Gene Expression Profiling of Human Skin Donor Site Wound Healing to Guide Novel Regenerative Therapies

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Academic Dissertation

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Original publications

This thesis is based on the following original publications:

I Nuutila K, Siltanen A, Peura M, Bizik J, Kaartinen I, Kuokkanen H, Nieminen T, Harjula A, Aarnio P, Vuola J, Kankuri E. Human skin transcriptome during superficial cutaneous wound healing. *Wound Repair Regen*. 2012;20:830-9.

II Nuutila K, Siltanen A, Peura M, Harjula A, Nieminen T, Vuola J, Kankuri E, Aarnio P. Gene expression profiling of negative-pressure-treated skin graft donor site wounds. *Burns*. 2013;39:687-93.

III Nuutila K, Kankainen M, Harjula A, Vuola J, Kankuri E. Characterizing the wound healing response that drives human epidermal maturation on transcriptome level. Submitted manuscript.

IV Nuutila K, Peura M, Suomela S, Hukkanen M, Siltanen A, Harjula A, Vuola J, Kankuri E. Recombinant human collagen III gel for transplantation of autologous skin cells in porcine full-thickness wounds. *J Tissue Eng Regen Med*. 2013 Jan 30.

Main abbreviations

AMP	Antimicrobial peptide
BM	Basement membrane
CE	Cornified envelope
CEA	Cultured epithelial autograft
ECM	Extra cellular matrix
EDC	Epidermal differentiation complex
GF	Growth factor
IL	Interleukin
KRT	Keratin
MMP	Matrix metalloproteinases
MSC	Mesenchymal stem cell
NPWT	Negative-pressure wound therapy
PCR	Polymerase chain reaction
POD	Postoperative day
rhCol-III	Recombinant human collagen III
STSG	Split-thickness skin grafting
SC	Stratum corneum
STE	Skin tissue engineering
TE	Tissue engineering

Abstract

Information on the molecular details of human skin wound healing has been limited due to several types of challenges faced when data from basic research are adapted for use in clinical studies. Comprehensive genomewide data on gene behaviour during the various phases of wound healing have not been available, although many techniques, such as gene expression microarrays and RNA sequencing would, have made it possible.

The aim of my doctoral thesis was to examine human skin regeneration and to develop ways to enhance wound healing, especially in burn wound treatment. The main goal was to characterize the gene expression transcriptome of human donor site wounds over time. Skin biopsies were collected from patients before and after split-thickness skin graft harvesting. The first biopsy of intact skin was obtained immediately before the site was wounded by the graft harvest. Immediately thereafter, a second biopsy was performed to serve as a sample an of acute wound. Later, the next biopsies were obtained on the 3rd, 7th, 14th and 21st postoperative days. The skin graft donor site wound was chosen because it is controllably made in the operating room and represents a normal, uncomplicated, and consistently healing superficial skin wound. Negative pressure was applied to some of the donor sites to clarify the effects of this widely used therapy on gene expression in a healing wound. Moreover, this approach enabled the initial evaluation of the gene expression profiling principle for clinical research purposes.

The tissue samples collected were homogenized, and ribonucleic acid (RNA) was isolated. Thereafter, genomewide microarrays were performed on all samples. The gene profiles at different time points were compared, and data were presented as fold-change alterations in gene expression. As a result, the data reveals various types of gene expression patterns relating to, loss of expression due to tissue removal, down-regulation or induction of expression as the wound-healing cascade advances or regain of expression according to tissue regeneration. The results also give molecular level information on the effects of negative-pressure wound therapy.

These studies provide the first insights into the transcriptome during normal human superficial cutaneous wound healing. The data reveal novel genes associated with epidermal wound healing. Moreover, they provide a fundamental background for future studies that can be utilized in the clinical evaluation of the effects of therapies.

1. Introduction

Wound-healing capability is essential for survival. Millions of people around the world suffer from severe nonhealing wounds and hundreds of millions of euros are spent annually on treatments that unfortunately are too often inefficient (Sen et al., 2009). Two major causes tightly associated with compromised wound healing, namely population aging and the increased prevalence of obesity, have caused an alarming increase in this load. These concerns further stress the desperate need to find efficient new solutions for wound repair (Graham et al., 2003).

The complex process of wound healing is divided into three overlapping phases of inflammation, new-tissue formation and tissue remodelling (Reinke & Sorg, 2012). It has been studied for decades, and over the years researchers have been able to establish many molecular components involved in its underlying mechanisms. However, comprehensive studies on molecular detail are an unfortunate rarity in humans (Cole et al., 2001; Greco et al., 2010; Smiley et al., 2005). Many wound-healing data are still obtained from different animal models (Sullivan et al., 2001; Zcharia et al., 2005). Although rodents provide valuable information, the fact that a mouse is not a man remains.

It is challenging to study the process of wound healing in humans. Naturally, research must always be conducted on patients' terms and conditions. Opportunities to have control wounds and to obtain numerous and ample tissue samples for analysis, as in animal studies are very limited. The lack of proper controls complicates objective evaluation of therapies; all patients are different and all wounds are different. In addition, several other factors, such as age and accompanying diseases, strongly influence the efficacy of wound healing (Broughton et al., 2006).

As mentioned, novel therapies alongside traditional wound care are required. And, indeed, novel approaches such as various cell -, growth factor (GF) - and gene therapies are emerging. Several bioengineered skin products, as well as cell therapy products, are already on the market and have been proven to be helpful (Günter & Machens, 2012). The next challenge is to make these treatments available. Yet, the promising therapies have failed, due to their complexity or, even more commonly due to their ridiculously expensive prices. Thus, products should be practical for doctors

and nurses to use and, more importantly they should be affordable and effective. Future evolution in automated cell culture techniques, as well as rationalization of bureaucratic protocols, will help to solve these issues.

The goal of the first three studies in my doctoral dissertation was to provide novel insight into the molecular associations behind human cutaneous wound healing by mapping the gene expression profile of repair over time. The approach was to use skin graft donor site wounds that heal reliably, are clean and of standard depth for the characterization of gene expression. Punch biopsies of patients' wounds were collected over time, and the transcriptome at each time point was obtained, using commercially available comprehensive gene expression microarray chips. This approach will enable the established theories of the wound-healing cascade to be examined, challenged and extended, using the full human genome. In addition to giving abundant new information for research purposes, these data will be utilized as a clinical research tool to enable objective evaluation of human donor site wound healing.

In the fourth study the properties of human recombinant collagen III (rhCol-III) as a dermal filler and a carrier of autologous cells were examined. Cell viability inside the gel was examined *in vitro* and the *in vivo* properties were evaluated in porcine full-thickness wounds.

2. Review of the literature

2.1 Skin structure, function and homeostasis

Skin is the largest organ in the human body. It has a layered structure that is essential for a multitude of functions (Figure 1). Skin also contributes to our appearance and shape. It greatly affects our mental wellbeing through aesthetic and cultural influences. More importantly, the skin serves as a shield that protects us from the outside world. All layers of the skin (epidermis, dermis and hypodermis) serve as sentinels against various physical, biological and chemical threats (McLafferty et al., 2012). Skin is an important part of the immune system and contains components of both the innate and adaptive immune systems. Moreover, with its numerous nerve endings, the skin serves as a sensory organ by mediating sensations of heat, pressure, and pain to the central nervous system. Through various exocrine glands and a highly versatile capacity to regulate its blood flow, the skin contributes to regulation of body temperature (Hannuksela et al., 2003). The skin is also an endocrine organ and participates in the production and metabolism of vitamin D_3. Finally, the skin helps us to express feelings by reflecting the various actions of the autonomic nervous system, such as through vasolidation-blushing (Calonje et al., 2011).

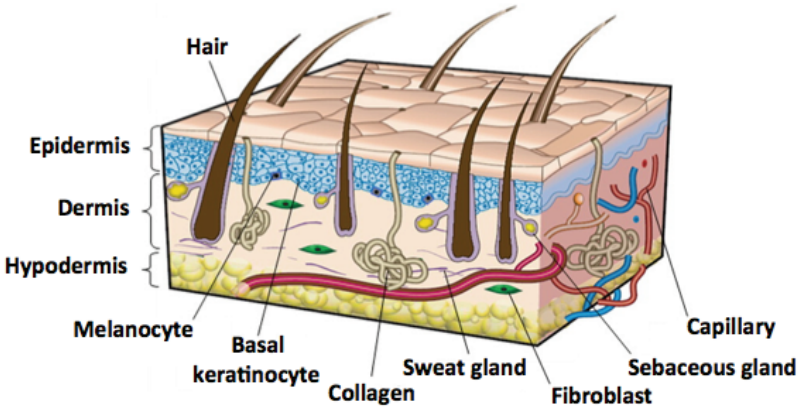
Skin is constantly stressed by threats from the outside world. Production of antimicrobial peptides (AMPs) that contribute to the killing of bacteria, viruses, and fungi is the primary protection system against microbes, and during microbial invasion expression of AMPs increases. The AMPs protect either directly by killing microbes or indirectly by signalling host response through GF, chemotactic and angiogenic activity (Schauber & Gallo, 2009). Indirectly acting AMPs are also known as *alarmins*. Many cell types (such as keratinocytes, sebocytes and mast cells) that reside in the skin produce AMPs. Moreover, inflammatory cells patrolling or infiltrating the skin also contribute to production of AMPs (Chan et al., 2012). For killing, AMPs target various microbial structures in the membranes or in the cytoplasm. They can also cause targeted disruption of bacterial metabolic pathways. Their initial targeting to the microbes relies on their positive charge against negatively charged microbes. There are over 20 cutaneous AMPs of which β-defensins and cathelicidins are the most well known (Yang & Oppenheim, 2004). AMPs are

structurally very diverse and thus their antimicrobial activity is the only admission requirement. Some of the AMPs are secreted in the uppermost layer the of epidermis, such as the *Escherichia coli*-killing S100 calcium-binding protein A7 (S100A7, psoriasin) and ribonuclease 7 (RNASE7). However, most of the AMPs reside in the living layers of the skin (Brogden, 2005).

In addition, the skin's immune system consists of antigen-presenting Langerhans cells, lymphocytes, mast cells and keratinocytes. Langerhans cells are dendritic cells that stretch out and establish connections with keratinocytes. They register early injury or infection and relay the message to other members of the immune defence. Langerhans cells together with various lymphocytes induce the adaptive immune response. As for keratinocytes, in the absence of injury they secrete several inhibitory cytokines, such as GF and interleukins (ILs), and in response to injury they stimulate inflammation and activate Langerhans cells (Williams & Kupper, 1996). Mast cells are important effector cells in the induction of an inflammatory response. They also take part in the pathogenesis of various inflammatory disorders, such as immunoglobulin E (IgE)-mediated allergies and autoimmune disorders (Maurer et al., 2006).

Skin has a strong ability to renew itself. However, for it to function normally there must be a balance between generation of new cells and desquamation of dead cells. In a continuously undergoing process, new epidermal cells are produced in the basal layer, while old dead keratinocytes are sloughed off from the surface. The life of a newborn keratinocyte begins in the basal layer, once it commits to the predetermined programme of differentiation (Figure 2) (Blanpain & Fuchs, 2009). Subsequently, the cell begins its journey towards the surface of the skin. It migrates through the various layers, during which the supply of nourishment decreases; the cell accumulates keratin (KRT), and eventually dies. After about 1 month, the terminally differentiated, already dead cell reaches the surface and is sloughed off. Taken together on a cell population level, this process is called epidermal homeostasis, and epidermal stem cells are responsible for its maintenance. In the skin, these stem cells reside in the hair follicles, in the *stratum basale* of the epidermis, and in the sebaceous glands and sweat glands (Fuchs, 2007; Rittié et al., 2013).

A.



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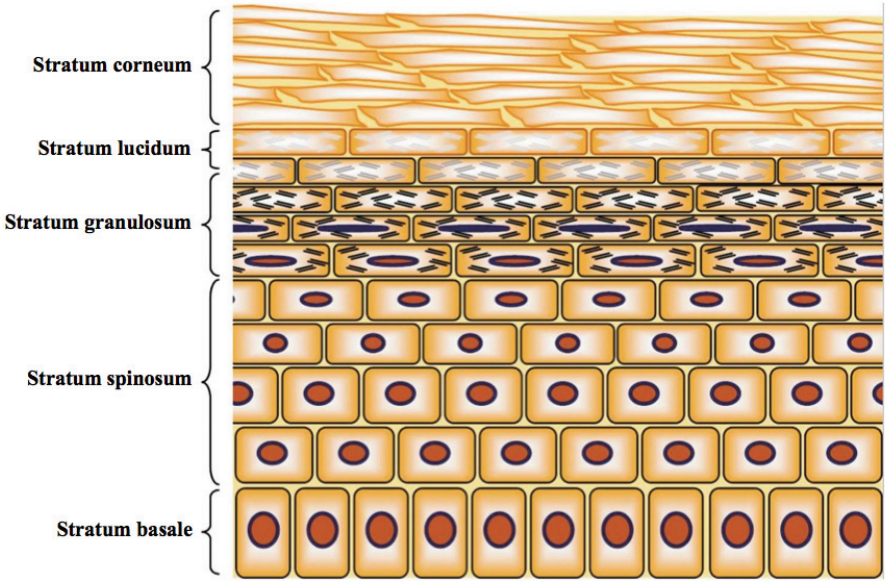

Figure 1. Structure of skin. A. Different layers of the skin and its appendages (Adapted from Böttcher-Haberzeth et al., 2010). B. Structure of the epidermis (Adapted from Polat et al., 2010).

2.1.1 Epidermis

The epidermis is the outermost layer of the skin (Figure 1). The majority of its cells, about 90%, are keratinocytes, but it also contains melanocytes, Langerhans cells, Merkel cells and some inflammatory cells. It is avascular and thus dependent on the blood vessels of the underlying dermis to secure its metabolic demands. The thickness of the epidermis varies from 0.5 mm in the eyelids to 4 mm in the palms of the hands or even thicker in the soles of the feet. Counting outward from the dermis, the five histologically distinguishable layers of the epidermis are: *stratum basale*, *stratum spinosum*, *stratum granulosum*, *stratum lucidum*, and *stratum corneum* (SC) (Figure 1B) (Calonje et al., 2011). The epidermis renews itself through cell division in *stratum basale* from where cells start their journey towards the surface of the skin. Each layer represents a different stage in the life of an epidermal cell (Blanpain & Fuchs, 2006).

To form a barrierlike structure, the epidermal cells inter connect by different types of intercellular junctions. In the living layers of the epidermis (from the *stratum basale* to the *stratum granulosum*), the cells link with each other via desmosomes, adherens junctions and tight junctions (Morita et al., 2011). In the SC, cell-cell connections are formed by corneodesmosomes. Adherens junctions are intercellular protein structures that are located at cell-cell junctions in all layers of the epidermis, except the SC. In addition to keeping epithelial cells together, they regulate many cellular processes, such as cell shape, cell division, growth, apoptosis and barrier function. They are composed of cadherin and catenin components. The transmembrane part of the junction consists of Ca^{2+}-dependent E- and P-cadherins, while the plaque is made of plakoglobin (δ-catenin), p120 (γ-catenin), α-catenin and β-catenin (Brandner et al., 2010). Desmosomes constitute the major intercellular adhesion complex in the epidermis, since they anchor KRT intermediate filaments to the cell membrane and link adjacent keratinocytes. There are three main desmosomal protein components: the desmosomal cadherins, the armadillo family of nuclear and junctional proteins, and the plakins. The desmosomal plaque proteins (desmoplakin, plakoglobin, desmoglein, desmocollin, plakophillin, KRT) and transmembranous molecules create a structural and signalling bridge between the cells (Green & Simpson, 2007). Desmosomes connect the cells in all layers of the epidermis, except in the SC, where the morphology and layered structure of the desmosomes become altered, and they

are transformed into corneodesmosomes. They also lose the intercellular part of the junction, and the intracellular plaque embeds within the cross-linked cornified envelope (CE). Corneodesmosomes remain functional and eventually are the main structure that must be broken before the differentiated cells can slough off in desquamation (Jonca et al., 2011). Tight junctions are cell-cell junctions that in the skin are mainly located in the *stratum granulosum* layer of the epidermis. They are composed of transmembrane proteins (e.g. claudin 1 - 24, occludin, tricellulin), junction adhesion molecules, and intracellular scaffold proteins (e.g. zona occludens proteins 1 - 3 (ZO-1-3) and cingulin). Tight junctions form the main barrier against solute permeation in the human epidermis (Kirschner et al., 2010).

The *stratum basale* is the innermost layer of the epidermis, and normally comprises a single row of columnar keratinocytes that divide through mitosis (Figures 1B and 2). It is the only layer in which the epidermal cells can divide. The proliferation of basal cells is dependent on the underlying basement membrane (BM), which is rich in proteins and GFs (Fuchs, 2009). New epidermis is constantly formed by epidermal stem cells of this layer; the cells divide, commit to differentiation and migrate towards the upper layers. However, for the skin to function normally, the stem cells must accomplish a dual task of self-renewal and generation-differentiated cells. It is believed that there are two distinct strategies of cell division to maintain the balance between stem cells and their differentiated progeny: asymmetric and symmetric cell division. In asymmetric cell division, the stem cells divide in the basal layer and form two identical daughter cells, one of which remains as a stem cell while the other commits to a terminal differentiation programme. As for symmetric cell division, each stem cell divides symmetrically to form either two daughter stem cells or two differentiating cells. An evolved third strategy could be the sequential combination of these two: cells could divide either asymmetrically or symmetrically (Blanpain & Fuchs, 2009; Morrison & Kimble, 2006). Thus, in addition to stem cells the basal layer contains committed progenitor cells, also known as transit-amplifying cells. They divide frequently, but for a limited number of cycles, before they undergo terminal differentiation.

Many pathways and genes regulate skin homeostasis. Research so far has identified two of the most important pathways involved: Wnt and Notch. The Wnt pathway favours the maintenance of stem-cell characteristics and thus inhibits the switch to

transit-amplifying cells, whereas Notch signalling is believed to have a contrasting effect (Honeycutt & Roop, 2004; Okuyama et al., 2008). The proliferative potential of basal cells correlates with the expression of beta 1 integrin (ITGB1). Hence, stem cells express more ITGB1 than transit cells. c-Myc, transforming growth factors beta (TGF-β) and alpha (TGF-α), and p63 also regulate the proliferation of epidermal stem and transit cells. Cells in the basal layer typically express KRTs 5 and 14. These structural proteins together with tubulin and actin form the cytoskeleton of the epithelial cells (Blanpain & Fuchs, 2009; Watt, 1998). In addition, there are also melanocytes and Merkel cells in the basal layer. Melanocytes produce melanin, a pigment material that defines the colour of our skin. They comprise about 8% of the cells in the epidermis. The skin protects us from ultraviolet (UV) radiation by accelerating the function of melanocytes. In melanocytes, melanin is produced and stored in specialized cell organelles called melanosomes (Marks & Seabra, 2001). After the melanosomes are packed with melanin, the melanocytes release them to large numbers of nearby keratinocytes through a network of cytoplasmic extensions, or dendrites that open up throughout the intercellular spaces of the lower epidermis. When the skin is irradiated with ultraviolet B (UVB) radiation, more melanosomes form and their transport to the keratinocytes becomes more efficient (Hume & Seabra, 2011). As for the Merkel cells, they are oval receptor cells responsible for sensation of touch (Calonje et al., 2011).

From the basal layer, cells committed to terminal differentiation migrate first to the *stratum spinosum* where they change their shape to more round and spinous and also lose their ability to divide (Figures 1B and 2) (Fuchs, 2007). The *stratum spinosum* is 5 - 12 cells thick and the cells are attached to each other through adherens junctions, desmosomes and tight junctions. As the cells move through this layer, the junctions break and reform continuously (Kirschner & Brandner, 2012). Here, the cells switch off genes encoding KRTs 5 and 14 and begin expressing KRTs 1 and 10 to form even stronger intermediate filaments that link together with desmosomes. In the upper layers, KRT 2 expression is also switched on (Kurokawa et al., 2011). In addition, Langerhans cells lodge in this layer. These cells are formed of specialized dendritic cells of the immune system in the red bone marrow, from where they migrate to the *stratum spinosum*, and act as an important player in the immunologic defence against microbes. Moreover, vitamin D is produced in the two innermost layers of the

epidermis, in the *stratum basale* and *stratum spinosum*. UVB radiation transforms the skin's dehydrocholesterol into previtamin cholicalciferol, which develops further into vitamin D in the liver and kidneys (Bikle, 2011).

The next layer is the 3 - 5-cell-thick *stratum granulosum* (Figures 1B and 2). When the cells reach this layer, they have become longer and flattened and are known as granular cells. Here, the cells undergo apoptosis and lose their metabolic activity. They also lose their nucleus and other cell organelles and become comprised of fibrous KRT and keratohyalin. Despite this, the cells retain their transcriptional activity. The granular cells secrete proteins containing lysine and glutamate, which bond together in a process catalysed by transglutaminases (TMGs) 1, 2 and 5 and form the CE. The CE is a protein-rich structure surrounded by a lipid envelope that replaces the plasma membrane in the differentiated keratinocytes (Figure 2). It provides a vital mechanical and permeability barrier to the skin. There are several proteins present in the CE, including loricrin (LOR), involucrin (IVL) and the family of small proline-rich (SPRR) proteins, as well as the S100 proteins. Other CE proteins include filaggrin (FLG), late-cornified envelope (LCE) proteins, elafin (peptidase inhibitor 3 (PI3)) and various KRTs (such as KRT2, 9 and 10) (Candi et al., 2005). Very interestingly, many genes regulating important functions of late epidermal differentiation are located in the same cluster of genes in human chromosome 1q21, known as the epidermal differentiation complex (EDC) (Marenholz et al., 2001).

Genes in the EDC belong to three families encoding 1) precursor proteins of the CE, 2) calcium-binding proteins of the S100A family and 3) intermediate filament-associated proteins. IVL, LOR, SPRR proteins, and LCE proteins are the main precursors of the CE. The second group, the S100A family, consists of 17 genes and 6 pseudogenes, all of which contain EF-hand domains. Generally, the S100 proteins regulate different steps of the calcium signal transduction pathway and affect cell shape, cell cycle and differentiation. Genes in the third family are fusion genes evolved from the other two EDC gene families (Mischke et al., 1996). Members of the fused gene family include FLG, repetin (RPTN), hornerin (HRNR) and cornulin (CRNN), which are large cytoplasmic matrix proteins linked with intermediate filament proteins and smaller components of the CE. Importantly, many skin diseases, such as atopic dermatitis, psoriasis, ichthyosis vulgaris, Vohwinkel's syndrome and

progressive symmetric erythrokeratoderma, have been associated with mutations in the EDC genes for inherited predisposition (Kypriotou et al., 2012).

The next layer, the transparent *stratum lucidum* (Figures 1B and 2), exists only in areas where the epidermis is thickest, as in the palms of the hands or in the soles of the feet. It comprises 3 - 5 layers of flat and clearly dead keratinocytes that are made up mostly of KRT. This layer provides some degree of waterproofing to the skin (Calonje et al., 2011).

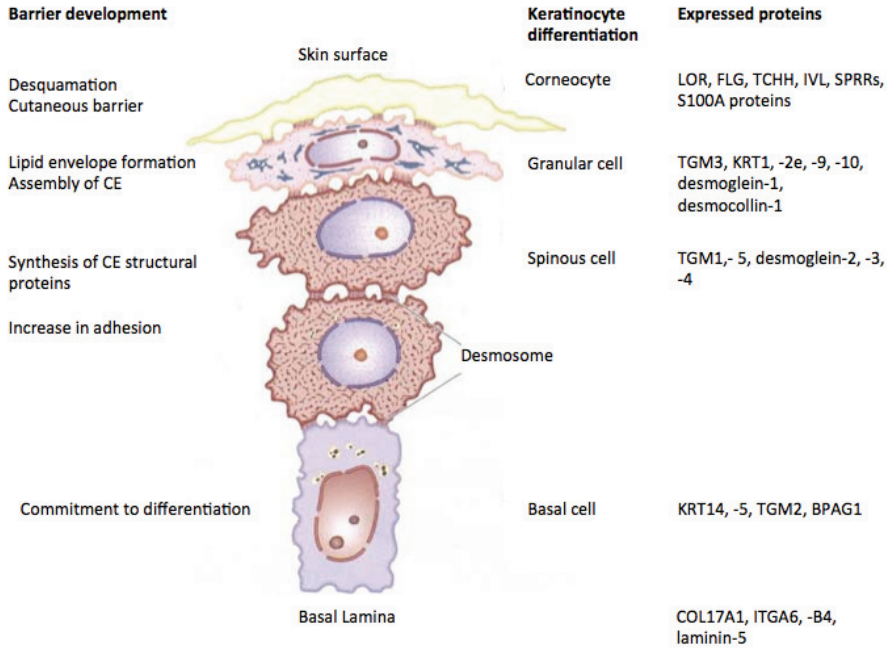

Figure 2. Skin homeostasis and barrier development (Adapted from http://www.pgbeautygroomingscience.com).

The SC is the endpoint of terminal differentiation (Figures 1B and 2). It is composed of 25 - 30 layers of flattened dead-cell remnants, called corneocytes. They are inter connected by corneodesmosomes. The cells contain KRT, which protects skin and the tissues below from heat and chemicals. They also lose the intercellular part of the junction, and the intracellular plaque embeds within the cross-linked CE. The corneodesmosomes remain functional and eventually are the last structures to be broken before the differentiated cells are sloughed off by desquamation (Menon et al., 2012).

As stated above, one of the main responsibilities for the skin is to offer mechanical strength and form an epidermal barrier. Terminal differentiation of the epidermal cells and most importantly the proper development and maintenance of the SC are the demands for barrier formation. An intact and healthy corneal layer efficiently blocks the entry of various microorganisms deeper into the body and reduces the absorption of chemicals from the environment (Madison, 2003). The SC almost completely prevents the evaporation of water through the skin. The corneocytes secrete their intracellular lipid, which attaches cells together and prevents them from drying. The lipid composition of the SC is an equimolar mixture of ceramides, cholesterol and free fatty acids (Yardley & Summerly, 1981). When cells push through the *SC* they lose their stickiness and slough off, either singly or in clumps (Wickett & Visscher, 2006).

2.1.2 Basement membrane

Cells of the basal layer reside on a BM, which is the border between the epidermis and dermis. The BM is a ubiquitous sheetlike deposition of the extracellular matrix (ECM). The major components of the BM are type IV collagen, laminin, heparin sulphate proteoglycan and entactin. Mesenchymal and epithelial cells secrete the components of the BM and are thus responsible for its synthesis and development (Ko & Marinkovich, 2010). Although its main function is to anchor the cells of the epidermis to the underlying dermis, interactions between the BM components and epithelial cells have an important impact on cell function, including proliferation, migration, and differentiation. The BM is also a significant factor in angiogenesis and, thus, its proteins accelerate the differentiation of endothelial cells. Structurally, it is divided into an upper *basal lamina* and a lower *reticular lamina*. The *basal lamina* itself consists of a complex network of interacting macromolecules and has two

layers: the lower *lamina densa* and upper *lamina lucida*. Many of these components are glycoproteins, and various collagens, laminins and integrins. The main adhesion unit at the dermal-epidermal junction is a hemidesmosome-anchoring, filament-anchoring fibril complex that forms a continuous structural link between the basal keratinocytes and subjacent dermal components (Breitkreutz et al., 2009). In addition, there are protein-protein interactions between these molecules to secure adhesion between the epidermis and subjacent dermis. The main component of the anchoring fibrils is type VII collagen and that of the *lamina densa* is type IV collagen. Lower reticular lamina is a thin layer of the ECM that sometimes lies below the basal lamina. It is mainly composed of collagenous fibres, and connects the basal lamina to the dermis. In addition, at the bottom of the *stratum basale* epidermal thickenings, undulating rete ridges anchor the epidermis to the papillary region of the dermis. These ridges also develop fingerprint patterns on the fingertips (Furuyama & Mochitate, 2000).

2.1.3 Dermis

The dermis is the zone between the hypodermis and epidermis. The major cell types of the dermis are fibroblasts, macrophages and adipocytes. Its main functions are to supply nutrients to the epidermis, regulate temperature and provide a mechanically tough elastic supporting layer. The dermis is also responsible for the skin's tensile strength. Structurally, it can be divided into two layers: an upper papillary layer and a lower reticular layer. Nerve endings and capillaries that nourish the epidermis are located in the superficial papillary layer. Beneath that is the reticular layer, which, like the dermis is made of strong connective, whose main structural elements are collagen and, elastic fibres. The most abundant component of the ECM in the dermis is collagen, especially collagen types I (80%) and III (10%). Although some mature collagens are rather stable and may remain unaltered in tissues for lengthy periods, a continuous process of loss and replacement of collagens is orchestrated by the matrix metalloproteinase (MMP) enzyme family. In the skin, MMPs are secreted by fibroblasts and keratinocytes and are divided into collagenases (e.g. MMP1), gelatinases (e.g. MMP2), matrilysins (e.g. MMP7), stromelysins (e.g. MMP13), and membrane-type MMPs (e.g. MMP14). They play an important role in tissue remodelling and therefore their expression is increased, e.g. during wound healing.

Matrix components, collagen and elastin provide strength and elasticity to the skin and are mainly secreted by fibroblasts (Calonje et al., 2011; Hannuksela et al., 2003).

The dermis is rich in blood vessels and is responsible for nourishing the acellular epidermis. Several arterioles extend from the subcutaneous fat to the dermis and form a network of blood vessels called the deep vascular plexus. These vessels deliver blood to the various parts of the dermis and subcutaneous tissue. In addition, small branches from this plexus nourish the hair follicles and sebaceous glands. Small arterioles (and their corresponding venules) connect the deep vascular plexus with another network of cutaneous blood vessels called the superficial vascular plexus, which provides blood to the epidermis. Apart from nourishment, cutaneous blood flow is also crucial to thermoregulation (McLafferty et al., 2012).

Skin appendages, various glands and hair follicles are also important components of the dermis. There are two kinds of glands in the dermis: sweat glands and sebaceous glands. Sebaceous glands are especially abundant on the face, neck and scalp and are often connected to the hair follicles. They secrete a greasy solution called sebum that covers the hair and skin and protects it from drying. It also controls epidermal water loss, and is believed to have some antimicrobial properties (Kanitakis, 2002). Sweat glands are divided into eccrine glands and apocrine glands. There are many more eccrine glands and they are found everywhere on the skin, but are particularly abundant on the face area, palms, and soles. Both physical and mental stress accelerates these glands' functions. Eccrine glands are believed to play an important role in epidermal repair by generating keratinocyte outgrowths that ultimately form new epidermis (Rittié et al., 2013). Apocrine glands are mostly found in the anogenital and axillary regions. Their function is not understood. Hair is produced in hair follicles that reside either in the dermis or in the subcutaneous fat layer, and are present on almost all surfaces of the human body (Calonje et al., 2011).

In addition to in the *stratum basale*, skin stem cells are also located in these appendages. The hair follicle stem cells are located in the bulging regions of the follicle and are responsible for the uninterrupted renewal of hair follicles. First, they differentiate into highly proliferative matrix cells that further differentiate either into hair shaft cells or inner root sheath cells. Hair stem cells are also capable of forming new epidermal cells, but this only occurs when the skin is wounded. In addition to

hair follicle stem cells, there are also melanocyte stem cells located in the hair follicles. These cells are involved in hair pigmentation. Stem cells in sebaceous glands differentiate into sebocytes that form new sebaceous glands and secrete sebum (Boehnke et al., 2012).

2.1.4 Hypodermis

Underneath the dermis is the hypodermis, also known as the *subcutis*, the deepest layer of the skin. It is fat tissue that is divided into small lobes developed by fibrous walls or septae. The cells found in this layer include adipocytes, fibroblasts and macrophages. The function of the hypodermis is to act as a ligament between the skin and other tissues, such as muscles. It protects the body from impacts, is also involved in thermoregulation and serves as a nutritional storage.

There are about 1 million sensory afferent nerve fibres in the skin. They are unevenly distributed in the body, most of them conveying tactile sensations from the face and hands/feet, with relatively few supplying the back. The main nerve trunks are in the hypodermis, where they divide into smaller branches that enter the dermis and in some cases even the basal layer of the epidermis (Reinisch & Tschachler, 2005). Neurons in the skin are divided according to their modality. There are touch-, pressure-, and vibration-sensing mechanoreceptors, cold- and warm-sensing thermoreceptors, as well as pain-sensing nocireceptors in the skin. Mechanoreceptor axons are all myelinated and they include the Pacinin corpuscle, Meissner corpuscle, Raffini corpuscle, Merkel disk and hair follicle receptor. Cold receptors have a small myelinated axon that reaches out to the epidermis; while warm receptors have a shorter unmyelinated axon. They both activate when the skin's temperature diverts from its normal levels. In addition, there are rapid nocireceptors, whose myelinated axons stretch to the epidermis side, and slow nocireceptors with shorter and unmyelinated axons (Boulais & Misery, 2008).

2.2 Wounds and healing

Chronic wounds and compromised wound healing are major concerns for the public health sector. Complex and lengthy treatments cause an increasing burden on healthcare expenses. Deep burns, chronic and other difficult-to-treat wounds require surgery and extended hospitalization periods. In the United States every year 6.5

million patients need treatment for chronic wounds and an estimated US $25 billion is spent annually. More worryingly, the burden is growing yearly mainly due to the increasing incidence of obesity and diabetes (Sen et al., 2009).

2.2.1 Acute wounds

Wounds are usually divided into acute and chronic wounds. Acute wounds are traumatic or surgical wounds and usually heal within a foreseeable period of time according to normal wound-healing processes. Acute wounds vary from superficial scratches to deep wounds that damage blood vessels, nerves, muscles or other tissues or internal organs (Percival, 2002).

Acute wound healing is a complex physiological process that is regulated by many different cell types, several GFs, cytokines and chemokines. Most of all, it is the body's inherent way of responding to an injury. During the healing process, cells such as many inflammatory cells, platelets, endothelial cells, fibroblasts and keratinocytes undergo distinct preprogrammed changes in their gene expression and phenotype. In consequence, the cells begin to migrate, proliferate and differentiate, which leads to inflammation response, new-tissue formation and finally tissue remodelling; in other words to the classical phases of wound healing (Figure 3) (Gurtner et al., 2008).

The wound-healing programme is an intricate interplay between several cell types involving various forms of intercellular signalling. The most important cells and GFs involved in wound healing are shown in Table 1. Most wounds heal rather quickly and efficiently within weeks, according to the phases of normal wound healing (Martin, 1997).

The immediate response to injury is *haemostasis*, which is achieved by formation of a fibrin mesh or clot in a process that is regulated by the coagulation cascade and constriction of vessel walls. Fibrin provides a temporary cover and acts as a matrix that aids cell migration during healing. In addition, it binds and stores GFs and cytokines (Gurtner et al., 2008). More importantly, platelets trapped inside the clot secrete several GFs, such as platelet-derived growth factors (PDGFs), epidermal growth factors (EGFs), insulinlike growth factors (IGFs) and TGF-β. The release of these signalling proteins accelerates the wound healing process by activating fibroblasts, endothelial cells and inflammatory cells (Enoch & Leaper, 2008).

Inflammation is the first actual phase of wound healing. It occurs immediately after cell damage and lasts for about 2 - 3 days (Figure 3). The immune defence is activated: the complement system activates, neutrophils migrate to the wound, platelets degranulate and bacteria degrade. The presence of various chemoattractants, such as complement components, TGF-β, ECM protein fragments and products from bacteria attracts neutrophils to the wound site within the first 24 - 48 hours after wounding. Infiltrating neutrophils clear the wound site of pathogens and are then cleared either passively in an eschar or through active phagocytosis by macrophages. Later on, about 2 days after the injury in the late phase of inflammation, monocytes emerge in the wound, also attracted by numerous specific chemoattractants. Monocytes infiltrate the wound area and differentiate into macrophages that initiate the formation of granulation tissue. Thus, being an important part of the inflammatory process macrophages also act as key regulatory cells for repair. The GFs and cytokines (e.g. TGF-α, TGF-β, PDGF, IGF, IL-1) that they produce are responsible for proliferation and production of ECM, fibroblasts, smooth-muscle cells and endothelial cells. (Singer & Clark, 1999)

New-tissue formation occurs 2 - 10 days after injury (Figure 3). An eschar is formed and epithelial cells migrate underneath. Keratinocytes migrate over the damaged dermis and new blood vessels form in angiogenesis, which is induced by several factors such as vascular endothelial growth factor A (VEGF-A), fibroblast growth factor 2 (FGF-2), PDGF, and TGF-β. Angiogenesis is necessary for formation of new granulation tissue. A legion of new capillaries provides nutrients and oxygen to the growing tissue and takes care of waste disposal. Equally important factors are macrophages that supply GFs for angiogenesis and fibroplasia and new ECM-producing fibroblasts. In 3 - 5 five days after injury, new granulation tissue is well established and replaces the fibrin clot (Gurtner et al., 2008). About 2 - 4 days after injury, fibroblasts appear in the wound. They migrate either from bone marrow or from the wound edges and several GFs, including PDGF and TGF-β, attract their movement. In the wound, fibroblasts form the new ECM by producing various ECM proteins (e.g. fibronectin, hyaluronan) and later on collagen and proteoglycans. Subsequently, macrophages stimulate fibroblasts in the wound and some differentiate into myofibroblasts. These cells contract and eventually close the wound together from the edges (Barrientos et al., 2008; Gurtner et al., 2008).

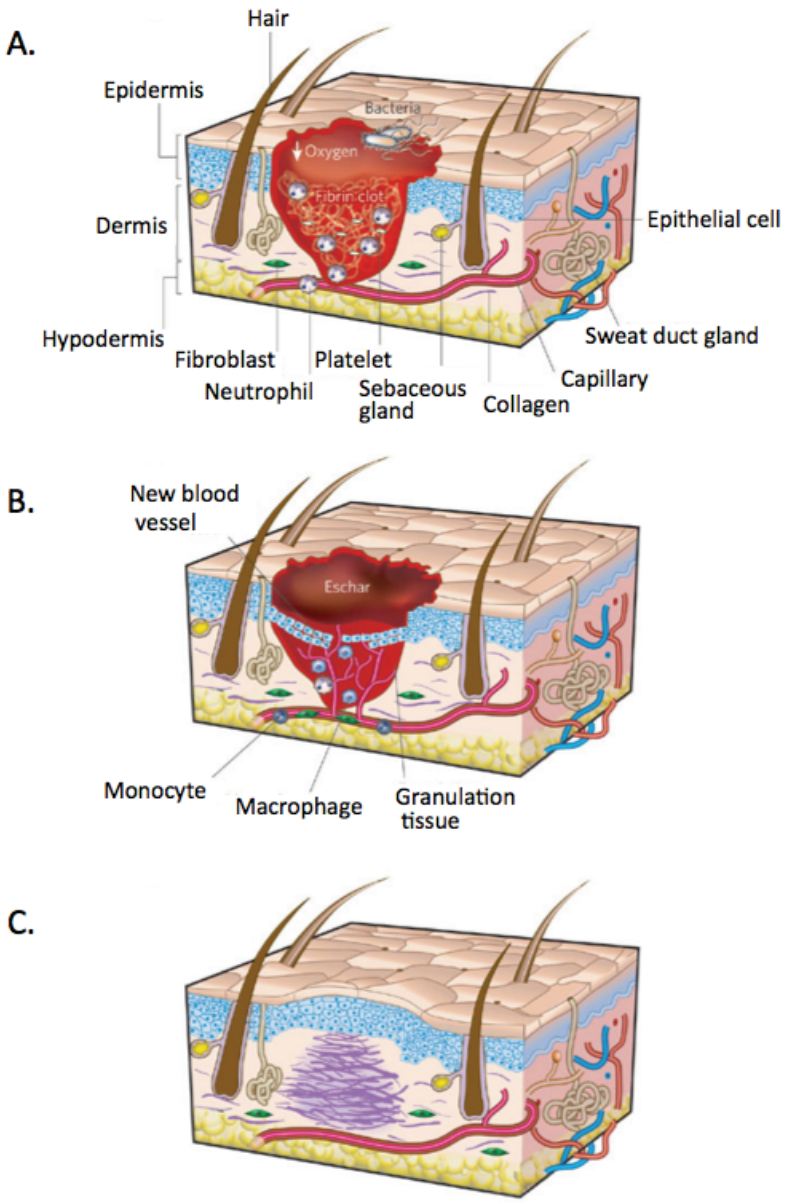

Figure 3. Classical steps in wound healing (Adapted from Gurtner, 2008): A. Inflammation, B. Tissue remodelling and C. New-tissue formation.

Table 1. Main wound-healing-related growth factors (GFs) and cytokines, their function, secreting cells, and their expression in both acute and chronic wounds (+, increased; -, decreased).
Abbreviations: EGF, epidermal GF; FGF, fibroblast GF; HGF, hepatocyte GF; KGF, keratinocyte GF; PDGF, platelet-derived GF; TGF, transforming GF; VEGF, vascular endothelial GF; IL, interleukin; TNF, tumour necrosis factor.

GF	Function	Cells	Acute	Chronic
EGF	Re-epithelialization	Platelets, Macrophages, Fibroblasts	+	-
FGF-1, 2	Granulation tissue formation, Re-epithelialization, Matrix formation, Remodelling	Keratinocytes, Mast cells, Fibroblasts, Endothelial cells, Smooth-muscle cells, Chondrocytes	+	-
HGF	Epithelial and endothelial cell proliferation, Hepatocyte motility	Mesechymal cells	+	-
KGF	Keratinocyte proliferation, differentiation, migration	Keratinocytes	+	-
PDGF	Inflammation, Granulation tissue formation, Re-epithelialization, Matrix formation, Remodelling	Platelets, Keratinocytes, Macrophages, Endothelial cells, Fibroblasts	+	-
TGF-β	Inflammation, Granulation tissue formation, Re-epithelialization, Matrix formation, Remodelling	Platelets, Keratinocytes, Macrophages, Lymphocytes, Fibroblasts	+	-
VEGF	Granulation tissue formation	Platelets, Keratinocytes, Macrophages, Endothelial cells, Smooth-muscle cells, Fibroblasts	+	-
Cytokines				
IL-1	Inflammation, Re-epithelialization	Neutrophils, Macrophages, Monocytes, Keratinocytes	+	+
IL-6	Inflammation, Re-epithelialization	Neutrophils, Macrophages	+	+
TNF-α	Inflammation, Re-epithelialization	Neutrophils, Macrophages	+	+

A major event in the *new-tissue formation* phase is *re-epithelialization*, which is a combination of keratinocytes migrating and proliferating in the wound. Basal keratinocytes migrate from the wound edges as well as from the skin appendages to the injured area to form a fragile cover over the wound, in a process called *epiboly*. Cells undergo phenotypic alterations to ease the migration towards the provisional wound matrix. Firstly, they must disassemble most of the intercellular desmosomes and hemidesmosomes, which involves the rearrangement of integrin receptors. They also retract their intracellular tonofilaments and KRT filaments and form focal contacts, as well as cytoplasmic actin filaments that allow cell movement. In addition, the ECM must be broken down to allow cell migration between the collagenous dermis and fibrinous eschar. To degrade the ECM, the epidermal cells produce MMPs (collagenases) and plasminogen activators. The normally firm attachment to the underlying dermis is now loosened and keratinocytes migrate in a *leapfrog* fashion across the provisional matrix. Some hours after the onset of migration, the cells begin to proliferate strongly to replace the cells that were lost in the injury. Finally, migration is halted by contact inhibition when the advancing keratinocytes from both edges of the wound meet. Further growth and differentiation of the cells re-establishes the stratified epidermis with the underlying basal lamina. Re-epithelialization begins within hours after injury and is regulated by several GFs.

One of the most important molecules associated with re-epithelialization is HGF (Table 1). It promotes both keratinocyte migration and proliferation. HGF mediates its functions by binding to a membrane receptor tyrosine kinase, c-Met, that is essential for wound repair. When activated by HGF, it induces a process called *invasive growth* that in wound healing regulates inflammation and tissue remodelling (Gentile & Comoglio, 2004). HGF's important role in re-epithelialization supports results with HGF knockout mice, which have showed a robust consistent delay in re-epithelialization of skin wounds when HGF is blocked. Other important re-epithelialization-related GFs include members of the FGF and EGF families (Chmielowiec et al., 2007). In addition to keratinocytes, epidermal stem cells from skin appendages participate in the repair process. In response to wounding, they commit to terminal differentiation and migrate into the wound area (Shaw & Martin, 2009).

The last stage—*tissue remodelling*—begins a couple of weeks after wounding and can last over 1 year (Figure 3). At this stage, most of the endothelial cells, macrophages and myofibroblasts go through apoptosis or leave the wound. The ECM of the damaged area consists mainly of collagen and other ECM proteins. During the first 6 - 12 months after the injury, the ECM regenerates constantly, which strengthens the tissue. This process is orchestrated by various MMPs, which fibroblasts and other cells secrete. Although the wound heals, it will never again meet the qualities of an unharmed skin (Gurtner et al., 2008; Singer & Clark, 1999).

2.2.2 Burns

Burn wounds are acute wounds that are caused by heat, chemicals, electricity, sunlight, radiation or friction. Burns can be categorized into superficial (I°), partial-thickness (II°) and full-thickness wounds (III°). The majority of burn wounds are superficial and involve only the epidermis. They are usually caused by UV radiation from the sun and heal well by themselves, without any sign of scarring (Johnsson & Richard, 2003).

Partial-thickness burns are further divided into superficial and deep. Superficial partial-thickness burns extend through the epidermis and reach the upper papillary layer of the dermis. These wounds become erythematous, and thin fluid-filled blisters develop within minutes of the injury. Body fluid leaks on to the wound surface, due to the loss of the epidermal barrier, making the wounds moist. Deep partial-thickness burns, on the other hand, reach as far as the lower reticular layer of the dermis. The wound area is moist and may form blisters, similarly to superficial partial-thickness burns. (Johnsson & Richard, 2003)

I° burns heal in 1 week without any additional procedures. Superficial II° burns undergo epithelialization similar to split-thickness donor sites from dermal appendages in 2 - 3 weeks with good functional and cosmetic results. Deep II° burns require weeks, even months, to re-epithelialize and result with prolonged pain and severe scarring. Skin grafting is needed to accelerate the wound cover. III° burns can heal only to some extent by epithelialization from the wound edges and by contraction, although they almost always need skin grafting (Herndon, 2012).

A full-thickness burn extends throughout the depth of the skin into the hypodermis (subcutaneous tissue). It may also damage muscle, bone or interstitial tissue. Soon

after the injury, fluids and proteins, leaking from capillaries into the interstitial space, cause oedema. An immediate immunologic response to injury in large burns causes the systemic inflammatory response syndrome (SIRS) and accelerated metabolic demands, needing aggressive nutritional support (Johnsson & Richards, 2003).

Deep and large burns require hospitalization and, without exception, surgical treatment. The gold standard is to excise the dead burnt tissue and replace it with split-thickness skin grafts (STSGs). In this process, the patient's own healthy skin is harvested and subsequently transferred to the burnt site after resection of the dead tissue. Although efficient, it is associated with substantial problems, such as poor-quality skin, infections, and scarring and lack of donor sites in large burns (Archer et al., 1998).

In addition, the donor site skin should be appropriate for the recipient site. If the graft is harvested from a different part of the body, there is risk of colour mismatch. Moreover, in the case of a very large burn, the areas available for harvesting are very limited. In such cases, the available graft is usually extended by meshing. Meshing improves wound drainage and thus draft-take. However it compromises both the aesthetic and functional outcomes. As an adjunct treatment, cadaver skin, xenografts, various biomaterials or special dressings are commonly used to treat burns (Lindford et al., 2010).

2.2.3 Chronic wounds

Sometimes wounds do not heal according to the normal healing process or the healing is remarkably delayed. Wounds are defined as chronic when they have failed to heal in 3 months. They are not capable of closure or of regaining the anatomy and function of healthy skin. Conventionally, chronic wounds do not occur in healthy people. They are very often related to conditions such as obesity, diabetes, and old age (Harding et al., 2002).

The biology of chronic wounds is still poorly understood. Chronic wounds do not follow the steps of the normal wound-healing process and remain in the inflammation phase. It is suggested that lack of GFs, imbalance between proteinases and their inhibitors or presence of too many senescent cells in the injured area may result in the inability of these wounds to heal. In addition, adequate angiogenesis is crucial for wound closure. This in turn regulates fibroblast ability to form granulation tissue, and

thus inadequate microvasculature can lead to chronic nonhealing wounds. This is especially common in diabetic patients (Mustoe et al., 2006).

The majority of chronic wounds fall into three categories: pressure ulcers, venous ulcers, and diabetic ulcers. It has been proposed that the pathogenesis of impaired wound healing is based on four factors: bacterial colonization of the wound, ischaemia, aging and reperfusion injury. Bacteria are substantially involved in the pathogenesis of chronic wounds. An open wound becomes contaminated within the first few days from the surrounding skin. By comparing the wound exudate of chronic wounds with that of clean surgical wounds, it has been indicated that chronic wound exudate contains greater amounts of proinflammatory cytokines and proteases (Peirce et al., 2000). In addition, GF levels are lower in chronic wound exudate. The second factor, ischaemia, also impairs wound healing. It is known that wounds in the lower extremities will not heal if the partial oxygen pressure is less than 30 mmHg. Partial oxygen pressure affects the local blood flow, which in turn affects the arterial flow, arteriovenous gradients, capillary density and local tissue consumption. Reperfusion injury has also been considered as one of the major factors leading to impaired healing. Tissue reperfusion injury occurs upon restoration of blood flow after an ischaemic period. The absence of oxygen and nutrients causes a reduction in tissue metabolism that results in inflammation and oxidative damage. Usually chronic wounds occur in older people. The average age of a chronic wound patient is over 60 years. Cells in chronic wounds are under constant and significant stress. The ability of aged patients to respond to altered cellular and systemic stress is weakened (Stojadinovic et al., 2005).

Treatment of chronic wounds has been very challenging due to the wounds' diverse aetiologies and pathological backgrounds. This has caused problems in identification of the specific molecular mechanisms behind impaired wound healing. Another challenging factor is that, chronic wounds do not exist in animals and thus knowledge has to come from the clinic. Chronic wounds are treated with different wound dressings that aim at promoting healing by making the wound environment more favourable (Lawall, 2012). Additionally, GFs can be administered topically to wounds to increase cell proliferation and angiogenesis. Autologous skin grafts and various biomaterials are commonly used, especially to treat venous ulcers (Peirce et al., 2000).

One of the treatment options for chronic wounds in the clinic is negative-pressure wound therapy (NPWT). It is commonly used to improve skin wound healing and to accelerate wound bed preparation, especially when dealing with chronic and other difficult-to-treat wounds (Orgill & Bayer, 2011). The effect of negative pressure on wound healing is partially mediated through modification of the wound environment into a more favourable state for healing by removing exudates and debris. In deep lesions, the negative pressure also aids wound closure. The advantages of NPWT over conventional dressings include decreased tissue oedema, stimulation and formation of new granulation tissue, increased local blood flow and decreased bacterial load (Schintler, 2012).

2.2.4 Fibrosis

Fibroblast proliferation and excessive growth of granulation tissue may cause scarring and wound contraction (Leask et al., 2004). Wounds, especially, those that develop keloids and hypertrophic scars, heal not through regeneration but through fibrosis. The pathogenesis of fibrosis is a combination of several factors. Individual age, genetic/racial background and hormonal imbalance greatly affect the prevalence of keloids and hypertrophic scars. These are manifested in the common notion that some people get scars easier than others. The location of the wound plays a role in scar formation, e.g. the shoulders and chest are the most common skin sites for keloid formation. In addition, the size of the wound or degree of a burn wound, inflammation, tension exerted in the wound area and, in case of iatrogenic wounds, the surgical technique used also impacts scar formation. These factors alone and together lead to abnormal cellular response; cells secrete an abnormal pattern of GFs and there are not enough molecules required for apoptosis or for remodelling of the ECM. The major GFs involved in tissue repair and fibrosis are TGF-β, PDGF and VEGF. They accelerate the deposition of the ECM by inducing the expression of ECM proteins, such as collagen and fibronectin. Although the production of the ECM is crucial to establishing and maintaining the structural and mechanical integrity of the skin, the excessive deposition may result in scar pathology. Additionally, GFs decrease the expression of MMPs that are involved in the breakdown of the ECM (Joo & Seomum, 2008). Normally, when tissue repair is completed, GF activity is turned off; however, in scar tissue it is overexpressed and poorly regulated (Ignotz & Massagué, 1986). Thus, increased GF production results in fibroblast proliferation,

neovascularization and increased collagen and fibronectin synthesis. Animal studies utilizing anti-TGF-β antibodies and TGF-β knockouts have resulted in less fibrosis and enhanced re-epithelialization. The overproduction of GFs and decreased MMP activity, in turn, lead to abnormal wound healing and eventually to the formation of a scar. In addition to cosmetic harm, a more serious problem with scar tissue is that it lacks the original functionality and elasticity of skin and causes contracture (Wolfram et al., 2009).

2.3 Skin tissue engineering

Tissue engineering (TE) is a field of science that combines engineering and medicine to find new ways to improve regeneration and regain organ or tissue function. In the concept of TE, cells, materials and biological factors are used separately or combined (Figure 4) (Berthiaume et al., 2011; Stoltz et al., 2012).

New tools are needed to lighten the massive and constantly increasing load of wounds and wound healing on both healthcare and the economy. In an ideal world, wounds would always heal rapidly and scar-free, but how could this goal be achieved? Skin tissue engineering (STE) explores the promising opportunities in this field, either alone or combined with traditional wound treatments. The main tasks of STE are to enhance the quality of healing and to reduce scarring (Wood, 2012). In addition, STE aims to introduce much needed help for the treatments of nonhealing wounds, as well as injuries such as large burns, when the possibilities for skin grafting are limited and substitutive material is needed to cover the damaged tissue (Mansbridge, 2008).

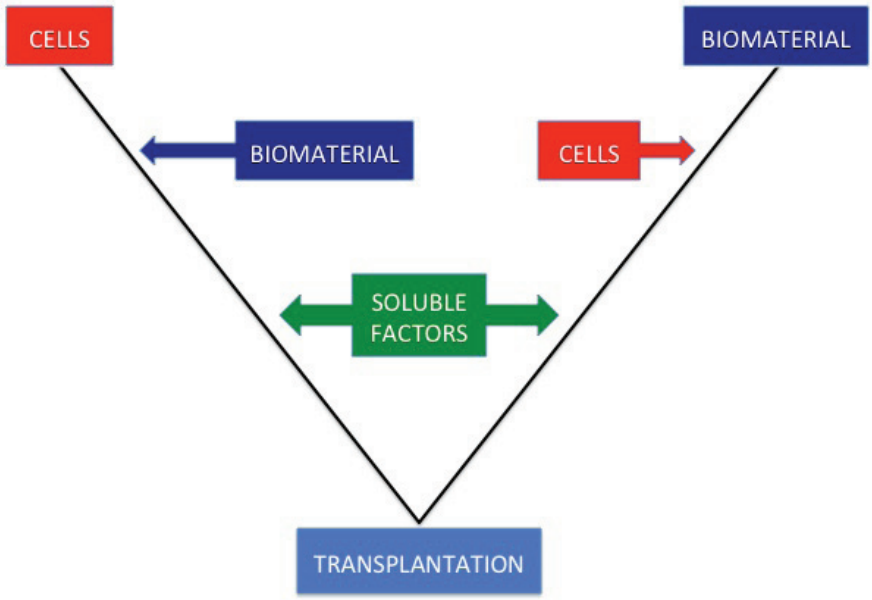

Figure 4. Concept of tissue engineering.

To be accepted by healthcare professionals, a good STE therapy must be practical as well as cost- and time-effective. When designing therapy, one should understand the aetiology and pathophysiology of the wound; in other words, which mechanisms to inhibit and which to induce, what structures should be mimicked and what replaced. Another crucial point to grasp is that there is no single solution that would optimally suit all defects and all causes.

In general, the cells that form the superficial epidermal protective layer are needed for a skin wound to heal. Equally important is the dermal component and the ECM, which can support epidermal cell proliferation, migration and differentiation (Böttcher-Haberzeth et al., 2010; Zhong et al., 2010).

2.3.1 Cell therapy

The first question with cell therapy is always whether to use autologous or allogeneic cells. Both have their pros and cons. Autologous cells offer a more permanent solution with fewer worries of any rejection or disease transfer. On the other hand their availability and preparation time often limit their use. Allogeneic cells would potentially be available when needed, but their benefits are mainly limited to factors secreted by the cells. Legislation concerning cell therapy is very strict and even stricter when dealing with allogeneic or cultured cells (European Medicine Agency, human cell-based medicinal products, CHMP/410869/06). The problem with cultured cells is that culturing may cause mutations and thus the altered cells could cause cancer when transplanted back to the patient. This is especially dangerous in the case of autologous cultures, because they are not recognized as foreign.

Keratinocyte transplantation is the best-known skin-related cell therapy. Since 1975, when Rheinwald and Green succeeded in cultivation and expansion of epidermal keratinocyte populations, cultured epithelial autografts (CEAs) have been used to treat burns throughout the world (Rheinwald & Green, 1975). Although a promising technique, it has struggled to gain acceptance and has never become a standard treatment. CEAs have many problems of which the time required for cell culturing is the most challenging (Carsin et al., 2000). It requires several weeks to expand the cells into a confluent sheet. It is also difficult to time the operations with the delivery of cultured cells (Wood et al., 2006). Other problems include poor adherence and high costs. In addition, CEAs are very fragile and difficult to handle upon transplantation

(Böttcher-Haberzeth et al., 2010; Chester et al., 2004).

The problems with CEAs ushered in development of several other techniques and solutions for keratinocyte transplantation. Cultures have been harvested as preconfluent, in order to reduce the time required for a confluent sheet to form (Johnen et al., 2008). In this approach, cells can be transplanted as a suspension or a spray on the damaged tissue. Commonly, preconfluent cultures are combined with scaffolds. The most straightforward way is to culture cells on the scaffold and transplant this engineered structure on to the wound. Biological polymers, such as collagen (Horch et al., 2000) or fibrin (Ronfard et al., 1991), have been used as delivery vehicles for keratinocytes. In these studies, isolated keratinocytes have been plated on or inside the material and cultured for several days before transplantation (Bannasch et al., 2000). The cell spray approach has also been used in several countries. The benefits of this technique include rapid application and feasibility of treating large areas without need of a scaffold. By spraying the cells it is possible to treat areas up to 800 times larger than the area harvested. Another benefit is the ability to perform the entire procedure bedside in a very short period of time. The drawbacks are poor attachment and loss of cells due to mechanical pressure while spraying. In addition, the amount of cells must be large enough to obtain any meaningful effect (De Angelis et al., 2013; Fredriksson et al., 2008; Gerlach et al., 2011; Hartmann et al., 2007; Kirsner et al., 2012). Currently, there is one commercial product, ReCell®, on the market that utilizes this technique. The ReCell® kit includes all the tools needed for isolating keratinocytes from the skin. Thus, the procedure can be performed on-site in approximately 1 hour (Wood et al., 2012).

In one study, Gravante et al. (2007) concluded that ReCell® is a feasible, simple and safe technique that gives results similar to those of STSG. However, it is best fitted for the treatment of smaller cosmetic skin conditions, such as vitiligo (Cervelli et al., 2009). To treat large areas, such as burns or chronic wounds, up-scaling is essential. In a recent multicentre, double-blind, randomized, placebo-controlled trial, Kirsner et al. (2012) treated chronic leg ulcers by spraying human allogeneic fibroblasts and keratinocytes. The study concluded that chronic wounds could be healed by spraying cells, even without any additional TE. The trial also indicated that an optimum dose would behalf a million cells every 14 days.

In addition to keratinocytes other cell types such as fibroblasts and mesenchymal stem

cells (MSCs) have been evaluated in the treatment of skin wounds (Boyce et al., 2002; Guenou et al., 2009; Ramanauskaitė et al., 2010). MSC therapy especially, has been considered as a potential form of skin therapy. The great benefit with stem-cell treatment is that autologous cells would be abundantly available from different sources such as from bone marrow or adipose tissue. Allogeneic MSC products, such as Stemedyne™-MSC and Revascor®, are already available commercially to treat cardiovascular diseases.

In addition to their capability of differentiation into skin cells, MSCs are believed to enhance dermal regeneration by several ways throughout the various stages of the wound-healing process (Salem & Thiemermann, 2010). In preclinical studies, MSC during the inflammation phase could modulate the immune response by inhibiting the recruitment, proliferation and biological activity of immune cells. In the wound environment, MSCs also secrete prostaglandins (PTGs), which favours regeneration (Jackson et al., 2012a). In the new-tissue formationphase, MSCs promote wound healing by secreting several GFs contributing to cell proliferation and angiogenesis. In addition, MSCs are believed to attenuate fibrosis (Jackson et al., 2012b). In the final stage of the healing process, MSCs can transdifferentiate into keratinocytes and fibroblasts and enhance re-epithelialization and production of new ECM. Of course, the big problem in dealing with stem cells is their uncontrolled differentiation and tumour risk (Semi et al., 2013).

2.3.2 Bioengineered skin

Engineering of a skin substitute has proven to be extremely challenging, due to the complex, multilayered, appendage-containing structure of skin. An ultimate goal of skingineering would be to succeed in building a dermoepidermal substitute that would vascularize rapidly, be biodegradable and support cell migration and proliferation. Equally as important, the matrix should be convenient to use as well as affordable (Pham et al., 2007; Wood, 2012). Materials such as collagen, fibrin and gelatin have been commonly used in skin bioengineering, both as dermal fillers as well as delivery vehicles for cells but do not fulfil all the abovementioned qualities (Bello et al., 2001; Metcalfe & Ferguson, 2007).

So far, Apligraf® is the only commercially available Food and Drug Admistration (FDA)-approved, bilayered dermoepidermal skin substitute containing living cells. It

has both dermal and epidermal layers and is histologically similar to normal human skin. Its dermal layer contains type I bovine collagen and allogeneic fibroblasts whereas the epidermal layer is made of allogeneic keratinocytes. Both cell types are obtained from neonatal foreskin. Apligraf® is currently used in the treatment of chronic wounds, and some promising results have been reported, especially in the treatment of venous ulcers (Barber et al., 2008; Edmonds et al., 2009; Fivenson et al., 2003).

Dermagraft® is another living cell-containing skin substitute. It is a meshed polyglactin scaffold seeded with allogeneic human fibroblasts. The fibroblasts proliferate in the scaffold and secrete dermal collagen, GFs, matrix proteins and cytokines. During the process, the scaffold material is gradually degraded. Dermagraft® is used as a temporary or permanent cover in the treatment of burns and chronic wounds. Clinical studies have shown that Dermagraft® is as good as an allograft in terms of wound infection, wound exudate, wound closure, wound-healing time and graft take (Edmonds et al., 1997; Hansbrough et al., 1997; Harding et al., 2013).

There are several acellular dermal substitutes on the market, which combined with skin grafts, have given promising results (Iorio et al., 2012). Integra® is among the most generally used acellular dermal scaffolds. It contains two layers; the bottom layer is made of bovine tendon collagen cross-linked with glycosaminoglycans. The structure supports capillary and cellular ingrowth (Pandya et al., 1998). The upper part is a semipermeable silicon layer that prevents water-vapour loss and adds strength to the scaffold. The idea of Integra® artificial skin is to act as an off-the-shelf dermis that can be combined with either laboratory-grown keratinocytes or STSG. However, there are some problems such as poor graft take on the top of Integra®, as well as the expensive price. An additional challenge is the two-step procedure, which means that STSG or cells cannot be placed on the top of the matrix before granulation tissue has grown through the matrix (Hansen et al., 2001; Iorio et al., 2012).

Matriderm® is another similar, cell-free dermal skin substitute. Its dermal component is composed of collagen and elastin, both of bovine origin. Matriderm® is also used as a two-step procedure together with STSG for the reconstruction of dermis in wound and burn surgery. Clinical trials have shown that in the treatment of burns,

Matriderm® promotes healing with satisfying aesthetic and functional results. Similar to the other materials, its indications are limited by its high costs (Böttcher-Haberzeth et al., 2010; Cervelli et al., 2011; Haslik et al., 2010; Keck et al., 2011).

Although many artificial skin products have been developed, the market still lacks a cost-effective, single-step, dermoepidermal skin substitute. Commercially available artificial skin products, their origins and indications are presented in Table 2.

2.3.3 Topical growth factor therapy

GFs play important roles in the process of normal wound healing (Werner & Grose, 2003). Thus, topical administration of GFs directly to the wound has been studied intensively as a potential regenerative treatment, especially for nonhealing wounds. Several GFs, EGF, PDGF, TGF-β, and FGF-7 (also called keratinocyte growth factor, KGF), have been considered as potential agents for therapy (Hardwicke et al., 2008; Mansbridge, 2008). However, there are only a few of FDA-approved products available for clinical use. Regranex gel ® contains human recombinant PDGFs and is produced, using recombinant deoxyribonucleic acid (DNA) technology (Wiemann et al., 1998). It promotes angiogenesis and accelerates wound healing in diabetic ulcers (Sibbald et al., 2003). Another product, called AutoloGel™, contains autologous PDGFs derived from the patient's own blood (Driver et al., 2006). In a randomized clinical trial, Carter et al. (2011) indicated that chronic wounds respond positively to AutoloGel™ treatment.

There are some limitations related to GF therapy. First of all, it is by no means certain whether the application of a single GF efficiently enhances the healing process. Might a cocktail of various GFs function more effectively? On the other hand, the production of recombinant GFs is very laborious and expensive. Another serious concern is that proteolytic enzymes in the wound exudate may lyse topically applied proteins. Thus, the amount of GFs administrated to the wound must be large to obtain any effect. For example, Regranex® treatment requires a dose of 100 $\mu g/cm^2$ every other day (Niezgoda et al., 2005).

2.3.4 Gene therapy

To address the problems of GF therapy, genetic approaches have been in focus. In gene therapy, foreign DNA is delivered into the host cells. As a wound-healing therapy the aim would be to stimulate and enhance the production of proteins, such as

GFs, secreted by the cells (Eming et al., 2007). There are two ways to deliver genes into target tissue, either *in vivo* or *ex vivo*. *Ex vivo* techniques are more expensive, laborious and time-consuming. First, the target cells are isolated from tissue and cultured in vitro; subsequently they are transfected and finally transplanted back into a recipient. *In vivo* techniques, in which genes are introduced directly to the target tissue, are more straightforward and more reasonable in price. In both approaches, transfection can be achieved using viral or nonviral genes. Viral gene transfer relies on the natural ability of viruses to penetrate into cells and express their genes in hosts. The most commonly used viruses for transfection are retro- and adenoviruses. Viral gene transfer in to aid cutaneous wound healing has been studied in several animal models with some promising results (Jeschke et al., 2005; Rosenthal et al., 1997; Vogt et al., 1994). Unfortunately, the biggest problem with this approach seems to be inflammation and infection risk caused by the virus, which can lead to impaired healing instead (Vogel, 2000). Nonviral gene transfer includes naked DNA transfection and liposomal transfection. Nonviral techniques are more simple and inexpensive. In addition there are no inflammation or infection risks. However, these methods are nonspecific and vary in the level of gene expression (Branski et al., 2007). So far, there are only a few commercial gene therapy products approved for clinical use in humans. Gendicine™ and Advexin™ are products based on p53 expression and indicated for treating cancer (Gabrilovich, 2006; Peng, 2005). Additionally, the adeno-associated viral vectors Oncorine™ and Cerepro™, also for cancer care, are emerging in the markets (Räty et al., 2008).

Table 2. Examples of commercial skin substitutes.

Product	Type of substitute	Composition	Indication
CellSpray®	epidermal	Cultured epithelial autograft suspension	For treatment of superficial burns. Can be used together with a dermal product to treat deep dermal burns
Epicel®	epidermal	Cultured epidermal autograft. Autologous keratinocytes grown from patient skin biopsy.	For treatment of deep dermal and full-thickness wounds, alone or in conjunction with STSG.
MySkin®	epidermal	Cultured epidermal autograft. Autologous keratinocytes grown from patient skin biopsy.	For the treatment of burns, ulcers and other nonhealing wounds.
ReCell®	epidermal	Autologous epidermal cell suspension	For treatment of burns, scars, hypopigmentation and vitiligo
Alloderm®	dermal	Processed human allograft with acellular dermal matrix and intact basement membrane	For the treatment of full-thickness burns and use in plastic and oral surgery
Dermagraft®	dermal	Bioabsorbable polyglactin mesh scaffold seeded with human allogeneic neonatal fibroblasts	For treatment of foot ulcers in diabetic patients
Integra®	dermal	Silicone, cross-linked bovine tendon, collagen type I and shark glycosaminoglycan	For treatment of burns and full-thickness wounds in conjunction with STSG
Matriderm®	dermal	Bovine collagen type I, II, V and elastin	For treatment of burns and full-thickness wounds in conjunction with STSG
EZ-Derm®	dermal	Porcine-derived xenograft in which collagen is cross-linked with aldehyde	For treatment of partial-thickness wounds
Apligraft®	dermoepidermal	Human allogeneic neonatal keratinocytes on neonatal fibroblasts containing bovine collagen type I	For treatment of burns and partial- and full-thickness ulcers
OrCel®	dermoepidermal	Human allogeneic neonatal keratinocytes on bovine collagen sponge containing human allogeneic neonatal fibroblasts	For treatment of burns and partial- and full-thickness ulcers

2.4 Translational wound-healing research

The biggest problems in clinical wound-healing research are the lack of corresponding control wounds and the difficulty in monitoring the healing in an objective manner. In addition, ethical questions arise when testing a new product or harvesting tissue samples from humans. Thus, preclinical studies with animals as well as *in vitro* studies in the laboratory are crucial to investigating the molecular-level mechanisms behind the wound-healing process. *In vitro* wound-healing models are essential tools for studying in-depth responses selectively in a given cell population, in terms of behaviour (such as differentiation capacity or barrier function), responses to stimuli (such as GFs) or cell-cell interactions. Animal models are fundamental in the development, efficacy and safety testing of new therapies or products. Moreover, they make it possible to mimic clinical problems in a controlled manner.

Several animals, such as mice, rats and rabbits, have been used in wound-healing research over the years (Arai et al., 2012; Benavides et al., 2009; Dorsett-Martin et al., 2004). However, preclinically the pig is the best animal to study and test therapies prior to adoption for clinical use. Porcine skin is very similar both in structure and function to human skin (Montagna & Yun, 1964). Both have an avascular epidermis that is multilayered and varies in thickness in different body parts. Porcine dermis resembles human dermis being divided into an upper papillary layer and lower reticular layer. In both human skin and pigskin, the ratio of the epidermis to dermis is similar. The vascular organization is also alike and, more importantly, wounds heal through epithelialization as in humans. The practical advantages compared with other animals are an appropriate size and less hairy body. Some differences do exist, however, such as that pigskin does not contain eccrine glands and the apocrine glands are different (Vardaxis et al., 1997).

2.5 Gene expression profiling in wound-healing research

Although skin wound healing has been studied for decades, the molecular mechanisms behind the process are still not completely clear and most of the molecular-level understanding has been derived from various animal models. Fortunately, RNA- and DNA-based techniques have enabled molecular-level information to be obtained even from a small sample of tissue. Since these methods

require only a small amount of patient skin, they make it possible to study wound healing directly in humans. With techniques such as polymerase chain reaction (PCR) and microarray, it is possible to investigate how the expression of different genes is altered in skin during trauma or disease. More importantly, these techniques offer an unbiased approach to the observation of healing. However, it should be recalled that these techniques may have limitations concerning heterogeneous cell types in a tissue sample.

2.5.1 Polymerase chain reaction

The PCR is a biochemical technology in which a specific region of DNA is amplified using a PCR machine *in vitro*. Currently, several variations of the basic PCR exist, including real-time PCR, reverse transcription PCR, nested PCR, quantitative PCR and assembly PCR (Sze, 2004). The PCR technique can be used for several applications, such as identification of genetic fingerprints, diagnosis of hereditary and infectious diseases, and cloning the DNA for sequencing. In wound-healing research, it is used to recognize gene transcriptional activity during repair. There are even specific wound-healing PCR kits on the market, which make it possible to simultaneously follow several particular wound-healing-targeted genes (Bartlett & Stirling, 2003). The role of GFs in wound repair has been emphasized and thus their expression is studied widely using PCR (Werner & Grose, 2003; Kondo & Ishida 2010). A good example of PCR in clinical wound-healing research is a study by Conway et al. (2007). They investigated the role of HGF with PCR in human wound healing by obtaining skin biopsies from chronic wound tissue, acute wound tissue and normal skin. Their study showed that both HGF and its receptor c-Met are expressed differently in acute and chronic wounds (Conway et al., 2007).

2.5.2 Microarray

Microarrays are another much used procedure for various gene expression studies. Microarray chips are composed of microscopic DNA spots, called probes that contain picomole amounts of a specific DNA sequence. The key method behind microarrays is hydridization between two DNA strands; the probes detect the target DNA that is complementary to its sequence. The difference between a microarray and PCR is that with this technology it is possible to examine expression of all genes in the genome at the same time.

So far, the utilization of microarrays in wound-healing research has been modest. However, some interesting studies do exist. Smiley et al. (2005) used microarrays to better understand the molecular differences between cultured skin substitutes and native skin. Their gene expression analysis revealed that entire clusters of genes were either up- or down-regulated upon combination of fibroblasts and keratinocytes in cultured skin grafts. Furthermore, several categories of genes were overexpressed in cultured skin substitutes compared with native skin, including genes associated with hyperproliferative skin or activated keratinocytes (Smiley et al., 2005). In another study, Chen et al. (2010) investigated why oral mucosal wounds heal more rapidly and with reduced scar formation than cutaneous wounds. They obtained biopsies of both oral mucosal and skin wounds and used microarrays to compare the transcriptomes in order to find critical differences in the healing response at these two sites. Their results demonstrated dramatically different reactions to injury between skin and mucosal wounds. In a study by Greco et al. (2010), the gene expression profiles of thermally injured human skin were observed with microarrays. For the analysis, skin samples were collected from 45 burn patients and the transcriptomes were compared with those from 15 control patients. Greco et al. found that the expression of several hundreds of genes was altered significantly between groups, including many calcium-binding proteins, cytokeratins, and chemokines. In one study, Deonarine et al. (2007) investigated gene expression changes in skin wounds by collecting punch biopsies before and after wounding from basal-cell carcinoma patients. They concluded that the initial response to a cutaneous wound induces transcriptional activation of proinflammatory stimuli that may alert the host defence. It would be advantageous to be able to adopt all existing and future microarray data for clinical use, as has already happened, e.g. in cancer biology, using expression signatures as diagnostic and prognostic tools (Quackenbush 2006; van de Vijver et al., 2002).

3. Aims of the study

There is clearly a clinical need for effective skin-healing treatments for the increasing incidence of chronic wounds and still inadequate solutions for major skin-loss trauma, especially burns. The main goal of this thesis was to use the relatively new technology of transcriptome array analysis, to study wound-healing trajectories with a genome-wide 'resolution' of gene expression changes underlying the physiological healing response over time. The main hypothesis was that transcriptome array analysis will allow the identification and confirmation of previously established molecular regulatory events, and identify new events, which may suggest or extend therapeutic strategies

The objectives of the individual studies were to:

I clarify what happens to gene expression in the acute, inflammation and proliferation phases of superficial cutaneous wound healing, to identify the genes that are activated over time, and to organize the genes into groups according to the similar patterns observed in their expression profiles.

II clarify the potential of the approach in a real clinical context, using NPWT as a model experiment.

III clarify the differences in gene expression between the intact skin and the already re-epithelialized donor site wound.

IV to pilot the introduction of a newly available type of collagen, which exemplifies the possible use of advanced biomaterial/cell therapy for skin reconstruction.

4. Materials and methods

4.1 Microarray studies

4.1.1 Patients and sample collection

Human skin tissue samples were obtained from the Burn Centre of Helsinki University Hospital, Helsinki, Finland and from the Department of Surgery of Satakunta Central Hospital, Pori, Finland. All samples were taken with a 3-mm diameter biopsy punch from patients scheduled to undergo STSG. Skin graft harvesting was carried out with a compressed air-driven dermatome (Zimmer®, Zimmer Holdings Inc., Warsaw, IN, USA). The graft sizes ranged from 10 to 25 cm x 7.5 cm with a fixed thickness of 10/1000 inches (0.25 mm). The donor site wounds were covered with OpSite® polyurethane dressing (Smith & Nephew plc, London, UK). The wounds were photographed after the graft harvesting and during dressing changes. Wounds with early signs of clinical infection (enhanced secretion, pain, and positive swap culture) were excluded from the study. The patient exclusion criteria included diabetes, skin disease, cortisone treatment, immune suppression, anticoagulation, bleeding disorder, and unstable heart disease. Figure 5 illustrates sample collection in various studies.

Study I

Three to four samples of each patient were collected from four different time points. The first sample was taken from the intact skin immediately before STSG from the area to be wounded. The second was obtained from the acute wound, immediately after STSG. The third and fourth samples were biopsied from the same wound through the polyurethane dressing on the 3rd and 7th PODs (Figure 5). A total of 40 biopsies were collected from 12 patients (five from Helsinki, three from Pori), out of which 25 biopsies from eight patients met the RNA yield and quality criteria for inclusion in the study. All patients were healthy males between 20 and 75 years of age.

Study II

Twelve biopsies from two time points were collected from six patients. The first sample was taken from the intact skin immediately before STSG from the area to be

wounded, and the second from the wound through the dressing on the 7th POD. The patients were randomly selected by cointoss to receive either NPWT or control therapy. For this study, four patients received NPWT and two were controls. Negative pressure at 100 mmHg was continuously applied to the NPWT patients from the 1st POD until the 7th (Figure 5). Two porous Polymem® Wic® (3×3 inches, Ferris Mfg. Corp., Burr Ridge, IL, USA) dressings were placed at the donor site. The suction tube was placed between these porous dressings. An OpSite® polyurethane dressing (Smith & Nephew) was then placed so as to cover the entire donor site wound. The dressings were changed on the 3rd and 7th PODs. Control therapy consisted of a wound dressing similar to that used for the NPWT patients, but the suction tubing from the wound was closed and no negative pressure was applied.

In addition, wound exudates were collected from all patients and analysed for leukocyte, erythrocyte and haemoglobin concentrations, as well as for volume. Statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). The NPWT samples were collected from the canister containing the evacuated fluid, and control samples were obtained by needle aspiration under the dressing. Exudates were obtained on the 1st, 2nd and 3rd PODs. The patients participating for this study were male and female between 20 and 75 years of age.

Study III

Three samples were collected from four patients. As in the previous studies the first was obtained from the intact skin before STSG. The latter two were taken on the 14th and 21st PODs (Figure 5). All patients were male burn patients between 20 and 75 years of age.

4.1.2 Sample processing

Tissue homogenization and RNA isolation

The human skin tissue biopsies obtained were immediately immersed in RNAlater® buffer according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). They were then incubated at +4 °C for 1 day, and subsequently stored at -80 °C until RNA isolation.

Before isolation of total RNA, the samples were homogenized, using the Precellys 24-tissue homogenizer with a Cryolys temperature controller unit (Bertin Technologies SA, Montigny-le-Bretonneux, France). The frozen samples were placed in the hard tissue-homogenizing tubes (Bertin Technologies) with small ceramic beads and 350 µl of RLT lysis buffer (Qiagen). The biopsies were processed 3 x 23 s at 6000 revolutions per minute (rpm). The temperatures ranged between -5 °C and +10 °C during the homogenization.

Thereafter, the total RNA was extracted from the homogenized tissue samples with an RNeasy® Mini Kit (Qiagen), according to the manufacturer's protocol. The RNA quality was measured using an Agilent 2100 bioanalyser (Agilent Technologies Inc., Palo Alto, CA, USA) to ensure RNA integrity. After the RNA isolation, the RNA integrity was measured for each sample to ensure that the RNA quality was adequate for the microarray process.

Histology

For histology, the tissue samples were placed in formalin and fixed for 7 days. Thereafter, the samples were transferred to 70% ethanol for storage until paraffin embedding. After embedding in paraffin blocks, 4-µm-thick sections were cut, using a microtome. The sections were stained with haematoxylin and eosin (H&E).

Microarray

The purified RNA was synthesized into double-stranded complementary DNA (cDNA) with a Superscript double-stranded cDNA synthesis kit (Affymetrix Inc., Santa Clara, CA). A Bioarray High Yield RNA transcript labeling kit (Affymetrix) was then used to synthesize the complementary RNA (cRNA). The cDNA and cRNA products were purified with a GeneChip sample cleanup module (Affymetrix). The cRNA products were then used for the fragmentation reaction and hybridized into the Affymetrix Human Genome U133 Plus 2.0 GeneChip Array (Affymetrix). After 16 hours, the chips were washed and stained with a GeneChip Fluidics Station 450 (Affymetrix), followed by chip scanning with a GeneChip Scanner 3000 7G (Affymetrix). In addition to the Affymetrix GeneChip array, Illumina Human HT-12 GeneChips (Illumina Inc., San Diego, CA, USA) were also used. In the first study, parallel experiments were done, using the Affymatrix as validation method, while the third study was executed, using only the Illumina Human HT-12 GeneChip. All

microarray data are Minimum Information About a Microarray Experiment-compliant (MIAME) (Brazma et al., 2001) and were deposited in the National Centre for Biotechnology Information's (NCBI) Gene Expression Omnibus with accession numbers GSE28914 and GSE33169 and can be accessed at http://www.ncbi.nlm.nih.gov/geo.

Quantitative real-time PCR

Before PCR, the total RNA was translated into cDNA using the RT^2 First Strand Kit (Qiagen). The PCR was used as a data validation method in the first study and was executed with the Human Wound Healing PCR 96-well array (Qiagen), according to the manufacturer's protocol using the CFX96 instrument (Bio-Rad Laboratories Inc., Hercules, CA, USA). The data were analysed using the PCR Array Data Analysis Web Portal (http://www.SABiosciences.com/pcrarraydataanalysis.php) (Qiagen).

Microarray data analysis

The Affymetrix and Illumina microarray data were analysed in a similar manner. The GeneChip signal intensity data were uploaded to GeneSpring GX software, version 11.0.1 (Agilent Technologies) and normalized to the median. Experimental grouping was then performed according to therapy and time point. The data were then interpreted to pool together the samples of the patients from each group. Finally, the data were presented as gene expression fold changes between groups.

The data were preprocessed with robust multiarray analysis (Irizarry et al., 2003) and median expression values were derived. Further statistical analyses were done, using the limma, BioMart and qvalue packages of the Bioconductor Project (Durinck et al., 2005; Gentleman et al., 2004; Smyth, 2004; Storey & Tibshinari, 2003). At first, Affymetrix type of probe set names given in the chip were converted to EnsEMBL gene names using BioMart (Durinck et al., 2005). Those probe sets matching multiple EnsEMBL entries and probe sets not matching any EnsEMBL entry were removed from the data. The probe sets matching the same EnsEMBL entry were averaged into a single expression estimate for that EnsEMBL entry. The significance of the differential expression was assessed, using the empirical Bayes-moderated paired *t*-statistics followed by *p*-value adjustment with the Storey's *Q*-value approach (Smyth, 2004; Storey & Tibshinari, 2003). Pairing of the patient samples was done by allowing patient-pair effects in the linear model. The arrays were quality-weighted

before statistical testing (Ritchie et al., 2006). Genes with *Q*-value-corrected *p*-values ≤ 0.05 were considered as significantly differentially expressed.

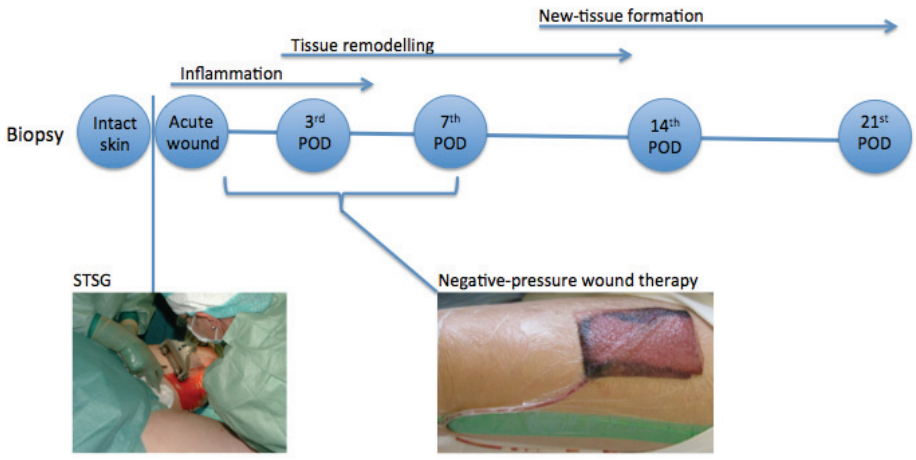

Figure 5. Schematics of the studies I - III.

4.2 Transplantation of skin cells inside collagen gel

4.2.1 Primary cell isolation and culture

A tissue sample was obtained from the dorsal skin of a landrace pig under general anaesthesia. The skin sample obtained was carefully washed with antibiotics containing phosphate-buffered saline (PBS), the subcutaneous fat was removed and the sample was minced. To separate the epidermis and dermis, the tissue samples were incubated overnight in PBS diluted with 2U/ml dispase (Dispase®, Roche Diagnostics Deutschland GmbH, Mannheim, Germany). After incubation, the epidermis was peeled off from the dermis, using needles.

The primary keratinocytes were isolated from the epidermis by further (about 5 min) incubation in 0.1% trypsin-PBS (Sigma-Aldrich Corp., St. Louis, MO, USA) at +37 °C. After incubation, the samples were pipetted strongly to the detach cells from the epidermis. The gained single-cell suspension obtained was then seeded on a rat-tail collagen-I (Sigma-Aldrich) coated cell culture dish and grown in defined keratinocyte serum-free medium (K-SFM; Invitrogen™, Life Technologies Corp., Carlsbad, CA,

USA) supplied with growth supplement (Life Technologies) and 1% penicillin-streptomycin (Life Technologies)

Primary fibroblasts where obtained from the remaining pieces of dermis. The samples were incubated in collagenase IV (1 mg/ml; Worthington Biochemical Corp., Lakewood, NJ, USA) and 0.1% trypsin (Sigma-Aldrich) solution (2 x 1 hour, +37°C). Thereafter, the cells were seeded in a coated cell culture dish and grown in Dulbecco's Modified Eagle Medium**:** Nutrient Mixture F-12 (DMEM / F-12) supplied with 10% bovine serum and 1% penicillin-streptomycin (all from Life Technologies).

4.2.2 Primary cell-containing collagen gel preparation

The rhCol-III was used as a carrier material for cells (FibroGen Inc, San Francisco, CA, USA). To develop a gel, the pH was adjusted from the initial 2 to 7.2 by adding sodium phosphate (Na_3PO_4) in a 9:1 ratio. Thereafter, the rhCol-III solution was transferred to 1ml syringes. To strengthen the gel, the concentration of the rhCol-III was fixed by centrifuging the syringes at 5000 g (3 x 5 min). After each centrifugation, the supernatant was discarded and 1 ml new rhCol-III solution was added to the syringe to gradually increase the gel volume.

Next, cells from the primary cultures were detached from the culture dishes, using trypsin (Sigma-Aldrich), and pipetted on the top of the syringe. Two types of syringes were prepared: 1) keratinocyte syringes (containing 0.5 x 10^6 keratinocytes) and 2) keratinocyte-fibroblast syringes (containing 0.25 x 10^6 of both cell types) (Figure 6 A). The cells were mixed inside the gel by centrifugation (350 g, 5 min). In coculture syringes, the fibroblasts were added first. In addition, acellular syringes, containing only rhCol-III, were prepared for controls. All syringes were then placed in the incubator (37 °C, 5% CO_2) and kept there overnight prior to application (Figure 6 B).

A confocal microscope (Leica Microsystems AG, Wetzlar, Germany) was used to ensure cell viability inside the gel. The cells were stained before mixing them inside the gel. CellTracker (Invitrogen) was used to stain the cytoplasm and DRAQ5 (Axxora LLC, San Diego, CA, USA) to stain the nuclei. The syringes were prepared and after the overnight incubation, the gel was injected onto a microscope slide. A confocal microscope was executed using a Leica TCS SP2 acoustic optical beam splitter (AOBS) system with a argon 488-nm or diode-pumped solid-state laser

(DPSS) 561-nm with excitation lines and an HCX PL APO CS 63 x/1.40 NA or 40 x/1.25 NA oil-immersion objective. The image stacks were collected through the sample, using sequential scanning and a standardized 120-nm z-sampling density.

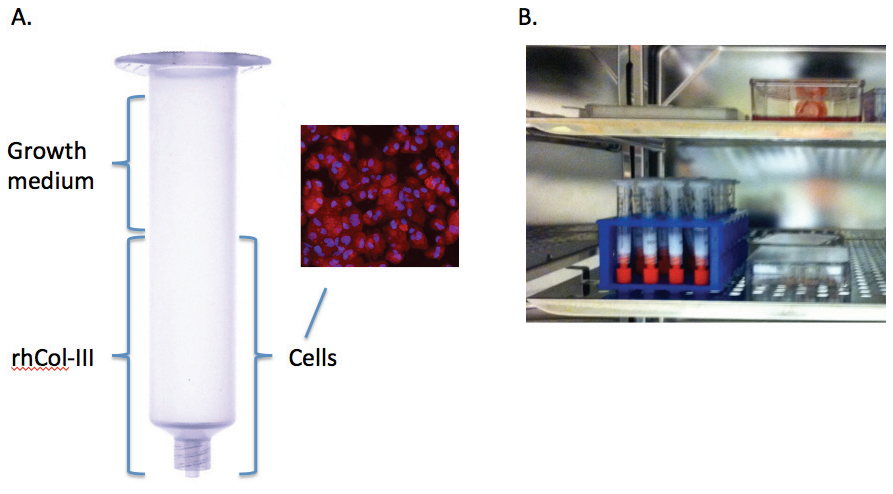

Figure 6. Concept of study IV.

4.2.3 Full-thickness wounds and therapy application

Two pathogen-free 2-month-old domestic landrace female pigs were anaesthetized, using an intramuscular injection of ketamine (4 - 6 mg/kg) and medetomidine (0.5 mg/kg). Anaesthesia was continued with intravenous (i.v.) infusion of propofol (4 - 10 mg^{-1} kg^{-1} h^{-1}).

Prior to wounding the back of the pig was shaved, washed thoroughly and cleaned with 0.5% chlorhexidine. Thereafter, 14 identical deep dermal wounds were made on the back of each pig, using an 8 mm biopsy punch, seven wounds on each side of the spine.

Therapy was applied by injecting the prepared syringes into the wounds: keratinocyte syringes (n = 8), keratinocyte-fibroblast syringes (n = 8), acellullar (n = 8). In addition, empty untreated wounds (n = 4) were used as an additional control for treated wounds. For each wound the treatment was randomized. After the application, the wounds were covered with an OpSite® polyurethane dressing (Smith & Nephew)

and healing was followed up for 5 days. On the 5th POD, the wounds were excised with ample margins and placed in 10% formaldehyde solution for fixation.

4.2.4 Histological analysis

After fixation, the wound samples were trimmed, embedded in paraffin and cut into 4µm-thick sections. To observe the development of granulation tissue and wound healing, the sections were stained with H&E, then viewed under a microscope and photographed (Leica IM500 and Leica QWIN software; Leica Microsystems AG, Heerbrugg, Switzerland). The amount of granulation tissue was measured from the bottom of the wound bed, and the wound healing was measured as the reduction in the empty area per wound section. To assure objectivity, the analyses were performed in blind fashion.

Paraffin sections were also used for immunohistochemical analysis. The sections were stained with mouse antihuman pancytokeratin antibody (diluted 1:40; EMD Millipore Corp., Billerica, MA, USA) to identify keratinocytes in the wound bed. DAB (3,3-diaminobenzidine) was used as the chromogenic substrate for the secondary antibody-conjugated horseradish peroxidase (HRP) for visualization of pancytokeratin-positive cells. Positive cells from the stained sections were identified using ImageJ software (ImageJ, National Institutes of Health; (NIH); http://rsb.info.nih.gov/ij).

Sirius Red staining was performed to calculate the amount of collagen in the wound bed. The previously stained sections were deparaffinized followed by a 1-h incubation in the dark with Sirius Red dye in saturated aqueous picric acid (1 mg/ml). After the incubation, the sections were washed three times in running tap water. The sections were then dehydrated in ethanol and xylene before being permanently mounted in DPX (Leica Microsystems). The total collagen content was calculated as the percentage of the Sirius Red-stained positive area from the total wound area in scanned images of the stained tissue sections with ImageJ software.

4.2.5 Statistical analysis

GraphPad Prism 4.0 (GraphPad Software) was used to analyse the data. Statistical analyses were performed, using Student's *t*-test (two-tailed). A p-value < 0.05 was considered statistically significant.

4.3 Ethics

In the microarray study, the protocols were approved by the Operative Ethics Committees of the Pirkanmaa Hospital District (approval number R09087, studies I and II) and the Hospital District of Helsinki and Uusimaa (DNRO 101/13/03/02/11, study III). All samples were obtained in accordance with the Declaration of Helsinki Principles and all patients gave their informed consent on participation in the studies.

In the study IV, all animals were maintained and treated in accordance with the *Principles of Laboratory Animal Care* (NIH Publication No. 8623, revised 1985). The study was approved by the Provincial State Office of Southern Finland (ESLH-2009-03831/Ym-23).

5. Results

5.1 Donor site wound healing

The purpose of the first three studies was to map the gene expression changes occurring during the various stages of human superficial cutaneous wound healing. Standard depth, clean and consistently healing donor site wounds were used as models for the studies. Biopsies were collected over time to represent various steps of the healing process. The first biopsy was obtained before wounding from the intact skin, and the following biopsies, respectively, from the wound in the acute phase, on the 3rd, 7th, 14th, and 21st PODs. In addition, the interest was to see how the effect of a clinical wound therapy would be reflected in terms of gene expression changes. For this purpose, we used NPWT, which also served as an example.

The behaviour of gene expression at the transcriptional level was investigated by comparing their messenger RNA (mRNA) levels at different time points. Finally, the data were presented as gene expression fold-changes between time points. Overall, clear changes in expression were manifested in thousands of genes, with the biggest changes being in the order of several hundredfold. The inter-individual variation in gene expression profiles between patients was very low, although the patients' ages and general conditions varied. In all studies the cut-off fold-change for a significant gene expression change was ≥ 2.0. Moreover, all the expression changes presented in the following chapters are statistically significant (p-value < 0.05).

In addition, one must keep in mind that in all comparisons with intact skin, a negative fold-change of a gene can be due to removal of the cells expressing it, while a positive fold-change of another gene can be due to enrichment of cells expressing that gene in the tissue sample.

5.1.2 Acute wounding

What happens to the skin at the molecular level after wounding?

This was studied by comparing the acute wound with the intact skin. In all, a statistically significant alteration in expression was observed for 8305 different genes (Figure 7). The genes most increased in expression (approx. fivefold) were KRT-associated proteins (KRTAPs). The most decreased ones were the CE proteins-

encoding genes, such as LCE 1 genes, sciellin (SCEL), envoplakin (EVPL), FLG, and LOR. Many genes of the keratin family (such as KRT1, KRT2, KRT23, KRT31 and KRT80) decrease, suggesting that the cells expressing them are removed in STSG. The most dominant gene in this respect was KRT2. It decreased in expression 430 times against intact skin. Interestingly, the genes next on the list, such as IL-37 and secreted lymphocyte antigen 6 complex/plasminogen activator urokinase receptor domain-containing 1 (SLURP1), decreased only 20 - 50 fold, suggesting that these genes are significantly, albeit less strongly than the KRTs, expressed in the epidermis.

5.1.3 Inflammation

Which are the genes most dominant during the inflammation phase of wound healing?

Gene expression in the inflammation phase was studied by comparing the samples collected on the 3rd POD with the acute wound samples. The inflammatory profile is characterized by high expression of ILs (such as IL-8), chemokine ligands, and receptors (such as chemokine (C-C motif) ligand 18 (CCL18), chemokine (C-X-C motif) ligand 5 (CXCL5), and C-X-C chemokine receptor type 4 (CXCR4)) as well as several leukocyte cell-surface antigens (such as cluster of differentiation 86 (CD86) and CD163). Additionally AMPs were very well presented. Genes, such as S100A7A and PI3 were among the most increased genes in this comparison. Overall, the expression of 10194 genes was altered in this comparison.

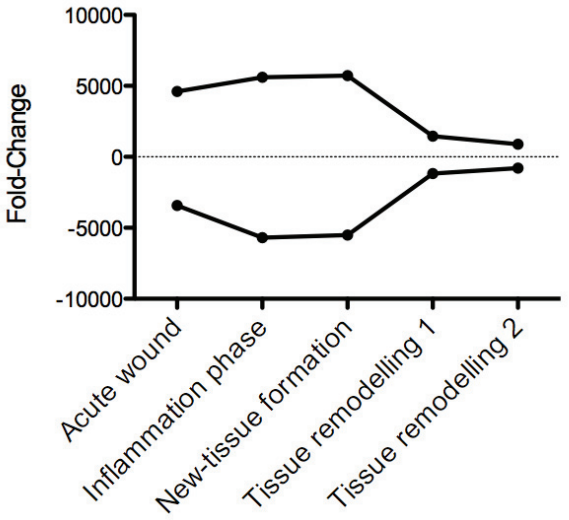

Figure 7. Number of genes that are altered in expression during the wound-healing cascade in comparison to intact skin. The curve above the X-axis decribes genes whose expression increased and the curve below genes whose expression decreased.

5.1.4 New-tissue formation

How are new-tissue formation and re-epithelialization reflected in gene expression?

To provide an insight to genes associated with new-tissue formation, the 3rd and 7th POD samples were compared with the acute wound. The expression of 12068 and 12275 genes changed respectively. Moreover, the 7th POD gene profile versus the 3rd POD profile was used to characterize the re-epithelialization phase.

The gene family most visible, in comparing the gene profiles of both the 3rd and 7th POD with acute the wound profile, was the MMP family. The MMPs with the highest expression were MMP1, MMP3 and MMP10. Overall, in comparing the 3rd and 7th POD, with the acute wound, the genes with the highest expressional increase and thus closely associated with resurfacing overall were CCL18, IL8, MMP1, PI3, serpin peptidase inhibitor, clade B (ovalbumin), member 3 (SERPINB3), SERPINB4, secreted phosphoprotein 1 (SPP1), SPRR3 and S100A7A.

Histologically some epithelial recovery was already visible on the 3rd POD but by the 7th POD, the wound was almost fully covered with new epithelium and a thin CE. Thus, the re-epithelialization phase was explicitly described in comparison between the 7th and 3rd POD samples. The expression of several KRTs (such as KRT1, KRT4, KRT10, KRT13, KRT78 and KRT80) was higher. Remarkably, these include those KRTs (KRT1, KRT10, KRT78 and KRT80) that were removed by STSG in acute epithelial wounding. The development of a new CE was also evident by higher expression of many major CE components (such as FLG, LCE2B, LCE3D, LOR, SPRR1A and SPRR3).

5.1.5 Tissue remodelling

How close is the gene expression profile of skin in the remodelling phase to that of intact skin?

The samples obtained on the 14th and 21st PODs were compared with the intact skin. At this phase of healing, the number of genes that are altered on expression decreases. The gene expression transcriptome slowly moves towards that of intact skin.

In all, 2632 genes expression changes at 14 days after STSG in comparison to the intact skin and only 1610 by 21 days (Figure 7). In addition, the expression changes were more moderate than those in the earlier stages of healing. Overall, the gene most altered was KRT2 with a negative fold-change of almost 200 on the 14th POD,

compared with the intact skin. The profiles at both time points were very similar, with AMPs being the genes most increased (such as S100A7A) and KRTs and CE-encoding genes being the most visible group of decreasing genes.

The data reveal important roles for distinct control mechanisms that decrease the inflammatory process and promote epidermal maturation. Some of the key processes are associated with lymphocyte chemotaxis, iron homeostasis, shift in cytokine and chemokine profiles, as well as retinoic acid and lipid metabolism.

5.1.5 Effect of NPWT on donor site wound healing

Can we see the impact of a therapy at the gene expression level of wound healing?

On the 7th POD patients receiving NPWT were compared with those not receiving NPWT. By this time point, the treated patients had been receiving the therapy continuously for 6 days.

The results indicate that clear and statistically significant distinctions were present in the comparison data. Many genes that increase in the NPWT-treated patients were associated with cell proliferation and inflammation. The gene most increased was IL24, with a 10-fold difference between groups and the second in order was prostaglandin-endoperoxide synthase 2 (PTGS2) with an 8-fold induction. In addition, several other ILs and their receptors (such as IL1RL1, IL8, IL1A, and IL13RA2) were among the genes most increased as well as many MMPs (such as MMP3 and MMP10).

The most interesting finding was that the genes most decreased were CE genes, all of which encode proteins of the SC. The gene most increased was FLG, with a fold-change difference of 27. Other major CE components, such as FLG2, LOR, LCE1 and SLURP1 were all decreased. Another apparent group of decreased genes were the KRTs. Decrease in five different KRTs, all associated with keratinocyte differentiation (KRT1, KRT2, KRT10, KRT13 and KRT15) was evident. These findings suggest that the effect of NPWT is specifically targeted to inhibition of maturation of the epidermal layer. Thus, the differentiation-associated homeostasis of the epidermis may be delayed when NPWT is used.

In addition, the wound exudate compositions were examined in both the NPWT and control patients. The exudates were collected from 11 patients for measurement of

leukocyte, erythrocyte and haemoglobin concentrations. The exudate volumes were also measured. The number of leukocytes on the 3rd POD was significantly smaller in exudates from wounds treated with NPWT (NPWT vs. control, $p < 0.05$). The number of erythrocytes in the NPWT wounds was smaller on the 1st POD, but clearly larger on the 2nd and 3rd PODs. The exudate volume was significantly ($p < 0.05$) larger in the NPWT wounds, suggesting that the NPWT was effectively administered.

5.1.6 Gene expression data validation

To validate the microarray gene expression data, some of the samples (eight biopsies from three patients) were analysed on two platforms (Affymetrix and Illumina), differing in array technology. The Affymetrix and Illumina arrays are based on completely different techniques and can thus be considered as separate platforms for the evaluation of gene expression. They differ in oligonucleotide physical attachment, probe selection and assay principle.

The gene expression data from both microarrays were compared with the corresponding data of the same samples. Of the top 100 genes identified as increased on the Affymetrix platform, 97%, 98% and 100% were identified as increased on the Illumina in the acute phase and on the 3rd and 7th PODs, respectively. Similarly, 96%, 99% and 98% of the decreased genes on the Affymetrix were identified as decreased on the Illumina (Table 3).

To strengthen the validation, a quantitative real-time PCR array (Qiagen) was performed on one patient's intact skin sample and 7th POD sample. This commercial PCR array provides gene expression profiles of 84 genes associated with the wound-healing response. These genes expression alterations between the 7th POD and the intact skin were presented as fold-change differences similar to those in the microarray analysis. The results from the PCR arrays were very similar to those of the microarray results and, thus, further confirmed the data (Table 3).

Table 3. Analysis technique validation: 7th POD vs. intact skin comparison

Regulation: UP		**Fold-change**	
Gene	PCR	Affymetrix	Illumina
MMP1	535	594	2690
CXCL1	58	25	79
SERPINE1	47	24	39
IL6	21	8	216
CXCL2	18	16	68
IL1B	17	12	318
CXCL5	10	14	165
WISP1	7	2	42
ITGB6	7	2	6
MMP9	5	10	18
Regulation: DOWN		**Fold-change**	
Gene	PCR	Affymetrix	Illumina
ITGA2	4	2	2
FGF2	4	2	2
EGF	3	2	3
FGF10	3	1	1
MAPK1	2	1	14
IL6ST	2	2	68
TGFBR3	3	3	2
CSF3	2	2	3
CDH1	2	-	1
MAPK3	2	1	1

5.2 *In vivo* transplantation of primary skin cells inside the collagen gel

The use of the rhCol-III gel for transplantation of autologous keratinocytes and fibroblasts was evaluated in porcine full-thickness wounds. Five days after the transplantation, the wounds were removed for histological analysis. The formation of granulation tissue, as well as the amounts of keratinocytes and collagen, were determined.

5.2.1 Cell viability inside the gel

Cell viability inside the rhCol-III gel was confirmed with a confocal microscope. After an overnight incubation, the gel containing the dyed cells was photographed under the microscope. The observation proved that both keratinocytes and fibroblasts remained viable before transplantation.

5.2.2 Full-thickness wound healing

The formation of granulation tissue was evaluated from the H&E-stained wound sections of each wound. The thickness of the granulation tissue was 0.08 ± 0.03 pixels (px) in the rhCol-III, 0.08 ± 0.03 px in the keratinocyte, 0.08 ± 0.03 px in the fibroblast-keratinocyte groups and 0.06 ± 0.02 px in the untreated group. Thus, the results indicate that all treatments increased granulation tissue formation compared with the controls. No significant differences were observed between the treatment groups. Nevertheless, the analysis suggest that the rhCol-III gel improved granulation tissue formation, but the addition of cells showed no effects, at least not during this 5-day follow-up.

The number of keratinocytes in the wound bed was measured as another marker for wound healing. The keratinocytes were identified by immunohistochemical pancytokeratin staining of sections of each wound. The pancytokeratin-positive area was $0.76\% \pm 0.17\%$ of the wound area in the rhCol-III, $1.39\% \pm 0.20\%$ in the keratinocyte, $1.02\% \pm 0.13\%$ in the fibroblast-keratinocyte and $0.83\% \pm 0.13\%$ in the untreated groups. The results suggest that the autologous cultured keratinocytes transplanted inside the rhCol-III gel were present in the wound 5 days after transplantation. The difference between cell treatment groups was evidenced by less pancytokeratin positivity, although the difference between groups did not attain statistical significance. Significant differences in the amount of pancytokeratin-positive cells in the wound were found only in the keratinocyte group between both the rhCol-III gel and the empty wound.

For determining the amount of collagen in the wound, the sections were stained with Sirius Red and photographed with a polarized light microscope. The images showed that the amount of collagen was most increased in wounds treated with rhCol-III gel or rhCol-III gel containing only keratinocytes. The number of Sirius Red-positive collagen fibrils was $22.0\% \pm 1.7\%$ per wound area in the rhCol-III, $26.4\% \pm 2.1\%$ in the keratinocyte, $11.0\% \pm 2.0\%$ in the fibroblast-keratinocyte and $10.3\% \pm 0.9\%$ in the untreated group. Thus, the measurements show that changing the cell-type composition of the rhCol-III gel from keratinocytes only to a 50/50 fibroblast-keratinocyte mixture dramatically altered the amount of collagen detectable by Sirius Red staining in the wounds.

In conclusion, the results indicate that there are cell-type-dependent differences in the stability of rhCol-III *in vivo*. The fibroblast-containing gel was effectively removed from the wound, whereas gels without cells or with keratinocytes only remained intact. The results demonstrate that the properties of the rhCol-III gel for skin cell transplantation can be significantly altered in a cell-type-dependent manner.

6. Discussion

6.1 Donor site wound-healing studies

So far only a limited amount of data are available on what happens in terms of gene expression in the wound during various stages of cutaneous wound healing. Most of the molecular-level wound-healing data have been obtained from various animal models. Several studies have clarified the role of specific genes in various wound models in animals. Lees et al. (2013) studied the expression of a tropomyosin isoform in a mouse model and showed more rapid wound healing when the gene was deleted. In another mouse model, Sun et al. (2013) concluded that IL19 is important for cutaneous wound healing because it up-regulates KGF expression. Additionally, *in vitro* gene expression studies have been performed with various cell cultures (Kolář et al., 2012). The role of GFs and cytokines especially has been the subject of intense focus (Kiwanuka et al., 2012; Lin et al., 2005).

Few results are available on gene expression in human wound healing. Some of these reports are based on samples that were collected for another purpose or alternatively obtained from nonstandardized wounds (Deonarine et al., 2007, Smiley et al., 2005). A comprehensive look at normal wound healing over time has been lacking.

To execute a prospective, molecular-level wound-healing study in the clinic is not without problems. One of the biggest challenges in clinical wound-healing research is the lack of appropriate controls. To overcome this issue, STSG harvesting of donor site wounds was used for this study. Donor site wounds provide an excellent model for studying the mechanisms of wound healing in the clinic, since being surgically made, they are always standard in depth and size. They are noninfected and clean, and moreover these wounds tend to heal very well, which enables the observation of normal skin wound healing over time (Orgill, 2009).

To find novel genes associated with wound healing and to map gene profiles of thousands of genes, gene expression microarrays were considered as the best option. They enable the entire genome to be addressed and require only a small amount of tissue (Villaseñor-Park & Ortega-Loayza, 2013). The fact that a 3-mm punch biopsy is sufficient to clarify the entire genome was the determining factor in making this

study happen. As a method, the microarray is relatively expensive, which limited the amount of samples processed in these studies. On the other hand, it is more affordable than next-generation sequencing. Another technique for studying gene expression is the PCR (Bartlett & Stirling, 2003), which is even more sensitive than the microarray. It is however very much limited in the number of genes it can detect and offers few opportunities to find new gene associations.

The microarray technique has been in use for more than 20 years (Chang, 1983) and the data it produces have been considered reliable. Nevertheless, to confirm the reliability of the data, selected samples were processed with two different microarray platforms (Affymetrix, Illumina) and single-gene expressions were compared with PCR. The microarray results were more than 95% similar and the PCR of the selected genes strengthened the credibility of the results even further (Table 3). Barnes et al. (2005) made very similar findings in a study comparing the Affymetrix and Illumina platforms and showed that these platforms yield highly similar data. Interestingly, Affymetrix and Illumina are technically very different. They differ in oligonucleotide physical attachment, probe selection and in design procedure. The Affymetrix arrays are produced by *in situ* synthesis of 25-mer oligonucleotides, while in Illumina process the oligonucleotides are attached to microbeads that are then introduced into microarrays, using a random self-assembly mechanism (Lockhart et al., 1996). The Affymetrix uses multiple probes for each gene together with single-base mismatch probes intended as controls for non-specific hybridization. The Illumina system produces about 30 copies of the same oligonucleotide on the array, which provides an internal technical replication that Affymetrix lacks. Finally, multiple Illumina arrays are placed on the same physical substrate, meaning that hybridization and other steps are performed in a parallel manner, while the Affymetrix arrays are processed separately (Gundersson et al., 2004). An important matter considering the microarray platforms is the price. The Illumina is more cost-effective than the Affymetrix. Thus, its Human HT12 is the most affordable human array on the market. Affymetrix human arrays are roughly twice the Illumina prices (www.helsinki.fi/fugu).

In all, 49 biopsies from 18 patients and from six different time points were used for these studies. However, more samples were collected, but not all met the quality requirements for the microarray process. The RNA integrity number (RIN) (Webster, 2006) was used as the main determinant for samples suitability for the microarray. To

obtain enough good-quality RNA from the acute wound samples was very challenging. Success with these samples was random, although they were collected in a similar manner by the same surgeons and processed by the same researcher. Thus, the entire epidermis with only minor involvement of the dermis was harvested. This is crucial, because when the various time points are compared with intact healthy skin, a negative fold-change in a gene can result from removal of the cells expressing it, while a positive fold-change of another gene can be due to enrichment of cells expressing that gene in the remaining dermis.

In our model, the wounds were very standardized (size and depth always the same) and were made with an air-driven dermatome machine and, more importantly located in the same part of the body (anterior thigh), in all patients. Thus, the skin was similar in thickness among patients. The sample collection was performed with identical 3 mm biopsy punches and always obtained from the middle of the wound. The repeated 3-mm biopsies healed well and caused only inconspicuous scars, well accepted by the patient.

Our data revealed, as expected, the involvement of thousands of genes during the first 3 weeks of donor site healing. More importantly, the data showed which genes were most dominant and additionally revealed that specific groups of genes are more important than others. One of these gene groups was the AMPs, which are known to play important roles in skin immune defence. Our data revealed that many of them also increase highly at different stages of wound repair. PI3, also known as skin derived antileukoproteinase (SKALP) or elafin (Wiedow et al., 1990) various S100 calcium-binding proteins (Zimmer et al., 1995) and defensin beta 4A (DEFB4) (Gallo & Hooper, 2012) were among the genes most induced at all our time points. PI3 was first found in psoriatic skin, and its expression in healthy human epidermis is low (van Bergen et al., 1996). It inhibits serine proteases and influences on cellular proliferation, inflammation and infections (Shaw & Wiedow, 2011). Our data identified this possible regulatory function of PI3, and we can now closely link this gene with all phases of cutaneous wound healing. The S100A genes are known to be involved in the regulation of many cellular processes, such as the cell cycle and differentiation (Eckert et al., 2004), suggesting possible regulatory functions in wound healing.

Epidermal differentiation and development of the protective barrier were very evident in our data. Many CE-encoding gene expressions were altered during the study period follow-up. In addition to the PI3 and S100A already mentioned, these genes included SPRR3, LCE3, LOR and FLG. The CE is an insoluble epidermal protective barrier that is composed of terminally differentiating stratified squamous epithelial cells, which functions together with the lipid envelope as the first line of defence in skin. The composition, shape and thickness of the CE vary according to the tissue and its state of maturation (Kalinin et al., 2002; Kypriotou et al., 2012). Interestingly, the CE-linked genes that were manifested in our data with altered expression are located in the same gene cluster in chromosome 1q21. This cluster is called the epidermal EDC. The EDC is comprised of many genes that encode structural and regulatory proteins critical for keratinocyte differentiation and development of the SC (Henry et al., 2012). In addition, the expressional changes in the various KRTs, especially KRT2, can be considered as reflecting keratinocyte differentiation and development of new CE. KRT2 is expressed largely in the upper spinous layer of the epidermis and has been associated with keratinocyte differentiation (Bloor et al., 2003). Our data suggest that KRT2 is a good marker gene for healthy intact skin. In the acute phase, when the epidermis is removed, KRT2 decreased 430-fold; on the 3rd and 7th PODs it is decreased even more: over 700-fold. Then, 14 days after the operation, KRT2 was decreased 200-fold and on the 21st POD, when the wound is clearly closed, KRT2 is still decreased 50-fold. This shows that the maturation of skin after wounding requires longer periods of time than in our study. However, in large burns when donor site skin is limited, surgeons may already use the same donor site 3 weeks after the initial STSG.

Several GFs (such as EGF, FGF, HGF, PDGF and VEGF) are generally considered to be the most crucial factors in the wound-healing process (Behm et al., 2012). Very interestingly, mRNA expression of these GFs remained rather static during our follow-up. Thus, our data may indicate that superficial wounding does not alter the expression of these genes, in other words these genes are not regulated on a transcriptional level in epidermal wound healing, or that the microarray technique is not sensitive or specific enough to detect the various forms, modifications, and sizes of each GF mRNA. A future alternative to further investigate the regulation of GF expression in this clinical wound-healing model would be to utilize sensitive RNA-

sequencing techniques (Maher et al., 2009; Mutz et al., 2013). Further information could be provided by various approaches including epigenetics, proteomics and metabolomics (Adamski & Suhre, 2013; Brinton et al., 2012; Ballestar & Esteller, 2008). On the other hand, GF signalling is also dependent on the corresponding receptors on target cells (Alberts et al., 2002). Many GFs are produced by fibroblasts that reside in the dermis and wounds in these studies have been superficial. Thus, wound depth can affect GF expression. GFs may also be constantly present in skin and may accumulate in the ECM.

In conclusion, the data obtained from these studies offer the first large-scale perception into the molecular details of normal, superficial, human, skin wound healing over time. Moreover, the data enable the researcher to follow any gene's behaviour during the first 3 weeks of the healing process. It is important to understand the aetiology of normal healing in detail to better understand chronic and other difficult-to-treat wounds. The generated gene library of normal skin wound healing can be utilized as an objective tool when observing the effects of various therapies. Now that the background data are available the number of controls can be minimized, which saves money and time.

A good example of utilization of these data was our study that focused on clarifying how NPWT affects gene expression during wound healing. Although NPWT had been in routine use for years and was recognized as effective, the molecular mechanisms behind this therapy were unknown (Gregor et al., 2008). In comparing the data of NPWT patients with nontreated patients, we concluded that NPWT enhances the specific inflammatory gene expression associated with granulation, epithelial migration and wound healing. Interestingly the data also revealed that NPWT's continued use inhibits epithelial differentiation.

6.2 Full-thickness wound healing study

TE provides an array of new approaches for the treatment of deep dermal wounds. Hence, cell therapies and novel biomaterials either alone or combined are believed to be the answer for problems associated with chronic wound healing (Wong & Gurtner, 2012).

In the skin, collagen provides cellular support and plays an important role in affecting cell attachment, migration, proliferation, differentiation, and survival. It is also an important factor in the wound-healing process (Li et al., 2007). Thus, several types of collagen products have been popular in various TE applications, such as skin, cartilage, cornea and bone TE (Cen et al., 2008; Ferreira et al., 2012; Glowacki & Mizuno, 2008). Recombinant collagens, especially have been considered to show potential, due to their biocompatibility and safety (Yang et al., 2004). Recombinant processes allow the manufacture of human collagens that are pure and free of animal components. In addition, these techniques enable the production of all types of collagens, including types that exist only superficially in natural tissue. The most cost-effective and scalable process for manufacturing recombinant collagens is to use genetically engineered microorganisms, such as bacteria (e.g *E. coli*) and yeasts (e.g *Pichia pastoris*) (Báez et al., 2005; Ramshaw et al., 2009).

To study novel biomaterials in animal models is crucial before they can be adopted for clinical use. Only animal models enable the systematic research on the effects of the material in living tissue. Additionally, tissue samples at the end of the study can be collected for many more detailed analyses, including histology and RNA-based experiments. The porcine model was chosen due to similarities between porcine and human skin (Montagna & Yun, 1964). In addition, pigs were considered better than rodents, due to their larger size. In addition, we had experience with pigs from our previous studies. The problem with pigs is that they grow very rapidly and even full-thickness wounds tend to heal rather quickly.

We made full-thickness wounds on the backs of pigs to investigate the suitability of rhColl-III for use as a dermal filler as well as for a vehicle in which to transplant cells. Our approach was to prepare collagen-containing syringes with and without cells. The cells were centrifuged inside the collagen and incubated in the syringe overnight. The syringes proved very handy and could potentially be used as an off-the-shelf product

for clinicians. The cells remained viable inside the collagen and it was very convenient to apply the material from the syringe.

The results of our study support the use of rhCol-III gel as a favourable material for cell delivery in the treatment of full-thickness wounds. Moreover, its kinetics in the wound can be altered in a carrier cell-type-dependent manner and are more sustained when combined with autologous keratinocytes than with fibroblasts. However, these results are preliminary and further studies with longer follow-up times and with larger numbers of animals and wounds would be needed to truly ensure the potential of rhCol-III in TE of full-thickness wounds.

7. Summary and conclusions

The purpose of my PhD project was to study human skin regeneration and to develop means for enhancing compromised wound healing, especially in the treatment of burns. The first three studies focused on investigating and clarifying the gene expression transcriptome in donor site wounds before and after skin graft harvesting and during the healing process. The fourth study focused on full-thickness wound healing in a porcine model.

The main take-home messages of these studies were:

I we were able to validate a protocol for following transcriptome changes in the clinical setting. The data show that thousands of genes are associated with superficial human skin wound healing over time and up to 900-fold changes in gene expression during donor site wound healing.

II The effect of NPWT is clearly detectable in the gene profile of treated wounds. In the early phases of wound healing, NPWT enhances the expression of genes associated with inflammation, epithelial migration and wound healing. On the other hand, its continued use may inhibit epithelial differentiation.

III In the new-tissue formation phase of wound healing, the expression of several thousand of genes differs from that of intact skin. KRT- and CE-encoding genes are decreased the most, whereas AMPs, MMPs and collagens are the most increased.

IV The rhCol-III gel is suitable as a delivery vehicle for primary skin cells. In addition, the gel improves full-thickness wound healing by promoting early granulation tissue formation.

Taken together, new features of the epidermal wound-healing trajectory were identified (I-III), while other aspects in epidermal regeneration may not be as evident as previously believed. Moreover, it enables an objective evaluation of the effects of wound-healing therapies. We also showed that (IV) the use of rhCol-III gel as a cell therapy vehicle for cultured autologous keratinocytes and fibroblasts in an experimental full-thickness wound model were effective.

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9. References

Adamski J, Suhre K. Metabolomics platforms for genome wide association studies--linking the genome to the metabolome. *Curr Opin Biotechnol*. 2013;24:39-47.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. *Molecular Biology of the Cell*. 4th edition. New York: Garland Science; 2002. Extracellular Control of Cell Division, Cell Growth, and Apoptosis.

Arai K, Yamazaki M, Maeda T, Okura T, Tsuboi R. Influence of various treatments including povidone-iodine and healing stimulatory reagents in a rabbit ear wound model. *Int Wound J*. 2012;9

Archer SB, Henke A, Greenhalgh DG, Warden GD. The use of sheet autografts to cover extensive burns in patients. *J Burn Care Rehabil*. 1998;19:33-8.

Báez J, Olsen D, Polarek JW. Recombinant microbial systems for the production of human collagen and gelatin. *Appl Microbiol Biotechnol*. 2005;69(3):245-52.

Ballestar E, Esteller M. Epigenetic gene regulation in cancer. *Adv Genet*. 2008;61:247-67.

Bannasch H, Horch RE, Tánczos E, Stark GB. Treatment of chronic wounds with cultured autologous keratinocytes as suspension in fibrin glue. *Zentralbl Chir*. 2000;125:79-81.

Barber C, Watt A, Pham C, Humphreys K, Penington A, Mutimer K, Edwards M, Maddern G. Influence of bioengineered skin substitutes on diabetic foot ulcer and venous leg ulcer outcomes. *J Wound Care*. 2008;17:517-27.

Barnes M, Freudenberg J, Thompson S, Aronow B, Pavlidis P. Experimental comparison and cross-validation of the Affymetrix and Illumina gene expression analysis platforms. *Nucleic Acids Res*. 2005;33:5914-23.

Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen*. 2008;16:585-601.

Bartlett JM, Stirling D. A short history of the polymerase chain reaction. *Methods Mol Biol*. 2003;226:3-6.

Behm B, Babilas P, Landthaler M, Schreml S. Cytokines, chemokines and growth factors in wound healing. *J Eur Acad Dermatol Venereol*. 2012;26:812-20.

Bello YM, Falabella AF, Eaglstein WH. Tissue-engineered skin. Current status in wound healing. *Am J Clin Dermatol*. 2001;2:305-13.

Benavides F, Oberyszyn TM, VanBuskirk AM, Reeve VE, Kusewitt DF. The hairless mouse in skin research. *J Dermatol Sci*. 2009;53:10-8.

Berthiaume F, Maguire TJ, Yarmush ML. Tissue engineering and regenerative medicine: history, progress, and challenges. *Annu Rev Chem Biomol Eng*. 2011;2:403-30.

Bikle DD. Vitamin D metabolism and function in the skin. *Mol Cell Endocrinol*. 2011;347:80-9.

Blanpain C, Fuchs E. Epidermal stem cells of the skin. *Annu Rev Cell Dev Biol*. 2006;22:339-73.

Blanpain C, Fuchs E. Epidermal homeostasis: a balancing act of stem cells in the skin. *Nat Rev Mol Cell Biol*. 2009;10:207-17.

Bloor BK, Tidman N, Leigh IM, Odell E, Dogan B, Wollina U, Ghali L, Waseem A. Expression of keratin K2e in cutaneous and oral lesions: association with keratinocyte activation, proliferation, and keratinization. *Am J Pathol*. 2003;162:963-75.

Boehnke K, Falkowska-Hansen B, Stark HJ, Boukamp P. Stem cells of the human epidermis and their niche: composition and function in epidermal regeneration and carcinogenesis. *Carcinogenesis*. 2012;33:1247-58.

Boulais N, Misery L. The epidermis: a sensory tissue. *Eur J Dermatol*. 2008;18:119-27.

Boyce ST, Kagan RJ, Yakuboff KP, Meyer NA, Rieman MT, Greenhalgh DG, Warden GD. Cultured skin substitutes reduce donor skin harvesting for closure of excised, full-thickness burns. *Ann Surg*. 2002;235:269-79.

Brandner JM, Haftekand M, Niessen CM. Adherens Junctions, Desmosomes and Tight Junctions in Epidermal Barrier Function. *Open Dermatology J*. 2010;4:14-20

Branski LK, Pereira CT, Herndon DN, Jeschke MG. Gene therapy in wound healing: present status and future directions. *Gene Ther*. 2007;14:1-10.

Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FC, Kim IF, Markowitz V, Matese JC, Parkinson H, Robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R, Vilo J, Vingron M. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. *Nat Genet*. 2001;29:365-71.

Breitkreutz D, Mirancea N, Nischt R. Basement membranes in skin: unique matrix structures with diverse functions? *Histochem Cell Biol*. 2009;132:1-10.

Brinton LT, Brentnall TA, Smith JA, Kelly KA. Metastatic biomarker discovery through proteomics. *Cancer Genomics Proteomics*. 2012;9:345-55.

Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol*. 2005;3:238-50.

Broughton G, Janis JE, Attinger CE. Wound healing: an overview. *Plast Reconstr Surg*. 2006;117:1e-S-32e-S.

Böttcher-Haberzeth S, Biedermann T, Reichmann E. Tissue engineering of skin. *Burns*. 2012;36:450-60.

Calonje B, Brenn T, Lazar, McKee P. *McKee's Pathology of the Skin*, 4th Edition. Saunders, 2011. The Structure and Function of Skin.

Candi E, Schmidt R, Melino G. The cornified envelope: a model of cell death in the skin. *Nat Rev Mol Cell Biol*. 2005;6:328-40.

Carsin H, Ainaud P, Le Bever H, Rives J, Lakhel A, Stephanazzi J, Lambert F, Perrot J. Cultured epithelial autografts in extensive burn coverage of severely traumatized patients: a five year single-center experience with 30 patients. *Burns*. 2000;26:379-87.

Carter MJ, Fylling CP, Li WW, de Leon J, Driver VR, Serena TE, Wilson J. Analysis of run-in and treatment data in a wound outcomes registry: clinical impact of topical platelet-rich plasma gel on healing trajectory. *Int Wound J*. 2011;8:638-50.

Cen L, Liu W, Cui L, Zhang W, Cao Y. Collagen tissue engineering: development of novel biomaterials and applications. *Pediatr Res*. 2008;63(5):492-6.

Cervelli V, De Angelis B, Balzani A, Colicchia G, Spallone D, Grimaldi M. Treatment of stable vitiligo by ReCell system. *Acta Dermatovenerol Croat*. 2009;17:273-8.

Cervelli V, Brinci L, Spallone D, Tati E, Palla L, Lucarini L, De Angelis B. The use of MatriDerm® and skin grafting in post-traumatic wounds. *Int Wound J*. 2011;8:400-5.

Chan JK, Roth J, Oppenheim JJ, Tracey KJ, Vogl T, Feldmann M, Horwood N, Nanchahal J. Alarmins: awaiting a clinical response. *J Clin Invest*. 2012;122:2711-9.

Chang TW. Binding of cells to matrixes of distinct antibodies coated on solid surface. *J Immunol Methods.* 1983;65:217-23.

Chen L, Arbieva ZH, Guo S, Marucha PT, Mustoe TA, DiPietro LA. Positional differences in the wound transcriptome of skin and oral mucosa. *BMC Genomics*. 2010;11:471.

Chester DL, Balderson DS, Papini RP. A review of keratinocyte delivery to the wound bed. *J Burn Care Rehabil*. 2004;25:266-75.

Chmielowiec J, Borowiak M, Morkel M, Stradal T, Munz B, Werner S, Wehland J, Birchmeier C, Birchmeier W. c-Met is essential for wound healing in the skin. *J Cell Biol*. 2007;177:151-62.

Cole J, Tsou R, Wallace K, Gibran N, Isik F. Early gene expression profile of human skin to injury using high-density cDNA microarrays. *Wound Repair Regen*. 2001;9:360–370.

Conway K, Ruge F, Price P, Harding KG, Jiang WG. Hepatocyte growth factor regulation: an integral part of why wounds become chronic. *Wound Repair Regen*. 2007;15:683-92.

De Angelis B, Migner A, Lucarini L, Agovino A, Cervelli V. The use of a non cultured autologous cell suspension to repair chronic ulcers. *Int Wound J*. 2013.

Deonarine K, Panelli MC, Stashower ME, Jin P, Smith K, Slade HB, Norwood C, Wang E, Marincola FM, Stroncek DF. Gene expression profiling of cutaneous wound healing. *J Transl Med*. 2007;5:11.

Dorsett-Martin WA. Rat models of skin wound healing: a review. *Wound Repair Regen*. 2004;12:591-9.

Driver VR, Hanft J, Fylling CP, Beriou JM; Autologel Diabetic Foot Ulcer Study Group. A prospective, randomized, controlled trial of autologous platelet-rich plasma gel for the treatment of diabetic foot ulcers. *Ostomy Wound Manage*. 2006;52:68-70, 72, 74.

Durinck S, Moreau Y, Kasprzyk A, Davis S, De Moor B, Brazma A, Huber W. BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. *Bioinformatics*. 2005;21:3439-40.

Eckert RL, Broome AM, Ruse M, Robinson N, Ryan D, Lee K. S100 proteins in the epidermis. *J Invest Dermatol*. 2004;123:23-33.

Edmonds ME, Foster AV, McColgan M. 'Dermagraft': a new treatment for diabetic foot ulcers. *Diabet Med*. 1997;14:1010-1.

Edmonds M; European and Australian Apligraf Diabetic Foot Ulcer Study Group. Apligraf in the treatment of neuropathic diabetic foot ulcers. *Int J Low Extrem Wounds*. 2009;8:11-8.

Eming SA, Krieg T, Davidson JM. Gene therapy and wound healing. *Clin Dermatol*. 2007;25:79-92.

Enoch S, Leaper DJ. Basic science of wound healing. *Surgery*. 2008;26:31-37.

Ferreira AM, Gentile P, Chiono V, Ciardelli G. Collagen for bone tissue regeneration. *Acta Biomater*. 2012;8:3191-200.

Fivenson D, Scherschun L. Clinical and economic impact of Apligraf for the treatment of nonhealing venous leg ulcers. *Int J Dermatol*. 2003;42:960-5.

Fredriksson C, Kratz G, Huss F. Transplantation of cultured human keratinocytes in single cell suspension: a comparative in vitro study of different application techniques. *Burns*. 2008;34:212-9.

Fuchs E. Scratching the surface of skin development. *Nature*. 2007;445:834-42.

Fuchs E. Finding one's niche in the skin. *Cell Stem Cell*. 2009;4:499-502.

Furuyama A, Mochitate K. Assembly of the exogenous extracellular matrix during basement membrane formation by alveolar epithelial cells in vitro. *J Cell Sci*. 2000;113:859-68.

Gabrilovich DI. INGN 201 (Advexin): adenoviral p53 gene therapy for cancer. Expert *Opin Biol Ther*. 2006;6:823-32.

Gallo RL, Hooper LV. Epithelial antimicrobial defence of the skin and intestine. *Nat Rev Immunol*. 2012;12:503-16.

Gentile A, Comoglio PM. Invasive growth: a genetic program. *Int J Dev Biol*. 2004;48:451-6.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol*. 2004;5:R80.

Gerlach JC, Johnen C, Ottoman C, Bräutigam K, Plettig J, Belfekroun C, Münch S, Hartmann B. Method for autologous single skin cell isolation for regenerative cell spray transplantation with non-cultured cells. *Int J Artif Organs*. 2011;34:271-9.

Glowacki J, Mizuno S. Collagen scaffolds for tissue engineering. *Biopolymers*. 2008;89(5):338-44.

Graham ID, Harrison MB, Nelson EA, Lorimer K, Fisher A. Prevalence of lower-limb ulceration: a systematic review of prevalence studies. *Adv Skin Wound Care*. 2003;16:305-16.

Gravante G, Di Fede MC, Araco A, Grimaldi M, De Angelis B, Arpino A, Cervelli V, Montone A. A randomized trial comparing ReCell system of epidermal cells delivery versus classic skin grafts for the treatment of deep partial thickness burns. *Burns*. 2007;33:966-72.

Greco JA, Pollins AC, Boone BE, Levy SE, Nanney LB. A microarray analysis of temporal gene expression profiles in thermally injured skin. *Burns.* 2010;36:192–204.

Green KJ, Simpson CL. Desmosomes: new perspectives on a classic. *J Invest Dermatol*. 2007;127:2499-515

Gregor S, Maegele M, Sauerland S, Krahn JF, Peinemann F, Lange S. Negative pressure wound therapy: a vacuum of evidence? *Arch Surg*. 2008;143:189-96.

Guenou H, Nissan X, Larcher F, Feteira J, Lemaitre G, Saidani M, Del Rio M, Barrault CC, Bernard FX, Peschanski M, Baldeschi C, Waksman G. Human embryonic stem-cell derivatives for full reconstruction of the pluristratified epidermis: a preclinical study. *Lancet*. 2009;374:1745-53.

Gunderson KL, Kruglyak S, Graige MS, Garcia F, Kermani BG, Zhao C, Che D, Dickinson T, Wickham E, Bierle J, Doucet D, Milewski M, Yang R, Siegmund C, Haas J, Zhou L, Oliphant A, Fan JB, Barnard S, Chee MS. Decoding randomly ordered DNA arrays. *Genome Res*. 2004;14:870-7.

Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. *Nature*. 2008;453:314-21.

Günter CI, Machens HG. New strategies in clinical care of skin wound healing. *Eur Surg Res*. 2012;49:16-23.

Hannuksela M, Peltonen S, Reunala T, Suhonen R. Ihotaudit. Kustannus oy Duodecim, 2011.

Hansbrough JF, Mozingo DW, Kealey GP, Davis M, Gidner A, Gentzkow GD. Clinical trials of a biosynthetic temporary skin replacement, Dermagraft-Transitional Covering, compared with cryopreserved human cadaver skin for temporary coverage of excised burn wounds. *J Burn Care Rehabil*. 1997;18:43-51.

Hansen SL, Voigt DW, Wiebelhaus P, Paul CN. Using skin replacement products to treat burns and wounds. *Adv Skin Wound Care*. 2001;14:37-44.

Harding KG, Morris HL, Patel GK. Science, medicine and the future: healing chronic wounds. *BMJ*. 2002;324:160-3.

Harding K, Sumner M, Cardinal M. A prospective, multicentre, randomised controlled study of human fibroblast-derived dermal substitute (Dermagraft) in patients with venous leg ulcers. *Int Wound J*. 2013;10:132-7.

Hardwicke J, Schmaljohann D, Boyce D, Thomas D. Epidermal growth factor therapy and wound healing--past, present and future perspectives. *Surgeon*. 2008;6:172-7.

Hartmann B, Ekkernkamp A, Johnen C, Gerlach JC, Belfekroun C, Küntscher MV. Sprayed cultured epithelial autografts for deep dermal burns of the face and neck. *Ann Plast Surg*. 2007;58:70-3.

Haslik W, Kamolz LP, Manna F, Hladik M, Rath T, Frey M. Management of full-thickness skin defects in the hand and wrist region: first long-term experiences with the dermal matrix Matriderm. *J Plast Reconstr Aesthet Surg*. 2010;63:360-4.

Henry J, Toulza E, Hsu CY, Pellerin L, Balica S, Mazereeuw-Hautier J, Paul C, SerreG, Jonca N, Simon M. Update on the epidermal differentiation complex. *Front Biosci*. 2012;17:1517-32.

Herndon D. Total Burn Care, 4th Edition. Saunders;2012.

Honeycutt KA, Roop DR. c-Myc and epidermal stem cell fate determination. *J Dermatol*. 2004;31:368-75.

Horch RE, Debus M, Wagner G, Stark GB. Cultured human keratinocytes on type I collagen membranes to reconstitute the epidermis. *Tissue Eng*. 2000;6:53-67.

Hume AN, Seabra MC. Melanosomes on the move: a model to understand organelle dynamics. *Biochem Soc Trans*. 2011;39:1191-6.

Ignotz RA, Massagué J. Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol Chem*. 1986;261:4337-45.

Iorio ML, Shuck J, Attinger CE. Wound healing in the upper and lower extremities: a systematic review on the use of acellular dermal matrices. *Plast Reconstr Surg*. 2012;130:232S-41S.

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res*. 2003;31:e15.

Jackson WM, Nesti LJ, Tuan RS. Concise review: clinical translation of wound healing therapies based on mesenchymal stem cells. *Stem Cells Transl Med*. 2012a;1:44-50.

Jackson WM, Nesti LJ, Tuan RS. Mesenchymal stem cell therapy for attenuation of scar formation during wound healing. *Stem Cell Res Ther*. 2012b;3:20.

Jeschke MG, Schubert T, Krickhahn M, Polykandriotis E, Klein D, Perez-Polo JR, Przkora R, Herndon DN. Interaction of exogenous liposomal insulin-like growth factor-I cDNA gene transfer with growth factors on collagen expression in acute wounds. *Wound Repair Regen*. 2005;13:269-77.

Johnen C, Steffen I, Beichelt D, Bräutigam K, Witascheck T, Toman N, Moser V, Ottomann C, Hartmann B, Gerlach JC. Culture of subconfluent human fibroblasts and keratinocytes using biodegradable transfer membranes. *Burns*. 2008;34:655-63.

Johnson RM, Richard R. Partial-thickness burns: identification and management. *Adv Skin Wound Care*. 2003;16:178-87; quiz 188-9.

Jonca N, Leclerc EA, Caubet C, Simon M, Guerrin M, Serre G. Corneodesmosomes and corneodesmosin: from the stratum corneum cohesion to the pathophysiology of genodermatoses. *Eur J Dermatol*. 2011;21:35-42.

Joo CK, Seomun Y. Matrix metalloproteinase (MMP) and TGF beta 1-stimulated cell migration in skin and cornea wound healing. *Cell Adh Migr*. 2008;2:252-3.

Kalinin AE, Kajava AV, Steinert PM. Epithelial barrier function: assembly and structural features of the cornified cell envelope. *Bioessays*. 2002;24:789-800.

Kanitakis J. Anatomy, histology and immunohistochemistry of normal human skin. *Eur J Dermatol*. 2002;12:390-9.

Keck M, Haluza D, Lumenta DB, Burjak S, Eisenbock B, Kamolz LP, Frey M. Construction of a multi-layer skin substitute: Simultaneous cultivation of keratinocytes and preadipocytes on a dermal template. *Burns*. 2011;37:626-30.

Kirschner N, Houdek P, Fromm M, Moll I, Brandner JM. Tight junctions form a barrier in human epidermis. *Eur J Cell Biol*. 2010;89:839-42.

Kirschner N, Brandner JM. Barriers and more: functions of tight junction proteins in the skin. *Ann N Y Acad Sci*. 2012;1257:158-66.

Kirsner RS, Marston WA, Snyder RJ, Lee TD, Cargill DI, Slade HB. Spray-applied cell therapy with human allogeneic fibroblasts and keratinocytes for the treatment of chronic venous leg ulcers: a phase 2, multicentre, double-blind, randomised, placebo-controlled trial. *Lancet*. 2012;380:977-85.

Kiwanuka E, Junker J, Eriksson E. Harnessing growth factors to influence wound healing. *Clin Plast Surg*. 2012;39:239-48.

Ko MS, Marinkovich MP. Role of dermal-epidermal basement membrane zone in skin, cancer, and developmental disorders. *Dermatol Clin*. 2010;28:1-16.

Kolář M, Szabo P, Dvořánková B, Lacina L, Gabius HJ, Strnad H, Sáchová J, Vlček C, Plzák J, Chovanec M, Cada Z, Betka J, Fík Z, Pačes J, Kovářová H, Motlík J, Jarkovská K, Smetana K Jr. Upregulation of IL-6, IL-8 and CXCL-1 production in dermal fibroblasts by normal/malignant epithelial cells in vitro: Immunohistochemical and transcriptomic analyses. *Biol Cell*. 2012;104:738-51.

Kondo T, Ishida Y. Molecular pathology of wound healing. *Forensic Sci Int*. 2010;203:93-8.

Kurokawa I, Takahashi K, Moll I, Moll R. Expression of keratins in cutaneous epithelial tumors and related disorders--distribution and clinical significance. *Exp Dermatol*. 2011;20:217-28.

Kypriotou M, Huber M, Hohl D. The human epidermal differentiation complex: cornified envelope precursors, S100 proteins and the 'fused genes' family. *Exp Dermatol*. 2012;21:643-9.

Lawall H. Treatment of chronic wounds. *Vasa*. 2012;41:396-409.

Leask A, Denton CP, Abraham DJ. Insights into the molecular mechanism of chronic fibrosis: the role of connective tissue growth factor in scleroderma. *J Invest Dermatol*. 2004;122:1-6.

Lees JG, Ching YW, Adams DH, Bach CT, Samuel MS, Kee AJ, Hardeman EC, Gunning P, Cowin AJ, O'Neill GM. Tropomyosin Regulates Cell Migration during Skin Wound Healing. *J Invest Dermatol*. 2013;133:1330-9.

Li J, Chen J, Kirsner R. Pathophysiology of acute wound healing. *Clin Dermatol*. 2007;25:9-18.

Lin ZQ, Dong YZ, Zhang XD, Wang T, Sun KL, Niu WY. Effect of interleukin-6 on gene expression of certain cytokines during wound healing process of mouse skin. *Yi Chuan Xue Bao*. 2005;32:46-51.

Lindford AJ, Frey I, Vuola J, Koljonen V. Evolving practice of the Helsinki Skin Bank. *Int Wound J*. 2010;7:277-81.

Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, Brown EL. Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol*. 1996;14:1675-80.

Madison KC. Barrier function of the skin: "la raison d'être" of the epidermis. *J Invest Dermatol*. 2003;121:231-41.

Maher CA, Kumar-Sinha C, Cao X, Kalyana-Sundaram S, Han B, Jing X, Sam L, Barrette T, Palanisamy N, Chinnaiyan AM. Transcriptome sequencing to detect gene fusions in cancer. *Nature*. 2009;458:97-101.

Mansbridge J. Skin tissue engineering. *J Biomater Sci Polym Ed*. 2008;19:955-68.

Marenholz I, Zirra M, Fischer DF, Backendorf C, Ziegler A, Mischke D. Identification of human epidermal differentiation complex (EDC)-encoded genes by subtractive hybridization of entire YACs to a gridded keratinocyte cDNA library. *Genome Res*. 2001;11:341-55.

Marks MS, Seabra MC. The melanosome: membrane dynamics in black and white. *Nat Rev Mol Cell Biol*. 2001;2:738-48.

Martin P. Wound healing-aiming for perfect skin regeneration. *Science*. 1997;276:75-81.

Maurer M, Lopez Kostka S, Siebenhaar F, Moelle K, Metz M, Knop J, von Stebut E. Skin mast cells control T cell-dependent host defense in Leishmania major infections. *FASEB J*. 2006;20:2460-7.

McLafferty E, Hendry C, Alistair F. The integumentary system: anatomy, physiology and function of skin. *Nurs Stand*. 2012;27:35-42.

Menon GK, Cleary GW, Lane ME. The structure and function of the stratum corneum. *Int J Pharm*. 2012;435:3-9.

Metcalfe AD, Ferguson MW. Tissue engineering of replacement skin: the crossroads of biomaterials, wound healing, embryonic development, stem cells and regeneration. *J R Soc Interface*. 2007;4:413-37.

Mischke D, Korge BP, Marenholz I, Volz A, Ziegler A. Genes encoding structural proteins of epidermal cornification and S100 calcium-binding proteins form a gene complex ("epidermal differentiation complex") on human chromosome 1q21. *J Invest Dermatol*. 1996;106:989-92.

Montagna W, Yun JS. The skin of the domestic pig. *J Invest Dermatol*. 1964;42:11-21.

Morita K, Miyachi Y, Furuse M. Tight junctions in epidermis: from barrier to keratinization. *Eur J Dermatol*. 2011;21:12-7.

Morrison SJ, Kimble J. Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature*. 2006;441:1068-74.

Mustoe TA, O'Shaughnessy K, Kloeters O. Chronic wound pathogenesis and current treatment strategies: a unifying hypothesis. *Plast Reconstr Surg*. 2006;117:35S-41S.

Mutz KO, Heilkenbrinker A, Lönne M, Walter JG, Stahl F. Transcriptome analysis using next-generation sequencing. *Curr Opin Biotechnol*. 2013;24:22-30.

Niezgoda JA, Van Gils CC, Frykberg RG, Hodde JP. Randomized clinical trial comparing OASIS Wound Matrix to Regranex Gel for diabetic ulcers. *Adv Skin Wound Care*. 2005;18:258-66.

Okuyama R, Tagami H, Aiba S. Notch signaling: its role in epidermal homeostasis and in the pathogenesis of skin diseases. *J Dermatol Sci*. 2008;49:187-94.

Orgill DP. Excision and skin grafting of thermal burns. *N Engl J Med*. 2009;360:893-901.

Orgill DP, Bayer LR. Update on negative-pressure wound therapy. *Plast Reconstr Surg*. 2011;127:105S-115S.

Pandya AN, Woodward B, Parkhouse N. The use of cultured autologous keratinocytes with integra in the resurfacing of acute burns. *Plast Reconstr Surg*. 1998;102:825-30.

Peirce SM, Skalak TC, Rodeheaver GT. Ischemia-reperfusion injury in chronic pressure ulcer formation: a skin model in the rat. *Wound Repair Regen*. 2000;8:68-76.

Peng Z. Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers. *Hum Gene Ther*. 2005;16:1016-27.

Percival NJ. Classification of Wounds and their Management. *Surgery (Oxford)*. 2002;20:114-117.

Pham C, Greenwood J, Cleland H, Woodruff P, Maddern G. Bioengineered skin substitutes for the management of burns: a systematic review. *Burns*. 2007;33:946-57.

Polat BE, Blankschtein D, Langer R. Low-frequency sonophoresis: application to the transdermal delivery of macromolecules and hydrophilic drugs. *Expert Opin Drug Deliv*. 2010;7:1415-32.

Quackenbush J. Microarray analysis and tumor classification. *N Engl J Med*. 2006;354:2463-72.

Ramanauskaitė G, Kašėta V, Vaitkuvienė A, Biziulevičienė G. Skin regeneration with bone marrow-derived cell populations. *Int Immunopharmacol*. 2010;10:1548-51.

Ramshaw JA, Peng YY, Glattauer V, Werkmeister JA. Collagens as biomaterials. *J Mater Sci Mater Med*. 2009;20 Suppl 1:S3-8

Reinisch CM, Tschachler E. The touch dome in human skin is supplied by different types of nerve fibers. *Ann Neurol*. 2005;58:88-95.

Reinke JM, Sorg H. Wound repair and regeneration. *Eur Surg Res*. 2012;49:35-43.

Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell*. 1975;6:331-43.

Ritchie ME, Diyagama D, Neilson J, van Laar R, Dobrovic A, Holloway A, Smyth GK. Empirical array quality weights in the analysis of microarray data. *BMC Bioinformatics*. 2006;7:261.

Rittié L, Sachs DL, Orringer JS, Voorhees JJ, Fisher GJ. Eccrine sweat glands are major contributors to reepithelialization of human wounds. *Am J Pathol*. 2013;182:163-71.

Ronfard V, Broly H, Mitchell V, Galizia JP, Hochart D, Chambon E, Pellerin P, Huart JJ. Use of human keratinocytes cultured on fibrin glue in the treatment of burn wounds. *Burns*. 1991;17:181-4.

Rosenthal FM, Cao L, Tanczos E, Kopp J, Andree C, Stark GB, Mertelsmann R, Kulmburg P. Paracrine stimulation of keratinocytes in vitro and continuous delivery of epidermal growth factor to wounds in vivo by genetically modified fibroblasts transfected with a novel chimeric construct. *In Vivo*. 1997;11:201-8.

Räty JK, Pikkarainen JT, Wirth T, Ylä-Herttuala S. Gene therapy: the first approved gene-based medicines, molecular mechanisms and clinical indications. *Curr Mol Pharmacol*. 2008;1:13-23.

Salem HK, Thiemermann C. Mesenchymal stromal cells: current understanding and clinical status. *Stem Cells*. 2010;28:585-96.

Schauber J, Gallo RL. Antimicrobial peptides and the skin immune defense system. J *Allergy Clin Immunol*. 2009;124:R13-8.
Schintler MV. Negative pressure therapy: theory and practice. *Diabetes Metab Res Rev*. 2012;28:72-7.

Semi K, Matsuda Y, Ohnishi K, Yamada Y. Cellular reprogramming and cancer development. *Int J Cancer*. 2013;132:1240-8.

Sen CK, Gordillo GM, Roy S, Kirsner R, Lambert L, Hunt TK, Gottrup F, Gurtner GC, Longaker MT. Human skin wounds: a major and snowballing threat to public health and the economy. *Wound Repair Regen*. 2009;17:763-71.

Shaw L, Wiedow O. Therapeutic potential of human elafin. *Biochem Soc Trans*. 2011;39:1450-4.

Shaw TJ, Martin P. Wound repair at a glance. *J Cell Sci*. 2009;122:3209-13.

Sibbald RG, Torrance G, Hux M, Attard C, Milkovich N. Cost-effectiveness of becaplermin for nonhealing neuropathic diabetic foot ulcers. *Ostomy Wound Manage*. 2003;49:76-84.

Singer AJ, Clark RA. Cutaneous wound healing. *N Engl J Med.* 1999;341:738-46.

Smiley AK, Klingenberg JM, Aronow BJ, Boyce ST, Kitzmiller WJ, Supp DM. Microarray analysis of gene expression in cultured skin substitutes compared with native human skin. *J Invest Dermatol*.2005*;*125*:*1286–1301.

Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004;3.

Stojadinovic O, Brem H, Vouthounis C, Lee B, Fallon J, Stallcup M, Merchant A, Galiano RD, Tomic-Canic M. Molecular pathogenesis of chronic wounds: the role of beta-catenin and c-myc in the inhibition of epithelialization and wound healing. *Am J Pathol*. 2005;167:59-69.

Stoltz JF, Decot V, Huseltein C, He X, Zhang L, Magdalou J, Li YP, Menu P, Li N, Wang YY, de Isla N, Bensoussan D. Introduction to regenerative medicine and tissue engineering. *Biomed Mater Eng*. 2012;22:3-16.

Storey JD, Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci U S A*. 2003;100:9440-5.

Sullivan TP, Eaglstein WH, Davis SC, Mertz P. The pig as a model for human wound healing. *Wound Repair Regen.* 2001*;*9*:*66-76.

Sun DP, Yeh CH, So E, Wang LY, Wei TS, Chang MS, Hsing CH. Interleukin (IL)-19 promoted skin wound healing by increasing fibroblast keratinocyte growth factor expression. *Cytokine*. 2013

Sze D. PCR Technology: Current Innovations. *Australian J Med Sci*. 2004;25:192.

van Bergen BH, Andriessen MP, Spruijt KI, van de Kerkhof PC, Schalkwijk J. Expression of SKALP/elafin during wound healing in human skin. *Arch Dermatol Res*. 1996;288:458-62.

van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van der Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, Bernards R. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med*. 2002;347:1999-2009.

Vardaxis NJ, Brans TA, Boon ME, Kreis RW, Marres LM. Confocal laser scanning microscopy of porcine skin: implications for human wound healing studies. *J Anat*. 1997;190:601-11.

Villaseñor-Park J, Ortega-Loayza AG. Microarray technique, analysis, and applications in dermatology. *J Invest Dermatol*. 2013;133:e7.

Vogel JC. Nonviral skin gene therapy. *Hum Gene Ther*. 2000;11:2253-9.

Vogt PM, Thompson S, Andree C, Liu P, Breuing K, Hatzis D, Brown H, Mulligan RC, Eriksson E. Genetically modified keratinocytes transplanted to wounds reconstitute the epidermis. *Proc Natl Acad Sci U S A*. 1994;91:9307-11.

Watt FM. Epidermal stem cells: markers, patterning and the control of stem cell fate. *Philos Trans R Soc Lond B Biol Sci*. 1998;353:831-7.

Webster MJ. Tissue preparation and banking. *Prog Brain Res*. 2006;158:3-14.

Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. *Physiol Rev*. 2003;83:835-70.

Wickett R, Visscher M. Structure and function of the epidermal barrier, American *Journal of Infection Control*. 2006;34:98-110

Wiedow O, Schröder JM, Gregory H, Young JA, Christophers E. Elafin: an elastase-specific inhibitor of human skin. Purification, characterization, and complete amino acid sequence. *J Biol Chem*. 1990;265:14791-5.

Wieman TJ, Smiell JM, Su Y. Efficacy and safety of a topical gel formulation of recombinant human platelet-derived growth factor-BB (becaplermin) in patients with chronic neuropathic diabetic ulcers. A phase III randomized placebo-controlled double-blind study. *Diabetes Care*. 1998;21:822-7.

Williams IR, Kupper TS. Immunity at the surface: homeostatic mechanisms of the skin immune system. *Life Sci*. 1996;58:1485-507.

Wolfram D, Tzankov A, Pülzl P, Piza-Katzer H. Hypertrophic scars and keloids--a review of their pathophysiology, risk factors, and therapeutic management. *Dermatol Surg*. 2009;35:171-81.

Wong VW, Gurtner GC. Tissue engineering for the management of chronic wounds: current concepts and future perspectives. *Exp Dermatol*. 2012;21:729-34.

Wood FM, Kolybaba ML, Allen P. The use of cultured epithelial autograft in the treatment of major burn wounds: eleven years of clinical experience. *Burns*. 2006;32:538-44.

Wood F. Tissue engineering of skin. *Clin Plast Surg*. 2012;39:21-32.

Wood FM, Giles N, Stevenson A, Rea S, Fear M. Characterisation of the cell suspension harvested from the dermal epidermal junction using a ReCell® kit. *Burns*. 2012;38:44-51.

Yang C, Hillas PJ, Báez JA, Nokelainen M, Balan J, Tang J, Spiro R, Polarek JW. The application of recombinant human collagen in tissue engineering. *BioDrugs*. 2004;18:103-19.

Yang D, Oppenheim JJ. Antimicrobial proteins act as "alarmins" in joint immune defense. *Arthritis Rheum*. 2004;50:3401-3.

Yardley HJ, Summerly R. Lipid composition and metabolism in normal and diseased epidermis. *Pharmacol Ther*. 1981;13:357-83.

Zcharia E, Zilka R, Yaar A, Yacoby-Zeevi O, Zetser A, Metzger S, Sarid R, Naggi A, Casu B, Ilan N, Vlodavsky I, Abramovitch R. Heparanase accelerates wound angiogenesis and wound healing in mouse and rat models. *FASEB J.* 2005;19:211-221.

Zhong S, Zhang Y, Lim C. Tissue scaffolds for skin wound healing and dermal reconstruction. *WIREs Nanomedicine and Nanobiotechnology* .2010;2:510-525.

Zimmer DB, Cornwall EH, Landar A, Song W. The S100 protein family: history, function, and expression. *Brain Res Bull*. 1995;37:417-29.