	p-value (GT vs DM)	p-value (GT vs STSG)	p-value (DM vs STSG)	Min p-value across comparisons	Max average expression
DNAH10	3	31,486	41,226	23,802	2	11,978	8,231	5,820	4	0,921	1,499	0,749	0,287	0,093	0,021	0,021	31,486
KRT34	2	0,070	0,039	0,027	2	8,246	11,636	8,228	3	0,018	0,010	0,006	0,213	0,050	0,136	0,050	8,246
DSG1	4	2,675	1,033	0,516	3	1,545	0,822	0,475	4	3,979	1,204	0,602	0,091	0,076	0,015	0,015	3,979
CASP14	4	1,551	0,763	0,382	3	0,479	0,183	0,105	4	2,665	1,384	0,692	0,034	0,104	0,023	0,023	2,665
KRT16	4	1,010	0,543	0,271	3	1,923	0,388	0,224	4	2,560	2,976	1,488	0,029	0,173	0,367	0,029	2,560
ENO1	4	0,176	0,097	0,048	2	0,154	0,160	0,113	4	1,134	0,587	0,293	0,417	0,009	0,046	0,009	1,134
SPTBN1	2	0,604	0,783	0,554	2	0,379	0,188	0,133	4	0,120	0,096	0,048	0,365	0,117	0,038	0,038	0,604
PKM	4	0,168	0,070	0,035	2	0,130	0,172	0,122	4	0,564	0,211	0,106	0,350	0,006	0,034	0,006	0,564
PRDX2	4	0,342	0,154	0,077	2	0,181	0,169	0,120	4	0,495	0,095	0,048	0,152	0,072	0,019	0,019	0,495
LYZ	3	0,373	0,140	0,081	2	0,116	0,015	0,011	3	0,229	0,292	0,169	0,046	0,242	0,321	0,046	0,373
S100A9	4	0,084	0,061	0,031	2	0,063	0,016	0,011	2	0,347	0,152	0,107	0,270	0,015	0,056	0,015	0,347
TPH1	4	0,078	0,035	0,017	3	0,061	0,048	0,028	4	0,261	0,166	0,083	0,297	0,037	0,051	0,037	0,261
SFN	4	0,034	0,016	0,008	2	0,118	0,125	0,089	3	0,200	0,161	0,093	0,103	0,044	0,296	0,044	0,200
ATOH1	4	0,198	0,067	0,034	2	0,140	0,092	0,065	2	0,059	0,027	0,019	0,212	0,028	0,178	0,028	0,198
TGM1	4	0,102	0,072	0,036	2	0,127	0,118	0,084	4	0,198	0,066	0,033	0,380	0,049	0,187	0,049	0,198
DERMIS																	
Protein	N (GT)	Average (GT)	SD (GT)	SEM (GT)	N (DM)	Average (DM)	SD (DM)	SEM (DM)	N (STSG)	Average (STSG)	SD (STSG)	SEM (STSG)	p-value (GT vs DM)	p-value (GT vs STSG)	p-value (DM vs STSG)	Min p-value across comparisons	Max average expression
KRT11	4	150,956	60,264	30,132	4	86,093	28,998	14,499	4	70,916	37,007	18,504	0,050	0,032	0,271	0,032	150,956
KRT16C	4	2,145	1,330	0,665	4	0,281	0,138	0,069	4	1,459	1,753	0,877	0,016	0,278	0,114	0,016	2,145
KRT16	4	1,136	0,340	0,170	4	0,521	0,460	0,230	4	0,716	0,554	0,277	0,037	0,122	0,304	0,037	1,136
COL6A2	4	0,254	0,157	0,079	4	0,629	0,984	0,492	3	1,081	0,560	0,323	0,240	0,017	0,256	0,017	1,081
KRT17	4	1,023	1,470	0,735	4	0,297	0,064	0,032	4	0,837	0,534	0,267	0,181	0,410	0,046	0,046	1,023
PRDX2	4	0,777	0,570	0,285	4	0,204	0,081	0,040	4	0,197	0,075	0,037	0,047	0,045	0,446	0,045	0,777
BLMH	4	0,234	0,250	0,125	4	0,220	0,107	0,054	4	0,504	0,213	0,106	0,462	0,075	0,027	0,027	0,504
FN1	4	0,166	0,169	0,084	3	0,482	0,249	0,144	3	0,284	0,191	0,110	0,050	0,213	0,168	0,050	0,482
IGHM	2	0,029	0,027	0,019	2	0,231	0,081	0,057	2	0,114	0,120	0,085	0,039	0,126	0,184	0,039	0,231
COL12A1	3	0,023	0,008	0,005	3	0,195	0,081	0,047	3	0,073	0,071	0,041	0,011	0,147	0,061	0,011	0,195
ATP5A1	4	0,055	0,035	0,017	3	0,063	0,066	0,038	3	0,179	0,085	0,049	0,422	0,021	0,066	0,021	0,179
DSC3	2	0,148	0,027	0,019	3	0,036	0,014	0,008	4	0,053	0,061	0,031	0,004	0,058	0,334	0,004	0,148
TKT	2	0,137	0,081	0,057	3	0,095	0,016	0,009	4	0,052	0,009	0,005	0,209	0,039	0,003	0,003	0,137
PRSS1	2	0,136	0,004	0,003	2	0,099	0,091	0,064	3	0,046	0,047	0,027	0,312	0,042	0,220	0,042	0,136
PLEC	1	0,118	-	-	3	0,035	0,018	0,010	2	0,088	0,002	0,002	-	-	0,015	0,015	0,118

**Table IX. Top 10 upstream regulator proteins**

EPIDERMIS						
Upstream regulator	Entrez gene name	Molecule type	p-value of overlap	Target molecules in dataset		
ESRRG	estrogen related receptor gamma	ligand-dependent nuclear receptor	8.51E-08	ATP5F1B, ENO1, MDH2, PKM, TPI1		
MAPT	microtubule associated protein tau	other	1.67E-06	ATP5F1B, EIF4A2, ENO1, GDI2, PKM, RAB10, TPI1		
STK11	serine/threonine kinase 11	kinase	5.07E-06	ENO1, MDH2, PKM, PNP, RAB10, TPI1		
ESRRA	estrogen related receptor alpha	ligand-dependent nuclear receptor	8.71E-06	ATP5F1B, ENO1, KRT34, PKM, TPI1		
MYC	MYC proto-oncogene, bHLH transcription factor	transcription regulator	8.93E-06	ATOH1, ENO1, GDI2, GGH, LYZ, PKM, PRDX2, RAB10, TGM1, TPI1		
TAZ	tafazzin	enzyme	1.69E-05	KRT34, PKM, SERPINB7, TPI1		
PTP4A1	protein tyrosine phosphatase 4A1	phosphatase	3.35E-05	EIF6, GDI2, PKM, PRDX2		
EFNA4	ephrin A4	kinase	3.74E-05	DSG1, KRT16, TGM1		
EFNA3	ephrin A3	kinase	4.01E-05	DSG1, KRT16, TGM1		
EFNA5	ephrin A5	kinase	4.60E-05	DSG1, KRT16, TGM1		

DERMIS						
Upstream regulator	Entrez gene name	Molecule type	p-value of overlap	Target molecules in dataset		
MAPT	microtubule associated protein tau	other	2.30E-07	ANXA6, ATP5F1A, EEF2, HSPA1A/HSPA1B, PLEC, TPI1, TUBB2B		
PTP4A1	protein tyrosine phosphatase 4A1	phosphatase	2.58E-07	COL6A2, EEF2, HSPA1A/HSPA1B, PRDX2, TKT		
Tgf beta	transforming growth factor-β	group	9.87E-07	CLU, COL12A1, COL6A2, FN1, KRT1, KRT16		
MYC	MYC proto-oncogene, bHLH transcription factor	transcription regulator	5.62E-06	ANXA6, CLU, ECM1, EEF2, FN1, GGH, PRDX2, TKT, TPI1		
APP	amyloid beta precursor protein	other	8.66E-06	ANXA6, ATP5F1A, CLU, FN1, HSPA1A/HSPA1B, PRDX2, TPI1, TUBB2B		
KRT14	keratin 14	other	1.21E-05	DSC3, KRT1, KRT16		
SMYD1	SET and MYND domain containing 1	transcription regulator	3.32E-05	ATP5F1A, TKT, TPI1		
CD38	CD38 molecule	enzyme	3.67E-05	ANXA6, FN1, TPI1, TUBB2B		
BCL2L1	BCL2 like 1	other	6.13E-05	COL6A2, ECM1, FN1		
CDK4/6	cyclin dependent kinase 4/6	group	7.99E-05	KRT1, PRSS1, TPI1		

### 5.3.1 GENE EXPRESSION ANALYSES

In the study III using Ingenuity Pathway Analysis (IPA) software the pathway and network analysis were performed for epidermal differentially expressed proteins of STSGs of burn patients. Analysis of the upregulated proteins showed showed following functions possibly linked to DNAH10: epithelial cell proliferation, hair cell differentiation and epithelial barrier formation.

The promoter regions of DNAH10 were analysed by using TRANSFAC® to predict transcription factors of DNAH10. In this analysis and prediction of the best three hits for transcription factors yielded following transcription factors: AP-2, KLF-6 and ZF-5, which all regulate cell proliferation and differentiation.

The DNAH10 gene has been shown to have ten splice variants. Six out of these transcripts are coding proteins. Two of them, DNAH10-210 and DNAH10-201, are coding large dynein proteins (500 kDa) and three of them, namely DNAH10-205, -207, and -209, code smaller DNAH10 proteins (35-115kDa). Three of transcripts yield protein products that include unique peptide sequence “LESIFIGDIRSQLPEEAKKFDNIDKVF”: DNAH10-205, DNAH10-201 and DNAH10-210. In our proteomic analysis in mass-spectrometry we also identified a unique DNAH10 peptide that could be encoded by a shorter transcript variant DNAH10-205.

The RNA sequencing data of lesional and non-lesional psoriatic skin showed a down regulation of DNAH10 in lesional psoriasis whereas in immunohistochemistry there was a clear increase in DNAH10 in lesional psoriasis compared to non-lesional and healthy control skin ( $p < 0,0001$  between healthy psoriasis skin and psoriasis lesion, psoriasis lesion mean  $0,1794 \pm 0,1147$  SD, healthy psoriasis skin mean  $0,07966 \pm 0,008032$  SD,  $p < 0,0001$  between psoriasis lesion and healthy control, healthy control mean  $0,07267 \pm 0,09042$  SD).

### 5.3.2 CELL STIMULATION EXPERIMENTS

In order to better understand the possible role/ functions and regulation of DNAH10 primary human keratinocytes (PHK) were cultured and stimulated with different agents.

A three-hour- treatment of primary keratinocyte cell culture with Mitomycin-C resulted in increase in expression of DNAH10 compared to non-treated keratinocyte cell cultures after 72- hour- culture medium incubation. Mitomycin C is an antineoplastic agent blocking DNA synthesis and thereby cell proliferation.

Cell stimulation of pro-inflammatory cytokines TNF- $\alpha$  and growth factor TGF- $\beta$ 1 resulted in a decrease in expression of DNAH10 in primary human keratinocytes after 24-hour-incubation. TGF- $\beta$ 1 decreased the expression of DNAH10 less than TNF- $\alpha$  but their combination showed the strongest decrease in DNAH10 expression.

**Table X.** Summary of results of burn patients study III + VSS

Parameter	STSG	ADM	IGT
VSS	2,5 -> 0,8	2,5 -> 1,3	2,9 -> 0,8
Ki67(MIB-1)	++	+	+++
Col IV	++	+++	+
CASP14	+++	++	++
DNAH10	+	+++	++
Epidermis DEP top 15 highest expression mean values	10/15 KRT34+	1/15 KRT34++	4/15 KRT34+++
Dermis DEP top 15 highest expression mean values	3/15	4/15	8/15
Expression mean values			
DNAH10	0,921	11,978	31,486
KRT1	70,916	86,093	150,956
KRT6C	1,459	0,281	2,145
KRT16	0,716	0,521	1,136

## **6. DISCUSSION**

This research is based on materials retrieved from a prospective study on ten patients with third-degree burns. Two different types of materials were used in test areas and the purpose was to evaluate if the different wound bed compositions in the early healing phase have an effect on wound healing process and/or on the outcome of a skin graft.

Covering the excised burn wound either with a temporary material or with dermal components containing permanent material permits a delay in final epidermal coverage. This is crucial in cases where there is a shortage of donor sites when re-harvesting of the sites is inevitable. It is important that these covering materials fulfill the requirements of barrier function from outside in. Regarding inside out barrier function, preventing the escape of fluids, would be profitable but seldom available in temporary dressings. The gold standard is to use STSGs as final covers of excised burns even after pre-treatment of the wound bed. Despite the commonness of STSG use in surgery, relatively little is known about the wound healing of the skin graft at the cellular or molecular level in humans.

### **6.1 THE EFFECTS OF DIFFERENT TREATMENT MATERIALS ON WOUND HEALING AND ON STSGS, AND LONG-TERM OUTCOME ONE YEAR AFTER SURGERY, STUDIES I & II**

The focus of study I was on clinical, histological, and immunohistochemical evaluation. In study II, the focus was on the healing of STSGs during transition from inflammation phase to proliferation phase and in remodeling phase evaluated with image-assisted analysis.

Viscose cellulose sponge Cellonex™ a by-product engineered by the wood industry was used in these studies as a temporary wound cover. It was changed as a wound dressing twice before removal and replacement with STSG. The purpose was to induce granulation tissue formation on the wound bed to optimize the environment for skin grafting. The suitability of the material for wound temporizing before harvesting the skin graft was also tested. In previous studies, the material has been shown to act as a chemoattractant for cells that are involved in the repair process. Even doing so, the cellulose sponge has been shown not to interfere with the normal wound healing process (207-210). Chemoattraction of cells into cellulose sponge has been explained by absorption of proteins of blood into the material. Platelets from the clot secrete cytokines and growth factors attracting inflammatory cells to the site (211). When implanted in rodent studies the viscose cellulose sponge has been shown to elicit moderate foreign-body reaction. The same cellulose sponge material coated with hydroxyapatite was shown to attract circulating hematopoietic and mesenchymal progenitor cells in a rodent study (212).

The other material tested was an acellular dermal matrix (ADM), a dermal substitute Integra®. It is a synthetic combination of collagen type I, the most

abundant ECM protein in dermis, from bovine hide and glycosaminoglycan chondroitin-6-sulfate from shark's cartilage and on top a polysiloxane membrane mimicking epidermis for barrier function. This ADM offers components of natural dermal ECM as a template for the migration of the cells and for the ingrowth of tissue with vasculature. The host cells are expected to remodel and degrade the matrix while simultaneously producing replacing components to achieve ECM with closer resemblance to normal skin than to a scar.

### **6.1.1 BIOCOMPATIBILITY AND BIODEGRADABILITY**

In order for a material to be biocompatible with the host tissue it should perform its desired function without causing adverse effects (213). In these studies the materials performed as expected, promoting wound healing and functioning as a barrier without causing undesirable effects. In permanent substitutes, the degradation time is of great importance. If the matrix is degraded too early the contractile cells are released too early causing wound contraction. If the degradation is prolonged the foreign-body reaction and macrophages are activated causing inflammation resulting scarring (213). ADM used in these studies is stated to be degraded approximately within 30 days (Summary of Safety and Effectiveness Data-FDA). Chondroitin-6-sulfate (C6S) inhibits proteolytic enzymes. It takes part in the binding and regulating growth factors. It also influences chemotaxis, migration, hyperplasia, and differentiation of cells. C6S in the dermal substitute could reduce biodegradation of collagen caused by proteolytic enzymes, which increases physical stability and could facilitate the migration of cells from the surrounding tissue into the collagen scaffold. (214) Similarly to the study of Jeng et al. (215) in the current study the remnants of ADM were suspected to be still visible in samples at 3 months but no immunohistochemical staining was performed to confirm the finding. The viscose cellulose sponge used in this study is not intended to be used as a dermal substitute in humans due to its slow degradation by non-enzymatic hydrolysis and it eliciting foreign-body reactions.

### **6.1.2 INFLAMMATION**

The mechanism of action of these two materials is almost the opposite in wound healing. The cellulose sponge is known to elicit a local inflammatory reaction and foreign-body reaction (211) whereas a component of ADM, chondroitin-6-sulfate exhibits anti-inflammatory properties. Chondroitin sulfate (CS) has been shown to decrease inflammatory signaling by reducing NF- $\kappa$ B pathway activation in macrophages and therefore it has been hypothesized that CS could be beneficial in diseases with a strong inflammatory component with preliminary evidence of improving moderate to severe psoriasis. CS has also been reported to reduce pro-inflammatory cytokines like IL-6 and IL-1 $\beta$ , as well as inhibiting collagen type I degradation by MMP-1 (216). Chondroitin has also been suggested to mask the sites of binding on collagen preventing platelet adhesion and thereby reducing the resulting inflammation (169).

In the current studies (I and II), the above-mentioned properties of the materials were confirmed. Cellulose sponge treated test areas exhibited the most of all types of leukocytes during the time before permanent covering of the wound with STSGs, whereas ADM-treated test areas showed the least inflammatory cells. The low to moderate inflammatory cell infiltration to the ADM site is in accordance with other studies (217-219). Inflammation was not particularly prominent in any of the test areas though. The inflammatory response in cellulose-sponge treated areas may have been additionally increased by the possible contamination of the wound bed during dressing changes. The prolonged inflammation of the wound bed combined with delayed onset of epithelialization has been seen as an unwanted feature in burn surgery predisposing to hypertrophic scarring. Bacterial colonization prolonging inflammation and stimulating scar formation has been found to be a risk factor for hypertrophic scarring with level II evidence.(220).

Both neutrophils and M1 macrophages show antimicrobial activity. Early-stage macrophages also control induction of granulation tissue and myofibroblast differentiation whereas M2 macrophages have been shown to decrease their expression of inflammatory cytokines and increase anti-inflammatory signaling (145). In study II, CD163 staining was used to identify M2 macrophages, both M2c representing macrophages that suppress proinflammatory cytokines and M2d representing regulatory macrophages (221). One week after grafting STSGs onto cellulose sponge induced granulation tissue (IGT) presented the most M2 macrophages and the ADM the least of the test groups. This is in accordance with findings of total numbers of leukocytes and of macrophages which both had the highest counts in the IGT group and the lowest counts in the ADM group of the test groups in study I. Especially on days 7 and 14, the cellulose-sponge treated areas showed markedly more neutrophils than the other test areas. After permanent covering of the wound bed with STSG the wound bed contamination of microbes ceased and the barrier function was restored, resulting less triggers for pro-inflammatory signaling, creating a microenvironment favorable for macrophage conversion into the M2 phenotype. Macrophages may alter their phenotype according to their microenvironment (222). According to a rodent study of Agrawal et al. the shifting from M1 to M2 polarization altered back and forth in the same ADM during healing in (223). This shifting back and forth in ADM could not be detected in the current study.

### **6.1.3 PROLIFERATION PHASE**

#### ***Angiogenesis, granulation tissue and revascularization of STSGs***

Angiogenesis in healing wounds is closely associated with the production of vascular endothelial growth factor (VEGF), which promotes neovascularization by enhancing endothelial cell proliferation (224). Macrophages have a crucial role in angiogenesis by producing VEGF-A (221). It has been suggested that M1 macrophages stimulate capillary sprouting, whereas M2 macrophages assist in vessel stabilization through pericyte recruitment (221). Macrophages have been shown to

guide and bridge angiogenic sprouts enabling endothelial cell tip fusion or anastomosis between two tips and to facilitate the co-operation between pericytes and endothelial cells. Macrophages are also able to transdifferentiate into endothelial cells, smooth muscle cells and pericytes (225). Mid-stage macrophages have been shown to play a role in transition of granulation tissue into scar tissue (145).

Cellulose sponge has been used both in animal experiments and in treating traumatic injuries and burns to promote granulation tissue formation (207,226-228). The current findings are in accordance with these studies. In study I in hot spot counting by Chalkley count, the IGT group exhibited the highest density of capillaries of all test areas during days 7 and 14, whereas in the ADM group the angiogenesis of the material proceeded more slowly showing the least staining of the test groups during days 7 and 14. In all patients, the ADM had vascularized by two weeks as had been seen in another study in the acute setting (219), whereas in reconstructive cases the vascularization has been shown to take longer (229,230). M2 macrophages are known promote angiogenesis, tissue re-modelling, and tissue repair. It has been suggested that macrophage influx and angiogenesis are spatially correlated (221). In study II the higher incidence of M2 in the IGT also coincided with stronger vascular response.

In burn surgery there is a controversy regarding granulation tissue formation. Increase in vascularity contributes to a good graft take, but prior studies have associated the increased vascularity with high inflammatory cell density and eventually skin fibrosis or hypertrophic scarring (231). On the other hand, granulation tissue provides an infrastructure for epithelialization (232) and has also been suggested to be a source of dermal stem cells (232). There is no clear cut-off point of beneficial granulation tissue. We currently rely on clinical judgement and bacterial cultures to guide the removal of granulation tissue completing the surgical treatment after the primary burn excision. In developing countries the removal of granulation tissue in burns is sometimes considered unnecessary (233). A study by Dhar et al. found no significant difference in the microbiological profile, graft take, or esthetic appearance of the grafts between surgical removal and non-removal of the granulation tissue (234). In another study on chronic-burn wound patients, failure to remove the granulation tissue had no adverse effects on the graft take. Non-removal of granulation tissue was associated with less intraoperative blood loss, less oozing of tissue fluids conserving the proteins especially important in malnourished patients, and reduced time for hemostasis (233).

### ***Graft take and ADM take***

Before the ingrowth of vasculature took place there was no supply of oxygen and nutrition into the ADMs and STSGs other than through imbibition (fluid absorption) of plasma accompanied with sporadic cells. The graft survival and graft take relies on the restoration of perfusion. Despite the commonness of STSG use, the physiological (re)vascularization process is poorly understood (156,235). It is not fully clear how the revascularization of the skin graft occurs, whether the endothelial cells of the wound bed migrate and populate the graft, or how the cells from the graft intrude into the wound bed. According to animal studies, the current view supports

the idea of ingrowth of newly formed blood vessels from the wound bed into the graft, which are subsequently connected to the existing vasculature by inosculation “plug-in”. The perfusion of graft capillaries in mice has been seen by intravital microscopy already on day 3 and the restoration of circulation by day 5 (235). In the current study, the timely graft take was good irrespective of the treatment area. No complications preventing graft take like hematoma, seroma, or infection, were detected. One STSG on ADM was lost due to erroneous upside-down placement of the STSG and it was successfully replaced one week later.

ADMs were vascularized by angiogenesis from the wound bed. Angiogenesis into the ADM seemed to be slower than the formation of granulation tissue with abundant vascularity on the cellulose sponge-treated test areas but all ADMs vascularized well within fourteen days. This is in accordance with other studies in acute settings (219), whereas in later reconstructions the vascularization is decelerated until four weeks (230) or later. This time delay interval before vascularization of a skin substitute limits its use and predisposes the material and wound bed to infection. On the other hand in many situations the time delay is appreciated and this wound temporising can be continued for weeks, even months by absence of infection while awaiting the possibility to re-harvest the donor sites. Some current dermal substitutes permit STSG transplantation simultaneously relying on the graft imbibition through the substitute but the risk of delay of plugging in of STSG and thereby the risk of graft loss is increased.

In study II, in image analysis one week after transplantation the STSGs on IGT showed the most stained total area and the largest average size of vessels with significant difference compared to the other test areas. In this image analysis the vessel density was also higher in cellulose-sponge-treated areas than in other test areas, but the difference remained non-significant. This all suggests that the cellulose sponge-induced active matrix of granulation tissue is supportive microenvironment for graft take and the proliferative phase of wound healing.

### ***Epithelialization***

All test areas were covered with unmeshed equally thick STSGs within two weeks after burn-wound excision and all grafts took (except one mentioned earlier, which was successfully re-grafted one week later). Papillary fibroblasts in granulation tissue are found to support epithelialization and formation of BM, and to produce less collagen and more proteoglycans resulting in improved differentiation and stratification of epidermis (34). In these studies all STSGs contained papillary dermis with fibroblasts but the granulation tissue in each test group was different. One year after excision the differentiation evaluated by differentiation marker CASP14 (study III) was higher on the control sites compared to the other test areas. In numbers of cell layers, no differences between the test groups were detected.

### **Keratinocyte proliferation**

One week after grafting, the keratinocytes in STSGs on IGT proliferated markedly more than on other test groups with highly significant differences. The increased proliferation of keratinocytes on IGT corresponded and coincided with higher numbers of M2 macrophages as well as with increased vascularity compared

to other test areas. The STSGs on IGT likely had good supply of oxygen and nutrition as well as the supply of inflammatory cells especially monocyte/macrophages from circulation in addition to local tissue macrophages. Rich vascularity also guaranteed an adequate amount of endothelial cells to produce growth factors in conjunction with macrophages and fibroblasts of granulation tissue to promote keratinocyte proliferation. Factors known to promote keratinocyte proliferation include growth factors and cytokines like: IL-1, -6, and -8; TGF- $\alpha$ ; GM-CSF; and amphiregulin (236). These factors are produced by keratinocytes, but partly also by macrophages, endothelial cells, and fibroblasts. The constant cross-talk between keratinocytes, fibroblasts, inflammatory cells, and endothelial cells is vital in order for wound healing to proceed properly. By three months there was no difference between the test areas in proliferation of keratinocytes since the inflammation ceased with decreased secretion of growth factors and cytokines. In study III, at one year the keratinocyte proliferation was again significantly higher in IGT than in ADM treatment sites. The reason for this is unclear. There is a wide variety of possible explanations such as chronic irritation or inflammation (237), differences in epidermal barrier function, in T-memory cells or if it was due to epidermal stem cells remembering earlier stronger inflammation with the changes in their chromosomes, overproduction of growth factors, abnormal response to lymphokines and cytokines, disturbances in IL-1 cascade (238), tissue stiffening (239), abnormal keratinocyte proliferation and differentiation, dermal infiltration of immune cells, increased capillary density and wide capillaries with increased permeability enhancing recruitment of inflammatory cells (240), histamine (241) which were not examined in this study.

### ***Changes in BM***

Even though the re-epithelialization was not an issue in these studies changes in BM composition were discovered. Collagen type IV in BM was found to be low or non-existing in most of the samples by day three and up to 3 months, at which time its' expression was lowest. By 12 months, collagen type IV was almost a continuous line in most of the samples. The STSGs of ADM group showed the most staining of collagen type IV and the STSGs on IGT the least staining at twelve months compared to the other test areas. Immunohistochemical staining of laminin-5 was also tested and yielded no staining results. Either the antibody tested did not work in the samples, or there was no laminin-5 present. The reason for missing or decreased type IV collagen in BM of STSGs after transplantation is unclear. The possible explanations could be either that the dermatome affects the BMs during the harvesting of STSGs via an unknown mechanism, or that this is the natural course of healing of STSGs. It is known that upon tissue injury pro-inflammatory and pro-fibrotic mediators such as IL-1, IL-6, TNF- $\alpha$ , and TGF- $\beta$  are released (128). As a result, secretion of metalloproteases like MMP-2 and MMP-9 is increased in conjunction with migration of macrophages to the site of injury releasing MMP-12 which all degrade type IV collagen. Fragmented BM allows entry and exit of inflammatory cells like macrophages, and fibroblasts forming fibrotic tissue. Moreover, the breakdown of BM and increased expression of TGF- $\beta$ 1 have been

suggested to promote epithelial-mesenchymal transformation (242) and likely also contribute to myofibroblasts transformation increasing scar formation. MMP-2-mediated proteolytic alteration of type IV collagen is important for the migration of macrophages and fibroblasts (128). In studies I and III, an inverse relationship was observed between keratinocyte proliferation evaluated of MIB1 and expression of BM collagen type IV between the different treatment areas. One possible explanation could be that the proliferating cells produce growth factors and cytokines that induce the release and synthesis of proteases degrading collagen type IV simultaneously. Horch (243) and Langdon (244) both observed the disappearance and later re-appearance of BM after skin grafting in different settings.

### ***Fibroblast proliferation***

In study I the numbers of fibroblasts was highest at three weeks after operation and at 3 month follow-up the control group showed an increased amount myofibroblasts compared to other test groups, but the differences were not significant. ADM had almost no proliferating fibroblasts on d7 and d14. In study II, one week after grafting, the dermal cells of the superficial dermis proliferated more in the groups of pre-treated wound beds compared to the control group suggesting that the new additional vascularized ECM promoted fibroblast proliferation. At three months, the situation had changed to just opposite. The superficial fibroblasts in the control group proliferated markedly more (nearly ten times more) and approximately twice as much as one week after grafting. The reason for the late increase of dermal cell proliferation of the control site is unclear.

## ***Remodeling***

### **Myofibroblasts and wound contraction**

None of the test areas showed major contraction, but the measurements were not accurate enough to draw any conclusions. Compared to the other test groups, the control group showed the most myofibroblasts after excision, between day 3 and 3 months. In addition to inflammation, other factors promoting myofibroblast activation also increase the risk for scarring. Inflammatory signals and profibrotic growth factors activate fibroblasts (245). It may well be that ADM decreases wound contraction as suggested by others by attracting myofibroblasts to bind to the scaffold instead of binding to surrounding tissue to contract the wound and by binding profibrotic TGF- $\beta$  by GAGs in the scaffold (246,247).

The neodermis of ADM has been shown become thicker during early remodeling and then gradually decrease in thickness as the maturation proceeds over a few months (229). In study I the thickness of neodermis was still increased both in ADM and the control site at one year whereas the increase of the thickness of IGT halted already at three months.

In normal wound healing after re-epithelialization, myofibroblasts disappear via apoptosis one to two weeks after injury. The restoration of well-established epidermis plays a role in inhibiting wound contraction. The faster the epidermis is regenerated, the more wound contraction is suppressed, whereas delay in re-

epithelialization may result in hypertrophic scarring. Early grafting with unmeshed sheet grafts has been shown to reduce granulation tissue formation and scarring, and to result in optimal functional and cosmetic outcomes (172) as in this study. In studies I and II, the timing of re-epithelialization failed to impact additional scar formation, but the degradation of collagen type IV of BM may have contributed to scar formation by promoting epithelial-mesenchymal transdifferentiation.

### ***Clinical scar assessment- cosmetic and functional outcome***

In this study, the original Burn Scar Index, the Vancouver Scar Scale (VSS) was used. The scale was later slightly amended in regard to pigmentation. Occupational therapists were accustomed to assessing scars with the original scale and therefore it was used. In clinical assessments with VSS, the scar quality seemed to improve the most in IGT group and the least in ADM group. In total scores, the ADM group performed slightly worse than the other groups, but the differences between the test groups were not significant. There were both palpable and visual differences between treatment sites in some patients but there were no clear trends and none of the treatment sites seemed to have a preferable outcome. Since the number of patients in the study is rather low, the interpretation of results may not be reliable and must be considered with caution.

In contrary to many other studies (248,249) that employed the assessment methods used here, ADM did not seem to perform better than STSG alone as a control site. The one-year time point may have been too early to detect final differences especially for ADM-treated areas since the maturation of ADM may have still been in progress. The assessment methods were also subjective and therefore not optimal to detect fine differences. Nonetheless, no major or clinically relevant differences were detected. In earlier studies of ADM reconstructions, better outcomes have been achieved in comparison to the situation prior to reconstruction (229,249-252). In an objective evaluation study using a Cutometer, ADM was claimed to be more pliable and elastic, a parameter not measured in VSS (248).

The main hypothesis was that the use of ADM would result in better long-term outcome, both functionally and cosmetically. The hypothesis could not be proven using VSS assessment and histological evaluations. In study I the ADM test group did not perform markedly better than the other test groups. Nonetheless, ADM also performed no worse than other materials, too. Histological and immunohistochemical differences at the one-year time point were minimal between the test groups.

VSS has been criticized for poor inter-rater reliability, non-specificity, deficiency in objectivity, not taking patients' symptoms into account, but also having no generally accepted interpretations of the scores (181) e.g. which scoring would define a hypertrophic scar. As in the study of Heimbach et. al., patients preferred ADM due to less itching compared to STSG, allograft, xenograft and synthetic dressings, a symptom not taken into account by VSS (249). All of these claims above are valid and there is an urgent need for better objective tools for scar assessment.

## 6.2 THE LONG-TERM EFFECTS OF DIFFERENT EARLY WOUND-BED TREATMENT MATERIALS DETECTED IN PROTEOMICS AND REFLECTED TO PSORIASIS, STUDY III

Study III focused on the long-term outcome of protein level analysis.

To examine and to compare the long-term effect of three different dermal compositions on STSGs at the protein level, one year after surgery of a deep burn wound, mass-spectrometry, MaxQuant 1.4.0.8 software and UniProt human database were used to identify and to quantitate proteins.

### 6.2.1 PROTEOMIC FINDINGS IN SKIN SAMPLES OF BURN PATIENTS

The number of proteins identified in skin varies depending on the methods used. According to the Human Protein Atlas [www.proteinatlas.org](http://www.proteinatlas.org), transcriptomic analysis has identified 14857 proteins in human skin (status 12.1.2020). Proteomic techniques may be more sensitive and more precise than conventional methods, but the meaning and the significance of the findings often remain unclear. Proteomic analyses have been found that the proteins in different areas of skin vary, e.g. breast skin has some different proteins compared to skin of the leg (253). In study III, 481 proteins were identified by untargeted, non-labelled proteomics from skin samples taken at one-year follow-up. Significantly different expression between the test-groups was found in 12% of the proteins identified, 30 proteins of epidermis, and 24 proteins of dermis. The most surprising finding was in a protein in the epidermis with a 34-fold difference between the control group and the IGT group, namely in a motor protein axonemal heavy chain dynein 10 (DNAH10).

#### **DNAH10**

DNAH10 protein has not been listed in the Human Protein Atlas <https://www.proteinatlas.org/search/keratinocytes> (status checked 12th January, 2020) as being detected in keratinocytes. Dyneins in motile cilia function in cell signaling and driving ciliary motility, inner arm dyneins regulating the beating pattern and frequency DNAH10 belonging to inner arm dyneins. In mRNA expression of DNAH10 level is very low, however the proteomics and immunohistochemistry employed in this study detected protein expression in skin. The immunoreactivity was localized to keratinocytes and sweat glands (glandulae sudoriferae). In the literature, DNAH10 is assigned as an inner arm dynein, which regulates the beating pattern and frequency. The current results, however, suggest cell-cycle- and cytokinedependent cytoplasmic expression of DNAH10. Due to the preliminary nature of the current study's results, care should be taken in their interpretation. Some of the study limitations are listed at the end of this chapter.

In phylogenetic studies, DNAH10 has been linked to the axoneme of motile cilia as an inner arm heavy chain force-generating motor protein taking part in bending microtubule in axoneme. It has been identified in respiratory cilia of airway epithelium and in skin in sweat glands. In this study, immunohistochemical stains

demonstrated DNAH10 expression in keratinocytes as well as in sweat glands. Nonetheless, almost all basal keratinocytes have been found to carry a primary cilium both in normal and in psoriatic skin. The ciliary structure is progressively reduced in keratinocytes towards the surface and no primary cilia were found in the granular layer (76). In the primary cilia of keratinocytes, no dynein arms were observed. Primary cilia have been found to be less common in mitotic than in non-mitotic tissue and they have been suggested to be involved in the regulation of mitosis (76). The major tasks of primary cilia include the recognition of extracellular signals, such as growth factors, hormones, and nutrients (240). The cilium membrane contains numerous receptors, ion channels, and signaling-pathway components, such as Shh and Wnt receptors. Primary cilia are important in signal transduction during development, cell migration, the cell cycle, and apoptosis. Ciliogenesis is promoted by various cellular stresses, such as UV radiation, heat shock, actin destabilization, loss of mechanical stresses, and serum starvation. Ciliogenesis is highly linked with the arrest of cell growth and proliferation. Differentiation of stem cells requires the presence of primary cilia and is associated with intra flagellar transport. The absence of primary cilia inhibits the differentiation of mesenchymal stem cells.(254)

### **OTHER PROTEOMIC FINDINGS**

In epidermis, seven out of nine of the most differently expressed proteins (DEPs) were most expressed in the control group, whereas the same number of DEPs were expressed the lowest in the ADM group. The functions of these proteins vary and associations between these proteins cannot (yet) be described. The expression of KRT34, a type I hair keratin heterodimerizing with type II keratins to form hair and nails, seemed to be increased in pre-treated groups compared to controls, suggesting that additional new ECM under the STSG may create a more supportive environment for hair follicles than STSG alone.

In dermis, four out of seven of the most differently expressed proteins were keratins, most likely from hair follicles, all expressed higher in the IGT group compared to other groups. The presence of these keratins in hair follicles has been described in various studies (253,254). KRT77, also known as KRT 1b, is expressed in the skin and eccrine sweat glands. KRT6, 16, and 17 are normally not expressed in the interfollicular epidermis but they are rapidly induced in response to various triggers e.g., wounding, infection, as well as in the setting of chronic hyperproliferative diseases e.g., psoriasis and cancer (20,21). The induction of KRT6/16/17 occurs at the expense of the KRT10 and KRT1 keratin pair, which inhibits cell cycle progression and promotes differentiation in normal keratinocytes.

### **6.2.2 VALIDATIONS**

To validate the correct laser dissection of different skin layers, all proteins identified in mass-spectrometry were clustered with Gene Cluster 3.0 software which resulted in two main branches corresponding to proteins of the epidermis and dermis. The validation of proteomic results was gained from IHC samples for DNAH10 and CASP14. The results of validation were not identical as expected due to different

methodology, but were similar to proteomic findings. DNAH10 was expressed the least in the test areas of control sites. The results of CASP14, a differentiation marker of keratinocytes, also corresponded with the results of proteomics. The control sites exhibited the highest expression of CASP14 of all test areas. The low number of patients studied limits the reliability of the results. Since the study was designed as comparison within patients also, the patients served as their own controls.

### **6.2.3 IPA AND TRANSFAC® ANALYSES**

All DEPs between different test groups were analyzed using Ingenuity Pathway Analysis (IPA) software pathway and network analysis to discover biological networks associated with these results from proteomics. Proliferation, differentiation, and barrier function were found to be significantly associated with the reported findings.

TRANSFAC® analysis provided likely transcription factors analyzed by promoter regions. The promoter region of DNAH10 was utilized to predict the binding sites for transcription factors. Within this promoter region, the most binding sites were found for transcription factors KLF6, AP-2, and ZF-5, all regulating cell proliferation and differentiation (255-260). Taken together, these associations linked DNAH10 with keratinocyte cell cycle control.

### **6.2.4 DNAH10 MRNA SPLICE VARIANTS**

Ten mRNA splice variants have been identified as transcribed from the DNAH10 gene. Six of these yield protein products. No DNAH10 gene expression was detected in the mRNA sequencing studies in psoriasis patients, suggesting that DNAH10 protein expression might be controlled at a post-translational level. The rather large size of the DNAH10 protein (515 kDa) may contribute to slow cycling and expression changes of the protein but the size of the protein varies by the splice variants: the transcripts DNAH10-210 and DNAH10-201 produce large proteins of 500 kDa, whereas the transcript variants DNAH10-205, -207, and -209 yield smaller proteins ranging from 35 to 115 kDa. The unique DNAH10 peptide identified by mass-spectrometry could be encoded by a shorter transcript variant DNAH10-205. This transcript may be subject to nonsense-mediated decay (NMD), a surveillance system serving quality control of newly synthesized mRNA (261). There are many different ways that the mRNA can escape NMD quality control (262). DNAH10-205 may escape and contribute to the NMD pathway to DNAH10 or DNAH10-205 expression in the long-term memory response.

Several transcripts are known to be transcribed from the DNAH10 gene and the protein products may also be subject to several kinds of post-translational modification. These processes are likely contributors to the expression patterns of DNAH10 tissue, cellular, and subcellular levels. The current discovery provides DNAH10 expression with a new clinical association with which its expression and regulation can be studied further in preclinical and clinical settings.

### **6.2.5 DNAH10 EXPRESSION IN INFLAMMATORY SKIN PATHOLOGY PSORIASIS**

Psoriasis and wound healing share several features. Clinically, both patient groups suffer from similar symptoms, such as dry skin that may crack and bleed, itching, and burning sensation. Both patients with psoriasis and burn patients with STSGs also show decreased or defective barrier function of skin at least up until one year after injury. Inflammation and epithelial hyperproliferation are characteristic landmarks for both, as well as angiogenesis/vascularization. All these processes are also influenced by dermis. Its immune cells, including skin-resident T cells, dendritic cells, and fibroblasts, secrete cytokines and chemokines that modulate these processes.

Biological processes such as proliferation, differentiation, and barrier function are significantly associated with the observation of DNAH10 in STSGs of burn wounds. This presents a remarkable resemblance to the pathological features of psoriasis (263-265) and previous work on psoriasis and keratinocytes (206,266,267); DNAH10 protein expression is also affected in active psoriatic epidermis. As dermal inflammation is also present at early stages of psoriasis-plaque formation, the increased protein expression of DNAH10 found in this study in the active sites of psoriatic inflammation, thus further links the protein regulation of DNAH10 with inflammation; and its likely dysregulation in psoriasis.

This hypothesis was tested on samples collected using dermatome (thus superficial, epidermis-enriched and similar in thickness to STSGs) from patients with psoriasis. These samples have been mRNA-sequenced previously, thus ample data was available to evaluate dynein expression at the mRNA level (266,267). The RNA sequencing data of lesional and non-lesional psoriatic skin showed a down regulation of DNAH10 in lesional psoriasis. The IHC provided clear evidence to support the hypothesis of increased expression of DNAH10 protein in the epidermis of psoriatic lesions compared to unaffected skin from the same individuals and to healthy controls. The tendency towards an aberrant dual pattern of DNAH10 staining across the depth of the epidermis as observed in normal control epidermis, psoriatic epidermis, and during wound healing, suggests that DNAH10 expression is activated at specific times during keratinocyte differentiation. The expression of axonemal dyneins has been linked to cell differentiation and correlated with the appearance of a ciliated phenotype (268).

### **6.2.6 DNAH10 IN CELL STIMULATION EXPERIMENTS**

This study sought to confirm the inverse correlation of proliferation and expression of DNAH10 in keratinocytes by cell stimulation experiments of cultured primary human keratinocytes. Keratinocytes were stimulated with Mitomycin-C, a cell cycle inhibitor blocking DNA synthesis. This resulted in increased DNAH10 in the inhibited keratinocytes compared to controls. Protein p53, the “guardian of the genome,” has been associated with dyneins. Giannakakou et al. have suggested that dyneins together with functional microtubules transport p53 and facilitate its accumulation in the nucleus after DNA damage (269). As a result of cellular stress

such as hypoxia or DNA damage, p53 accumulates in the nucleus, and activates and functions as a transcription factor. Treatment with Mitomycin-C may cause stress for keratinocytes. In this study, it seemed that DNAH10 was accumulated into the cytoplasm of keratinocytes. Other possibilities to explain the accumulation of DNAH10 might include rapid increased synthesis or it was not degraded in that time frame, suggesting that DNAH10 would be involved in proliferation. It could also be that DNAH10 was transported into dynein arms of cilia to be preassembled in the cytoplasm before transport into cilia (94).

Further investigations are needed to find out whether the DNAH10 accumulated into the cytoplasm originated from cilia (primary, motile, or nodal), or from intraflagellar transport or from cytoplasm.

The effect of the pro-inflammatory mediators TNF- $\alpha$  and TGF- $\beta$ 1 on DNAH10 expression in keratinocytes was examined. TNF- $\alpha$  is a pro-inflammatory cytokine produced by human keratinocytes. As primary inflammatory response to injury or other inflammatory stimulus, keratinocytes produce IL1 $\alpha$  and TNF- $\alpha$  (270). In keratinocytes, TNF alpha regulates immune and inflammatory responses, and also tissue remodeling, cell motility, cell cycle, and apoptosis (271). In addition to activating keratinocytes TNF- $\alpha$  and IL1 $\alpha$  also activate dermal fibroblasts and endothelial cells and attract neutrophils, macrophages, mast cells, and skin-specific memory T-cells. TNF- $\alpha$  also promotes tissue repair by inducing synthesis of BM components and collagen-degrading proteases, and it induces actin cytoskeleton regulators and integrins, enhancing keratinocyte motility and attachment. TNF- $\alpha$  may also sensitize or prime keratinocytes to other stimuli like cytokines and growth factors to react quickly (271). TNF $\alpha$  is known to be one of the main executor cytokines in psoriasis.

TGF- $\beta$ 1 is known to have both pro-inflammatory and anti-inflammatory properties affecting different immune cells thereby regulating the inflammatory response (270). TGF- $\beta$ 1 is also needed for maturation and migration of LCs. LCs and macrophages activate T cells to proliferate and differentiate into memory/effector T cells. As the healing progresses the inflammation is suppressed by TGF- $\beta$ 1, which has inhibitory effects on both innate and adaptive immune cell function (270). TGF- $\beta$ 1 is also known to inhibit keratinocyte proliferation, whereas it promotes keratinocyte migration. Blocking of TGF- $\beta$ 1 signaling has been shown to result in keratinocyte hyperproliferation.

Cell stimulation by pro-inflammatory cytokines TNF- $\alpha$  and growth factor TGF- $\beta$ 1 resulted in decreased expression of DNAH10 in primary human keratinocytes after a 24-hour-incubation. TGF- $\beta$ 1 decreased the expression of DNAH10 less than TNF- $\alpha$  but their combination showed the strongest decrease in DNAH10 expression in keratinocytes. The findings indirectly suggest that the decrease in keratinocyte DNAH10 after stimulation with pro-inflammatory and/or pro-fibrotic signals resulted in either increased consumption or decreased synthesis of DNAH10 in keratinocytes. This warrants further studies.

Cells residing in the epidermis have been shown to maintain signatures at the molecular level (these could be epigenetic modifications on DNA, or changes in RNA or protein expression) in response to inflammatory injury and wound healing.

The cells currently associated with this type of memory include CD8+ tissue-resident T-cells and Treg cells. The work reported here associates keratinocytes with this type of long-term molecular persisting response after wound healing, and especially with the formation of dermis.

### ***Limitations of Study I and II to draw conclusions***

Test areas were small and close to each other, thus the cellular signaling of the neighbor area could have affected the results. The number of patients is quite small even though all the treatments were compared with-in the same patient. The clinical scar assessment tool was too crude to detect fine differences between the treatment groups and, due to the tool itself, it was subjected to some degree subjectivity. The inter-rater differences were sought to be reduced to a minimum by limiting the number of raters and by having the same person throughout the study. The occupational therapist was a part of team that took care of the patients so any kind of blinding was impossible. In the early phase before covering the wound bed with STSGs the differentiation between the test sites was obvious. VSS does not actually measure patients' functional restrictions caused by the scar, nor does it evaluate patients' symptoms such as pain and itching, which have significant effects on quality of life. Nonetheless, many of the scar assessment scales and tools have been validated by comparing their results to VSS.

### ***Limitations of Study III***

The location of where the skin graft was harvested was not taken into account, which might have affected the results, but within each patient the grafts were taken from the same location to cover ADM and IGT areas.

The use of proteomic techniques is not easy in skin tissue due to an abundance of protein cross-linking and high lipid content making isolation and digestion of proteins for analyses challenging (192). The method may also be too sensitive and the interpretation of results of clinical work is difficult to make.

Especially the discovery of DNAH10 relied on a single specific peptide. Conventionally in proteomics, proteins identified by a single peptide are filtered out of the results. The dramatic change in expression of DNAH10, however, drew interest and it was chosen to be validated using immunohistochemistry. The limitation of IHC results is that they rely on use of a single antibody and the lack of use of blocking peptide in the experiments. These issues will be addressed in future experiments. The antibody was validated in the Human protein atlas at the time and showed specificity to only its own antigen in a protein array. The discovery of DNAH10 must also be validated in the fresh skin samples in the future as well as its' functional associations should be further studied using molecular biology tools such as CRISPR-Cas9 genome editing and gene under- and overexpression in primary and model keratinocytes.

## 7. CONCLUSIONS

This study of treating deep burn wounds after excision, with three different materials, yielded the following conclusions:

### Study I & II

- The early wound healing was affected by different covering materials: Cellulose sponge treatment promoted inflammatory response, whereas ADM presented a more suppressed inflammatory response. Early moderate inflammation had no adverse long-term effects.
- Granulation tissue induced by cellulose sponge provided a supportive environment for a skin graft.
- The use of ADM possibly retards early wound healing accompanied with decreased and/or delayed fibroplasia.
- The differences in early wound healing failed to affect the clinical functional or cosmetic outcome after one year. ADM performed equally well after one year compared to the other materials tested. The main hypothesis was proven to be wrong.
- Both ADM and cellulose sponge are useful tools if wound temporising before skin graft re-harvesting is needed.

### Study III

- Persistent differences in the expression of certain tissue-specific proteins, depending on the early wound bed treatment, shows that early treatment influences subsequent tissue proteome, although it may remain below the macroscopic phenotype.
- The finding of DNAH10 in keratinocytes is a novelty that has not been described previously and fails to fit into the categories of functions and locations of dyneins described previously. DNAH10 has been described to be a heavy chain two-headed inner-arm dynein found in motile cilia, but motile cilia have not been identified in keratinocytes.
- DNAH10 was found to be present in primary human keratinocytes in clinical patient material in the skin of burn patients, in psoriatic lesions, and the level of DNAH10 in cultured primary keratinocytes was modulated by mitomycin-C, TNF- $\alpha$  or TGF- $\beta$ 1, and a combination of the latter. Long-term persistent epidermal DNAH10 expression was associated with transient regenerative and inflammatory dermal signaling, and DNAH10 expression is increased in inflamed psoriatic skin. These discoveries associated epidermal protein expression of DNAH10 selectively on initially dermis-manipulated sites still one year after the intervention, and linked DNAH10 with dermal epidermal signaling, epidermal memory of a wound healing response, and dermal inflammation.

## 8. FUTURE PROSPECTS

This thesis provides an example of the role and value of objective comparative studies of different treatment modalities through the intra-patient controlled study protocols used here. More objective comparative studies of different treatment modalities for burns treatment are needed to be able to provide patients with the best evidence-based care. It would be important in the future to be able to compare the results between different centers also internationally and also for developing the quality of care in terms of patient-centered care and the whole quality of care for burn patients.

It seems that some long-term memories of past events like inflammation during wound healing of a STSG are sustained in cells, reflecting their ECM environment. Although there is no ongoing inflammation in the burn-patient samples, “the inflammation-experienced memory,” which is based on DNA chromatin changes, maintains DNAH10 expression. It remains unknown whether these possibly epigenetic changes may show decades later in skin function, for example via transepidermal water loss, transepidermal resistance, or amount of filaggrin in the epidermis. The expression of axonemal dyneins has already been linked to cell differentiation and correlated with the appearance of a ciliated phenotype. The results described here indicate a need for elucidating the roles of primary cilia and the axoneme in the epidermis and the functional role of the axonemal dyneins during epidermal differentiation. This elucidation will increase understanding of the intricate signaling networks and signaling pathways orchestrating the formation and integrity of the skin. Research is especially warranted for understanding the role and expressional control of this signaling machinery after wound healing. It would also be of great value to investigate DNAH10 expression in experimental animal models, as well as to further elucidate the expression patterns of DNAH10 using in vitro 3D epidermal constructs allowing for genetic manipulation, gene overexpression, and knock-outs. Moreover, the contributions of the various gene products and putative cleavage protein products of DNAH10 should be studied in more detail. According to the preliminary data presented in this thesis, the transcriptional, post-transcriptional, translational, and post-translational regulation of DNAH10 seems to be of extreme interest when further studying the role of this specific dynein in cell biology and different pathologies.

We do not know yet enough about the role and regulation of DNAH10 in physiological or pathophysiological contexts. However, the results associating DNAH10 protein with an epidermal delayed response to wound healing as well as psoriasis suggest it indeed could serve a significant biological role. In order to be able to have a closer look at the intracellular level of regulation, we need to build specific yet simple research methodology. RNA sequencing and various protein level tools could provide some answers. Since the low Mw DNAH10 proteins are detected, also by us using Western Blotting, in keratinocytes although current evidence suggests negligible differences in mRNA expression, expressional regulation was considered to occur at the post-translational level. Furthermore, also the high Mw species of DNAH10 is abundantly detected in keratinocytes. Given the

experimental challenges like antibody specificity, the expressional regulation of DNAH10 low Mw species was considered to occur at a post-translational level. It will be extremely interesting to determine at which level the regulation actually occurs. Sensitive and specific RNA and protein methodologies should be used. It will also be interesting to determine whether the proteolytic enzyme activity associated with keratinocyte differentiation affects DNAH10 processing. For example, there are putative MMP-specific enzyme cleavage sites on the high Mw DNAH10 that can yield the low Mw species.

## 9. ACKNOWLEDGEMENTS

This work has been carried out during past twenty years with all ups and downs at the Helsinki University Burn Centre, Department of Plastic Surgery as well as in the Department of Pathology, Haartman Institute, University of Helsinki. This work would not have been possible without the block grants provided by the Helsinki University Central Hospital Research Fund and MATINE, Instrumentarium.

My deepest gratitude goes to my supervisors, Professor Jyrki Vuola and Docent Esko Kankuri, for their enthusiastic endurance and support.

I warmly thank the wonderful support provided by Docent Ilkka Kaartinen and MA PhD Julian Dye who kindly reviewed my thesis and made constructive comments to improve the text; and Emeritus Prof. Sirpa Asko-Seljavaara and Prof. Erkki Tukiainen for their enthusiasm and support towards the research.

Enormous thanks for the assistance I received in performing the research, from Mrs. Riitta Töllikkö, Mrs. Pia Willman, Mrs. Oili Ask and other occupational therapists who helped with the patients and made the clinical assessments possible; Mrs. Lahja Eurajoki who provided practical advice and help in the laboratory; and PhD Antti Siltanen who provided help and useful advice in the laboratory. Thank you MD Jussi Valtonen and Docent Andrew Lindford for sharing the anxiety in the lab; and PhD Katariina Laurila for exchanging on-call duty during critical moments. I am indebted to my colleagues who took patient samples when I was unavailable.

I wish to thank my colleagues and staff at the Department of Plastic Surgery, and in Hyvinkää and in Lohja Hospitals for their friendship and the great atmosphere at work.

In particular, Mr. Timo Pessi and Mr. Kari Haikonen provided statistical advice and assistance; and Docent Timo Muhonen shared valuable comments and ideas for study I.

I express my deep gratitude to my co-authors: Professor Tom Böhling and MD Maarit Sarlomo-Rikala for your kindness and your endless hours at the microscope evaluating the slides; Docent Susanna Juteau for ongoing support and fruitful conversations during our sessions around the microscope; PhD Kristo Nuutila for the help and assistance in the laboratory; PhD Viljar Jaks for the help and enjoyable conversations with constructive comments; PhD Mariliis Klaas for the excellent collaboration; Docent Outi Elomaa for valuable comments and help in the revisions; and Professor Juha Kere for constructive comments and collaboration.

I warmly thank Dr Jennifer Rowland for extremely efficient and fast English revision of my article and this thesis and Sole Lätti for the illustrations in article number III and in this thesis.

I'm indebted to "Wound sisters" Kirsi Isoherranen, Milla Kallio, Heli Kavola, and Heini Halme for the support, friendship and endurance in "the world of wounds."

I owe my deepest gratitude to my beloved husband Eero, our daughter Maria, and our son Oskari, and to my joyful furry friends for keeping me sane Hetja & Jack. Thank you so much for bringing the joy and liveliness to my life.

## 10. REFERENCES

- (1) Lee SH, Jeong SK, Ahn SK. An update of the defensive barrier function of skin. *Yonsei Med J* 2006 Jun 30;47(3):293-306.
- (2) Matsui T, Amagai M. Dissecting the formation, structure and barrier function of the stratum corneum. *Int Immunol* 2015 Jun;27(6):269-280.
- (3) Simpson CL, Patel DM, Green KJ. Deconstructing the skin: cytoarchitectural determinants of epidermal morphogenesis. *Nat Rev Mol Cell Biol* 2011 Aug 23;12(9):565-580.
- (4) Watt FM, Fujiwara H. Cell-extracellular matrix interactions in normal and diseased skin. *Cold Spring Harb Perspect Biol* 2011 Apr 1;3(4):10.1101/cshperspect.a005124.
- (5) Nguyen AV, Soulika AM. The Dynamics of the Skin's Immune System. *Int J Mol Sci* 2019 Apr 12;20(8):10.3390/ijms20081811.
- (6) Metral E, Bechetoille N, Demarne F, Damour O, Rachidi W. Keratinocyte stem cells are more resistant to UVA radiation than their direct progeny. *PLoS One* 2018 Sep 12;13(9):e0203863.
- (7) Otsuka M, Egawa G, Kabashima K. Uncovering the Mysteries of Langerhans Cells, Inflammatory Dendritic Epidermal Cells, and Monocyte-Derived Langerhans Cell-Like Cells in the Epidermis. *Front Immunol* 2018 Jul 30;9:1768.
- (8) Lu CP, Polak L, Rocha AS, Pasolli HA, Chen SC, Sharma N, et al. Identification of stem cell populations in sweat glands and ducts reveals roles in homeostasis and wound repair. *Cell* 2012 Jul 6;150(1):136-150.
- (9) Blanpain C, Fuchs E. Epidermal homeostasis: a balancing act of stem cells in the skin. *Nat Rev Mol Cell Biol* 2009 Mar;10(3):207-217.
- (10) Sotiropoulou PA, Blanpain C. Development and homeostasis of the skin epidermis. *Cold Spring Harb Perspect Biol* 2012 Jul 1;4(7):a008383.
- (11) Quek LS, Grasset N, Jasmen JB, Robinson KS, Bellanger S. Dual Role of the Anaphase Promoting Complex/Cyclosome in Regulating Stemness and Differentiation in Human Primary Keratinocytes. *J Invest Dermatol* 2018 Aug;138(8):1851-1861.
- (12) Williams SE, Beronja S, Pasolli HA, Fuchs E. Asymmetric cell divisions promote Notch-dependent epidermal differentiation. *Nature* 2011 Feb 17;470(7334):353-358.

- (13) Wikramanayake TC, Stojadinovic O, Tomic-Canic M. Epidermal Differentiation in Barrier Maintenance and Wound Healing. *Adv Wound Care (New Rochelle)* 2014 Mar 1;3(3):272-280.
- (14) Garcia MA, Nelson WJ, Chavez N. Cell-Cell Junctions Organize Structural and Signaling Networks. *Cold Spring Harb Perspect Biol* 2018 Apr 2;10(4):10.1101/cshperspect.a029181.
- (15) Yokouchi M, Kubo A. Maintenance of tight junction barrier integrity in cell turnover and skin diseases. *Exp Dermatol* 2018 Aug;27(8):876-883.
- (16) Feingold KR. Lamellar bodies: the key to cutaneous barrier function. *J Invest Dermatol* 2012 Aug;132(8):1951-1953.
- (17) MacLeod AS, Hemmers S, Garijo O, Chabod M, Mowen K, Witherden DA, et al. Dendritic epidermal T cells regulate skin antimicrobial barrier function. *J Clin Invest* 2013 Oct;123(10):4364-4374.
- (18) Clayton K, Vallejo AF, Davies J, Sirvent S, Polak ME. Langerhans Cells-Programmed by the Epidermis. *Front Immunol* 2017 Nov 29;8:1676.
- (19) Zhang X, Yin M, Zhang LJ. Keratin 6, 16 and 17-Critical Barrier Alarmin Molecules in Skin Wounds and Psoriasis. *Cells* 2019 Aug 1;8(8):10.3390/cells8080807.
- (20) Wang F, Zieman A, Coulombe PA. Skin Keratins. *Methods Enzymol* 2016;568:303-350.
- (21) Freedberg IM, Tomic-Canic M, Komine M, Blumenberg M. Keratins and the keratinocyte activation cycle. *J Invest Dermatol* 2001 May;116(5):633-640.
- (22) Haines RL, Lane EB. Keratins and disease at a glance. *J Cell Sci* 2012 Sep 1;125(Pt 17):3923-3928.
- (23) Breitkreutz D, Mirancea N, Nischt R. Basement membranes in skin: unique matrix structures with diverse functions? *Histochem Cell Biol* 2009 Jul;132(1):1-10.
- (24) Breitkreutz D, Koxholt I, Thiemann K, Nischt R. Skin basement membrane: the foundation of epidermal integrity--BM functions and diverse roles of bridging molecules nidogen and perlecan. *Biomed Res Int* 2013;2013:179784.
- (25) Jayadev R, Sherwood DR. Morphogenesis: Shaping Tissues through Extracellular Force Gradients. *Curr Biol* 2017 Sep 11;27(17):R850-R852.
- (26) Yurchenco PD. Basement membranes: cell scaffoldings and signaling platforms. *Cold Spring Harb Perspect Biol* 2011 Feb 1;3(2):10.1101/cshperspect.a004911.

- (27) Krieg T, Aumailley M. The extracellular matrix of the dermis: flexible structures with dynamic functions. *Exp Dermatol* 2011 Aug;20(8):689-695.
- (28) Breitskreutz D, Koxholt I, Thiemann K, Nischt R. Skin basement membrane: the foundation of epidermal integrity--BM functions and diverse roles of bridging molecules nidogen and perlecan. *Biomed Res Int* 2013;2013:179784.
- (29) Morrissey MA, Sherwood DR. An active role for basement membrane assembly and modification in tissue sculpting. *J Cell Sci* 2015 May 1;128(9):1661-1668.
- (30) Nielsen MM, Witherden DA, Havran WL. gammadelta T cells in homeostasis and host defence of epithelial barrier tissues. *Nat Rev Immunol* 2017 Dec;17(12):733-745.
- (31) Salimi M, Ogg G. Innate lymphoid cells and the skin. *BMC Dermatol* 2014 Nov 26;14:18-5945-14-18.
- (32) Tracy LE, Minasian RA, Caterson EJ. Extracellular Matrix and Dermal Fibroblast Function in the Healing Wound. *Adv Wound Care (New Rochelle)* 2016 Mar 1;5(3):119-136.
- (33) Uitto J, Olsen DR, Fazio MJ. Extracellular matrix of the skin: 50 years of progress. *J Invest Dermatol* 1989 Apr;92(4 Suppl):61S-77S.
- (34) Rippa AL, Kalabusheva EP, Vorotelyak EA. Regeneration of Dermis: Scarring and Cells Involved. *Cells* 2019 Jun 18;8(6):10.3390/cells8060607.
- (35) Wang J, Dodd C, Shankowsky HA, Scott PG, Tredget EE, Wound Healing Research Group. Deep dermal fibroblasts contribute to hypertrophic scarring. *Lab Invest* 2008 Dec;88(12):1278-1290.
- (36) Yue B. Biology of the extracellular matrix: an overview. *J Glaucoma* 2014 Oct-Nov;23(8 Suppl 1):S20-3.
- (37) Maquart FX, Monboisse JC. Extracellular matrix and wound healing. *Pathol Biol (Paris)* 2014 Apr;62(2):91-95.
- (38) Walker C, Mojares E, Del Rio Hernandez A. Role of Extracellular Matrix in Development and Cancer Progression. *Int J Mol Sci* 2018 Oct 4;19(10):10.3390/ijms19103028.
- (39) Xue M, Jackson CJ. Extracellular Matrix Reorganization During Wound Healing and Its Impact on Abnormal Scarring. *Adv Wound Care (New Rochelle)* 2015 Mar 1;4(3):119-136.
- (40) Pelham RJ, Jr, Wang Y. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc Natl Acad Sci U S A* 1997 Dec 9;94(25):13661-13665.

- (41) Davidson CD, Wang WY, Zaimi I, Jayco DKP, Baker BM. Cell force-mediated matrix reorganization underlies multicellular network assembly. *Sci Rep* 2019 Jan 9;9(1):12-018-37044-1.
- (42) Liu F, Mih JD, Shea BS, Kho AT, Sharif AS, Tager AM, et al. Feedback amplification of fibrosis through matrix stiffening and COX-2 suppression. *J Cell Biol* 2010 Aug 23;190(4):693-706.
- (43) Huang X, Yang N, Fiore VF, Barker TH, Sun Y, Morris SW, et al. Matrix stiffness-induced myofibroblast differentiation is mediated by intrinsic mechanotransduction. *Am J Respir Cell Mol Biol* 2012 Sep;47(3):340-348.
- (44) Prager-Khoutorsky M, Lichtenstein A, Krishnan R, Rajendran K, Mayo A, Kam Z, et al. Fibroblast polarization is a matrix-rigidity-dependent process controlled by focal adhesion mechanosensing. *Nat Cell Biol* 2011 Nov 13;13(12):1457-1465.
- (45) Kang L, Liu X, Yue Z, Chen Z, Baker C, Winberg PC, et al. Fabrication and In Vitro Characterization of Electrochemically Compacted Collagen/Sulfated Xylorhamnoglycuronan Matrix for Wound Healing Applications. *Polymers (Basel)* 2018 Apr 9;10(4):10.3390/polym10040415.
- (46) Widgerow AD. Cellular/extracellular matrix cross-talk in scar evolution and control. *Wound Repair Regen* 2011 Mar-Apr;19(2):117-133.
- (47) Sephel GC, Davidson JM. Elastin production in human skin fibroblast cultures and its decline with age. *J Invest Dermatol* 1986 Mar;86(3):279-285.
- (48) Tracy LE, Minasian RA, Caterson EJ. Extracellular Matrix and Dermal Fibroblast Function in the Healing Wound. *Adv Wound Care (New Rochelle)* 2016 Mar 1;5(3):119-136.
- (49) Li Y, Liu Y, Xia W, Lei D, Voorhees JJ, Fisher GJ. Age-dependent alterations of decorin glycosaminoglycans in human skin. *Sci Rep* 2013;3:2422.
- (50) Soares da Costa D, Reis RL, Pashkuleva I. Sulfation of Glycosaminoglycans and Its Implications in Human Health and Disorders. *Annu Rev Biomed Eng* 2017 Jun 21;19:1-26.
- (51) Trowbridge JM, Gallo RL. Dermatan sulfate: new functions from an old glycosaminoglycan. *Glycobiology* 2002 Sep;12(9):117R-25R.
- (52) Mizumoto S, Kosho T, Yamada S, Sugahara K. Pathophysiological Significance of Dermatan Sulfate Proteoglycans Revealed by Human Genetic Disorders. *Pharmaceuticals (Basel)* 2017 Mar 27;10(2):10.3390/ph10020034.
- (53) Zhang W, Ge Y, Cheng Q, Zhang Q, Fang L, Zheng J. Decorin is a pivotal effector in the extracellular matrix and tumour microenvironment. *Oncotarget* 2018 Jan 3;9(4):5480-5491.

- (54) Merline R, Moreth K, Beckmann J, Nastase MV, Zeng-Brouwers J, Tralhao JG, et al. Signaling by the matrix proteoglycan decorin controls inflammation and cancer through PDCD4 and MicroRNA-21. *Sci Signal* 2011 Nov 15;4(199):ra75.
- (55) Sarkiri M, Fox SC, Fratila-Apachitei LE, Zadpoor AA. Bioengineered Skin Intended for Skin Disease Modeling. *Int J Mol Sci* 2019 Mar 20;20(6):10.3390/ijms20061407.
- (56) Varkey M, Ding J, Tredget EE. Advances in Skin Substitutes-Potential of Tissue Engineered Skin for Facilitating Anti-Fibrotic Healing. *J Funct Biomater* 2015 Jul 9;6(3):547-563.
- (57) Chen SX, Zhang LJ, Gallo RL. Dermal White Adipose Tissue: A Newly Recognized Layer of Skin Innate Defense. *J Invest Dermatol* 2019 May;139(5):1002-1009.
- (58) Tokuyama E, Nagai Y, Takahashi K, Kimata Y, Naruse K. Mechanical Stretch on Human Skin Equivalents Increases the Epidermal Thickness and Develops the Basement Membrane. *PLoS One* 2015 Nov 3;10(11):e0141989.
- (59) Werner S, Smola H. Paracrine regulation of keratinocyte proliferation and differentiation. *Trends Cell Biol* 2001 Apr;11(4):143-146.
- (60) Alberts B, Johnson A, Lewis J. An overview of the cell cycle. *Molecular Biology of the Cell*. 4th edition ed. New York: Garland Science; 2002.
- (61) Cooper GM. The Eukaryotic Cell Cycle. In: Sunderland MA, editor. *The Cell: A Molecular Approach*. . 2nd ed.: Sinauer Associates; 2000.
- (62) Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. DNA Replication Mechanisms. *Molecular Biology of the Cell*. 4th ed.: Garland Science; 2002.
- (63) Ekundayo B, Bleichert F. Origins of DNA replication. *PLoS Genet* 2019 Sep 12;15(9):e1008320.
- (64) National Institute of General Medical Sciences. *The New Genetics, How Genes Work?* . NIH Publication 2010 April 2010(10-662).
- (65) Kimball SR, Jefferson LS. New functions for amino acids: effects on gene transcription and translation. *Am J Clin Nutr* 2006 Feb;83(2):500S-507S.
- (66) Sonenberg N, Hinnebusch AG. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* 2009 Feb 20;136(4):731-745.
- (67) Tse LH, Wong YH. GPCRs in Autocrine and Paracrine Regulations. *Front Endocrinol (Lausanne)* 2019 Jul 12;10:428.
- (68) Nair A, Chauhan P, Saha B, Kubatzky KF. Conceptual Evolution of Cell Signaling. *Int J Mol Sci* 2019 Jul 4;20(13):10.3390/ijms20133292.

- (69) Schneider A, Klingmuller U, Schilling M. Short-term information processing, long-term responses: Insights by mathematical modeling of signal transduction. Early activation dynamics of key signaling mediators can be predictive for cell fate decisions. *Bioessays* 2012 Jul;34(7):542-550.
- (70) Nair A, Chauhan P, Saha B, Kubatzky KF. Conceptual Evolution of Cell Signaling. *Int J Mol Sci* 2019 Jul 4;20(13):10.3390/ijms20133292.
- (71) Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. *Physiol Rev* 2003 Jul;83(3):835-870.
- (72) Stone WL, Varacallo M. *Physiology, Growth Factor*. StatPearls Treasure Island (FL): StatPearls Publishing LLC; 2019.
- (73) Kim SH, Turnbull J, Guimond S. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. *J Endocrinol* 2011 May;209(2):139-151.
- (74) Veland IR, Awan A, Pedersen LB, Yoder BK, Christensen ST. Primary cilia and signaling pathways in mammalian development, health and disease. *Nephron Physiol* 2009;111(3):p39-53.
- (75) Popociv N, Wilson E. Cell surface receptors
- In: McQueen C, editor. *Comprehensive Toxicology*. 2nd ed.: Elsevier; 2010. p. Chapter 2.06.
- (76) Simpson CL, Patel DM, Green KJ. Deconstructing the skin: cytoarchitectural determinants of epidermal morphogenesis. *Nat Rev Mol Cell Biol* 2011 Aug 23;12(9):565-580.
- (77) Bielefeld KA, Amini-Nik S, Alman BA. Cutaneous wound healing: recruiting developmental pathways for regeneration. *Cell Mol Life Sci* 2013 Jun;70(12):2059-2081.
- (78) Chermnykh E, Kalabusheva E, Vorotelyak E. Extracellular Matrix as a Regulator of Epidermal Stem Cell Fate. *Int J Mol Sci* 2018 Mar 27;19(4):10.3390/ijms19041003.
- (79) Burgess DJ. Development: Differentiation goes up a Notch. *Nat Rev Genet* 2011 Apr;12(4):226.
- (80) Liu T, Zhang L, Joo D, Sun SC. NF-kappaB signaling in inflammation. *Signal Transduct Target Ther* 2017;2:10.1038/sigtrans.2017.23. Epub 2017 Jul 14.
- (81) Meng X, Qiu L, Song H, Dang N. MAPK Pathway Involved in Epidermal Terminal Differentiation of Normal Human Epidermal Keratinocytes. *Open Med (Wars)* 2018 May 10;13:189-195.

- (82) Chermnykh E, Kalabusheva E, Vorotelyak E. Extracellular Matrix as a Regulator of Epidermal Stem Cell Fate. *Int J Mol Sci* 2018 Mar 27;19(4):10.3390/ijms19041003.
- (83) Mirvis M, Stearns T, James Nelson W. Cilium structure, assembly, and disassembly regulated by the cytoskeleton. *Biochem J* 2018 Jul 31;475(14):2329-2353.
- (84) Hua K, Ferland RJ. Primary cilia proteins: ciliary and extraciliary sites and functions. *Cell Mol Life Sci* 2018 May;75(9):1521-1540.
- (85) Elofsson R, Andersson A, Falck B, Sjoborg S. The ciliated human keratinocyte. *J Ultrastruct Res* 1984 Jun;87(3):212-220.
- (86) Hoey DA, Downs ME, Jacobs CR. The mechanics of the primary cilium: an intricate structure with complex function. *J Biomech* 2012 Jan 3;45(1):17-26.
- (87) Joukov V, De Nicolo A. The Centrosome and the Primary Cilium: The Yin and Yang of a Hybrid Organelle. *Cells* 2019 Jul 10;8(7):10.3390/cells8070701.
- (88) Venkatesh D. Primary cilia. *J Oral Maxillofac Pathol* 2017 Jan-Apr;21(1):8-10.
- (89) Basten SG, Giles RH. Functional aspects of primary cilia in signaling, cell cycle and tumorigenesis. *Cilia* 2013 Apr 29;2(1):6-2530-2-6.
- (90) Satir P, Pedersen LB, Christensen ST. The primary cilium at a glance. *J Cell Sci* 2010 Feb 15;123(Pt 4):499-503.
- (91) Wheway G, Nazlamova L, Hancock JT. Signaling through the Primary Cilium. *Front Cell Dev Biol* 2018 Feb 8;6:8.
- (92) Anvarian Z, Mykytyn K, Mukhopadhyay S, Pedersen LB, Christensen ST. Cellular signalling by primary cilia in development, organ function and disease. *Nat Rev Nephrol* 2019 Apr;15(4):199-219.
- (93) Veland IR, Awan A, Pedersen LB, Yoder BK, Christensen ST. Primary cilia and signaling pathways in mammalian development, health and disease. *Nephron Physiol* 2009;111(3):p39-53.
- (94) Kobayashi D, Takeda H. Ciliary motility: the components and cytoplasmic preassembly mechanisms of the axonemal dyneins. *Differentiation* 2012 Feb;83(2):S23-9.
- (95) Kamiya R, Yagi T. Functional diversity of axonemal dyneins as assessed by in vitro and in vivo motility assays of *Chlamydomonas* mutants. *Zoolog Sci* 2014 Oct;31(10):633-644.
- (96) Beckers A, Ott T, Schuster-Gossler K, Boldt K, Alten L, Ueffing M, et al. The evolutionary conserved FOXJ1 target gene *Fam183b* is essential for motile cilia in

- Xenopus but dispensable for ciliary function in mice. *Sci Rep* 2018 Oct 2;8(1):14678-018-33045-2.
- (97) Horani A, Ustione A, Huang T, Firth AL, Pan J, Gunsten SP, et al. Establishment of the early cilia preassembly protein complex during motile ciliogenesis. *Proc Natl Acad Sci U S A* 2018 Feb 6;115(6):E1221-E1228.
- (98) Bloodgood RA. Sensory reception is an attribute of both primary cilia and motile cilia. *J Cell Sci* 2010 Feb 15;123(Pt 4):505-509.
- (99) Karak S, Jacobs JS, Kittelmann M, Spalthoff C, Katana R, Sivan-Loukianova E, et al. Diverse Roles of Axonemal Dyneins in *Drosophila* Auditory Neuron Function and Mechanical Amplification in Hearing. *Sci Rep* 2015 Nov 26;5:17085.
- (100) Roberts AJ, Kon T, Knight PJ, Sutoh K, Burgess SA. Functions and mechanics of dynein motor proteins. *Nat Rev Mol Cell Biol* 2013 Nov;14(11):713-726.
- (101) Pfister KK. Distinct functional roles of cytoplasmic dynein defined by the intermediate chain isoforms. *Exp Cell Res* 2015 May 15;334(1):54-60.
- (102) Lorenz HP, Adzick NS. Scarless skin wound repair in the fetus. *West J Med* 1993 Sep;159(3):350-355.
- (103) Amini-Nik S, Yousuf Y, Jeschke MG. Scar management in burn injuries using drug delivery and molecular signaling: Current treatments and future directions. *Adv Drug Deliv Rev* 2018 Jan 1;123:135-154.
- (104) Pastar I, Stojadinovic O, Yin NC, Ramirez H, Nusbaum AG, Sawaya A, et al. Epithelialization in Wound Healing: A Comprehensive Review. *Adv Wound Care (New Rochelle)* 2014 Jul 1;3(7):445-464.
- (105) Guo S, Dipietro LA. Factors affecting wound healing. *J Dent Res* 2010 Mar;89(3):219-229.
- (106) Larouche J, Sheoran S, Maruyama K, Martino MM. Immune Regulation of Skin Wound Healing: Mechanisms and Novel Therapeutic Targets. *Adv Wound Care (New Rochelle)* 2018 Jul 1;7(7):209-231.
- (107) Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen* 2008 Sep-Oct;16(5):585-601.
- (108) Chen D, Hao H, Fu X, Han W. Insight into Reepithelialization: How Do Mesenchymal Stem Cells Perform? *Stem Cells Int* 2016;2016:6120173.
- (109) Havran WL, Jameson JM. Epidermal T cells and wound healing. *J Immunol* 2010 May 15;184(10):5423-5428.

- (110) Toulon A, Breton L, Taylor KR, Tenenhaus M, Bhavsar D, Lanigan C, et al. A role for human skin-resident T cells in wound healing. *J Exp Med* 2009 Apr 13;206(4):743-750.
- (111) Venuprasad K, Kong YC, Farrar MA. Control of Th2-mediated inflammation by regulatory T cells. *Am J Pathol* 2010 Aug;177(2):525-531.
- (112) Nguyen AV, Soulika AM. The Dynamics of the Skin's Immune System. *Int J Mol Sci* 2019 Apr 12;20(8):10.3390/ijms20081811.
- (113) Greaves NS, Ashcroft KJ, Baguneid M, Bayat A. Current understanding of molecular and cellular mechanisms in fibroplasia and angiogenesis during acute wound healing. *J Dermatol Sci* 2013 Dec;72(3):206-217.
- (114) Moore AL, Marshall C, Nauta A, Lorenz HP, Longaker MT. Scarless Wound Healing: From Experimental Target to Clinical Reality  
. In: Atala A, Lanza R, Mikos A, Nerem R, editors. *Principles of Regenerative Medicine*. Third Edition ed.: Elsevier; 2019. p. 65--92.
- (115) Yang S, Sun Y, Geng Z, Ma K, Sun X, Fu X. Abnormalities in the basement membrane structure promote basal keratinocytes in the epidermis of hypertrophic scars to adopt a proliferative phenotype. *Int J Mol Med* 2016 May;37(5):1263-1273.
- (116) Liu T, Qiu C, Ben C, Li H, Zhu S. One-step approach for full-thickness skin defect reconstruction in rats using minced split-thickness skin grafts with Pelnac overlay. *Burns Trauma* 2019 Aug 13;7:19-019-0157-0. eCollection 2019.
- (117) Tan S, Khumalo N, Bayat A. Understanding Keloid Pathobiology From a Quasi-Neoplastic Perspective: Less of a Scar and More of a Chronic Inflammatory Disease With Cancer-Like Tendencies. *Front Immunol* 2019 Aug 7;10:1810.
- (118) Feng J, Mantesso A, De Bari C, Nishiyama A, Sharpe PT. Dual origin of mesenchymal stem cells contributing to organ growth and repair. *Proc Natl Acad Sci U S A* 2011 Apr 19;108(16):6503-6508.
- (119) Jackson WM, Nesti LJ, Tuan RS. Concise review: clinical translation of wound healing therapies based on mesenchymal stem cells. *Stem Cells Transl Med* 2012 Jan;1(1):44-50.
- (120) Jackson WM, Nesti LJ, Tuan RS. Mesenchymal stem cell therapy for attenuation of scar formation during wound healing. *Stem Cell Res Ther* 2012 May 31;3(3):20.
- (121) Kim H, Hyun MR, Kim SW. The Effect of Adipose-Derived Stem Cells on Wound Healing: Comparison of Methods of Application. *Stem Cells Int* 2019 Sep 17;2019:2745640.

- (122) Li P, Guo X. A review: therapeutic potential of adipose-derived stem cells in cutaneous wound healing and regeneration. *Stem Cell Res Ther* 2018 Nov 8;9(1):302-018-1044-5.
- (123) Yu G, Ye L, Tan W, Zhu X, Li Y, Jiang D. A novel dermal matrix generated from burned skin as a promising substitute for deep-degree burns therapy. *Mol Med Rep* 2016 Mar;13(3):2570-2582.
- (124) Shevchenko RV, Sibbons PD, Sharpe JR, James SE. Use of a novel porcine collagen paste as a dermal substitute in full-thickness wounds. *Wound Repair Regen* 2008 Mar-Apr;16(2):198-207.
- (125) Rowan MP, Cancio LC, Elster EA, Burmeister DM, Rose LF, Natesan S, et al. Burn wound healing and treatment: review and advancements. *Crit Care* 2015 Jun 12;19:243-015-0961-2.
- (126) Nguyen AV, Soulika AM. The Dynamics of the Skin's Immune System. *Int J Mol Sci* 2019 Apr 12;20(8):10.3390/ijms20081811.
- (127) Cao PF, Xu YB, Tang JM, Yang RH, Liu XS. HOXA9 regulates angiogenesis in human hypertrophic scars: induction of VEGF secretion by epidermal stem cells. *Int J Clin Exp Pathol* 2014 May 15;7(6):2998-3007.
- (128) Sand JM, Larsen L, Hogaboam C, Martinez F, Han M, Rossel Larsen M, et al. MMP mediated degradation of type IV collagen alpha 1 and alpha 3 chains reflects basement membrane remodeling in experimental and clinical fibrosis--validation of two novel biomarker assays. *PLoS One* 2013 Dec 23;8(12):e84934.
- (129) Ogawa R. Keloid and Hypertrophic Scars Are the Result of Chronic Inflammation in the Reticular Dermis. *Int J Mol Sci* 2017 Mar 10;18(3):10.3390/ijms18030606.
- (130) Kruglikov IL, Scherer PE. Dermal Adipocytes: From Irrelevance to Metabolic Targets? *Trends Endocrinol Metab* 2016 Jan;27(1):1-10.
- (131) Eming SA, Martin P, Tomic-Canic M. Wound repair and regeneration: mechanisms, signaling, and translation. *Sci Transl Med* 2014 Dec 3;6(265):265sr6.
- (132) Grieb G, Bucala R. Fibrocytes in Fibrotic Diseases and Wound Healing. *Adv Wound Care (New Rochelle)* 2012 Feb;1(1):36-40.
- (133) Lugenbiel P, Wenz F, Govorov K, Syren P, Katus HA, Thomas D. Atrial myofibroblast activation and connective tissue formation in a porcine model of atrial fibrillation and reduced left ventricular function. *Life Sci* 2017 Jul 15;181:1-8.
- (134) Fuchs PF, Heindryckx F, Calitz C, Binet F, Øie Solbak S, Danielson H, et al. Fibrin fragment E potentiates TGF- $\beta$ -induced myofibroblast activation and recruitment. *bioRxiv* 2019.

- (135) Klingberg F, Hinz B, White ES. The myofibroblast matrix: implications for tissue repair and fibrosis. *J Pathol* 2013 Jan;229(2):298-309.
- (136) Klingberg F, Hinz B, White ES. The myofibroblast matrix: implications for tissue repair and fibrosis. *J Pathol* 2013 Jan;229(2):298-309.
- (137) Baranyi U, Winter B, Gugerell A, Hegedus B, Brostjan C, Laufer G, et al. Primary Human Fibroblasts in Culture Switch to a Myofibroblast-Like Phenotype Independently of TGF Beta. *Cells* 2019 Jul 13;8(7):10.3390/cells8070721.
- (138) Deitch EA, Wheelahan TM, Rose MP, Clothier J, Cotter J. Hypertrophic burn scars: analysis of variables. *J Trauma* 1983 Oct;23(10):895-898.
- (139) Ogawa R. Keloid and Hypertrophic Scars Are the Result of Chronic Inflammation in the Reticular Dermis. *Int J Mol Sci* 2017 Mar 10;18(3):10.3390/ijms18030606.
- (140) Chen D, Hao H, Fu X, Han W. Insight into Reepithelialization: How Do Mesenchymal Stem Cells Perform? *Stem Cells Int* 2016;2016:6120173.
- (141) Yang S, Sun Y, Geng Z, Ma K, Sun X, Fu X. Abnormalities in the basement membrane structure promote basal keratinocytes in the epidermis of hypertrophic scars to adopt a proliferative phenotype. *Int J Mol Med* 2016 May;37(5):1263-1273.
- (142) Machesney M, Tidman N, Waseem A, Kirby L, Leigh I. Activated keratinocytes in the epidermis of hypertrophic scars. *Am J Pathol* 1998 May;152(5):1133-1141.
- (143) Limandjaja GC, van den Broek LJ, Waaijman T, van Veen HA, Everts V, Monstrey S, et al. Increased epidermal thickness and abnormal epidermal differentiation in keloid scars. *Br J Dermatol* 2017 Jan;176(1):116-126.
- (144) Limandjaja GC, van den Broek LJ, Waaijman T, van Veen HA, Everts V, Monstrey S, et al. Increased epidermal thickness and abnormal epidermal differentiation in keloid scars. *Br J Dermatol* 2017 Jan;176(1):116-126.
- (145) Rousselle P, Montmasson M, Garnier C. Extracellular matrix contribution to skin wound re-epithelialization. *Matrix Biol* 2019 Jan;75-76:12-26.
- (146) Cao PF, Xu YB, Tang JM, Yang RH, Liu XS. HOXA9 regulates angiogenesis in human hypertrophic scars: induction of VEGF secretion by epidermal stem cells. *Int J Clin Exp Pathol* 2014 May 15;7(6):2998-3007.
- (147) Wilgus TA, Ferreira AM, Oberyszyn TM, Bergdall VK, Dipietro LA. Regulation of scar formation by vascular endothelial growth factor. *Lab Invest* 2008 Jun;88(6):579-590.

- (148) Gao Y, Zhou J, Xie Z, Wang J, Ho CK, Zhang Y, et al. Mechanical strain promotes skin fibrosis through LRG-1 induction mediated by ELK1 and ERK signalling. *Commun Biol* 2019 Oct 4;2:359-019-0600-6. eCollection 2019.
- (149) Gao Y, Zhou J, Xie Z, Wang J, Ho CK, Zhang Y, et al. Mechanical strain promotes skin fibrosis through LRG-1 induction mediated by ELK1 and ERK signalling. *Commun Biol* 2019 Oct 4;2:359-019-0600-6. eCollection 2019.
- (150) Tokuyama E, Nagai Y, Takahashi K, Kimata Y, Naruse K. Mechanical Stretch on Human Skin Equivalents Increases the Epidermal Thickness and Develops the Basement Membrane. *PLoS One* 2015 Nov 3;10(11):e0141989.
- (151) Stamati K, Mudera V, Cheema U. Evolution of oxygen utilization in multicellular organisms and implications for cell signalling in tissue engineering. *J Tissue Eng* 2011;2(1):2041731411432365.
- (152) Chambers AC, Leaper DJ. Role of oxygen in wound healing: a review of evidence. *J Wound Care* 2011 Apr;20(4):160-164.
- (153) Memar MY, Yekani M, Alizadeh N, Baghi HB. Hyperbaric oxygen therapy: Antimicrobial mechanisms and clinical application for infections. *Biomed Pharmacother* 2019 Jan;109:440-447.
- (154) Broughton G, 2nd, Janis JE, Attinger CE. Wound healing: an overview. *Plast Reconstr Surg* 2006 Jun;117(7 Suppl):1e-S-32e-S.
- (155) Anderson K, Hamm RL. Factors That Impair Wound Healing. *J Am Coll Clin Wound Spec* 2014 Mar 24;4(4):84-91.
- (156) Augustine R, Kalarikkal N, Thomas S. Advancement of wound care from grafts to bioengineered smart skin substitutes. *Prog Biomater* 2014 Dec;3(2-4):103-113.
- (157) Bacakova L, Zikmundova M, Pajorova J, Broz A, Filova E, Blanquer A, et al. Nanofibrous Scaffolds for Skin Tissue Engineering and Wound Healing Based on Synthetic Polymers. *Current and future aspects of nanomedicine: IntechOpen*; 2019.
- (158) Sun G. Pro-Regenerative Hydrogel Restores Scarless Skin during Cutaneous Wound Healing. *Adv Healthc Mater* 2017 Dec;6(23):10.1002/adhm.201700659. Epub 2017 Sep 25.
- (159) Varkey M, Visscher DO, van Zuijlen PPM, Atala A, Yoo JJ. Skin bioprinting: the future of burn wound reconstruction? *Burns Trauma* 2019 Feb 12;7:4-019-0142-7. eCollection 2019.
- (160) Wurzer P, Keil H, Branski LK, Parvizi D, Clayton RP, Finnerty CC, et al. The use of skin substitutes and burn care-a survey. *J Surg Res* 2016 Apr;201(2):293-298.

- (161) Orgill DP, Butler C, Regan JF, Barlow MS, Yannas IV, Compton CC. Vascularized collagen-glycosaminoglycan matrix provides a dermal substrate and improves take of cultured epithelial autografts. *Plast Reconstr Surg* 1998 Aug;102(2):423-429.
- (162) Sarkiri M, Fox SC, Fratila-Apachitei LE, Zadpoor AA. Bioengineered Skin Intended for Skin Disease Modeling. *Int J Mol Sci* 2019 Mar 20;20(6):10.3390/ijms20061407.
- (163) Sheikholeslam M, Wright MEE, Jeschke MG, Amini-Nik S. Biomaterials for Skin Substitutes. *Adv Healthc Mater* 2018 Mar;7(5):10.1002/adhm.201700897. Epub 2017 Dec 22.
- (164) Debels H, Hamdi M, Abberton K, Morrison W. Dermal matrices and bioengineered skin substitutes: a critical review of current options. *Plast Reconstr Surg Glob Open* 2015 Feb 6;3(1):e284.
- (165) Yannas IV, Burke JF. Design of an artificial skin. I. Basic design principles. *J Biomed Mater Res* 1980 Jan;14(1):65-81.
- (166) Branski LK, Herndon DN, Pereira C, Mlcak RP, Celis MM, Lee JO, et al. Longitudinal assessment of Integra in primary burn management: a randomized pediatric clinical trial. *Crit Care Med* 2007 Nov;35(11):2615-2623.
- (167) Stern R, McPherson M, Longaker MT. Histologic study of artificial skin used in the treatment of full-thickness thermal injury. *J Burn Care Rehabil* 1990 Jan-Feb;11(1):7-13.
- (168) Yannas IV, Orgill DP, Burke JF. Template for skin regeneration. *Plast Reconstr Surg* 2011 Jan;127 Suppl 1:60S-70S.
- (169) Canonico S, Campitiello F, Della Corte A, Padovano V, Pellino G. Treatment of Leg Chronic Wounds with Dermal Substitutes and Thin Skin Grafts. In: Gore M, editor. *Skin Grafts*: Intechopen; 2013.
- (170) Ho J, Walsh C, Yue D, Dardik A, Cheema U. Current Advancements and Strategies in Tissue Engineering for Wound Healing: A Comprehensive Review. *Adv Wound Care (New Rochelle)* 2017 Jun 1;6(6):191-209.
- (171) Savoji H, Godau B, Hassani MS, Akbari M. Skin Tissue Substitutes and Biomaterial Risk Assessment and Testing. *Front Bioeng Biotechnol* 2018 Jul 26;6:86.
- (172) Boyce ST, Lalley AL. Tissue engineering of skin and regenerative medicine for wound care. *Burns Trauma* 2018 Jan 24;6:4-017-0103-y. eCollection 2018.
- (173) Shevchenko RV, James SL, James SE. A review of tissue-engineered skin bioconstructs available for skin reconstruction. *J R Soc Interface* 2010 Feb 6;7(43):229-258.

- (174) Shpichka A, Butnaru D, Bezrukov EA, Sukhanov RB, Atala A, Burdukovskii V, et al. Skin tissue regeneration for burn injury. *Stem Cell Res Ther* 2019 Mar 15;10(1):94-019-1203-3.
- (175) Cheshire PA, Herson MR, Cleland H, Akbarzadeh S. Artificial dermal templates: A comparative study of NovoSorb Biodegradable Temporising Matrix (BTM) and Integra((R)) Dermal Regeneration Template (DRT). *Burns* 2016 Aug;42(5):1088-1096.
- (176) Vig K, Chaudhari A, Tripathi S, Dixit S, Sahu R, Pillai S, et al. Advances in Skin Regeneration Using Tissue Engineering. *Int J Mol Sci* 2017 Apr 7;18(4):10.3390/ijms18040789.
- (177) Dixit S, Baganizi DR, Sahu R, Dosunmu E, Chaudhari A, Vig K, et al. Immunological challenges associated with artificial skin grafts: available solutions and stem cells in future design of synthetic skin. *J Biol Eng* 2017 Dec 13;11:49-017-0089-9. eCollection 2017.
- (178) Hughes OB, Rakosi A, Macquhae F, Herskovitz I, Fox JD, Kirsner RS. A Review of Cellular and Acellular Matrix Products: Indications, Techniques, and Outcomes. *Plast Reconstr Surg* 2016 Sep;138(3 Suppl):138S-47S.
- (179) Foley E, Robinson A, Maloney M. Skin Substitutes and Dermatology: A Review. *Curr Derm Rep* 2013(2):101--112.
- (180) Roques C, Teot L. A critical analysis of measurements used to assess and manage scars. *Int J Low Extrem Wounds* 2007 Dec;6(4):249-253.
- (181) Thompson CM, Sood RF, Honari S, Carrougher GJ, Gibran NS. What score on the Vancouver Scar Scale constitutes a hypertrophic scar? Results from a survey of North American burn-care providers. *Burns* 2015 Nov;41(7):1442-1448.
- (182) Nguyen TA, Feldstein SI, Shumaker PR, Krakowski AC. A review of scar assessment scales. *Semin Cutan Med Surg* 2015 Mar;34(1):28-36.
- (183) Corica GF, Wigger NC, Edgar DW, Wood FM, Carroll S. Objective measurement of scarring by multiple assessors: is the tissue tonometer a reliable option? *J Burn Care Res* 2006 Jul-Aug;27(4):520-523.
- (184) Fearmonti R, Bond J, Erdmann D, Levinson H. A review of scar scales and scar measuring devices. *Eplasty* 2010 Jun 21;10:e43.
- (185) Draaijers LJ, Tempelman FR, Botman YA, Tuinebreijer WE, Middelkoop E, Kreis RW, et al. The patient and observer scar assessment scale: a reliable and feasible tool for scar evaluation. *Plast Reconstr Surg* 2004 Jun;113(7):1960-5; discussion 1966-7.

- (186) Bae SH, Bae YC. Analysis of frequency of use of different scar assessment scales based on the scar condition and treatment method. *Arch Plast Surg* 2014 Mar;41(2):111-115.
- (187) Kantor J. Reliability and Photographic Equivalency of the Scar Cosmesis Assessment and Rating (SCAR) Scale, an Outcome Measure for Postoperative Scars. *JAMA Dermatol* 2017 Jan 1;153(1):55-60.
- (188) Mundy LR, Miller HC, Klassen AF, Cano SJ, Pusic AL. Patient-Reported Outcome Instruments for Surgical and Traumatic Scars: A Systematic Review of their Development, Content, and Psychometric Validation. *Aesthetic Plast Surg* 2016 Oct;40(5):792-800.
- (189) Pham TD, Karlsson M, Andersson CM, Mirdell R, Sjoberg F. Automated VSS-based Burn Scar Assessment using Combined Texture and Color Features of Digital Images in Error-Correcting Output Coding. *Sci Rep* 2017 Dec 1;7(1):16744-017-16914-0.
- (190) Lee KC, Dretzke J, Grover L, Logan A, Moiemmen N. A systematic review of objective burn scar measurements. *Burns Trauma* 2016 Apr 27;4:14-016-0036-x. eCollection 2016.
- (191) Perry DM, McGrouther DA, Bayat A. Current tools for noninvasive objective assessment of skin scars. *Plast Reconstr Surg* 2010 Sep;126(3):912-923.
- (192) Bliss E, Heywood WE, Benatti M, Sebire NJ, Mills K. An optimised method for the proteomic profiling of full thickness human skin. *Biol Proced Online* 2016 Jul 21;18:15-016-0045-y. eCollection 2016.
- (193) Hibbert SA, Ozols M, Griffiths CEM, Watson REB, Bell M, Sherratt MJ. Defining tissue proteomes by systematic literature review. *Sci Rep* 2018 Jan 11;8(1):546-017-18699-8.
- (194) Javad F, Day PJ. Protein profiling of keloidal scar tissue. *Arch Dermatol Res* 2012 Sep;304(7):533-540.
- (195) Ong CT, Khoo YT, Mukhopadhyay A, Masilamani J, Do DV, Lim IJ, et al. Comparative proteomic analysis between normal skin and keloid scar. *Br J Dermatol* 2010 Jun;162(6):1302-1315.
- (196) Swindell WR, Remmer HA, Sarkar MK, Xing X, Barnes DH, Wolterink L, et al. Proteogenomic analysis of psoriasis reveals discordant and concordant changes in mRNA and protein abundance. *Genome Med* 2015 Aug 4;7(1):86-015-0208-5. eCollection 2015.
- (197) Paiva-Lopes MJ, Delgado Alves J. Psoriasis-associated vascular disease: the role of HDL. *J Biomed Sci* 2017 Sep 14;24(1):73-017-0382-4.

- (198) Rashmi R, Rao KS, Basavaraj KH. A comprehensive review of biomarkers in psoriasis. *Clin Exp Dermatol* 2009 Aug;34(6):658-663.
- (199) Mezentsev A, Nikolaev A, Bruskin S. Matrix metalloproteinases and their role in psoriasis. *Gene* 2014 Apr 25;540(1):1-10.
- (200) Jiang S, Hinchliffe TE, Wu T. Biomarkers of An Autoimmune Skin Disease--Psoriasis. *Genomics Proteomics Bioinformatics* 2015 Aug;13(4):224-233.
- (201) Swindell WR, Remmer HA, Sarkar MK, Xing X, Barnes DH, Wolterink L, et al. Proteogenomic analysis of psoriasis reveals discordant and concordant changes in mRNA and protein abundance. *Genome Med* 2015 Aug 4;7(1):86-015-0208-5. eCollection 2015.
- (202) Arima K, Ohta S, Takagi A, Shiraishi H, Masuoka M, Ohtsuka K, et al. Periostin contributes to epidermal hyperplasia in psoriasis common to atopic dermatitis. *Allergol Int* 2015 Jan;64(1):41-48.
- (203) Bai J, Liu Z, Xu Z, Ke F, Zhang L, Zhu H, et al. Epigenetic downregulation of SFRP4 contributes to epidermal hyperplasia in psoriasis. *J Immunol* 2015 May 1;194(9):4185-4198.
- (204) Abhishek S, Palamadai Krishnan S. Epidermal Differentiation Complex: A Review on Its Epigenetic Regulation and Potential Drug Targets. *Cell J* 2016 Spring;18(1):1-6.
- (205) Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* 2012 Jun 28;9(7):676-682.
- (206) Tervaniemi MH, Katayama S, Skoog T, Siitonen HA, Vuola J, Nuutila K, et al. NOD-like receptor signaling and inflammasome-related pathways are highlighted in psoriatic epidermis. *Sci Rep* 2016 Mar 15;6:22745.
- (207) Viljanto J. A new method for treatment of open wounds. *Ann Chir Gynaecol Fenn* 1972;61(2):94-100.
- (208) Viljanto J, Jaaskelainen A. Stimulation of granulation tissue growth in burns. *Ann Chir Gynaecol Fenn* 1973;62(1):18-24.
- (209) Viljanto J. Cellstic: A device for wound healing studies in man. Description of the method. *J Surg Res* 1976 Feb;20(2):115-119.
- (210) Viljanto J, Rajamaki A. Cellular patterns in the early phase of healing wounds in children. *Scand J Plast Reconstr Surg* 1976;10(2):83-89.
- (211) Tommila M, Jokilampi A, Penttinen R, Ekholm E. Cellulose - A Biomaterial with Cell-Guiding Property. In: van de Ven T, Godbout L, editors. *Cellulose - Medical, Pharmaceutical and Electronic Applications: IntechOpen*; 2013.

- (212) Tommila M, Jokilammi A, Terho P, Wilson T, Penttinen R, Ekholm E. Hydroxyapatite coating of cellulose sponges attracts bone-marrow-derived stem cells in rat subcutaneous tissue. *J R Soc Interface* 2009 Oct 6;6(39):873-880.
- (213) Williams DF. On the mechanisms of biocompatibility. *Biomaterials* 2008 Jul;29(20):2941-2953.
- (214) Kim H, Son D, Choi TH, Jung S, Kwon S, Kim J, et al. Evaluation of an amniotic membrane-collagen dermal substitute in the management of full-thickness skin defects in a pig. *Arch Plast Surg* 2013 Jan;40(1):11-18.
- (215) Jeng JC, Fidler PE, Sokolich JC, Jaskille AD, Khan S, White PM, et al. Seven years' experience with Integra as a reconstructive tool. *J Burn Care Res* 2007 Jan-Feb;28(1):120-126.
- (216) Vallieres M, du Souich P. Modulation of inflammation by chondroitin sulfate. *Osteoarthritis Cartilage* 2010 Jun;18 Suppl 1:S1-6.
- (217) Burke JF, Yannas IV, Quinby WC, Jr, Bondoc CC, Jung WK. Successful use of a physiologically acceptable artificial skin in the treatment of extensive burn injury. *Ann Surg* 1981 Oct;194(4):413-428.
- (218) Michaeli D, McPherson M. Immunologic study of artificial skin used in the treatment of thermal injuries. *J Burn Care Rehabil* 1990 Jan-Feb;11(1):21-26.
- (219) Stern R, McPherson M, Longaker MT. Histologic study of artificial skin used in the treatment of full-thickness thermal injury. *J Burn Care Rehabil* 1990 Jan-Feb;11(1):7-13.
- (220) Butzelaar L, Ulrich MM, Mink van der Molen AB, Niessen FB, Beelen RH. Currently known risk factors for hypertrophic skin scarring: A review. *J Plast Reconstr Aesthet Surg* 2016 Feb;69(2):163-169.
- (221) Corliss BA, Azimi MS, Munson JM, Peirce SM, Murfee WL. Macrophages: An Inflammatory Link Between Angiogenesis and Lymphangiogenesis. *Microcirculation* 2016 Feb;23(2):95-121.
- (222) Chan T, Pek EA, Huth K, Ashkar AA. CD4(+) T-cells are important in regulating macrophage polarization in C57BL/6 wild-type mice. *Cell Immunol* 2011;266(2):180-186.
- (223) Agrawal H, Tholpady S, Capito AE, Drake DB, Katz AJ. Macrophage phenotypes correspond with remodeling outcomes of various acellular dermal matrices. *Open Journal of Regenerative Medicine* 2012 4th December;1(2):51--59.
- (224) Vural E, Berbee M, Acott A, Blagg R, Fan CY, Hauer-Jensen M. Skin graft take rates, granulation, and epithelialization: dependence on myeloid cell hypoxia-inducible factor 1alpha. *Arch Otolaryngol Head Neck Surg* 2010 Jul;136(7):720-723.

- (225) Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. *Nat Med* 2015 Jun;21(6):628-637.
- (226) Viljanto J, Jaaskelainen A. Stimulation of granulation tissue growth in burns. *Ann Chir Gynaecol Fenn* 1973;62(1):18-24.
- (227) Viljanto J. Cellstic: A device for wound healing studies in man. Description of the method. *J Surg Res* 1976 Feb;20(2):115-119.
- (228) Viljanto J, Rajamaki A. Cellular patterns in the early phase of healing wounds in children. *Scand J Plast Reconstr Surg* 1976;10(2):83-89.
- (229) Moiemens NS, Staiano JJ, Ojeh NO, Thway Y, Frame JD. Reconstructive surgery with a dermal regeneration template: clinical and histologic study. *Plast Reconstr Surg* 2001 Jul;108(1):93-103.
- (230) Moiemens NS, Vlachou E, Staiano JJ, Thawy Y, Frame JD. Reconstructive surgery with Integra dermal regeneration template: histologic study, clinical evaluation, and current practice. *Plast Reconstr Surg* 2006 Jun;117(7 Suppl):160S-174S.
- (231) Zhai Q, Zhou F, Ibrahim MM, Zhao J, Liu X, Wu J, et al. An immune-competent rat split thickness skin graft model: useful tools to develop new therapies to improve skin graft survival. *Am J Transl Res* 2018 Jun 15;10(6):1600-1610.
- (232) Chen Z, Dai T, Chen X, Tan L, Shi C. Activation and regulation of the granulation tissue derived cells with stemness-related properties. *Stem Cell Res Ther* 2015 Apr 29;6:85-015-0070-9.
- (233) Krishna D, Kumar S, Sharma U,D. Impact of nonscraping of granulation tissue on outcomes after skin grafting. *Indian J Burns* 2017(25):33--37.
- (234) Dhar S, Saraf R, Gupta AK, Raina B. Comparative study of skin grafting with and without surgical removal of granulation tissue in chronic burn wounds. *Burns* 2007 Nov;33(7):872-878.
- (235) Lindenblatt N, Platz U, Althaus M, Hegland N, Schmidt CA, Contaldo C, et al. Temporary angiogenic transformation of the skin graft vasculature after reperfusion. *Plast Reconstr Surg* 2010 Jul;126(1):61-70.
- (236) Kondo S. The roles of keratinocyte-derived cytokines in the epidermis and their possible responses to UVA-irradiation. *J Investig Dermatol Symp Proc* 1999 Sep;4(2):177-183.
- (237) Deo PN, Deshmukh R. Pathophysiology of keratinization. *J Oral Maxillofac Pathol* 2018 Jan-Apr;22(1):86-91.

- (238) Gniadecki R. Regulation of keratinocyte proliferation. *Gen Pharmacol* 1998 May;30(5):619-622.
- (239) Kenny FN, Drymoussi Z, Delaine-Smith R, Kao AP, Laly AC, Knight MM, et al. Tissue stiffening promotes keratinocyte proliferation through activation of epidermal growth factor signaling. *J Cell Sci* 2018 May 16;131(10):10.1242/jcs.215780.
- (240) Benhadou F, Mintoff D, Del Marmol V. Psoriasis: Keratinocytes or Immune Cells - Which Is the Trigger? *Dermatology* 2019;235(2):91-100.
- (241) Glatzer F, Gschwandtner M, Ehling S, Rossbach K, Janik K, Klos A, et al. Histamine induces proliferation in keratinocytes from patients with atopic dermatitis through the histamine 4 receptor. *J Allergy Clin Immunol* 2013 Dec;132(6):1358-1367.
- (242) Stahl PJ, Felsen D. Transforming growth factor-beta, basement membrane, and epithelial-mesenchymal transdifferentiation: implications for fibrosis in kidney disease. *Am J Pathol* 2001 Oct;159(4):1187-1192.
- (243) Horch RE, Corbei O, Formanek-Corbei B, Brand-Saberi B, Vanscheidt W, Stark GB. Reconstitution of basement membrane after 'sandwich-technique' skin grafting for severe burns demonstrated by immunohistochemistry. *J Burn Care Rehabil* 1998 May-Jun;19(3):189-202.
- (244) Langdon RC, Cuono CB, Birchall N, Madri JA, Kuklinska E, McGuire J, et al. Reconstitution of structure and cell function in human skin grafts derived from cryopreserved allogeneic dermis and autologous cultured keratinocytes. *J Invest Dermatol* 1988 Nov;91(5):478-485.
- (245) Hinz B. Formation and function of the myofibroblast during tissue repair. *J Invest Dermatol* 2007 Mar;127(3):526-537.
- (246) Yannas IV, Orgill DP, Burke JF. Template for skin regeneration. *Plast Reconstr Surg* 2011 Jan;127 Suppl 1:60S-70S.
- (247) Bansky R. The use of dermal template INTEGRA in a scalpation injury of hand. *Bratisl Lek Listy* 2005;106(6-7):221-225.
- (248) Nguyen DQ, Potokar TS, Price P. An objective long-term evaluation of Integra (a dermal skin substitute) and split thickness skin grafts, in acute burns and reconstructive surgery. *Burns* 2010 Feb;36(1):23-28.
- (249) Heimbach DM, Warden GD, Luterman A, Jordan MH, Ozobia N, Ryan CM, et al. Multicenter postapproval clinical trial of Integra dermal regeneration template for burn treatment. *J Burn Care Rehabil* 2003 Jan-Feb;24(1):42-48.
- (250) Stiefel D, Schiestl C, Meuli M. Integra Artificial Skin for burn scar revision in adolescents and children. *Burns* 2010 Feb;36(1):114-120.

- (251) Moiemem N, Yarrow J, Hodgson E, Constantinides J, Chipp E, Oakley H, et al. Long-term clinical and histological analysis of Integra dermal regeneration template. *Plast Reconstr Surg* 2011 Mar;127(3):1149-1154.
- (252) Chou TD, Chen SL, Lee TW, Chen SG, Cheng TY, Lee CH, et al. Reconstruction of burn scar of the upper extremities with artificial skin. *Plast Reconstr Surg* 2001 Aug;108(2):378-84: discussion 385.
- (253) Mikesch LM, Aramadhaka LR, Moskaluk C, Zigrino P, Mauch C, Fox JW. Proteomic anatomy of human skin. *J Proteomics* 2013 Jun 12;84:190-200.
- (254) Bae JE, Choi H, Shin DW, Na HW, Park NY, Kim JB, et al. Fine particulate matter (PM<sub>2.5</sub>) inhibits ciliogenesis by increasing SPRR3 expression via c-Jun activation in RPE cells and skin keratinocytes. *Sci Rep* 2019 Mar 8;9(1):3994-019-40670-y.
- (255) Sydor S, Manka P, Best J, Jafoui S, Sowa JP, Zoubek ME, et al. Kruppel-like factor 6 is a transcriptional activator of autophagy in acute liver injury. *Sci Rep* 2017 Aug 14;7(1):8119-017-08680-w.
- (256) Andreoli V, Gehrau RC, Bocco JL. Biology of Kruppel-like factor 6 transcriptional regulator in cell life and death. *IUBMB Life* 2010 Dec;62(12):896-905.
- (257) Moser M, Imhof A, Pscherer A, Bauer R, Amselgruber W, Sinowatz F, et al. Cloning and characterization of a second AP-2 transcription factor: AP-2 beta. *Development* 1995 Sep;121(9):2779-2788.
- (258) Pellikainen JM, Kosma VM. Activator protein-2 in carcinogenesis with a special reference to breast cancer--a mini review. *Int J Cancer* 2007 May 15;120(10):2061-2067.
- (259) Obata T, Yanagidani A, Yokoro K, Numoto M, Yamamoto S. Analysis of the consensus binding sequence and the DNA-binding domain of ZF5. *Biochem Biophys Res Commun* 1999 Feb 16;255(2):528-534.
- (260) Numoto M, Niwa O, Kaplan J, Wong KK, Merrell K, Kamiya K, et al. Transcriptional repressor ZF5 identifies a new conserved domain in zinc finger proteins. *Nucleic Acids Res* 1993 Aug 11;21(16):3767-3775.
- (261) Kurosaki T, Maquat LE. Nonsense-mediated mRNA decay in humans at a glance. *J Cell Sci* 2016 Feb 1;129(3):461-467.
- (262) Dyle MC, Kolakada D, Cortazar MA, Jagannathan S. How to get away with nonsense: Mechanisms and consequences of escape from nonsense-mediated RNA decay. *Wiley Interdiscip Rev RNA* 2020 Jan;11(1):e1560.
- (263) Nestle FO, Kaplan DH, Barker J. Psoriasis. *N Engl J Med* 2009 Jul 30;361(5):496-509.

- (264) Coto E, Santos-Juanes J, Coto-Segura P, Alvarez V. New psoriasis susceptibility genes: momentum for skin-barrier disruption. *J Invest Dermatol* 2011 May;131(5):1003-1005.
- (265) Wolf R, Orion E, Ruocco E, Ruocco V. Abnormal epidermal barrier in the pathogenesis of psoriasis. *Clin Dermatol* 2012 May-Jun;30(3):323-328.
- (266) Katayama S, Skoog T, Jouhilahti EM, Siitonen HA, Nuutila K, Tervaniemi MH, et al. Gene expression analysis of skin grafts and cultured keratinocytes using synthetic RNA normalization reveals insights into differentiation and growth control. *BMC Genomics* 2015 Jun 25;16:476-015-1671-5.
- (267) Tervaniemi MH, Katayama S, Skoog T, Siitonen HA, Vuola J, Nuutila K, et al. Intracellular signalling pathways and cytoskeletal functions converge on the psoriasis candidate gene CCHCR1 expressed at P-bodies and centrosomes. *BMC Genomics* 2018 Jun 4;19(1):432-018-4810-y.
- (268) Andrews KL, Nettesheim P, Asai DJ, Ostrowski LE. Identification of seven rat axonemal dynein heavy chain genes: expression during ciliated cell differentiation. *Mol Biol Cell* 1996 Jan;7(1):71-79.
- (269) Giannakakou P, Sackett DL, Ward Y, Webster KR, Blagosklonny MV, Fojo T. P53 is Associated with Cellular Microtubules and is Transported to the Nucleus by Dynein. *Nat Cell Biol* 2000 Oct;2(10):709-717.
- (270) Glick AB, Perez-Lorenzo R, Mohammed J. Context-dependent regulation of cutaneous immunological responses by TGFbeta1 and its role in skin carcinogenesis. *Carcinogenesis* 2008 Jan;29(1):9-14.
- (271) Banno T, Gazel A, Blumenberg M. Effects of tumor necrosis factor-alpha (TNF alpha) in epidermal keratinocytes revealed using global transcriptional profiling. *J Biol Chem* 2004 Jul 30;279(31):32633-32642.