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APPLICATIONS OF HUMAN SKIN *IN VITRO*

Susanna Lönnqvist

Division of Clinical Sciences
Department of Clinical and Experimental Medicine
Medical Faculty
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Siis älä siitä huoli, näinhän täällä käy

Aurinkokin paistaa, vaikkei sitä näy

(Eppu Normaali, 1993)

So don't you worry about it, that's the way things go

The sun is still shining, even though it doesn't show

SUPERVISOR

Gunnar Kratz, Professor

Department of Clinical and Experimental Medicine
Linköping University

CO-SUPERVISOR

Magnus Berggren, Professor

Department of Science and Technology
Linköping University

FACULTY OPPONENT

Jyrki Vuola, Associate Professor

Institute of Clinical Medicine
Helsinki University

ABSTRACT

Chronic wounds are a substantial problem in today's health care and place significant strains on the patient. Successful modelling of the wound healing process is pivotal for the advancement of wound treatment research. Wound healing is a dynamic and multifactorial process involving all constituents of the skin. The progression from haemostasis and inflammation to proliferation of epidermal keratinocytes and dermal fibroblasts, and final scar maturation can be halted and result in a chronic wound that fails to re-epithelialise. The wound healing process constitutes an example of dynamic reciprocity in tissue where cellular changes take place on cues from the extracellular matrix and vice versa when tissue homeostasis is disturbed. The extracellular matrix provides a structural context for the resident cells and the epidermal keratinocytes, and a functioning interplay between the two tissue compartments is crucial for successful wound healing to take place. Work included in this thesis has applied viable human full thickness skin *in vitro* to investigate the re-epithelialisation process and barrier function of intact skin. The use of full thickness skin *in vitro* can take into account the contextual aspect of the process where the epidermal keratinocytes are activated and obtain a migratory phenotype, and are continuously dependent on the cues from the extracellular matrix and support of the dermis. When utilising skin for studies on re-epithelialisation, circular standardised full thickness wounds were created and cultured for up to four weeks in tissue culture. In paper I, the organisation of a thick neoepidermis was investigated in the *in vitro* wound healing model when resident cells were provided with a porous suspended three dimensional gelatin scaffold. In paper II we investigated the use of a fluorescent staining conventionally used for proliferation studies to facilitate the tracing of transplanted epidermal cells in *in vitro* wounds, in order to improve and expand the use of the model. In paper III the model was utilised to investigate the treatment approach of acidification of wounds to evaluate the suitability of such intervention

in regards to keratinocyte function and re-epithelialisation. Studies on re-epithelialisation with the aid of the *in vitro* wound healing model provided insight in neoepidermal structure with porous gelatin scaffolding in the wound, a novel methodological approach to tracing cells and response to constrained wound healing environment. In paper IV, intact human skin was evaluated for modelling the cytotoxic response after exposure to a known irritant compound. To study barrier function, intact skin was exposed to irritants by restricting exposure topically, and full thickness skin *in vitro* was found suitable for modelling cytotoxicity responses. Employing human full thickness skin *in vitro* makes use of the actual target tissue of interest with epidermal and dermal cells, and full barrier function.

Populärvetenskaplig sammanfattning

Då huden skadas och ett sår bildas startas en mängd processer för att hejda ytterligare skada, begränsa infektion och reparera vävnaden för att återskapa den starka barriär som huden bildar mot omvärlden. Sår läkning är en dynamisk process som involverar alla celler och komponenter i huden. Läkningen startar med att blodflödet stillas och ett inflammatoriskt svar tar vid för att rengöra det skadade området. Cellerna i sårets kanter och i underliggande vävnad aktiveras för att reparera skadan. Översta hudlagret, epidermis, består av flera lager av epidermala keratinocyter. Denna celltyp ser till att den strukturella barriären som huden utgör är intakt genom att det i nedersta lagret av epidermis hela tiden bildas nya keratinocyter. Dessa genomgår sedan en process som till slut bildar ett flerskiktat lager av keratinocyter med döda och tåliga keratinocyter allra ytterst. Vid uppkomsten av ett sår aktiveras keratinocyterna och får egenskaper de annars inte uppvisar. De blir mobila då deras mål är att flytta sig ut från sårkanterna, bli flera och till slut täcka över såret med keratinocyter och slutligen ny epidermis. Samtidigt som keratinocyterna täcker in såret jobbar cellerna i det undre hudlagret dermis med att utsöndra komponenter för att möjliggöra keratinocyternas vandring ut från sårkanterna. Dessa celler heter dermala fibroblaster, och de fortsätter med att utsöndra beståndsdelar för den nya vävnaden och bidrar till att bilda de delar som till slut blir ett ärr i huden. De epidermala keratinocyterna och de dermala fibroblasterna är beroende av varandra i sår läkningsprocessen, och de signalerar till varandra och omgivande vävnad under hela förloppet.

Avhandlingens arbeten har riktat in sig på att undersöka hur man kan använda mänsklig hud i laboratoriet för att undersöka sår läkningsprocessen och barriärfunktionen hos hud. Genom att använda sig av hud i en laboratorieuppställning kan man studera händelseförloppet och delar av det genom att ändra vissa detaljer, införa behandling eller ändra miljön som huden och

cellerna i den hålls i. Användning av mänsklig hud möjliggör iakttagandet av samarbetet mellan keratinocyterna och fibroblasterna, och de olika delarna av huden de finns i, vilket inte är möjligt i förenklade modeller av hud. Att hitta bra och representativa sätt att följa sårhäkning är viktigt för att hitta mekanismer vid bildning av sår som inte läker och behandling av dem.

Populaaritieteellinen tiivistelmä

Kun iho vahingoittuu ja syntyy haava, käynnistyy samanaikaisesti useita eri prosesseja haavan parantamiseksi: lisävaurioita pitää välttää, infektioriskiä pienentää ja fyysinen suoja pitää luoda uudestaan. Haavan parantuminen on dynaaminen prosessi johon osallistuvat kaikki ihon eri solutyypit ja osat. Parantuminen alkaa verenvuodon pysähtymisellä ja tulehdusvasteella, jotta vaurioitunut kudos pysyy puhtaana. Ihosolut haavan reunoissa ja alla olevassa kudoksessa aktivoituvat. Ihon ylin kerros, orvaskesi, koostuu keratinosyyteistä. Tämä solutyyppi pitää huolen ihon rakenteellisesta suojasta siten, että orvaskeden alimmassa kerroksessa syntyy jatkuvasti uusia keratinosyyttejä. Nämä solut käyvät läpi eri kehitysmuotoja, jotka johtavat kerroksittaiseen orvaskeden rakenteeseen, ja muodostavat lopuksi orvaskeden ylimmän kerrokseen, jonka solut ovat kuolleita ja hyvin kestäviä. Kun iho vahingoittuu, keratinosyytit aktivoituvat ja ryhtyvät toimimaan normaalista poikkeavalla tavalla. Keratinosyytit muuttuvat liikkuviksi ja niiden tavoitteena on siirtyä haavan reunasta peittämään haava- aluetta. Keratinosyyttejä syntyy lisää ja lopulta koko haavan alue on niiden peitossa ja uuden orvaskeden muodostuminen voi alkaa. Samaan aikaan kun keratinosyytit vaeltavat haavojen reunoilta poispäin, solut alla olevassa haavakudoksessa tuottavat kudososia jotka mahdollistavat tämän vaelluksen. Nämä solut ovat fibroblasteja, sidekudoksessa yleisesti esiintyvää solutyyppiä. Fibroblastit jatkavat uuden sidekudoksen osien tuottamisen ja avustavat niiden osien kanssa, jotka lopulta muodostavat arven ihossa. Keratinosyytit ja fibroblastit ovat riippuvaisia toisistaan koko haavan parantumisen keston ajan ja kommunikoivat toistensa kanssa prosessin kuluessa.

Tässä väitöskirjassa keskitytään tutkimaan miten ihmisihoa voi käyttää laboratoriossa jotta haavan parantumista ja ihon suojan muodostamista voitaisiin tutkia. Ihon käyttö laboratoriossa mahdollistaa haavan parantumisen eri vaiheiden

tutkimisen, koska prosessin eri osia voi silloin muunnella tai niihin voi vaikuttaa hallitusti. Ihon käyttö mahdollistaa myös keratinosyyttien ja fibroblastien yhteistyön tutkimisen, mikä ei ole mahdollista yksinkertaisemmissa iho-malleissa. Hyvien ja edustavien mallintamismuotojen löytyminen on tärkeää kroonisten haavojen ja niiden hoitomuotojen tutkimisessa.

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LIST OF PAPERS

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Paper I

Susanna Lönnqvist, Jonathan Rakar, Kristina Briheim, Gunnar Kratz

Biodegradable gelatin microcarriers facilitate re-epithelialization of human cutaneous wounds – an *in vitro* study in human skin

PLoS One 2015 Jun 10;10(6)

Paper II

Susanna Lönnqvist, Maria Karlsson, Gunnar Kratz

Tracing transplanted human keratinocytes and melanocytes with carboxyfluorescein hydroxysuccinimidyl ester (CFSE) staining

Manuscript

Paper III

Susanna Lönnqvist, Peter Emanuelsson, Gunnar Kratz

Influence of acidic pH on keratinocyte function and re-epithelialisation of human *in vitro* wounds

Journal of Plastic Surgery and Hand Surgery 2015 Jun 7:1-7

Paper IV

Susanna Lönnqvist, Kristina Briheim, Gunnar Kratz

Non-occlusive topical exposure of human skin *in vitro* as model for cytotoxicity testing of irritant compounds

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David Nilsson, Gunnar Kratz, Magnus Berggren

Matrix-Addressing of an Electronic Surface Switch Based on a Conjugated
Polyelectrolyte for Cell Sorting

Advanced Functional Materials 2015 Oct 21

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Grenegård, Gunnar Kratz

Differentiation of human dermal fibroblasts towards endothelial cells

Differentiation 2013 Feb;85(3):67-77

Jonathan Rakar, Susanna Lönnqvist, Pehr Sommar, Johan Junker, Gunnar Kratz

Interpreted gene expression of human dermal fibroblasts after adipo-, chondro-,
and osteogenic phenotype shifts

Differentiation 2012 Nov;84(4):305-13

Abbreviations

α -SMA	α -smooth muscle actin
ANOVA	analysis of variance
bFGF	basic fibroblast growth factor
BPE	bovine pituitary extract
BSA	bovine serum albumin
Ca^{2+}	calcium cation
cDNA	complementary deoxyribonucleic acid
CFSE	carboxyfluorescein hydroxysuccinimidyl ester
CLDN1	claudin 1
COL	collagen
Ct	cycle threshold
DAPI	4', 6-diamino-2-phenylindole
dbcAMP	$\text{N}^6,2'$ -O-Dibutyryl adenosine 3',5'-cyclic monophosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ECVAM	European centre for validation of alternative methods
EDC	epidermal differentiation complex
EDTA	ethylenediamine tetra acetic acid
EGF	epidermal growth factor
FAK	focal adhesion kinase
FCS	foetal calf serum
FM	fibroblast medium
GM-CSF	granulocyte-macrophage colony stimulating factor
H&E	haematoxylin and eosin staining
HMBS	hydroxymethylbilane synthase
HSP27	heat shock protein 27
HSPB1	heat shock 27 kDa protein 1
ICAM-1	intercellular adhesion molecule 1
IFN- γ	interferon γ
IL	interleukin
KER	keratinocyte
KGF	keratinocyte growth factor
Ki-67	nuclear antigen Ki-67

KLK	kallikrein-related peptidase
KRT	keratin
KSFM	keratinocyte serum free medium
MEL	melanocyte
MGM	melanocyte growth medium
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MTT	3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
OCT	optimal cutting temperature compound
PBS	phosphate buffered saline
PC-1	PC-1 ^{TM} supplement
PCL	polycaprolactone
PDGF	platelet derived growth factor
PFA	paraformaldehyde
PGA	polyglycolic acid
PLA	polylactic acid
PTK2	protein tyrosine kinase 2
qRT-PCR	quantitative real time polymerase chain reaction
RGD	arginine-glycine-aspartate tripeptide
RMP	revolutions per minute
RNA	ribonucleic acid
S100A	S100 calcium binding protein A family
SDS	sodium dodecyl sulphate
TGF- α	transforming growth factor α
TGF- β	transforming growth factor β
TIMP1	tissue inhibitor of matrix metalloproteinase 1
TNF- α	tumour necrosis factor α
UV	ultraviolet
VEGF	vascular endothelial growth factor
ZO-1	zona occludens 1

INTRODUCTION

The skin constitutes a physical and molecular barrier against dehydration, pathogens and toxins. *Stratum corneum*, the outermost layer of the skin, forms a lipophilic barrier and together with a band of tight junctions and the innate immune system, comprise the first line of defence against the strains of the outside world. The layered structure compartmentalises the functions of the skin. The uppermost layer, the epidermis, forms the first line of barrier. The dermis underneath provides mechanical strength and possesses barrier, sensory and immune functions. The hypodermis forms the structural integrity of the tissue and provides vascularisation (Figure 1).

The structure of the epidermis is linked to the life cycle of the keratinocyte

The epidermis is a keratinised stratified squamous epithelium composed predominantly of keratinocytes as well as melanocytes, Langerhans' cells and Merkel cells. The epidermis consists of four layers: *stratum basale* with proliferative keratinocytes, *stratum spinosum*, *stratum granulosum*, and the *stratum corneum* (1). An additional layer between *granulosum* and *corneum* called *stratum lucidum* is present in areas of skin highly subjected to friction such as the palms and soles (2). The keratinocytes of the epidermis stem from epidermal stem cells which are present in the *stratum basale*. By dividing they give rise to the transit amplifying cell compartment that ultimately undergoes terminal differentiation and cell death to form the *stratum corneum* of densely packed cell membranes of keratinocytes (3). Keratinocytes express specific sets of keratins during their passage through the epidermis. Keratins are elastic fibrous proteins that form cytoplasmic intermediate filaments and are expressed in heterodimeric pairs with one acidic (type I) and one basic (type II) keratin (4). Basal keratinocytes express keratin 5 (type II) and keratin 14 (type I), and differentiating keratinocytes

express keratin 1, 2 (type II), and 10 (type I) (5). The keratin filament network anchors keratinocytes to the basement membrane through hemidesmosomes in *stratum basale* (6) and to adjacent keratinocytes through desmosomes, intercellular junction complexes associated with intermediate filaments (7), in all layers of epidermis (8). The *stratum granulosum* (Figure 1) consists of three layers of cells and is characterised by the presence of keratohyalin granules consisting of keratin binding proteins, such as filaggrin and loricrin. Together with mainly keratin 1 and 10, the proteins form a dense network of keratin filaments bundles (1). A strand of tight junctions is present in the second layer of cells in the *stratum granulosum*. This structure can be found on the apical side of most sheets of simple epithelium in vertebrates and functions as a sealing to control the paracellular pathways (9) for ions, small molecules and water (10). In the epidermis the cell-cell junctions consist of different transmembrane proteins like claudins (11) and cytosolic plaque proteins like zona occludens (ZO) proteins, out of which claudin 1 (CLDN1) has been shown to contribute strongly to the integrity of the junctional complexes (10). Knock-out mice for CLDN1 die within the first day after birth due to transepithelial water loss (12). The *stratum granulosum* is a transitional zone of the epidermis where the separation of metabolically active layers and the dead *stratum corneum* takes place.

The stratification of the epidermis is tightly connected to the life cycle of the keratinocyte. The keratinocytes of the uppermost layer of the *stratum granulosum* undergo terminal differentiation and a form of programmed cell death called cornification. The end product is the formation of the *stratum corneum* composed of 10-20 layers of enucleated cells forming a supracellular interconnected structure consisting of corneocytes (13). The formation of the *stratum corneum* takes place in three major steps: the replacement of intracellular contents of the keratinocytes by a proteinaceous cytoskeleton, the formation of the cornified envelope and the secretion of extracellular lipids. At the transition zone of the *stratum granulosum*

Figure 1. (A) Haematoxylin and eosin (H&E) staining of human skin with stratified, cornified epidermis and papillary and reticular dermis. (B) Schematic representation of the layers and cells of the epidermis. (C) H&E staining of hair follicle. (D) Schematic drawing of human skin with superficial epidermis, dermis with hair follicle and vessels, and subcutaneous fat of the hypodermis. Images captured or drawn by author, except (B) based on Servier Medical Art (licensed under Creative Commons).

keratinocytes undergo enucleation and morphologically change into flat polygonal cells. The organelles and the keratohyalin granules are degraded. The mechanisms of nuclear degradation and the following disassembly of the organelles are not well understood (14). The dephosphorylation of profilaggrin has been proposed as an initiating step in disassembling the keratin and keratin binding proteins (1, 15). The transition from residing in the *stratum granulosum* to partake in the formation of the *stratum corneum*, is initiated by an increase in intracellular Ca^{2+} (16), which has been shown to induce *in vitro* differentiation of keratinocytes as well (17). In addition, caspase 14 is involved in the process and is activated in the granular layer and contributes to the degradation of filaggrin (15). The keratinocytes form cornified envelopes where the plasma membranes are replaced by protein products of the epidermal differentiation complex (EDC) genes that are highly crosslinked by calcium activated transglutaminases specifically expressed in the cornifying layers (18). The EDC locus on chromosome 1q21 contains genes like loricrin, involucrin and the S100A family, and their expression is used as late maturation markers of the epidermis. The dense assembly of the transglutaminase crosslinked proteins is essential for the barrier function of the skin (19). The final stages of the life cycle of granular keratinocytes include the formation of the cornified envelope and the secretion of the lamellar granules, membrane bound cytoplasmic organelles found in *stratum spinosum* and *granulosum*. The lamellar granules, or lamellar bodies, fuse with the plasma membrane and thereby contribute to the hydration of the epidermis by secreting their contents into the extracellular space. The ceramides, free fatty acids and cholesterol of the lamellar granules seal the superficial granular layers and are responsible for the hydrophobic barrier of the epidermis (20).

The cornified keratinocytes of the *stratum corneum* are joined together through corneodesmosomes, an adhesion complex similar to the desmosome, but characterised by the presence of a unique extracellular component known as

corneodesmosin (21). Produced by keratinocytes and secreted by granular keratinocytes through the lamellar granule pathway, it is localised in the extracellular structures of the corneodesmosomes and cross-linked to the cornified cell envelopes (20). Corneodesmosin is gradually proteolysed under transition in the *stratum corneum* and ultimately cleaved by kallikrein-related peptidases (KLKs) and cathepsins (22). This leads to the desquamation of the epidermal keratinocyte, the final step in the keratinocyte's role as the structural component of the barrier of the epidermis (23).

Other cell types in the epidermis include the pigment producing melanocytes, Langerhans' cells and Merkel cells. Melanocytes are located in the *stratum basale* (24) and make up approximately 5 % of the cells of the epidermis (25). One epidermal-melanin unit consists of one dendritic melanocyte and an average of 36 keratinocytes (26). Once formed, melanin containing granules, called melanosomes, are translocated to the dendritic tips of the melanocytes by active transport (27). The melanosomes are transferred to keratinocytes by shedding vesicle transport and the melanosomes are trapped by microvilli on keratinocytes and incorporated into the cytosol. Once inside the cell, the melanosomes are dispersed with the ultimate goal to form a perinuclear cap in the keratinocytes to protect DNA from UV-light induced damage (28).

The only immune cells resident in the epidermis are the Langerhans' cells. As immature dendritic cells, they phagocytose actively in *stratum spinosum*. In the case of an injury and infection Langerhans' cells migrate to the peripheral lymph nodes, lose the antigen processing capabilities but upregulate major histocompatibility complex (MHC) and present antigens at a high level (29).

Merkel cells are mechanosensory cells located in the basal layer and form synapse-like connections to somatosensory afferents in the dermis where they convey touch

and pressure sensations (30). Merkel cells have been shown to be essential for the fine tuning of the pressure sensation which enables light touch discrimination of textures and edges (31).

The dermis

The connective tissue that constitutes the dermis is produced by dermal fibroblasts. Additional cell types present are macrophages and adipocytes. The dermis harbours nerves, glands, hair follicles and blood vessels that supply the skin and participate in thermal regulation. The most abundant proteins of the extracellular matrix (ECM) of connective tissues, and the whole human body, are collagens (32). By forming trimeric triplehelices that are organised into fibrils, the network formed by collagens has a high structural integrity that accounts for the toughness of skin. The fibril forming collagens I and III are present in the dermis. Basement membranes, and the *stratum basale*, contain collagen IV, which connects to collagens in the dermis via the anchoring fibril collagen VII (33). The elastic properties of the dermis are on account of the presence of micro fibrils of fibrillin with a core of cross-linked elastin. The elastic fibres provide compliance and recoil properties to the tissue and complements the collagen fibrils to form a resilient tissue construction (34). A dermal ECM protein with high importance for cell adhesion and migration is fibronectin (35, 36). The integrin binding RGD motif (arginine-glycine-aspartate tripeptide) was discovered in fibronectin. Fibronectin is an example of how ECM proteins can bind other ECM constituents, growth factors, receptors and adhesion proteins (37), and contribute to both structure and function of the ECM. Integrins are heterodimeric transmembrane receptors that can arrange into 24 different combinations and have high affinities for molecules in the ECM (38). The extrafibrillar matrix of the ECM beyond the fundamental structural proteins consists of glycosaminoglycans like hyaluronic acid, and proteoglycans. Proteoglycans contribute to the resistance to compression of the

dermis by being highly hydrophilic due to the positively charged polysaccharide chains (39, 40).

The structural division of the dermis in two layers is based on the packing of the connective tissue. The most superficial part of the dermis, the papillary dermis, is characterised by areolar connective tissue organised in dermal papillae forming projections into the epidermal side. Tactile receptors, the Meissner corpuscles (41), and free nerve endings are present in the papillae that are responsible for the conduction of sensations of e.g. pain. Somatosensory afferents from the reticular dermis underneath connects to Merkel cells in the basal layer of the epidermis through Merkel discs located in conjunction with the basement membrane, forming Merkel cell-neurite complexes making up a direct contact between the two tissue compartments (30, 42). The reticular dermis is a dense network of collagens and coarse elastic fibres making up the bulk of the skin and providing for its integrity and extensibility. Interspersed in the spaces between the connective tissue bundles are adipocytes, glands, nerves and the hair follicles. Epidermal cells are present in two of the structures of the hair follicle: in the matrix surrounding the connective tissue papilla and in the bulge of the hair follicle (Figure 1). Keratinocytes and melanocytes are resident in the matrix of the hair follicle and the origin of the epithelial cells is the epidermal stem cells of the bulge of the hair follicle (43). The bulge cells only contribute to the structure of the epidermis in the case of re-epithelialisation of a wound where the cells respond quickly to damage but give rise to a transit amplifying population that later is replaced by strictly epidermal keratinocytes (44).

The dermo-epidermal junction

The epidermis and dermis are separated by a basement membrane (Figure 1) (45). The membrane forms the physical division between the tissue compartments, restricts molecular transport between them and dictates polarity of the basal

keratinocytes which is of importance for the directionality of the differentiation and maturation of the epidermis (46). The dermo-epidermal junction is unique within basement membranes in its structure due to the presence of anchoring complexes. These structures establish integrin-mediated connections to link basal keratinocytes to the basement membrane. Integrin combinations expressed by basal keratinocytes are $\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha 6\beta 4$, out of which $\alpha 4\beta 6$ is strictly expressed on the basal side of the keratinocytes, opposing the basement membrane (47) and exclusively expressed in the hemidesmosomes in mature epidermis (48). The anchoring complex proteins are the link between the cytokeratin intermediate filaments of the basal keratinocytes and the basement membrane (Figure 2). The anchoring filaments are 2-4 nm in diameter and predominantly consist of laminin 5. Laminins are heterotrimeric basement membrane proteins composed of α , β and γ chains. Two nomenclatures are used today: laminin 5 was the fifth trimer composition discovered, but new nomenclature names laminins after chain numbers rendering laminin 5 and laminin 322 the same protein (49). Laminin 5 connects the hemidesmosomes through integrin $\alpha 6\beta 4$ to other laminins and collagen VII in the basement membrane compartment. Collagen VII in turn links the fibrous matrix elements (collagens I, III and V and fibrillins) and thereby anchors the complete complex to the papillary dermis. The anchoring complex

Figure 2. Organisation of the basement membrane with connections to the basal keratinocyte via integrins, and the collagens in papillary dermis. COL: collagen. Adapted from Uitto *et al.* 2001³¹

system provides the structural integrity needed for the frictional forces that skin is subjected to (47, 50).

When the barrier is breached

When wounding occurs, a myriad of processes are initiated to restore the barrier of the skin. Wound healing is a dynamic process involving all cell types in the skin, soluble factors and the ECM (51). A broad subdivision of the process is the inflammatory phase, proliferative phase and tissue remodelling phase (Figure 3).

The inflammatory phase is a direct response to the disruption of the tissue and the resident blood vessels. The blood clot formation achieves haemostasis and provides the cells in the wound with a provisional ECM. Platelets and injured cells start secreting mediators that recruit monocytes, fibroblasts and leukocytes to the site (52, 53). Infiltrating neutrophils remove debris and bacteria from the site and monocytes are recruited by ECM fragments and transforming growth factor β (TGF- β). Once activated, the macrophages release platelet derived growth factor (PDGF) (54) and vascular endothelial growth factor (VEGF), essential for formation of the granulation tissue (55). Granulation tissue is the provisional ECM formed during the wound healing process by major rearrangement of the ECM and that will eventually form the novel stroma (56, 57). Other monocyte and macrophage derived growth factors in the early inflammatory stage of wound healing are TGF- α , interleukin 1 (IL-1) and TGF- β (58) (Figure 3 B).

Epidermal keratinocytes are presented with an alternative pathway to differentiation upon injury, the pathway of activation. The activation of keratinocytes to hyperproliferative and migratory cells (59) is affected by extracellular stimuli and signals, and characterised by differential expression of keratins. The activation of keratinocytes leads to proliferation, migration, upregulation of cell surface receptors and production of basal membrane

components, all important for the re-epithelialisation process. Re-epithelialisation commences within hours of injury (58, 60, 61). Activated keratinocytes express keratins 6, 16 and 17 (5, 62). The accumulation of keratin 6 and 16 in wound edge keratinocytes coincides with the rearrangement of keratin 5 and 14 filaments changing from pancytoplasmic to concentrated at the edge of the cell, directed away from the migrational leading edge. The major cytoarchitectural changes precede the migration of keratinocytes out into the wound bed which also requires changes in the structure and number of desmosomes (63). The initiation of re-epithelialisation also requires downregulation of hemidesmosomal links between the epidermis and the basement membrane to allow lateral movement of activated keratinocytes (58, 64).

Epidermal keratinocytes are on constant stand-by for responding to attacks on the barrier. Sequestered in the cytoplasm, keratinocytes carry IL-1, both α and β forms (65). IL-1 is the key initiator of keratinocyte activation and is rapidly processed and released after injury (66). Keratinocytes express both types of IL-1 receptors and their antagonist making them highly responsive and sensitive to the effects of IL-1. IL-1 functions as an autocrine signal for keratinocytes to enhance the activation cycle (67) (Figure 4). IL-1 plays a crucial role for the paracrine signalling as it activates endothelial cells, fibroblasts and lymphocytes. IL-1 is a chemoattractant for lymphocytes and together with activation of endothelial cells and induction of selectin expression, IL-1 initiates lymphocyte extravasation (68). Cytokines and growth factors induced by IL-1 in keratinocytes include granulocyte-macrophage colony stimulating factor (GM-CSF) (69), tumour necrosis factor α (TNF- α) and transforming growth factor α (TGF- α), as well as IL-1. TNF- α has been shown to maintain the activation of keratinocytes (70) and elevated levels have been found in conditions like irritant contact dermatitis and infections (71). The maintained activation leads to production of several cytokines

like IL-3, 6 and 8, which contribute to the paracrine signalling of keratinocytes in the wound healing environment (5, 72).

A.

B. Day 3 Inflammation

Figure 3. (A) The overlapping stages of the wound healing process at different times after wounding. (B) Schematic overview of the inflammatory phase of wound healing with involved cell types and soluble factors. KGF: keratinocyte growth factor, PDGF: platelet derived growth factor, TGF: transforming growth factor, TNF: tumour necrosis factor, VEGF: vascular endothelial growth factor. (A) adapted from Komosinska-Vashev *et al.* 2014 ¹²² (B) from Singer *et al.* 1999 ³²

The signals form a positive feedback loop with increased production and upregulation of cell surface receptors and intercellular adhesion molecule 1 (ICAM-1) and integrins, essential for the migration of keratinocytes when the re-epithelialisation of the wound starts (5). The integrins expressed on activated keratinocytes allow interaction with fibrinogen and collagen type I which is present in the wound edge and interwoven with the fibrin in the blood clot. As the keratinocytes migrate to cover the wound bed they are guided by the array of integrins they express (73). The migrating keratinocytes create an optimal environment for migration in additional ways: they contribute to the degradation of the ECM, which is necessary for migration to take place, by producing collagenase and matrix metalloproteinases (MMPs) (74, 75). The new stroma, referred to as granulation tissue, starts filling out the wound bed after day four in the wound healing process. Produced by fibroblasts, the new connective tissue is rich in capillaries (58, 76). Both angiogenesis and vasculogenesis, with recruitment of bone marrow-derived progenitor cells, contribute to the vascularisation of the granulation tissue in the proliferative phase of wound healing (77). The formation of a vascular network is pivotal for tissue repair in terms of oxygenation and nutrient supply (78). Fibroblasts and macrophages continue moving into the wound as the provisional matrix is formed. Macrophages sustain the growth factor production to induce proliferation of all surrounding cell types and fibroblasts produce and deposit ECM rich in fibrin, fibrinogen and hyaluronic acid (51, 79). The provisional wound healing matrix and granulation tissue that enables migration of keratinocytes and fibroblasts, and oxygenation, is gradually replaced by fibroblasts that start depositing a collagenous matrix (80).

A contained proliferative burst is required to sustain the re-epithelialisation process. The keratinocytes behind the actively migrating wound edge start proliferating one to two days after injury (51). The inducing factors have not been established. The free edge effect has been proposed as one of the mechanisms

where the absence of neighbouring cell, in combination with growth factors, signals both migration and proliferation (58). As re-epithelialisation is completing, the basement membrane components collagen type IV and laminin reappear and fibronectin and fibrinogen are downregulated (57). At the late stage of re-epithelialisation where contraction of the restored basal membrane is required the activated keratinocytes obtain a contractile phenotype that is characterised by expression of keratin 17 (5, 81). Keratin 17 is present in basal layers of pseudostratified epithelia and myoepithelial cells, which have in common that they contract or change shape. The expression of keratin 17 in epidermis is restricted to the activated contractile keratinocyte and directly induced by interferon γ (IFN- γ) (38, 82). IFN- γ is an autocrine signal for lymphocytes and a paracrine signal for keratinocytes conveying the signal of the late inflammatory response (83). A de-activation signal in the form of TGF- β is expressed at this time in the wound healing process by dermal fibroblasts. TGF- β suppresses keratinocyte cell proliferation and directly induces basal-specific expression of keratin 5 and 14 (84). The effect of TGF- β on keratinocyte activity has been shown to be reversible and does not lead to terminal differentiation but reduces the growth rate back to a normal basal level (85, 86).

Figure 4. Keratin expression in keratinocytes and factors involved in the keratinocyte activation cycle. Adapted from Blumenberg *et al.* 2001 ⁵

The remodelling phase of the wound healing process is the long process of ECM reorganisation at the site of injury that will eventually lead to the formation of a

fibrous scar. The maturation of a scar can take years to complete. The initiation is characterised by the apoptosis of granulation tissue fibroblasts, a process for which no inducing signal is known (87, 88). With this, the formation of granulation tissue is stopped and the collagen type III that was deposited during the inflammatory phase is degraded. It is replaced by type I collagen oriented in small parallel bundles, different to the basket weave organisation of normal dermal collagen. This orientation of collagen I is responsible of the different texture and appearance of a cutaneous scar (89). During the remodelling phase, fibroblasts in the vicinity of the wounded area obtain a contractile phenotype referred to as myofibroblast (90). Myofibroblasts contribute to the closing of the wound by contracting the remodelling ECM and diminishing the wound area. Mature myofibroblasts express α -smooth muscle actin (α -SMA) stress fibres. The most well accepted inducer of myofibroblast activation is TGF- β 1 (91, 92). TGF- β 1 and 2 are key regulators of the inflammatory phase of wound healing, and play a role in promoting fibrous scar formation (93, 94). Excessive expression of TGF- β 1 and 2 has been shown to promote aberrant scarring, and dysregulation of the remodelling phase is manifested as hypertrophic or keloid scarring. In both cases the deposition of scar tissue is extensive, forming raised scars. Hypertrophic scars remain within the boundaries of the original injury whereas keloids grow beyond (95, 96). Myofibroblasts are also involved in the pathophysiology behind hypertrophic scars, especially following burn wounds, where myofibroblasts are numerous and heavily contract the scar. Scar contraction can be painful and disabling, and persists as a major complication following burns (91).

Non-healing wounds

Patients suffering from non-healing, or chronic, wounds are a large patient group in today's health care systems (97). The complications impose morbidity and mortality on mostly the elderly patient group and bring about suffering for long

term patients with pain and substantial effects on quality of life (98). Treatment times are often prolonged and the risk of recurrence is high, and it is estimated that 2 % of health care budgets in Scandinavia are consumed by costs associated with chronic wounds (99). A prevalence estimation of foot and leg ulcers on the Swedish general population is 0.12-0.2 % (100, 101).

The term chronic wound includes all wounds that fail to heal and include venous and arterial leg ulcers, diabetic foot ulcers and pressure ulcers (102-104). Cancer and radiotherapy can also lead to impaired healing (105), and all these preconditioning states are more prevalent in older adults. This patient group also undergoes surgery more often which increases the risk for chronic wound complications. Conditions that affect the vascular system affect the vasoregulation of the microcirculation of the skin which can lead to hypoperfusion (106). This reflects in changes in the inflammatory response in the skin during wound healing, low oxygen tension and poor nutrient delivery (107, 108). The resident cells in the chronic wound are also characterised by decreased proliferation rates and morphology resembling senescent cells. Fibroblasts isolated from venous leg ulcers have been shown to have a reduced response to PDGF and lower expression of TGF- β receptors compared with normal dermal fibroblasts. These are similar responses seen in fibroblast exposed to hypoxia, indicating that chronic wounds are hypoxic (107, 109, 110).

Chronic wounds are often characterised by being halted in the inflammatory phase of the wound healing process (111). An increase of cellular infiltrates that is persistent in a chronic wound leads to the prolonged presence of neutrophils and macrophages. This leads to the dysregulation of some of the key regulators of the inflammatory phase: IL-1 β and TNF- α have been shown to prolong the inflammatory phase and delay wound healing (112, 113). Venous stasis ulcers are the most prevalent lower limb ulceration (114). Venous hypertension is caused by

an obstruction or reflux, and the venous insufficiency resulting from it leads to dilation of capillaries and leakage of plasma proteins, fibrin deposition and a lower oxygen tension in the tissue. The effects are ischemia and hypoxia which leads to cell death and ulceration (115). The venous leg ulcers have many underlying pathogeneses but have in common the complications that are caused by prolonged inflammation times. This often leads to substantial microbial colonisation, a system that once established can sustain itself (116, 117). The chronic ulcers also have an aberrant expression of MMPs due to the elevated levels of IL- β 1 and TNF- α (112) and their locally expressed inhibitors (118) and inflammatory cytokines which degrade the ECM and vascular walls in the healing wound environment. In the case of diabetic foot ulcers, oxidative stress has been identified as one of the key problems. The stagnation in the inflammatory phase leads to continuous infiltration of neutrophils that release free radicals and inflammatory mediators, which causes cytotoxic effects on the surrounding tissues and delays wound closure (116, 119).

Wound treatment - dressings, skin substitutes and scaffolds

There are several types of different wound dressings for the treatment of non-healing wounds aimed to enhance wound healing. The foremost contribution of coverage of a wound with a dressing is the barrier effect it has and the maintenance of a moist wound healing environment (120). Providing a temporary barrier to reduce infection and minimise necrosis is the first line of action. Open wounds are frequently contaminated and chronic wounds are easily colonised with bacteria that can start spreading into the tissue, and infect the wound. The poor vascularisation and devitalised tissue that offers a favourable milieu for microorganisms contribute to the risk of infection. Debridement, cleansing and coverage are essential to minimise colonisation and prevent severe infection (121, 122). Moist or wet treatment of wounds has been shown to enhance the wound

healing process seen by a promotion of re-epithelialisation and reduction of inflammation. Before the 1960s and the introduction of the idea of moist wound healing, wounds were often treated dry with the notion that infection would be easier to combat in a dry environment (123), until Winter *et al.* showed accelerated re-epithelialisation of wounds on pigs treated with occlusive dressings (124). In 1963 Hinman and Maibach showed occlusion of experimental wounds to be beneficial when treating human wounds (123). Since then, moist dressings are standard care for chronic ulcerations (125). There is a vast range of dressings with a multitude of mechanisms of actions including incorporated antibiotics and growth factors (126-134). Dressings and occlusion have also been utilised to modulate components of the local wound environment, like pH value. With limited clinical evidence of enhanced wound healing and difficult interpretation of clinical trials with few randomised controlled trials available, simple dressings can be considered as a protective barrier to provide a suitable wound healing environment (111). An optimal wound dressing should fulfil certain criteria: provide hydration but remove exudate and be impermeable to microbes, but allow gas diffusion. The dressing should not release unfavourable agents or fibres, and not harm the periwound area when removed. Finally, the product should be easy to use and cost effective. Few dressings meet all requirements (134).

Efforts to improve wound healing are of course not restricted to chronic wound problematics. Burn patients are a large patient group with acute need for barrier restoration. Immediate measures taken are removal of necrotic tissue and wound coverage and subsequent autologous split or full thickness skin grafting with the goals of minimising bacterial colonisation to avoid sepsis, and to preserve as much of the unharmed tissue as possible (135). The problem with large burns is the scarcity of healthy donor sites for a graft, and donor site morbidity due to the poor general status of the patients. Cultured epidermal autografts were introduced in the 1980s (136). Keratinocyte sheets can be expanded and stratified in culture before

reintroducing them to the patient (137). A drawback is the long culture time required (135). To solve disadvantages of using epidermal autografts, autologous keratinocytes can be transplanted before reaching a confluent state as a single cell suspension, often with fibrin sealant (138, 139). The keratinocytes retain their proliferative state and contribute to re-epithelialisation (140). The greatest advantages are the shortening of culture times and the ease of handling. When transplanting subconfluent keratinocytes, the total cell viability is also higher compared with epidermal autografts (141). Autologous transplantation of keratinocytes, as epidermal autografts or in suspension, is a life saving measure that has improved burn wound care substantially, however the methodology does not support regeneration of the complete skin. The dermal component is still lacking, and transplantation sites are left with a thin, brittle skin that lacks thermoregulation, hair follicles and has sensory deficiencies (142).

Skin substitutes and scaffolds

There are several approaches to substituting the dermis, ranging from applying a scaffold for resident cells to populate as a way to induce guided tissue regeneration, to engineered constructs with living cell components (143-146). Initial tissue engineering efforts to replacing tissues utilised materials that were inert, with the notion that the material could not degrade and harm the host (147). Later development has led to the use of degradable scaffolds that aims to deliver cells, genes and/or proteins to the harmed tissue and gradually degrade, leaving space to the regenerated tissue. The ultimate scaffold will support tissue regeneration by preserving the tissue volume, and guide ingrowth and regeneration of the native tissue (148). The degradation rate of the scaffold should match the rate by which the new tissue is regenerated at the site of implantation and the scaffold should provide structural integrity to the damaged tissue for a certain period of time before adapting to the environment. Tissue guidance is allowed by an open scaffold system, where eventually the complete scaffold will be degraded or completely

integrated with the host (149). Scaffold design in terms of choice of material and porosity, besides the obvious biocompatibility, plays a large part in the suitability of the material for tissue engineering purposes. The target application dictates the requirements set on the material of choice, but in general terms the selection is based on the physical, mass transport and interaction properties (150). Moreover, mechanical strength of the material, gelation and diffusion properties need to be considered. Out of mass transport properties, diffusion is the most important one to provide oxygenation into the scaffold, and will depend on both size of the molecule and charge interactions with the scaffold material (151-153). The mechanical strength dictates the space filling properties of the material and the degradation rate that is crucial for optimal performance. The rigidity of the scaffold is also important in terms of mechanical input to the native cells (154-156). Porosity coheres with the gelation of a material and its subsequent topography and biological properties. As the scaffold is to be implanted, it should promote cellular function in order to work as a guiding scaffold. Adherence, proliferation and differentiation of cells should be supported (151).

Hydrogels have been extensively investigated in the field of tissue engineering of skin. The structure of gels with high wettability (≥ 30 % water content by weight) (151, 157) is similar to macromolecular components of connective tissue and suitable for implantation due to the low strain on native tissue they exert. Both synthetic and biological degradable polymers are utilised for hydrogel production. The most applied synthetic polymers include polylactic acid (PLA), polyglycolic acid (PGA) and polycaprolactone (PCL) (149). Synthetic polymer scaffolds with the aim to restore the epidermis have largely been unsuccessful, mainly due to the low cellular recognition and compatibility (158). Efforts are being made to combine the synthetic polymers with biopolymers to make use of preferable properties from both. Synthetic polymers have the advantage of fabrication where desirable physical features can be added (159) and the natural biopolymers carry a

biocompatibility that allows adherence of cells carrying adhesion molecules for natural components (151). Degradable biopolymers used in scaffolds for regeneration of skin explored today are mostly based on agarose, alginate, chitosan, fibrin, hyaluronic acid, gelatin and collagen.

Acellular constructs are designed to mimic ECM components and to provide a dermal scaffold to support dermal regeneration (160). Early studies on materials for wound dressings and skin tissue engineering understandably focused on collagen, and being the main component of the ECM there are several collagen based dressings on the market today in the form of gels, sheets and sponges. For tissue guidance, acellular dermal allografts are available commercially today. One of the most established is AlloDerm (LifeCell, Branchburg, NJ) (159) derived from human cadaveric skin that is acellularised, lyophilised and glycerolised. It naturally resembles the native dermal tissue (161). Today, AlloDerm is primarily applied to partial and full thickness burns, but also used for soft tissue replacement and reconstruction of abdominal wall defects (162). Cellular dermal allografts are the next level of complexity of skin substitutes, where a bovine or porcine collagen matrix is seeded with neonatal fibroblasts that remain viable for a limited time, but exert positive effects for regeneration of the dermal tissue (163). These constructs are focused on the aiding in regeneration of a dermis, but efforts are being made to provide constructs with both dermal and epidermal components. Integra (Integra LifeSciences, Plainsboro, NJ) is a synthetic bilayer product. It is a composite of collagen and chondroitin-6-sulfate of bovine origin with a silicone cover sheet (164). The matrix is engineered to have pores in the range of 20-50 μm . Three to four weeks after implantation for tissue guidance for dermal cells, the silastic cover is removed and an epidermal graft can be placed on the treated area. Both AlloDerm and Integra have been largely successful in providing resident cells with a space filling guiding scaffold, and more importantly in preservation of tissue volume after large trauma or burns. Nonetheless, they are hampered by high costs

and the need for several surgeries if an epidermal component is to be added. The prototypes for human allogenic skin substitutes including both dermal and epidermal components are Apligraf (Organogenesis, Canton, MA) (165) and OrCel (Ortec Intl.Inc., NY) (166). The dermal matrix is a bovine type I collagen gel with embedded neonatal fibroblasts. Neonatal keratinocytes are seeded on top of the constructs. These types of constructs are the most advanced products for treatment of wounds and present the closest resemblance to native tissue. The use of cellular allogenic composites is restricted due to high costs compared with conventional treatment strategies, a statement which in turn is debated when taking into account the quality of healing and loss of recurrence and later contacts with the health care system for the patients (162) .

Gelatin microcarriers

Bilayer constructs and dermal allografts represent scaffolding that are of a rigid nature and present a need for prefabrication. CultiSpher-S gelatin porous microcarriers (PerCell Biolytica, Sweden) are degradable microcarriers ranging in size from 70 – 170 μm in diameter (Figure 5). The carriers are used in suspension, enabling the use of the scaffold for any type or size of lesion. CultiSpher-S consist of porcine type A gelatin that is highly crosslinked. Gelatin is a derivative of collagen and therefore highly biocompatible and suitable as a biomaterial for guided regeneration of skin. The triple helix structure unique for collagen can be broken to obtain single chains, i.e. gelatin. Type A gelatin is obtained by acidic treatment of collagen (167). Gelatin has been widely investigated in different biomedical applications due to its natural origin, biocompatibility and degradability (168).

Figure 5. CultiSpher-S porous gelatin microcarrier. Size = 170 μm . Arrow indicates live keratinocyte.

The CultiSpher-S microcarriers have a porous structure which greatly enhances the surface area for cell adhesion with pores that range in size from 10-20 μm . The adhesion properties of the microcarriers makes them suitable for cell expansion for transplantation purposes or large scale biomolecule production (169) and several cell types have been successfully cultured on CultiSpher-S (170). The porosity of the microcarriers also contributes to the mass transfer properties of the scaffold by creating an open scaffold where nutrients and oxygen can pass freely.

Previous work utilising the CultiSpher-S microcarriers reflect the versatility of possible applications for porous gelatin carriers. The work includes investigations of microcarrier culture of primary keratinocytes aimed for transplantation and the transplantation of keratinocytes to full thickness wounds in rats (171, 172), the microcarriers as soft tissue guiding scaffold in mice (173) and humans (174), and engineering of bone and cartilage-like tissues with the aid of gelatin microcarriers (175, 176). The suitability of gelatin microcarriers for keratinocyte expansion and as transplantation vehicle has been established, accordingly in paper I the aim was to investigate the gelatin microcarriers for their scaffolding properties for epidermal cells without added cells. The foremost questions were which cells in the wounds would populate the scaffold and what organisation the tissue would obtain. Additionally, the use of CultiSpher-S in suspension in *in vitro* wounds was tested. A suspended three dimensional matrix that is malleable extends potential to use in different types and forms of skin lesions where no pre-fabrication of a scaffold would be needed.

Modelling skin and wound healing

There is no fully functional *in vitro* modelling system to model the complexity of the wound healing process of human skin. Basic molecular understanding of wound healing has primarily been discovered by the use of animal models. This hampers the transition from pre-clinical studies to clinical studies (177). The few

clinical studies in turn are difficult to implement due to the complex patient group and differences in underlying conditions that can affect the wound healing process. Animal models can never fully represent human wound healing and the use of animal models should always be restricted from an ethical point of view and (178). Modelling systems for investigating any physiological process or pathological responses are still of undisputed importance to identify biological targets and treatment strategies. For wound healing, numerous factors dictate the choice of model. General healing or only limited parts of the process can be of interest, or delayed or excessive healing, which all require different models to investigate (179).

The simplest of models to investigate wound healing responses is the use of single cell layers of keratinocytes in a scratch assay. This is merely a method to investigate keratinocyte proliferation and migration, and it has been used for investigating migration of keratinocytes in papers II and III. Using single cell layers and scratch assays limits the conclusions that can be drawn from the assay to concerning keratinocyte activation and migration only (180, 181). Studies on the contractile properties of fibroblasts or fibroblasts extracted at different stages of scar maturation are also performed with the aid of simpler *in vitro* models, often accompanied by contraction assays involving collagen gels (182). However, the complexity of wound healing with paracrine and autocrine interplay between the dermis and the epidermis cannot fully be reflected in cell assays. To add levels of complexity, skin substitutes have been developed and used for wound healing assays alongside wound treatment (183, 184). The organotypic models where individual components can be manipulated are an important tool for identifying specific factors or mechanisms of action, but remain incomplete in barrier function and ECM composition.

The benefit of using a whole animal model is the inclusion of circulatory and systemic responses. Model animals frequently used in pre-clinical studies of wound healing are rats and mice. Transgenic mouse models have contributed to the field in terms of specific gene products and their contribution to the wound healing process (185, 186) and by providing disease models (187, 188). The translational problem that stems from using rodents is that the biology of the skin and subsequently the wound healing process in rodents is different from human. The skin of rats and mice has fur and contains a subcutaneous layer of striated muscle, the panniculus carnosus. The overlaying skin is loosely attached to the supporting structure (189) and a combination of loose skin and its contractile properties makes wound contraction the main mode of action of healing in rodents (190). Excisional wounds in rodents require splinting to allow investigations on re-epithelialisation and granulation tissue effectively, which introduces an external impact on the wound healing process (191).

In terms of structure and physiology of the skin the ideal animal model for studying wound healing is the porcine model (192, 193). The wound healing process is in general similar in human and pig, and pig skin has a similar vasculature and the same proportions of epidermis and dermis as human skin. The physical size of the animal enables multiple wounds to be placed on one animal (179). Despite the benefits of employing pigs as model animals their use is restricted due to high costs and need for specialised facilities (194).

Experimental treatment strategies for wound healing in human subjects are performed at opportune conditions in clinical settings on chronic wound patients and on split-thickness donor sites (195). The infliction of incisional wounds have long been implemented (196) though the use of human subjects is always ethically constrained due to the pain and discomfort that experiments may cause, and the possibility of obtaining persisting scars. The use of human wound tissue samples

in combination with large animal models has been suggested (193). In a consensus statement, Baird *et al.* propose hypothesis construction to be based on human tissue samples and analysis by genomic and proteomic methods to identify targets and mechanisms that later would be investigated in animal models, and that tissue validation studies could contribute to more translationally relevant studies.

In research concerning the barrier function of the skin, human subjects have been exposed to known irritants by the means of occluded patch testing. The reaction in the skin has traditionally been evaluated on the basis of inflammatory symptoms (197). The use of human subjects is limited by the discomfort caused and subsequently that the exposure times have to be kept short. The clinical manifestations of irritants are difficult to determine visually, and one of the strongest arguments against patch testing has been the subjectivity of the analysis (198). The same concerns are raised in the use of the classic Draize test for toxicology and irritant testing. The Draize test is performed on small rodent eyes or skin and the adverse effects are recorded. Deemed cruel, the method has been challenged due to the inherent differences between humans and the model species (199). Alternative methods based on human reconstituted skin models are being developed, out of which few have reached the last validation steps according to the guidelines set by ECVAM, the European Centre for Validation of Alternative Methods. These lack the full barrier system of the skin which is of utmost importance for predictive results in irritant testing.

Human full thickness skin *in vitro*

The uniting factor in the four papers that constitute this thesis is the use of human full thickness skin *in vitro*. In papers I, II and III the human full thickness wound healing model has been used. In 1998, Kratz introduced a model based on tissue culture of viable human full thickness skin (200). Skin biopsies were cultured submerged in culture medium for up to four weeks, during which time cells

remained viable in the tissue. Standardised full thickness wounds are created in the tissue with the aid of biopsy punches. The full thickness wounds are prepared with a punch that is three or four millimetre in diameter. A biopsy is taken with a larger, six or eight millimetre punch, creating single biopsies with circular wounds in the centre. Wound edge keratinocytes are activated and migration takes place as the keratinocytes re-epithelialise the three to four millimetre wounds in an average of seven days (Figure 6 A). This takes place when wounds are cultured in a maintenance medium consisting of Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % foetal calf serum (FCS). The cultivation of wounds in 2 % FCS results in a viable cell population but no re-epithelialisation. The 2 % FCS culture condition could be considered as a non-healing environment with depletion of nutrients, and can be utilised to investigate treatment interventions for chronic wounds.

Figure 6. (A) Haematoxylin and eosin staining of a fully re-epithelialised *in vitro* wound after seven days of culture in Dulbecco's modified Eagle medium with 10 % foetal calf serum. (B) Representative image of wound edge of an *in vitro* wound presenting no re-epithelialisation. Scale bar = 500 μ m.

The progress of re-epithelialisation is monitored by tissue preparation and paraffin sectioning, or cryosectioning, followed by routine haematoxylin and eosin (H&E) staining (Figure 6). The re-epithelialisation can be measured or scored as complete or incomplete. In the occasion of a hair follicle present in the wound bed, the wound needs to be excluded from the analysis if only wound edge keratinocyte

contribution is of interest. Since epidermal cells are present in the sheet of the hair follicle the origin of the cells in the neoepidermal tongue cannot be determined. The tissue sections can further be investigated with histological or immunohistochemical methodology.

In paper IV, human full thickness skin was used to evaluate its suitability to test cytotoxic effects of irritants. To test cytotoxicity the skin was left intact as this would be the case in a normal exposure of human skin to harmful compounds. The benefit of using full thickness human skin is the intact barrier system that the epidermis and the dermis forms. Problems arising from using reconstituted skin models or skin cell assays are the vulnerability of these systems and the over-prediction this brings. The localisation on the body from which a sample for experiments is extracted plays an important role for irritant testing: thinner skin will be over-predictive for a reaction on a body localisation with thicker skin. This should be considered when planning for experiments. As selecting localisation of tissue sample is important for irritant testing, the same principles can be applied to inclusion in re-epithelialisation experiments. The foremost inclusion/exclusion criteria of interest would be any underlying condition that severely affects wound healing, like diabetes or other venous insufficiencies.

Skin, wound healing and reciprocity

Cutaneous wound healing is a dynamic process where the different phases overlap in time, and involve all constituents of the wound healing environment from resident cells to small fragments of the ECM. The reciprocity of the interactions between cells and the ECM has long been established and wounding is an example of the adaptability of the reciprocal interactions that take place when extensive disturbance to tissue homeostasis occurs. The term dynamic reciprocity was coined to describe how the interactions take place bi-directionally and change in response to cues from the microenvironment (201). The components of the ECM take part

in all steps of the wound healing process and not only as a temporary matrix (202). The first response to wounding, the haemostatic response, is a demonstration of direct interaction between components of the ECM and the initiating factors in the wound healing process. As wounding occurs, cells and platelets in the vasculature are directly exposed to ECM components and haemostasis is initiated (203). Another example of direct contact is the extravasation of neutrophils and monocytes, where binding of cells to the affected endothelial cells and the exposed ECM is crucial for extravasation to occur. Once in the damaged tissue, monocytes bind to fibronectin in the wound environment which initiates phagocytic responses (204) and differentiation into macrophages (205).

The ECM provides not only the structural integrity of the tissue but also the cellular context for the cells present in the skin (206). Cells require to be supported and connected to neighbouring cells or an ECM in order to function. The ECM proteins support functions of cells by the multiple modes of interaction they present, and the cells respond to changes in the ECM (207). Integrins are the key players in conveying biochemical and structural changes from the ECM to cells, and integrins possess inside-out and outside-in signalling capacity. By binding the RGD motif, integrins are involved in the crosstalk between ECM proteins and the actin cytoskeletons (208), cell adhesion and migration (209), as well as growth factor responses (210). The ECM itself is a repository for growth factors that can be locally released to influence cells in the surrounding as a rapid response when wounding occurs (211). Several ECM proteins present affinity for both cell adhesion and growth factors which in turn fine tunes the local availability of a growth factor to the vicinity of cell surface receptors (212). As important as a fast response to disturbed homeostasis is, is the modulation of the ECM when cellular functions need to be downregulated at the remodelling phase to avoid excessive scarring and damage to the tissue (211, 213).

Fibroblasts are responsible for the deposition of granulation tissue and the ECM during wound healing. From being quiescent cells in the dermis, fibroblasts are activated to migratory cells with biosynthetic tasks (214). Mechanical stimulation through cues from the ECM (215) and activation by TGF- β (216) signals resident fibroblasts to differentiate into contractile myofibroblasts. Keratinocyte-derived interactions have also been suggested, since fibroblast in co-culture with keratinocytes acquire contractile phenotypes after a short period of time (217). The interplay between keratinocytes and fibroblasts are prominent in the late inflammatory and the proliferative stages of the wound healing process (218). The importance of ECM and fibroblast support for keratinocyte proliferation are known and primary keratinocytes have long been cultured on feeder layers of irradiated fibroblasts. Demonstrated in the 1970s (219) the effects have been shown to be reciprocal: the keratinocytes influence the fibroblasts to express keratinocyte growth factor (KGF), IL-6 and GM-CSF to sustain a proliferative environment (220, 221). KGF expression in fibroblasts can also be induced *in vitro* by IL-1, TNF- α , PDGF and serum (222), factors that all are initiating factors in the cutaneous wound healing response. Recent work by Larjava *et al.* (223) showed keratinocytes to directly affect fibroblast function via secreted vesicles. Keratinocytes contain microvesicles that can be shed, and when stimulating fibroblasts with microvesicles, pro-granulation tissue genes were upregulated in the fibroblasts in a dose-dependent manner. A third of the regulated genes were involved in TGF- β signalling. Furthermore, microvesicles were shown to regulate release of MMPs, IL-6 and IL-8. The role of microvesicles in the wound healing context is still to be elucidated, but it is one example of the various ways of interplay between the two main compartments of the skin and the cells that reside therein.

The complex nature of the wound healing process and its dynamic reciprocity speaks for modelling systems that take into account the contextual aspect of

individual steps in the process. The use of human full thickness skin is a means of investigating the wound healing process *in vitro* without use of whole animals, yet still taking into account the epidermal and dermal contributions to re-epithelialisation. The dermis and its ECM supports the function of epidermal keratinocytes both under normal and wound healing conditions. By utilising human full thickness skin *in vitro* with all its structural components, a comprehensive look on re-epithelialisation and barrier function of the skin can be achieved.

AIMS OF THESIS

The overall aim of the work presented in this thesis was to investigate viable human full thickness skin *in vitro* to enable investigations on wound re-epithelialisation and irritant responses.

The specific aims of the included papers:

- I. To investigate the effect of providing resident cells in *in vitro* wounds with a porous gelatin microcarrier scaffold in suspension.
- II. To develop a method to investigate the fate of keratinocytes and melanocytes in the re-epithelialisation process when transplanted to human *in vitro* wounds in suspension or on gelatin microcarriers.
- III. To investigate the effects of acidic pH on re-epithelialisation of human *in vitro* wounds and keratinocyte function to evaluate the efficiency of lowering pH of the wound environment as adjuvant therapy for wound healing.
- IV. To investigate human full thickness skin *in vitro* as a modelling system for cytotoxic effects of irritant compounds topically applied to human skin.

MATERIAL AND METHODS

Primary cell culture and media

Keratinocytes and melanocytes were cultured in their respective maintenance medium (Table I) with medium changes three times per week. Fibroblast medium (FM) consisting of Dulbecco's Eagles modified medium (DMEM) supplemented with 10 % foetal calf serum (FCS) was used for washing tissue and cells during cell preparation, and for centrifuging cells after passaging with trypsin. Primary keratinocytes and melanocytes (Figure 7) were prepared from full thickness skin biopsies under sterile conditions. Subcutaneous fat was removed by sharp dissection and the skin was washed in sterile phosphate buffered saline (PBS). For keratinocyte preparation, skin was cut into strips measuring one centimetre wide and three centimetres long and submerged in a DMEM solution containing 25 U/mL dispase at 4 °C overnight. The epidermis was lifted off with forceps, minced with scissors, and placed in trypsin and ethylenediamine tetra acetic acid (EDTA) (0.25 % and 0.02 %, Gibco, Thermo Fisher Scientific, Waltham, MA) solution for 15 minutes at 37 °C. The solution was agitated every second minute by vortexing. The trypsin was inactivated with centrifugation at 300 G in FM. The resuspended epidermis-cell suspension was cultured in keratinocyte serum free medium (KSFM), and non-adherent cells and debris was removed by rinsing with KSFM two days after seeding. Melanocyte preparation required removal of subcutaneous fat. The remaining epidermal and dermal tissue was minced with scissors, and placed in trypsin-EDTA solution and agitated with a magnetic stirrer continuously for 40 minutes at 37 °C. After agitation, the tissue was left to sediment and the supernatant was collected, centrifuged in FM and resuspended in melanocyte growth medium (MGM). The procedure was repeated three to four times, and the supernatants were collected for seeding in appropriate culture vessels, depending

on the amount of starting material. Culture medium change took place the day after seeding.

Table I. Composition of cell culture medium

Medium	Abbreviation	Basal medium	Supplements	Manufacturer
Keratinocyte serum free medium	KSFM	KSFM with L-glutamine	25 µg/mL bovine pituitary extract (BPE) 1 ng/mL epidermal growth factor (EGF) 50 U/mL penicillin 50 µg/mL streptomycin	Gibco, Thermo Fisher Scientific
Melanocyte growth medium	MGM	PC-1 base medium	2 % PC-1 supplement 1 % L-glutamine 5 ng/mL basic fibroblast growth factor (bFGF) 24.6 g/mL N ⁶ ,2'-O-Dibutyryl adenosine 3',5'-cyclic monophosphate (dbcAMP) 50 U/mL penicillin 50 µg/mL streptomycin	Base: Lonza Supplements: Sigma-Aldrich
Fibroblast medium	FM	Dulbecco's Modified Eagle Medium	10 % foetal calf serum 50 U/mL penicillin 50 µg/mL streptomycin	Gibco, Thermo Fisher Scientific

Figure 7. (A) Colony of primary human keratinocytes. (B) Primary human melanocytes. Scale bars = 1000 µm.

Microcarriers and spinner flask culture

Porous gelatin microcarriers from PerCell Biolytica (Åstorp, Sweden) were applied to *in vitro* wounds in papers I and II. The aim of paper I was to investigate the scaffolding effects of the microcarriers. The CultiSpher-S microcarriers are based on highly crosslinked porcine gelatin type A and fully biodegradable (Figure 5). Cell culture on microcarriers requires agitation of the microcarriers suspended in culture medium which is done by using spinner flasks for culture (Figure 8) (Techne, Staffordshire, UK). The spinner flask is a basic bioreactor and one of the most commonly used in cell culture applications (224). Bioreactor design spans from microscale perfusion chambers to industrial size rotating wall vessels (225). The culture of cells on microcarriers in spinner flasks can be performed when the goal is to upscale the culture of adherent cells (226), since the use of spinner flasks reduces medium consumption

Figure 8. Spinner flask culture set-up with spinner flask and stirrer placed in incubator.

and hence the costs. The spinner culture also improves oxygenation and the distribution of nutrients for the cultured cells (224). For maximal cell proliferation to be achieved, mass transfer needs to be reached without the negative effects of the hydrodynamic forces present in the spinner culture (226). The cell damage in spinner flasks mainly rise from collisions between the microcarriers or with the agitator, or with interactions with liquid eddies when the cell-microcarrier suspension is agitated (227). An agitation rate enough to suspend the microcarriers can be sufficient for adequate oxygenation. However, if the spinning is the only mode of aeration of the culture the agitation speed might have to be increased. A

liquid area can absorb only a limited amount of oxygen and this has to be considered when the culture volume of the spinner culture is decided: a larger volume of liquid will decrease the liquid surface/volume ratio and result in decreased oxygenation and lower cell yield (226). Other considerations than agitation speed and culture volume that need to be addressed are the carrier and cell type to make sure the adhesion of the cells is adequate. The possible reactions of the cells to the forces in culture should also be considered and the integrity of the microcarriers themselves exposed to the planned agitation speeds and culture times should be monitored (224).

The property of the microcarriers as tools for cell transplantation was utilised in paper II where cells were expanded on microcarriers before transferred to *in vitro* wounds. Keratinocytes and melanocytes were mixed with microcarriers at a concentration of 50 000 cells/mg microcarriers. The suspension was added to spinner flasks and agitated at 35 RPM for five minutes every hour for the first 24 hours of culture to allow cell attachment. After the initial phase, the culture was agitated continuously and half of the culture medium volume was changed three times per week. When microcarriers were transplanted to *in vitro* wounds, the spinner flask was manually agitated to obtain a uniform solution to remove the microcarrier samples from.

Viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a colourimetric assay for assessing metabolic activity of cells. The assay is based on the activity of mitochondrial reductases, which can reflect the number of viable cells in a sample. The enzymes reduce the yellow MTT salt to purple insoluble formazan. Once dissolved in dimethyl sulfoxide (DMSO) or isopropanol, the produced formazan is measured

spectrophotometrically at 570 nm to quantify the absorbance (228). In paper II, MTT assays were performed on keratinocytes and melanocytes in adherent culture, and on both cell types cultured on microcarriers in spinner flask culture. In paper III, keratinocyte viability was investigated after exposure to acidic pH. Cultures were incubated in MTT (Sigma-Aldrich, St. Louis, MO) dissolved in DMEM 0.3 mg/mL for four hours in the dark, after which incubation with DMSO took place for 10 minutes. In paper IV, the MTT assay was employed to investigate the cytotoxic effect of irritant compounds applied to human full thickness skin. Skin biopsies were submerged in MTT solution for four hours and the formed formazan was dissolved in isopropanol overnight at room temperature.

Migration assay

Migration of carboxyfluorescein hydroxysuccinimidyl ester (CFSE) stained keratinocytes and melanocytes (paper II) and keratinocytes exposed to acidic pH (paper III) was investigated by performing a scratch assay (180). Cells were seeded and cultured overnight to a confluent layer. Prior to the migration assay, the cultures were incubated with mitomycin C (Roche Diagnostics, Basel, Switzerland) for two hours at 37 °C. Mitomycin is a DNA cross linker that will inhibit proliferation in the culture and therefore excludes the contribution of proliferation to re-population of the scratch (229). The cell layer was then scratched with a p200 pipet tip to obtain a cell free area, which was monitored over a series of hours to investigate the migration of the cells into the cell free area (Figure 9). Analysis of the migration assay includes tracing the remaining areas that are not re-colonised by cells in the image processing analysis software FIJI (230) and compare the results with time point zero hours. Results are presented as coverage of the initial cell free area in percent.

Figure 9. Scratch assay and progression of migration. Time point zero hours (left) with a distinct scratch in the confluent keratinocyte layer compared with time point 24 hours (right) of the same well where keratinocytes have re-populated the scratched area. Scale bar = 500 μm .

Quantitative real-time polymerase chain reaction

In paper III, the relative mRNA expression of certain genes of interest was investigated in keratinocytes exposed to pH 5.0 and pH 6.0 compared with untreated controls with the aid of quantitative real-time polymerase chain reaction (qRT-PCR) (231). Gene expression assays and reagents were purchased from Applied Biosystems (Foster City, CA). Total RNA was isolated from cells with High Pure Isolation Kit (Roche Diagnostics) and quantity and quality was measured with a NanoDrop 2000 (Thermo Fisher Scientific). RNA was transcribed to cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and PCR was run using TaqMan Fast 96-well plates in a HT7900 thermocycler (Applied Biosystems).

The use of Taq polymerase for DNA replication applications was a break through when introduced. Previously polymerase activity was restricted by the high temperatures required to denature the newly formed DNA strands to enable the single strands to act as templates for the next round of amplification. The Taq polymerase was discovered in the bacterium *Thermus aquaticus* and isolated by Chien et al. in 1976 (232). *Thermus aquaticus* is resident in hot springs so the polymerase could now withstand the temperatures up to 95 $^{\circ}\text{C}$ that are required for

a PCR, and enabled reaction in a single tube and no addition of polymerase in the middle of running the PCR (233). The primer assays used in paper III were TaqMan probe based gene expression assays. TaqMan probes are primer probes that were developed by Roche Molecular Diagnostics and Applied Biosystems enabling real time monitoring of the accumulation of PCR product during the reaction. The probes are designed with a fluorophore at the 5' end and a quencher at the 3' end which quenches the fluorescent signal when in close proximity of the reporter fluorochrome, i.e. when the probe is intact. As the polymerisation proceeds, the exonuclease activity of the Taq polymerase cleaves the probe on the target sequence and allows a fluorescence signal to be detected. This enables continuous monitoring of the amplification of target sequences during the exponential cycles of the PCR and the relative quantification of the expression of genes of interest.

To analyse the data from gene expression experiments in paper III, a method to establish relative expression termed the $2^{-\Delta\Delta C_t}$ method was used (234). The C_t value (cycle threshold value) of the gene of interest is the number of amplification cycles required to reach a threshold of fluorescence signal above the background and baseline signals. The basic assumption made is that one cycle difference in reaching the threshold represents a doubling of starting template in the PCR. The difference in C_t value, and thus the fold change, is obtained by relating the C_t value of the target gene to an endogenous control (hydroxymethylbilane synthase (HMBS) in paper III) and to an internal calibrator, in our case the untreated control cells. The application of endogenous controls normalises the expression and controls for variations in assays and experiments, as well as cDNA yield.

Human *in vitro* wound healing model

The *in vitro* wound healing model utilised in papers I, II and III is based on the culture of viable human full thickness skin. Preparation of *in vitro* wounds requires

full thickness skin, cleared from subcutaneous fat. Skin samples were washed and kept moist in PBS. The adipose tissue was removed with scissors. Full thickness wounds were prepared by using standard 3 mm biopsy punches (Figure 10 A and B) to create circular wounds. This resulted in standardised full thickness wounds spanning over the dermo-epidermal junction into the dermis. The wounds were separated from the larger skin specimen with a 6 mm biopsy punch, to create 6 mm biopsies with a central 3 mm wound (Figure 10 C). The wounds were cultured in individual wells in 24-well plates in FM with medium changes three times per week in all three projects (Figure 10 D). In paper I, wounds were incubated in cell culture inserts (Figure 10 E). The placement of the wound in the air-liquid interface induces stratification of the epidermis, with cornified keratinocytes in the superficial layers of the newly formed epidermis of a re-epithelialised wound. Wounds with hair follicles present were excluded from analysis.

Non-occlusive topical exposure

In paper IV, full thickness skin *in vitro* was used to model reactions in the skin after exposure to irritant compounds. Cut-off strip tubes in pairs or in sets of three were used to create confined areas of exposure on the skin (Figure 11). The rings were pressed into skin that was cleared from adipose tissue to create chambers with exposure areas of 0.80 cm². Sodium dodecyl sulphate (SDS) and other test substances, as well as PBS as DMEM controls were applied to the formed chambers and the samples were placed in an incubator. After pre-defined exposure times the centres of the exposed areas were extracted with a 3 mm biopsy punch. Biopsies were washed in PBS and submerged in 0.3 mg/mL MTT-solution (in DMEM) for four hours at 37 °C. As described earlier, the formed formazan was precipitated using isopropanol at room temperature overnight. The optical density was measured using a plate reader (Versamax, Molecular Devices). Results were presented as relative to control substance values and expressed in percent.

Figure 10. (A) Standard three and six millimetre biopsy punches for creation of *in vitro* wounds. (B) Full thickness skin sample with two *in vitro* wounds created with a three millimetre biopsy punch. (C) Single *in vitro* wound extracted from the whole skin sample with a six millimetre biopsy punch. (D) Submerged *in vitro* wound. (E) *In vitro* wound cultured in cell culture insert.

Figure 11. (A) Cut-off strip tubes in sets of three for creating restricted exposure areas on human skin for cytotoxicity testing. (B) Tubes pressed onto viable full thickness skin for non-occluded exposure to irritant.

Paraffin embedding and sectioning

Paraffin embedding of tissue samples and sectioning was performed in paper III. Paraffin embedding is routine methodology to obtain tissue samples of high morphological standard that can be stored for long periods of time. To begin the embedding process the *in vitro* wounds were fixated with 4 % formaldehyde (PFA) (HistoLab, Gothenburg, Sweden) for four hours. The fixation substance and length of fixation depends on the tissue sample and downstream application. Formaldehyde in aqueous solution forms methylene glycol which reacts with several side chains of proteins. The formed reactive side groups react with each

other or combine with hydrogen groups, thereby causing fixation of the proteins (235). After washing in PBS, tissue was dehydrated in ethanol. Lastly, the samples were submerged in Tissue clear, which is miscible with melted paraffin. After soaking in paraffin overnight, the samples were embedded in paraffin and left to cool to obtain hard tissue samples that can be sectioned, and stored at room temperature. Tissue was sectioned using a Leica RM2255 microtome (Leica, Wetzlar, Germany) in 10 µm thick sections and baked on glass slides at 56 °C overnight.

Cryosectioning

Cryosectioning of tissue biopsies in the size range of the *in vitro* wounds is a faster method to obtain tissue sections compared to paraffin embedding and sectioning. However, the process of cryosectioning may lead to loss of structural morphology compared with fixated paraffin samples. Cryosectioning was preferred in papers I and II due to the presence of microcarriers in the wounds: early experiments revealed loss of microcarriers in the dehydration process and cryosectioning, which requires less preparation and change of solutes, was chosen as sectioning method. Samples were attached to sample holders with optimal cutting temperature compound (OCT) (HistoLab) and snap frozen by submerging in liquid nitrogen. Sectioning was performed with a Leica CM3050 cryostat at -28 °C and tissue sections were placed on Superfrost plus glass slides (Thermo Fisher Scientific). The thickness of prepared sections was 10 µm for routine staining described below and 50 µm for confocal imaging in paper I.

Haematoxylin and eosin staining

Tissue staining with haematoxylin and eosin (H&E) was used in papers I, II and III to observe morphology and to investigate the degree of re-epithelialisation of the wounds. H&E is the most widely used staining method for tissue morphology investigations and histopathological assessments. Paraffin embedded sections in

paper III were deparaffinised in tissue clear, rehydrated in ethanol, and subsequently washed in de-ionised water prior to staining. Frozen sections in papers I and II were air dried and washed in PBS to remove the OCT. Sections were stained with haematoxylin, washed under running tap water and stained with eosin. After staining, sections were dehydrated in ethanol and mounted with mounting medium (Mountex, HistoLab) and a cover slip. The times for staining in haematoxylin and eosin solutions varied depending on what type of fixation and preservation was used, with generally shorter staining times for frozen sections.

Eosins are routinely used as counterstains to haematoxylin nuclear staining. They are acidic and stain basic positively charged structures, and by staining with eosin one can distinguish cytoplasm of different cell types and different types of connective tissue in varying shades of red and pink. The intense staining of red blood cells can also facilitate the localisation of small vessels in the dermis of the skin. The differentiation of the stain takes place when the slides are dehydrated in alcohol after staining and is essential to obtain information and comprehensive images of the tissue slide. The haematoxylin stains are extracted from the logwood and are not stains in themselves: haematein naturally formed by oxidation carries the colour properties. Additionally, a mordant is required to increase the affinity of haematein to tissue. Mayer's haematoxylin used in papers I, II and III is an alum haematoxylin where the added mordant is aluminium salt. The metal cation gives a net positive charge to the complex of dye and mordant, and binding of anionic structures such as nuclear chromatin is made possible. Haematoxylin stains nuclei in a red stain that is blued by washing in a slightly alkaline solution, most often tap water. This results in the dark blue colour that characterises the haematoxylin stain (235). All sections were visualised using an Olympus BX41 light/fluorescence microscope (Olympus, Solna, Sweden)

Immunohistochemistry

Immunohistochemical staining was utilised in papers I-III to visualise, localise and identify different proteins. The basic principle of immunohistochemistry is the use of targeted antibodies for a specific antigen of interest. The primary antibodies are targeted with secondary antibodies conjugated with a fluorochrome or substrate for enzymatic detection of the primary antibody. Fluorescent secondary antibodies were used in all three projects (Alexa Fluor 488 and 546, Molecular Probes, Thermo Fisher Scientific). In papers I and II, keratinocytes were targeted by staining with antibodies against both acidic and basic cytokeratins. In paper I, this was performed to visualise the neoepidermis of the wounds and to quantify the thickness, and in paper II for distinguishing resident keratinocytes from the CFSE-stained, transplanted keratinocytes. In paper I the markers of a mature epidermis were targeted to investigate the maturation of the thick neoepidermis seen in the wounds with administered microcarriers (Table II).

Cryosectioned tissue samples (paper I and II) were left to air dry for 30 minutes prior to immunohistochemical staining. Paraffin embedded slides were treated with Tissue clear and washed in 99.5 % ethanol and 95 % ethanol prior to staining. All sections were fixated with 4 % PFA except sections intended for staining with antibody against laminin 5. These were fixated with cold acetone as suggested by manufacturer of the antibodies. After washing in PBS, unspecific binding sites for primary antibodies were blocked with 2.5 % bovine serum albumin (BSA). Primary antibodies were applied in a humidified chamber for 60 minutes for 10 µm sections, and at 4 °C overnight for 50 µm sections. Secondary Alexa-conjugated antibodies were applied in a humidified chamber in the dark for 60 minutes. Slides were mounted with mounting medium Prolong Gold (Thermo Fisher Scientific) containing 4', 6-diamino-2-phenylindole (DAPI) for nuclear staining. Control samples with omitted primary antibodies were employed to ensure specificity of the secondary antibodies. The primary and secondary antisera

used in the studies have been used in previous studies and tested for specificity and cross-reactivity (236-239).

Table II. Antigens targeted with immunohistochemical staining in paper I

Antigen		Reference
Keratin 5	Keratin expressed by keratinocytes in the basal layer of epidermis, expressed concurrently with keratin 14	(240)
Keratin 10	Keratin expressed by keratinocytes in the superficial layers of epidermis, expressed concurrently with keratins 1 and 2	(241, 242)
Laminin, alpha 5	Extracellular anchoring filament and major component of hemidesmosomes in basal membranes	(46)
Ki-67	Nuclear antigen present only in proliferative cells	(243)

Carboxyfluorescein hydroxysuccinimidyl ester (CFSE) staining

Cell staining with 5(6) carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE) is conventionally used for proliferation measurements of lymphocytes. The non-fluorescent pro-dye passively moves over the cell membrane due to the presence of two acetate groups (244). Once inside the cell, intracellular esterases remove the acetate groups and a green fluorescence is yielded (Figure 12). The membrane permeability of the molecule is reduced and the succinimidyl groups present form covalent bonds with amino groups, mainly lysine residues. The now covalently linked conjugates of the stain can persist in lymphocytes over months and results in a fluorescent staining that is sequentially halved at every cell division.

In paper II we have employed CFSE-staining for passive staining of primary keratinocytes and melanocytes for tracing transplanted cells in tissue sections of *in vitro* wounds. Keratinocytes and melanocytes were stained adherently according to same protocol with caution to expose the stain solution or the stained cells to bright light. CFSE (Molecular Probes, Thermo Fisher Scientific) was diluted in DMSO to 5 mM, and diluted to working concentrations in pre-warmed (37 °C) PBS. Three millilitres of CFSE solution was added to adherent cells in a 75 cm²

flask and the flask was agitated for two minutes. Cells were incubated at 37 °C for 15 minutes and medium was changed to pre-warmed cell culture medium. After 30 minutes in the fresh medium cells can be harvested or left for expansion. Keratinocytes and melanocytes cultured on microcarriers in paper II were stained as described prior to attachment to the microcarriers.

Figure 12. Schematic description of the pro-dye of CFSE that can pass freely over the cell membrane, and that after cleavage by intracellular esterases obtains the fluorescent state and upon binding to intracellular proteins is retained in the intracellular compartment. Adapted from Parish, 1999 ²⁵⁸

Flow cytometry

Flow cytometry enables simultaneous measurement and analysis of several characteristics of cells or particles using light scatter properties. Standard applications are cell counting and cell sorting as well as analyses based on

fluorescently labelled markers of interest. Measurement of CFSE intensity with a flow cytometer was performed to establish the proliferation of the CFSE stained cells on the microcarriers and cells cultured adherently. Proliferation was investigated after 72 hours in spinner flask culture. The CFSE stain is covalently bound intracellularly and inherited at cell division as previously described. This enables proliferation measurements under the assumption that the amount of CFSE is halved at every cell division. By measuring the CFSE intensity of individual cells with a flow cytometer, populations of cells that have equal amounts of CFSE can be discerned. CFSE-stained keratinocytes and melanocytes cultured on microcarriers were detached by incubation with trypsin for five minutes at 37 °C followed by vortexing. Keratinocytes were passed through a 30 µm filter and melanocytes through a 10 µm filter, washed and resuspended in PBS. A control population for CFSE staining representing cells that have not divided was stained as previously described six hours before flow cytometric analyses. Adherently cultured CFSE-stained cells and six hour control cells were trypsinised, washed and resuspended in PBS. Flow cytometric measurements were performed with a Gallios flow cytometer (Beckman Coulter, Brea, CA) at 488 nm. Data was analysed with Kaluza software (Beckman Coulter). Single cells were included in the statistical analysis and cells overlapping the CFSE intensity of the six hour control were excluded to obtain percentage of cells that had divided.

Fluorescence microscopy and confocal microscopy

In papers I and II fluorescence microscopy was used to localise antigens stained with fluorescent secondary antibodies to identify proteins present in the re-epithelialised wounds. The sections were visualised with an Olympus BX41 light/fluorescence microscope. Images were captured with a DP70 CCD camera (Olympus) and overlay images were constructed in Adobe Photoshop CS5 (Adobe Systems, San Jose, CA).

In paper I, the neoepidermis of wounds with microcarriers was investigated with confocal imaging of the cytokeratin staining. Images were captured with a Zeiss LSM 700 microscope with Zeiss ZEN software (Carl Zeiss AG, Oberkochen, Germany) and images were constructed in FIJI (230).

Statistical analyses

Statistical analyses were performed in GraphPad Prism v. 5.0 (GraphPad, La Jolla, CA). All graphs were constructed in GraphPad Prism v. 5.0. A probability level of ≤ 0.05 was considered significant.

Paper I. Neoepidermal thickness was measured digitally and wounds with administered microcarriers were compared with control wounds without microcarriers. Means were compared using Student's t-test.

Paper II. A two-way analysis of variance (ANOVA) was performed to test significance of viability of CFSE-stained keratinocytes and melanocytes compared to non-stained control. A two-way ANOVA repeated measurements test was performed followed by Bonferroni's multiple comparisons test on the data from scratch assays on migration of CFSE-stained keratinocytes and melanocytes. The percentage of divided cells in microcarrier culture was compared with adherent control cells and groups were compared with Student's t-test.

Paper III. A Kolmogorov-Smirnov testing of the viability and migration data revealed normal distribution and groups per time point were compared with Student's t-test in both cases. Fold change in the PCR of respective groups were compared with controls cultured at normal pH with a Mann-Whitney U test.

Paper IV. Groups of the fifteen minute exposure experiments were compared with Student's t-test. One-way ANOVA was performed on experiments with more than

two groups. Non-linear regression analysis of cell viability and increasing concentration of SDS was performed.

Ethical considerations

All cells used were of human origin. Primary cells were prepared from discarded full thickness skin biopsies, rescued from routine reduction plasties or abdomenoplastic surgeries. The rescue of tissue did not influence the proceeding of the surgery in any way. All tissue samples were de-identified to ensure that samples cannot be traced back to the patient to adhere to ethical guidelines concerning the use of discarded human tissue (Law 2003:46).

SUMMARY OF PAPERS

The work presented in this thesis aimed at evaluating the use of human full thickness skin *in vitro*. When cultured in a maintenance medium containing 10 % FCS, the skin remains viable with cells that respond to wounding cues with activation and re-epithelialisation. Moreover, intact full thickness skin exhibits a complete barrier function with all components and constituting cells present. The human *in vitro* wound healing model was used to study neoepidermal organisation following administration of porous gelatin scaffolds. In this setting, a novel fluorescent staining was evaluated for the tracing of transplanted cells, and to evaluate the effects of modifying the wound healing environment. Finally, the use of intact skin to study cytotoxicity responses was evaluated.

Paper I: Biodegradable gelatin microcarriers facilitate re-epithelialisation of human cutaneous wounds – an *in vitro* study in human skin

In previous studies published by our group, The CultiSpher-S gelatin porous microcarriers have been used to investigate suitability as a dermal scaffold and as a carrier for keratinocytes transplanted to rats (172, 174). In paper I, the human wound healing model was used to test the effect of providing the resident cells in the wounds with a porous, malleable gelatin scaffold. The results revealed population of the scaffold after 21 days in culture. Wound edge keratinocytes formed a thick neoepidermis (Figure 13) that stratified when cultured in the air-liquid interface. The neoepidermis was analysed with regards to markers of mature epidermis: keratin 5, keratin 10, and laminin 5 (Figure 14 A and D) using immunohistochemical staining. Additionally, the expression of the proliferation marker nuclear antigen Ki-67 was investigated. The cytokeratin expression was found to be similar to the normal organisation of keratin 5 in the basal layers of the epidermis and keratin 10 in the superficial layers. There was no increase in the

Figure 13. (A) Neoepidermal thickness after 21 days in tissue culture. Control: $47.6 \mu\text{m} \pm 3.1$, with microcarriers: $128.0 \mu\text{m} \pm 17.4$ (mean $\pm$ SD). (B) Haematoxylin and eosin staining of *in vitro* wound with incorporated microcarrier scaffold presenting a thick neoepidermis. Scale bar = $500 \mu\text{m}$.

Figure 14. (A) Immunohistochemical staining against basal keratin 5 (green) and keratin 10 (red) of wound with incorporated CultiSpher-S microcarriers. (B) Immunohistochemical staining against keratin 5 (green) and keratin 10 (red) in normal unwounded skin. (C) Confocal image of microcarrier populated by keratinocytes, staining against pancytokeratin (red). (D) Immunohistochemical staining against laminin 5 (green) in wound with incorporated microcarriers, (E) normal unwounded skin and (F) control neoepidermis. Nuclear staining (blue) with 4, 6-diamidino-2-phenylindole (DAPI). Scale bars = $100 \mu\text{m}$.

expression of the proliferation marker Ki-67 at the wound edges of the experimental wounds compared with control wounds without carriers. This is

indicative of normal proliferation rates, and that the neoepidermis is the result of migration of wound edge keratinocytes. The neoepidermis in the wounds receiving microcarriers displayed a disorganised cell orientation. However, the keratinocytes within the scaffold expressed laminin 5 (Figure 14 D), an important anchoring filament in the basement membrane structure. The integrin expression in keratinocytes and the distribution in the epidermis contributes to the polarity of keratinocytes (245) and the integrins are associated with laminin 5. The expression of laminin 5 by keratinocytes that are incorporated into the lower parts of the microcarrier scaffold indicates an obtained polarity in the neoepidermis.

The porosity of the CultiSpher-S microcarriers serves two main purposes: it increases the surface area for cell attachment for adherent cells, and it allows the diffusion of nutrients and oxygen throughout the entire scaffold. The effect of the porosity of the scaffold biomaterial was investigated by culturing wounds with added gelatin in solution. This revealed incomplete re-epithelialisation of experimental wounds and points to the importance of the porous structure of CultiSpher-S as a guiding cue for wound edge keratinocytes. Structure-wise the microcarriers fulfil several of the demands placed on a biomaterial aimed for regenerative purposes (246) where the ultimate goal is to use biomaterials that closely resemble and mimic the niche of the cells that are to be stimulated to regenerate a certain type of tissue. Gelatin bears high molecular resemblance to collagen, the main component of dermis. Collagen type I and III form thin collagen bundles in the papillary dermis. The lower and major part of the dermis, the reticular layer, consists of thick bundles of collagen I (247). The *in vitro* wounds span over the dermo-epidermal junction into the loosely packed papillary dermis. When inspecting the interface between microcarriers and the dermal wound bed, no gap or capsule formation was observed. Previous work by Huss *et al.* (174) showed no adverse effects when CultiSpher-S microcarriers were injected into the dermis of healthy volunteers. The microcarriers were populated with fibroblasts

after 14 days and the gelatin degraded by 12 weeks. The microcarriers show here another important property for regenerative biomaterials: since the niche is changing over time, which certainly is true for a wound healing environment, the microcarriers can adapt to the environment yet provide an initial structural stability. If possible, a biomaterial should also support neovascularisation. This is true for the gelatin microcarriers and was shown in the study by Huss *et al.* where the neodermis was vascularized after 12 and 26 weeks (174). In the present study, we observed capillaries in the dermis in the vicinity of the microcarriers and no gap formation between the scaffold and the dermis. Keratinocytes had populated the complete carriers as seen by confocal imaging of microcarriers in the wounds (Figure 14 C). Taken together, these studies indicate that CultiSpher-S show biocompatibility and can function as a guiding scaffold for both epidermal and dermal regeneration.

Conclusions and future outlook

- Human *in vitro* wounds with administered microcarriers presented a thick neoepidermis with incorporation of the gelatin microcarrier scaffold
- Keratinocytes in the neoepidermis expressed keratins in a similar stratified organisation to native epidermis
- Keratinocytes within the microcarrier scaffold expressed basal membrane marker laminin 5
- The porosity of the microcarrier scaffold contributed to the organisation of the neoepidermis
- No gap formation took place in the interface between scaffold and the dermal wound bed

The *in vitro* wounds are cultured submerged in cell culture medium. In clinical practice acute and chronic wounds are treated under moist conditions, which has

been extensively demonstrated to improve healing outcome. Future human studies to evaluate the beneficial properties of a porous microcarrier scaffold on wound healing need be performed to obtain relevant information of the organisation and barrier properties of the neoepidermis in an *in vivo* environment.

CultiSpher-S microcarriers exhibit many beneficial properties for future applications: the carriers can be topically applied or injected, they are highly biomimetic and can be loaded with cells or growth factors. The loading properties strongly contribute to the possible development of a transplantable scaffold which could mimic the natural niche of the involved cells and adapt to and stimulate the wound healing process. The CultiSpher-S are malleable and require no pre-fabrication steps before application to any shape of wound, and are according to our findings suitable as a tissue guiding scaffold for both epidermis and dermis.

Paper II: Tracing transplanted human keratinocytes and melanocytes with carboxyfluorescein hydroxysuccinimidyl ester (CFSE) staining

The human *in vitro* full thickness skin wound healing model was utilised in paper I to investigate the potential of porous gelatin microcarriers as scaffolding for resident cells. The culture of adherent cells in this type of microcarrier culture is conventionally aimed to expand cells for subsequent transplantation, and the porous structure responsible for the scaffolding effects is optimised for a large adhesion surface and therefore a large number of cells. To use the wound healing model to further investigate *in vitro* wound healing requires a means for tracing transplanted cells in culture. The aim of paper II was to investigate the use of carboxyfluorescein hydroxysuccinimidyl ester (CFSE) as a tracing staining for primary keratinocytes and melanocytes. Clinically, keratinocytes are transplanted when treating burns, and melanocytes when treating hypopigmentation conditions

like vitiligo. The use of CFSE-staining has traditionally been proliferation monitoring in lymphocytes (248) and investigated with flow cytometry. We investigated viability, migration, and proliferation of CFSE-stained keratinocytes and melanocytes. We found CFSE- staining to not affect viability of either cell type. Viability of keratinocytes at the seven day time point with 5 μ M staining was $100.0 \% \pm 8.2$ relative to unstained control $100.0 \% \pm 7.4$. Viability of melanocytes at the same time point and concentration was $114.0 \% \pm 19.0$ relative to control $99.8 \% \pm 17.3$. CFSE has been shown to be potentially toxic and modulating surface protein conformation, which needs to be considered if surface proteins are the target of the investigations (249, 244) however, for primary keratinocytes and melanocytes the lowest staining concentration proposed by manufacturer was shown to be sufficient and safe. Migration was investigated with scratch assays and migration of both cell types was unaffected seen by a full repopulation of the scratched area within 24 hours. Proliferation of CFSE-stained keratinocytes and melanocytes was investigated with flow cytometric measurement, thus employing the CFSE-staining for its conventional purpose. Retained proliferation is a prerequisite for the microcarriers to be utilised clinically for transplantation since a large number of cells is required for these procedures. Proliferation rates were retained when the two cell types were cultured on microcarriers ($70.4 \% \pm 37.9$ divided cells in keratinocyte microcarrier culture compared with adherent control $88.8 \% \pm 5.4$, and $26.3 \% \pm 21.0$ compared with adherent control $20.46 \% \pm 17.2$ for melanocytes). Both keratinocytes and melanocytes showed great variability when cultured on microcarriers, with keratinocyte proliferation differing from one spinner flask culture to the other. Technical shortcomings could be the reason for the observed variability in keratinocyte cultures: the detachment of the CFSE-stained cells from microcarriers with trypsin was more problematic to perform on keratinocytes than on melanocytes. This corresponds to the level of adhesion the two cell types present in adherent culture on cell culture polystyrene, where

melanocytes are easily detachable requiring substantially less time for trypsinisation than keratinocytes. Filtration necessary for the subsequent flow cytometric measurements could also have played part in the problem of obtaining sufficient amounts of cells, and if keratinocytes were still attached to fragments of gelatin these cells would have been lost before analysis.

The CFSE-staining withstood cryosectioning procedures. Green fluorescent transplanted keratinocytes and melanocytes could be localised in tissue sections after seven, 14 and 21 days of tissue culture (Figure 15). Keratinocytes were

Figure 15. (A) Re-epithelialised wound after seven days of culture with carboxyfluorescein hydroxysuccinimidyl ester (CFSE) stained keratinocytes transplanted in suspension indicated by arrow heads. Dashed lines indicate wound edges. Scale bar = 500 μm . (B) *In vitro* wound at day seven of culture with transplanted CFSE-stained keratinocytes and CFSE-stained melanocytes on microcarrier scaffolds. Section stained with antibody against cytokeratin to distinguish native keratinocytes (red), transplanted double-stained keratinocytes (yellow, arrows) and transplanted melanocytes (green, arrow head). Scale bar = 200 μm . Nuclear staining (blue) with 4, 6-diamino-2-phenylindole (DAPI).

transplanted in suspension as well as on microcarrier scaffolds. The wounds receiving no microcarriers but CFSE-stained cells showed expected re-epithelialisation times and incorporation of transplanted keratinocytes in the re-epithelialised wound (Figure 15 A), thus no negative effect of the staining on re-epithelialisation could be observed. Co-transplantation of CFSE-stained keratinocytes and melanocytes was performed with the aid of microcarriers. Here the two transplanted cell types were distinguished from one another by immunohistochemical staining against cytokeratin and red fluorescent secondary antibody. The double stained keratinocytes could be seen in orange/yellow compared with the red stained native epidermis (Figure 15 B).

The application of CFSE-staining for tracing human primary keratinocytes and melanocytes was shown to be suitable and easily applicable. The staining is readily available, easy to use and requires no custom preparation of the cells. In the present study CFSE-staining was used for tracing human primary keratinocytes and melanocytes *in vitro*. CFSE-stained cells have been traced *in vivo* in mouse (250, 251), rat (252), and pig models (253) but further studies on toxicity of CFSE for humans need to be performed before *in vivo* applications can be considered.

Conclusions and future outlook

- Staining of human primary keratinocytes and melanocytes with 5 μ M CFSE did not significantly affect viability, migration or proliferation of the cells
- Administration of CFSE-stained cells to human *in vitro* wounds did not affect re-epithelialisation
- The CFSE-staining can withstand cryosectioning procedures
- CFSE-stained keratinocytes and melanocytes could be traced in tissue sections from *in vitro* wounds to up to three weeks

The proliferation measurement with the aid of flow cytometry of CFSE-staining is based on the dilution of the staining that occurs at every cell division. It is assumed that the fluorescence intensity is halved at every generation. CFSE fulfils several important requirements for a dye to perform with high resolution under these premises: the initial staining intensity is high, the variability of the fluorescence is low, there is minimal transfer of dye after staining and CFSE shows low toxicity for cells (254). Due to staining intensity and low variability in fluorescence, distinct peaks in fluorescence intensity can be obtained when investigating number of cell divisions with flow cytometry. In paper II we have investigated the presence of stained cells with retained fluorescent signal in tissue sections, unable to state how many cell divisions the observed cell has undergone. Measurements of staining intensity of traced cells in sections needs to be compared with newly stained cells, to be able to report number of divisions of transplanted cells that have taken place. The performance of CFSE as a staining for measuring fluorescence intensity in tissue sections needs to be evaluated. The application of CFSE-staining on human primary epidermal cells could be a valuable tool for investigating transplantation and the fate of transplanted cells owing to its simple processing, stable fluorescence and low impact on human primary keratinocytes and melanocytes.

Paper III: Influence of acidic pH on keratinocyte function and re-epithelialisation of *in vitro* wounds

Adjuvant therapies for modifying the wound healing environment and therefore hopefully aiding chronic wound healing include laser and ultrasound therapy, electrical stimulation, and treatment with hyperbaric oxygen, negative pressure therapy (255) and pH modulation (256). None of the treatment approaches have been shown to conclusively contribute to improved healing though negative pressure therapy has been extensively used in the clinical setting (257). Few

investigative reports are available on the impact on lowering wound pH on wound healing (258). The rationale behind the lowering of pH in the wound environment stems from the facts that intact skin is slightly acidic and that when wound healing proceeds, a slight acidosis takes place. Several proteases and bacteria are active in basic milieus, and chronic wounds display elevated pH levels that could contribute to the unfavourable wound healing environment present in chronic wounds (256). The aim of paper III was to utilise the *in vitro* wound healing model to investigate re-epithelialisation of *in vitro* wounds exposed to pH 5.0 and pH 6.0, and the effects on primary human keratinocytes in culture exposed to the lowered pH levels with functional assays and qRT-PCR.

In vitro wounds were cultured submerged in FM with 10 % FCS adjusted to pH 5.0 and pH 6.0 with 1 M hydrochloric acid. Wounds cultured at pH 5.0 showed no re-epithelialisation at any time point. Wounds and biopsy edges of wounds cultured at pH 6.0 showed moderate outgrowth compared with control wounds cultured at pH 7.4 (Figure 16 A, B and D). The findings in the functional assays were supportive of the tissue culture results where the responses of keratinocytes cultured at pH 5.0 to activation and migration cues were lost. These responses were partially retained in keratinocytes cultured at pH 6.0 represented by moderate re-epithelialisation of *in vitro* wounds.

Scratch assays revealed keratinocytes exposed to pH 6.0 to be able to repopulate the scratched area up to 80 % after 24 hours compared with no migration taking place in the cultures exposed to pH 5.0 (Figure 17). Reflected in the viability measurements where viability in the pH 5.0 culture was beneath 50 % of the normal controls at all time points, the poor migration was expected.

The qRT-PCR revealed differential expression of the investigated mRNA. The detrimental effect of pH 5.0 and the low RNA yields it resulted in, limited the time of exposure to lowered pH to six hours. Genes of interest were matrix

Figure 16. Haematoxylin and eosin staining of *in vitro* wound sections. (A) Neoepidermal outgrowth of wound cultured at pH 6.0 for eight days and (B) control wound at day four. (C) Wound edges of wound exposed to pH 5.0 with no outgrowth compared with (D) fully re-epithelialised control wound at day 12. Arrows indicate wound edges, arrow heads neoepidermal outgrowth. Scale bar = 500 μ m.

metalloproteinase 1 (MMP1), tissue inhibitor of matrix metalloproteinase 1 (TIMP1) (259), protein kinase 2 (PTK2) and heat shock 27 kDa protein 1 (HSPB1). HSPB1 is also known as HSP27, heat shock protein 27, and is a cytoprotective protein that localises to the nucleus when cells are stress induced and is involved in avoiding apoptosis, and actin network rearrangement (260). QRT-PCR showed upregulation of HSPB1 in cells exposed to pH 6.0. This could partly account for the retained capacity to migrate by the cells exposed to pH 6.0 which was seen in the scratch assay, coupled to the upregulation of PTK2 that was also revealed by the qRT-PCR. PTK2 (also known as FAK, focal adhesion kinase) binds the activated cytoplasmic tail of plasma membrane integrins (261) and is the mediator in several important pathways (262) wherein migration and adhesion in response to cues from the ECM through integrins are well charted (263). Capacity to migrate

at pH 6.0 is of importance if acidification is to be applied as a treatment strategy. However, values of the qRT-PCR present substantial variance but the results indicate upregulation of both PTK2 and HSPB1.

Keratinocytes cultured at pH 6.0 showed higher viability, an 80 % coverage of scratched area in the scratch assay, upregulation of HSPB1 and less aberrant expression of the junctional protein ZO-1 than keratinocytes cultured at pH 5.0, with a pattern of loss of response in cells at pH 5.0 and a recovery and adaptation of cells to the environment at pH 6.0. Considering the demanding environment pH 5.0 imposes the findings of poor cell function at pH 5.0 were expected, however the recovery of migration at pH 6.0 shows that keratinocytes under these conditions are responsive to cues from the environment to migrate.

Conclusions and future outlook

- No re-epithelialisation of *in vitro* wounds occurred under exposure to pH 5.0 compared to a minor outgrowth of an epithelial tongue in wounds cultured at pH 6.0
- Keratinocytes exposed to pH 6.0 upregulated the cytoprotective protein HSPB1 and PTK2, important for migration

Figure 17. Scratch assay performed on keratinocytes exposed to pH 6.0 and pH 5.0. At 18 hours keratinocytes cultured at pH 6.0 repopulated the scratched area to 80 %, no migration could be observed in cultures cultured at pH 5.0.

- Keratinocytes exposed to pH 6.0 in culture showed retained capacity to migrate compared with keratinocytes exposed to pH 5.0

Lowering the pH levels in the chronic wound is a relevant strategy to inhibit detrimental proteases active at high pH levels (264). In attempt to mimic the natural state of the wound, acidification could be beneficial for chronic wound environments that have stagnated in the inflammatory phase. According to our findings on keratinocyte function and re-epithelialisation of *in vitro* wounds exposed to pH 5.0 and 6.0 we conclude that lowering the pH in the wound environment below pH 6.0 is inadvisable due to the severely impaired function of keratinocytes at pH 5.0. The wound environment pH levels should be taken into account when choosing dressings for treatment of chronic wounds in regard to permeability of the dressing. Non-permeable dressings retain gases in the wound, including CO₂. The CO₂ will contribute to acidification of the wound whereas permeable dressings like hydrogel dressings permit gas exchange and can contribute to maintaining pH levels in the wound healing environment suitable for re-epithelialisation.

Paper IV: Non-occlusive topical exposure of human skin *in vitro* as model for cytotoxicity testing of irritant compounds

In paper IV, human full thickness skin was utilised to investigate cytotoxicity in skin when exposed to a known irritant. The aim was to elucidate whether intact skin with a fully functional barrier could be used to model the response. Irritants are difficult to test: the use of animals should be restricted from an ethical stand point, the Draize test has been challenged over the years. Moreover, the signs of irritation visible to the subjective investigator include erythema, oedema and spongiosis that are difficult to discern (265). This limits the use of human subjects where only a certain degree of discomfort is acceptable.

The first 15 minute exposure tests, which is standard testing time for short exposure, on intact human skin *in vitro* were carried out with PBS as negative control substance and SDS, the known irritant, diluted in PBS. The variance in response to PBS was found to be substantial and therefore PBS as control substance was changed to non-supplemented DMEM. The viability values obtained from the negative controls with DMEM were more stable, and long term (six hour) exposure experiments were carried out with DMEM as control and solvent. Non-linear regression analysis revealed an $R^2 = 0.76$ between increased concentration of SDS and decreased viability of keratinocytes in the exposed area. The exposure of intact skin to 2 % SDS only elicited a modest cytotoxic response (Figure 18 A and B). This points out the problems that arise when skin equivalents are used for cytotoxicity testing: the lack of full barrier protection of the skin equivalent models carries an over-prediction when testing irritants. There are several reconstituted human skin models, out of which EPISKIN (Episkin SNC, Chaponost, France) and EpiDerm (MatTek Corporation, Ashland, MA) are the most used (266). In early validation studies of the models the over-prediction rates were between 47 % and 60 %, even if accuracy rates were acceptable (267). The reconstituted skin models are based on normal human keratinocytes cultured on a collagen matrix and stratified by culturing in the air-liquid interface. The epidermal component of both systems is histologically similar to native epidermis but the models lack in barrier structure and a complete dermal component. The barrier capacity fails to reach the level of that of native tissue, and additionally all interaction between keratinocytes and fibroblasts. Native target tissue is preferable and there is a real possibility to use it in the case of human skin. In the presented model, tissue is viable and includes all cells present in the skin except homed immune cells (268). Most importantly the barrier components of *stratum corneum*, tight junction seals, an intact basement membrane and dermal cells are all present.

Figure 18. (A) Distribution of viability values of all six hour exposure experiments with full thickness skin exposed to 2 %, 10 % and 20 % sodium dodecyl sulphate (SDS). Viability values are relative to control Dulbecco's modified Eagle medium (DMEM). All group means differ significantly from control DMEM (mean $\pm$ SD, $n = 72$). (B) Non-linear regression analysis of increased concentration of SDS and decreased viability, $R^2 = 0.76$.

The foremost objection to using human skin in culture for testing of irritant potential is the inherent interpersonal variability. To counteract this, every experiment was performed on one skin sample. The irritant response measured by cytotoxicity was related to an internal positive control at all times. The positive control was 20 % SDS in DMEM. SDS is one of the most utilised known irritants in irritant testing, and 20 % is a frequently used concentration to elicit an irritant reaction (269). The reaction was then related to a negative control to obtain information on irritant potential, and also relate the investigated compound/concentration to the positive control to ensure the level of irritation the investigated compound evokes. In the application of human full thickness skin for

irritant testing cytotoxicity was chosen as parameter to investigate. It is a quantifiable parameter that directly reflects the damage to the keratinocytes. Disturbance of keratinocytes also initiates the subsequent events that lead to the manifestation of irritation by causing release of stored IL-1 α from the keratinocytes. A limitation of using human skin *in vitro*, or any other *in vitro* model, is the lack of circulation and a systemic response. However, a limited immune response in human skin *in vitro* is present in the form of stochastic presence of immune cells. Compared to reconstituted skin models this is still an advantage that the full thickness skin presents. By limiting the exposure to only topical the entry route of the investigated substance is the same that would occur in a real life situation and the immune activation starts with the release of cytokines from disrupted keratinocytes. The interaction of keratinocytes with melanocytes and dermal fibroblasts can take place in a model of full thickness skin, and the release of cytokines by keratinocytes and the subsequent effects on fibroblasts is of interest to develop the human full thickness irritation model further.

Conclusions and future outlook

- Viability staining of skin samples topically exposed to irritant yielded stable responses with non-supplemented Dulbecco's modified Eagle medium as negative control substance
- Non-linear regression analysis revealed $R^2 = 0.76$ between increased concentration of known irritant sodium dodecyl sulphate and decreased cell viability in the exposed areas

Many different approaches for toxicity testing are being developed to replace the use of animals. The next step in any development of a new methodology is the validation of the model. This includes systematic testing with known reference chemicals to determine the accuracy of the model and the intra- and inter

laboratory variability. The validation process should also establish the suitability of the model to test different classes of chemicals. The use of viable human skin as presented in paper IV needs to be validated to adhere to regulatory settings.

In the present study the means of exposure of the human full thickness skin was non-occluded, a mode of application that cannot easily be performed on human subjects. The presented model can be modified to investigate occluded exposure as well as repeated exposures. Repeated and occluded exposures are both relevant problem formulations for occupational hazard investigations. Human full thickness skin can be kept in culture for several weeks which enables the model to be utilised for long term exposures, another exposure scheme that is problematic to perform on human volunteers. Additionally, human skin *in vitro* can be pre-disposed, with e.g. water exposure, or abraded to investigate more specific question formulations where the quality of skin plays a part. The proposed model can be easily modified to provide more insight in irritant cytotoxicity in human skin.

CONCLUDING REMARKS

The work presented within this thesis has focused on applications of human full thickness skin *in vitro*. Human skin *in vitro* presents an adequate response to wounding visualised by the re-epithelialisation of standardised wounds and possesses a fully functional cutaneous barrier. Representative model systems for any physiological or pathological process is a necessity for relevant research focus and translational success. In the case of cutaneous wound healing, the need for a modelling system that takes into account the reciprocal interactions that take place during the complex process is crucial, as re-epithelialisation is highly dependent on extracellular matrix cues and fibroblast interaction. Investigations on wound healing and irritant response employing human skin *in vitro* makes use of the actual target tissue with all constituting cell types and matrix components present.

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