Department of Dermatology
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“A proteomic approach to investigate effects of octenidine dihydrochloride on human skin cells”

Masterarbeit / Master’s Thesis

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submitted by

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Vienna, January 2020

Acknowledgements

I first want to thank my supervisor Ao.Univ.-Prof^{in}. Dr^{in}. Adelheid Elbe-Bürger (Department of Dermatology, AKH Vienna) for giving me the opportunity to conduct my master's thesis in her lab; for her guidance, help and motivation during the practical work and especially her support during the writing procedure. Whenever I faced any troubles, no matter if private or work-related, her door was open and she tried to help.

I further want to thank Ao.Univ.-Prof. PD Dr. Michael Mildner (Department of Dermatology, AKH Vienna) for his ideas and support in realizing them in his lab.

I thank my internal supervisor Dr. Christoph Metzner (Department of Pathobiology, University of Veterinary Medicine Vienna) for reading and correcting my master's thesis and for his support during the writing process.

I want to express my gratitude to my colleagues Ana, Malgorzata, René, Tanya and Kathi for their scientific as well as non-scientific support, for always lending an ear and helping me to solve any kind of problems.

I finally thank my family, partner and friends for supporting me in this exciting and sometimes challenging phase of my life.

Content

1. Abbreviations

AMP	Antimicrobial peptide
APC	Antigen-presenting cell
BG	Birbeck granule
BP	Biological process
cDC	Conventional dendritic cell
CLA	Lymphocyte-associated antigen
CLR	C-type lectin receptor
CTCL	Cutaneous T cell lymphoma
Ctrl	Control
DC	Dendritic cell
dDC	Dermal dendritic cell
DTT	Dithiothreitol
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EpCAM	Epithelial cell adhesion molecule
FA	Formic acid
FXIIIA	Factor XIIIA
H&E	Hematoxilin and eosin
HRP	Horse radish peroxidase
HSC	Hematopoetic stem cell
IAA	Iodoacetamide
Id2	Transcription factor inhibitor of DNA binding 2
IL	Interleukin
ILC	Innate lymphoid cell
KC	Keratinocyte
kDA	Kilodalton
LC	Langerhans cell
LC-MS	Liquid chromatography mass spectrometry
L-CTCL	Leukemic CTCL
LFQ	Label-free quantification
LT	Lymphoid tissue
M	Molar
MF	Mycosis fungoides
MHC	Major histocompatibility complex
MMP	Matrix-metalloproteinase
NKT	Natural killer T cell
NLR	Nucleotide-binding oligomerization domain-like receptor
NOD	Nucleotide-binding oligomerization domain
NTS	Non tape-stripped
OCT	Octenidine dihydrochloride
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PRR	Pattern recognition receptor

RAG	Recombination activating gene
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptors
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
T_{CM}	Central memory T cells
TCR	T cell receptor
T_{EM}	Effector memory T cell
TFA	Trifluoroacetic acid
TIMP	Tissue inhibitor of metalloproteinases
TJ	Tight junction
TLR	Toll-like receptor
T_{REG}	Regulatory T cell
T_{RM}	Resident memory T cell
TS	Tape-stripped
Untr	Untreated

2. Introduction

2.1 Octenidine dihydrochloride (OCT)

OCT - a cationic and surface active antimicrobial compound - was introduced as a human skin antiseptic more than 30 years ago (Hübner et al. 2010a). OCT contains two non-interacting cationic centers, separated by a long aliphatic hydrocarbon chain (Fig. 1), enabling it to bind to negatively charged surfaces, such as microbial cell walls and eukaryotic cell membranes (Hübner et al. 2010a). Upon binding, OCT interacts with polysaccharides in the cell wall of microorganisms and induces leakage in the cytoplasmic membrane of eukaryotes (Ghannoum et al. 1990).

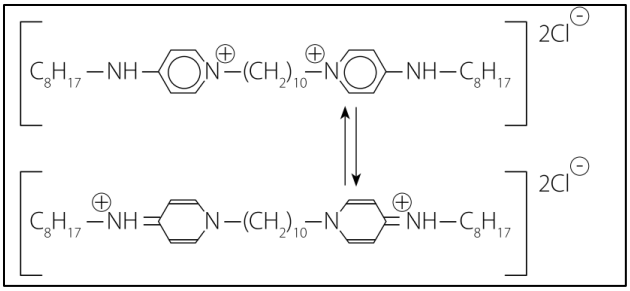

Figure 1. Chemical structure of OCT. The positively charged centers allow OCT to bind to negatively charged surfaces (Hübner et al. 2010a).

Based on its unspecific mode of action, OCT has a broad antimicrobial spectrum including gram-positive and -negative bacteria, chlamydiae, mycoplasma, fungi (Koburger et al. 2010), and simultaneously does not develop any microbial resistances (Gradel et al. 2005, Al-Doori et al. 2007). Moreover, it has been shown that OCT is even highly effective against biofilms isolated from laboratory strains of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (Hübner et al. 2010b), as well as against diverse bacterial wound bioburden in patients with a chronic wound (Cutting und Westgate 2012). Virucidal effects against enveloped viruses (e.g. herpes simplex) as well as antiviral action against f2- and MS2-coliphages have been shown, while there is no effect against non-enveloped viruses (e.g. adenovirus) described (von Rheinbaben und Wolff 2002). OCT differs from other antimicrobial substances as it lacks amide and ester structures, resulting in lower toxicity due to possible metabolites. Especially in terms of wound care, the broad pH range (1.6-12.2), where OCT shows antimicrobial activity is exceedingly beneficial, as the wound pH changes during the healing process. OCT does not adversely affect human epithelial or wound tissue (Metcalf et al. 2014). A recent study with *ex vivo* human skin demonstrated that OCT does neither morphologically alter

skin cell architecture, nor enhance apoptosis when locally applied to superficially wounded (= tape-stripped) skin. Further, it has been uncovered that OCT prevents migration of epidermal Langerhans cells (LCs) and inhibits the secretion of several cytokines (interleukin (IL)-8, IL-33, IL-10) *in vitro*, suggesting an anti-inflammatory capacity (Nikolić et al. 2019). *In vivo* studies revealed that OCT has very high tissue tolerability in wound tissue samples (Tietz et al. 2005), and beneficial effects on wound healing in humans. Patients, who underwent abdominoplasty, received both, a standard postoperative wound dressing on one wound side and an OCT-based hydrogel on the other side. The OCT-based wound dressing revealed improved wound healing associated with a lower incidence of hypertrophic scar formation (Fig. 2) (Matiasek et al. 2018).

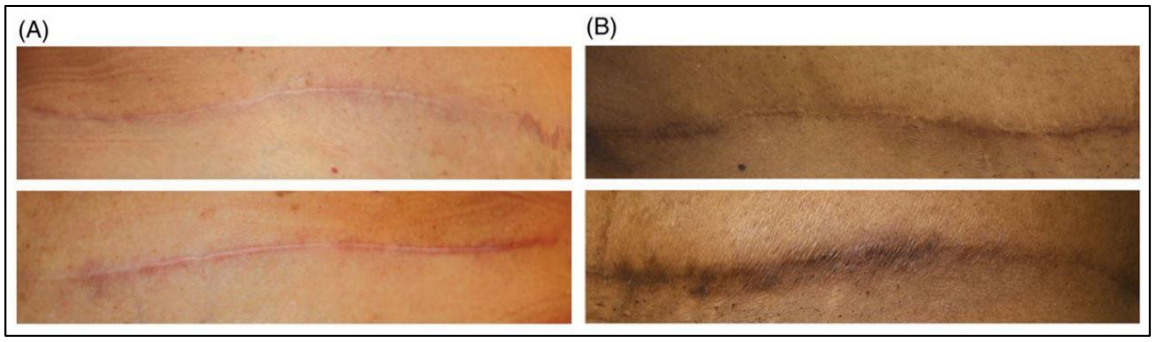

Figure 2. Improved wound healing upon OCT treatment 12 months after surgery. Shown are representative images for the treatment with an OCT-based hydrogel (top) and control (bottom) with lower incidence of hypertrophic scar formation in OCT-treated skin in a Caucasian (A; 40a) and a black (B; 47a) female patient (adapted from Matiasek et al. 2018).

Additionally, it has been reported recently that OCT is capable of reducing isolated bacterial and human proteases (Pavlík et al. 2019), which are known to be involved in the complex development of non-healing wounds and the subsequent formation of hypertrophic scars and keloids, which are characterized by inflammation and indefinite scar edges (Wang et al. 2018). Based on the findings that OCT has anti-inflammatory and protease-inhibitory capacities and positively influences wound healing, an *ex vivo* study has been designed to determine which proteins are contributing to the beneficial effects of OCT and is the topic of the thesis.

2.2 Human skin architecture

Human skin, as one of the largest organs, has a variety of crucial functions including temperature control, water content regulation, vitamin D production and energy storage. As a physical, chemical and antimicrobial barrier it protects the organism from mechanical injuries, microorganisms, substances, and radiation present in the environment (Dąbrowska et al. 2018). In addition to its function as a robust barrier against external insults, skin is an active immune organ that is composed of three compartments: hypodermis (panniculus adiposus), dermis and epidermis all of which harbor immune and non-immune cells (Fig. 3) (Pasparakis et al. 2014, Richmond und Harris 2014).

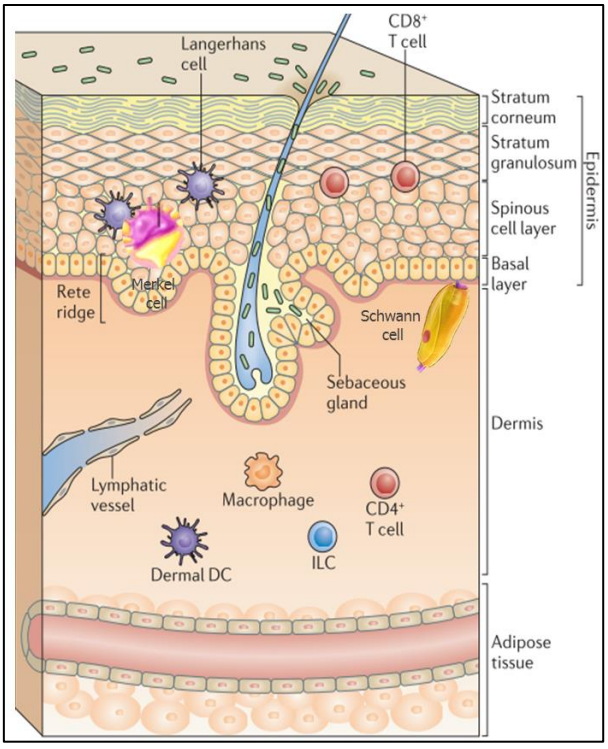

Figure 3. Architecture of human skin. The epidermis, mainly consisting of keratinocytes (KCs), is separated from the dermis by the basement membrane, which allows an exchange of fluid and cells. The dermis, the thickest skin compartment, is predominantly composed of connective fibers (collagen and elastic tissue). The adipose tissue (=hypodermis) harboring lipocytes and blood vessels serves as energy depot (adapted from Pasparakis et al. 2014).

2.2.1 Hypodermis

The hypodermis varies in thickness depending on the skin site. The lobules of lipocytes, which are interlaced with blood vessels and collagen, are functioning as a storehouse of energy (Kolarsick et al. 2011). Additionally, subcutaneous white adipose tissue (SWAT) has been associated with immune regulation and inflammation (Brembilla und Boehncke 2017, Nakatsuji et al. 2013). It has been shown that SWAT harbors a leukocyte population, comprised of approximately 50% macrophages and 25% T cells. The remaining leukocyte

populations consist of non-T cells, granulocytes, dendritic cells (DCs), and mast cells (Brüggen et al. 2019).

2.2.2 Dermis

The dermis is an integrated system of extracellular matrix (ECM) harboring fibroblasts, DCs, macrophages, T and B cells, natural killer T (NKT) cells, innate lymphoid cells (ILCs), as well as mast cells (Kashem et al. 2017). ECM, a non-cellular three-dimensional macromolecular network, is composed of collagens, proteoglycans/glycosaminoglycans, elastin, fibronectin, laminins, and several other glycoproteins (Theocharis et al. 2016). Dermal thickness varies from less than 0.5 mm to more than 5 mm. The main interstitial dermal collagens are type I and III collagen, whereas the principal basement membrane collagen (interface between epidermis and dermis) is type IV collagen (McGrath and Uitto 2010). The basement membrane zone allows the exchange of cells and fluid and holds the two layers together.

2.2.3 Epidermis

The epidermis is a continually renewing epithelium, subdivided into four layers or strata, starting with the basal layer (*stratum basale*) right above the dermis and proceeding upward through the squamous cell layer (*stratum spinosum*), the granular cell layer (*stratum granulosum*), and the cornified cell layer (*stratum corneum*) (Fig. 4) (Fuchs und Raghavan 2002).

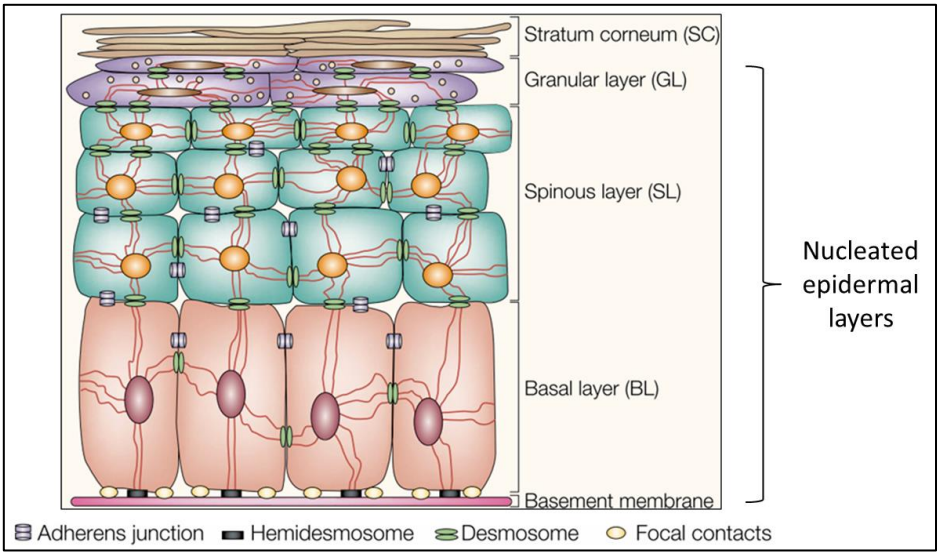

Figure 4. Epidermal layers. The epidermis consists of three nucleated layers (*stratum basale*, *stratum spinosum*, *stratum granulosum*) and an anucleated *stratum corneum*, where KCs change their phenotype to flat sharpened cells (adapted from Fuchs and Raghavan 2002).

KCs, representing the predominant cell type of the epidermis, are nucleated and viable from the basal to the granular layer where they undergo a morphological transformation into flat and anucleated squamous cells (Wickett and Visscher 2006). Although the cell-cell junctions and associated cytoskeletal proteins in the nucleated epidermal layers are crucial in terms of defence and protection, the physical barrier mainly consists of the *stratum corneum* (Proksch et al. 2008). Lipids are synthesized in keratinocytes (KCs), stored in lamellar bodies and released into the *stratum corneum* where they build intercellular layers. Keratins are the main components of the cytoskeleton in epithelial cells and huge numbers are expressed in different epithelia of humans and other vertebrates. They assemble into a web-like pattern of intermediate filaments and terminate at junctional desmosomes and hemidesmosomes. Filaggrin, a matrix protein, aggregates keratin filaments into tight bundles, promoting the collapse of the cell into a flattened shape (Baroni et al. 2012, Roop 1995). Filaggrin is synthesized as a giant precursor protein, profilaggrin (>400 kilo Dalton (kDa)), which is phosphorylated and insoluble. Profilaggrin, located in the *stratum granulosum,* has no keratin-binding activity. However, during the later stages of epidermal terminal differentiation, profilaggrin is dephosphorylated and proteolytically cleaved into multiple filaggrin monomers, which are able to bind keratin intermediate filaments, causing their aggregation. This process contributes to cellular compaction and barrier function. Loss-of-function mutations in FLG, the human gene encoding profilaggrin and filaggrin, have been identified as one potential cause of ichthyosis vulgaris (dry and scaly skin), atopic eczema as well as secondary allergic diseases such as asthma (Sandilands et al. 2009). Together, keratins and filaggrin constitute 80-90% of the protein mass of mammalian epidermis. Other structural proteins, such as involucrin, loricrin and keratolinin, are contained in cells of the uppermost spinous layer (Nemes and Steinert 1999). Apart from the *statum corneum* as first physical barrier, tight junctions (TJs) in the *stratum granulosum* are providing a second mechanical line of defence. TJs seal neighboring cells, control para-cellular pathways of molecules, and separate the apical from the basolateral parts of a cell (fence function). The most important TJ proteins in the human epidermis are occludin, claudins, and zonal occluding proteins (Brandner et al. 2015). Total epidermal thickness is depending from the anatomical location and varies from 4 mm or more (palms, soles) to 0.5 mm (eyelids). Also, the thickness of the *stratum corneum*, as well as size and morphology of corneocytes, strongly vary depending of the body site, demonstrating the importance of restricting to skin

from a distinct anatomical site to be used in one experimental setup (Gorcea and Moore 2019). Beside KCs, LCs, T cells, melanocytes, and Merkel cells are present in the epidermis.

Even though KCs are not immune cells in the accurate sense, they are capable of recognizing pathogens and mediating immune responses to discriminate between harmless commensal organisms and harmful pathogens (Lebre et al. 2007). The *stratum corneum*, contains an abundance of antimicrobial peptides (AMPs) secreted by epidermal KCs during their terminal differentiation (Harder et al. 2013). The best characterized AMPs in the skin are cathelicidins and β-defensins. Cathelicidins are protecting the skin through two distinct pathways: (i) direct antimicrobial activity, and (ii) initiation of a host response resulting in cytokine release, inflammation, angiogenesis, and re-epithelialization (Schauber and Gallo, 2008). β-defensins, small cationic peptides, are attracted to the negatively charged outer membranes of bacteria, fungi and enveloped viruses (Sørensen et al. 2008). Antimicrobial action is mediated via several mechanisms, including aggregation, pore formation, interference with cell wall synthesis, and prokaryotic membrane depolarization (Sahl et al. 2005). When the *stratum corneum* is perturbed, either after wounding or as a consequence of skin diseases such as eczema, pathogens can penetrate into the living layers of the epidermis (Gallo und Nakatsuji 2011). There they encounter the second line of defence which consists of pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and C-type lectin receptors (CLRs) all of which are expressed by KCs (Kumar et al. 2009). Activation of these receptors by pathogen-associated molecular patterns (PAMPs) boosts the production of AMPs and pro-inflammatory cytokines by KCs and recruits inflammatory cells to the site of pathogen entry (Kumar et al. 2009). Furthermore, KCs are an important source of chemokines and express chemokine receptors, allowing them to attract different immune cell types into the skin. In patients with psoriasis, activated KCs are capable of recruiting neutrophils to the inflamed epidermis by producing CXCL1 and CXCL8 (Albanesi et al. 2005).

2.3. Skin-resident immune cells

2.3.1. DCs

Professional antigen-presenting cells (APCs) in the skin include DCs, monocytes/macrophages, and B cells (Debes und McGettigan 2019). APCs have the capacity to enter skin from the peripheral circulation, and migrate through lymphatics to draining lymph nodes. As APCs sense, process and present antigens, they play an essential role in cutaneous inflammation (Kashem et al. 2017). Skin DCs are a heterogeneous population with functionally specialized subsets. They can can be divided into LCs, residing in the basal and suprabasal layers of the epidermis, are developmentally related to macrophages as well as dermal DCs (dDCs), that are located underneath the dermal-epidermal junction and are closely related to conventional DC (cDC) subsets found in lymphoid tissues (LT) (Nestle et al. 2009, Kashem et al. 2017). In LT, migratory DCs as well as blood-derived resident DCs contribute to the collective DC population and can be distinguished by differential expression levels of CD11c and HLA-DR, a major histocompatibility complex (MHC) class II cell surface receptor encoded by the human leukocyte antigen complex (Haniffa et al. 2015). For many years, DCs were thought to arise from bone marrow-derived hematopoietic stem cells (HSCs) through an intermediate monocyte stage (Van Furth 1982). However, there is now evidence that cDCs arise from bone marrow HSC-derived, DC-restricted precursors that do not undergo a monocyte intermediate stage (Merad et al. 2013). In humans, circulating DC precursors ($CD123^{lo/-}CD135^{+}CD116^{+}CD117^{+}CD115^{-}$) can give rise to all cDC subtypes (Lee et al. 2015). Human LCs and some dDCs express the MHC class I-related molecules CD1a, CD1b, and CD1c, which are involved in presentation of lipid antigens to T cells (de Jong et al. 2017). DCs that populate the dermal compartment include two main subsets, rare cDC1s ($XCR1^{+}$), and the more abundant cDC2s ($CD11b^{+}$), both also occurring in LT (Reynolds and Haniffa 2015). A specialized DC population is the plasmacytoid DC (pDC). They share a similar origin with cDCs and are circulating in blood and LT in steady state. In the skin they are only found under inflammatory conditions, where they are producing IFN-α (Reizis et al. 2011). LCs reside in the interfollicular and follicular epithelium and have the capacity to extend their dendrites through TJs to acquire antigen from the *stratum corneum* (Kubo et al. 2009). They express HLA-DR, langerin (CD207), the epithelial cell adhesion molecule (EpCAM) promoting LC migration, CD11c, e-cadherin and RTN1A (Clausen und Stoitzner 2015, Gschwandtner et al. 2018). Additionally, LCs possess characteristic Birbeck granules (BGs),

which are tennis racket-shaped cytoplasmic organelles (Kashem et al. 2017). Langerin, a type II membrane-associated C-type lectin, recognizes mannose residues via its single carbohydrate recognition domain. It is not only localized on the cell surface, but also intracellularly in close association with BGs. Transfection of langerin cDNA leads to BG formation in fibroblasts and anti-langerin antibodies, which bind to the LC surface, are internalized and delivered to BG. These observations indicate that langerin functions as an endocytic receptor for targeted transport of mannose-containing ligands to BGs (Mizumoto et al. 2004). Recently, glycosaminoglycan hyaluronic acid was uncovered as cellular ligand for langerin (van den Berg et al. 2015). Most studies on the role of dDCs or LCs have examined their capacity to stimulate naïve T cells (Fujita et al. 2009, Furio et al. 2010, Klechevsky et al. 2008) after they have left skin, migrated through afferent lymphatics and entered the T cell-rich area of the skin draining lymph node. However, given their respective strategic locations, it is far more likely that skin resident DCs will encounter resident memory T cells in skin, rather than a naïve T cell in lymph node (Seneschal et al. 2012). Recently, LC function has become more controversial, with some studies suggesting that their true role is not immune stimulation but rather immuno-regulation (Kaplan 2010, Lutz et al. 2010). Studies in LC-deficient mice indicated that LCs might actually reduce inflammation and mediate tolerance, especially in contact hypersensitivity reactions (Bobr et al. 2011, Bobr et al. 2011). Additionally, LCs as well as dDCs stay in strong interaction with regulatory T (T_{REG}) cells, which maintain immune homeostasis by suppressing self-reactive immune response (Ali and Rosenblum 2017).

2.3.2 Macrophages

In contrast to dDCs, macrophages are located throughout the whole dermis. They express factor XIIIA (FXIIIA) and the macrophage scavenger receptor CD163, which selectively characterizes monocytes and macrophages. Additionally, weak CD45 and HLA-DR expression could be shown for $FXIIIA^+$ and $CD163^+$ dermal macrophages (Zaba et al. 2007). The dynamics of macrophage and monocyte populations in the skin have not been thoroughly investigated and it is not clear whether the anatomical subdivision of tissue-resident and bone marrow-derived monocytes and macrophages has a functional correlate (Pasparakis et al. 2014). Their origin seems to be more complex than previously thought - with a pool that is established prenatally and a pool that develops after birth from blood monocytes that express high levels of CCR2 and of LY6C (Malissen et al. 2014). Dermal macrophages are

vastly superior at phagocytosis but inferior at T cell activation compared to LCs and cDCs (Tamoutounour et al. 2013), as they do not migrate to lymph nodes under normal conditions. However, macrophages have a major role in wound healing as they are capable of phagocytosing cell debris and necrotic tissue, and release reactive oxygen species (ROS) to eliminate invading pathogens (Yanez et al. 2017). Also, pro-inflammatory macrophages are capable of inhibiting fibroblasts or inducing them to produce matrix metalloproteinases (MMPs), which enhances ECM degradation (Zhu et al. 2016).

2.3.3 T cells

During thymocyte development, multipotent precursors from the bone marrow differentiate into αβ and γδ T cell lineages that are distinguished by their T cell receptor (TCR) and are characterized by diverse molecular programs. Further, successive stages of human thymocyte development can be categorized as double negative, double positive or single positive according to the expression of CD4 and CD8 co-receptors (Joachims et al. 2006). After positive and negative selection, T cells migrate to the periphery and recirculate between secondary lymphoid organs and blood by expressing the lymphoid homing receptors CCR7 and CD62L (Van Den Broek et al. 2018). In response to antigen encounter, effector and memory T cells are generated. Three major groups of memory T cells have been described: effector memory T cells (T_{EM}), central memory T cells (T_{CM}) and resident memory T cells (T_{RM}). While T_{EM} and T_{CM} cells are circulating populations, homing to secondary lymphoid organs and non-lymphoid tissues, T_{RM} cells reside in non-lymphoid tissues. The skin, an important site for immunization, hosts T_{RM} cells co-expressing either CD4 or CD8. Skin CD4 T_{RM} cells primarily reside in the dermis, while most $CD8^+$ T_{RM} cells are found in the epidermis (Lai et al. 2019). Initially it was thought that tissue-specific T cells remain in circulation until they are required at sites of inflammation. New discoveries revealed that ~98% of skin tropic memory T cells reside in the skin under non-inflamed conditions (Clark 2015). Besides the memory T cell marker CD45RO, resident skin T cells express cutaneous lymphocyte-associated antigen (CLA), and the chemokine receptors CCR4, CCR6, CCR8 and CCR10. While CLA has been identified as specialized cutaneous homing molecule for T cells, aforementioned chemokine receptors are vascular addressins, additionally contributing to the traffic of T cells to the skin (Clark et al. 2006). Importantly, most memory T cells in human mucosal, lymphoid and peripheral tissue sites such as skin express the putative T_{RM} cell marker CD69 (Farber et al. 2014). T cells found in the skin, that lack CD69 are thought to

be either $CCR7^+CD62^+$ T_{CM} cells or migratory $CCR7^+CD62L^-$ T_{EM} cells (McCully et al. 2018). $CD4^+$ T_{RM} cells and $CD8^+$ T_{RM} cells expressing CD103 are the predominant T cell subsets in the lungs, intestine, skin and bone marrow (Farber et al. 2014). Together, skin T_{RM} cells express CD45RO, CD69 and several homing molecules including CLA, CCR4, CCR6, CCR8, and CCR10. The most abundant skin T_{RM} cells are dermal $CD4^+$ T cells. T_{RM} cells provide rapid on-site immune protection upon differentiation and accumulation in tissues after pathogen infection but also contribute to numerous human inflammatory diseases. A problem occurs when T cells specific for allergens or auto-antigens enter peripheral tissue and differentiate into T_{RM} cells or when T_{RM} cells themselves become malignant (Clark 2015). As they do not recirculate, inflammatory lesions caused by T_{RM} cells persist long-term in a particular location and may often recur after treatment. Cutaneous T cell lymphomas (CTCL) are characterized by the formation of inflammatory skin lesions caused by malignant T cells. Patients with mycosis fungoides (MF), a benign skin limited variant of CTCL, have malignant T cells that are found only in well-demarcated fixed inflammatory skin lesions. Patients who progress often develop tumors and worsening skin disease but rarely develop circulating malignant T cells (Kim et al. 2003). Leukemic CTCL (L-CTCL) patients, in contrast, have malignant T cells that accumulate in the skin, blood, lymph nodes and sometimes other organs. Malignant T cells in MF lack CD62L and CCR7 expression and phenotypically resemble T_{RM} cells (Campbell et al. 2010), while malignant T cells in L-CTCL co-express CD62L and CCR7 along with the skin homing addressin CLA and CCR4 (Clark et al. 2012). Also psoriasis is a T_{RM}-mediated disease (Bhushan et al. 2002). Autoreactive pathogenic T cells are present in even the normal healthy appearing skin of patients with psoriasis, demonstrated by an experiment where normal appearing skin grafts from psoriatic patients led to the development of lesions in immunodeficient mice (Boyman et al. 2004). This further shows that lesions can develop in the complete absence of any recruitment of cells from blood.

$Foxp3^+CD4^+$ T_{REG} cells make up 20% of tissue-resident $CD4^+$ T cells (Ali and Rosenblum 2017). T_{REG} cells largely act to suppress pathogenic immune responses mediated by self-reactive cells, maintaining tissue immune homeostasis. However, the specialized functions of T_{REG} cells in the skin are not well defined. In healthy individuals, the majority of T_{REG} cells arise during thymic T cell maturation upon high recognition of self-antigen (Jordan et al. 2001). Alternatively, T_{REG} cells can be generated outside the thymus after naive $CD4^+$ T cells encounter peripheral antigens (Chen et al. 2003). An imbalance between pathogenic effector

T cells and T_{REG} cells results in chronic tissue inflammation and autoimmunity. T_{REG} cells in the skin are maintained and/or induced by interactions with both LCs and dDCs. Skin-homing T_{REG} cells are defined by expression of CCR4, CD103, CLA, and a-1,3-fuco-syltransferase (VIIFuT7) (Tubo et al. 2011, Malý et al. 1996). There is evidence that skin T_{REG} cells are linked with hair follicle biology. In humans, alopecia areata, an autoimmune disease characterized by aberrant hair follicle cycling, is connected to single nucleotide polymorphisms in regions encoding 'T_{REG} signature genes' (Petukhova et al. 2011, Conteduca et al. 2014). Additionally, T_{REG} cells are thought to be involved in wound healing as in mice a subset of highly activated T_{REG} cells accumulate in skin early after full thickness wounding (Nosbaum et al. 2015). Specific deletion of these cells early during the wound-healing process attenuates wound closure and re-epithelialization.

2.3.4 ILCs

ILCs are part of a family of innate immune cells that are involved in immunity and in tissue development and remodeling. They derive from a common lymphoid progenitor and their development is partially or completely dependent on the common γ-chain (γc or CD132), IL-7, Notch and the transcription factor inhibitor of DNA binding 2 (Id2) (Mjösberg und Eidsmo 2014). ILCs are defined by three main features: (i) absence of recombination activating gene (RAG)-dependent rearranged antigen receptors, (ii) lack of myeloid cell and DC phenotypical markers and (iii) their lymphoid morphology (Spits et al. 2013). ILCs can be divided into three subsets according to their expression of cytokines and transcription factors: group 1 ILCs (ILC1s and NK cells), group 2 ILCs (ILC2s) and group 3 ILCs (ILC3s and lymphoid tissue inducer cells). ILC1s produce IFN-γ and TNF-α. ILC2s produce IL-4, IL-5 and IL-13, and ILC3s produce IL-17A and/or IL-22 (Sonnenberg et al. 2013). ILC3s represent the most abundant ILCs in normal human skin, but are also associated with psoriasis, suggesting them as potential biomarker and therapeutic target in terms of epithelial inflammation (Villanova et al. 2014). To see how ILCs are embedded in the cutaneous immune network, a method for *in situ* mapping of ILCs in human skin was established (Brüggen et al. 2016). Identification of ILCs in human lesional skin from atopic dermatitis patients and psoriasis patients as well as from healthy donors was achieved by excluding expression markers of any other leukocyte population on the one hand, and by staining of transcription factors controlling ILC subpopulation development on the other hand. Additionally, a computed analysis algorithm allowing enumeration and topographic assessment of ILCs has been developed. The study

revealed a very sparse ILC population in healthy skin containing almost exclusively ILC1s and ILC3s. In contrast, inflamed skin harbored much larger numbers of ILCs. Atopic dermatitis skin was found to mainly host for ILC2s and ILC3s whereas psoriatic lesions predominately contained ILC1s and ILC3s. Topographically, ILCs were mainly clustered beneath the dermal-epidermal junction and exhibited a very close spatial relationship to T cells.

2.3.5 Mast cells

Mast cells are specialized for first-line surveillance functions in the skin storing pro-inflammatory mediators within their cytoplasmic granules which can be rapidly released upon mast cell contact with antigens. Their key role in host defense is demonstrated in dengue virus infection, in which skin mast cells contribute to an effective innate immune response by recruiting NK cells and NKT cells (St. John et al. 2011). Apart from their beneficial immunological function, mast cells play a central role in inflammatory and immediate allergic reactions (Amin 2012). The main activation trigger for mast cells in acute allergic reactions is IgE. Upon binding of antigen-specific IgE to its receptor FcεRI on mast cells and other effector cells causes the release of mediators in response to subsequent encounters with that specific antigen or with cross reactive antigens (Galli and Tsai 2012). Inflammatory mediators, such as histamine, heparin, proteases, chemotactic factors and cytokines act on the vasculature, smooth muscle, connective tissue, mucous glands and inflammatory cells (Borish und Joseph 1992). Histamine is not only released when the body encounters a toxic substance, it is also released when mast cells detect injury, causing vasodilatation. It has been suggested that histamine may also play a role in hypertrophic scar and keloid development, as elevated histamine levels may enhance the rate of collagen synthesis (Smith et al. 1987). Moreover, histamine levels are higher in the skin of patients with atopic dermatitis than in healthy individuals (Ruzicka and Glück 1983). It has been shown recently that exposure of KCs to histamine resulted in reduced expression of the late differentiation markers loricrin, filaggrin, keratin 1, and keratin 10 in monolayer culture as well as in an organotypic skin model (Gschwandtner et al. 2013). These findings suggest that histamine might aggravate atopic dermatitis by suppressing the differentiation of epidermal KCs, leading to an impaired barrier function of human skin. Beside FcεRIα, β and γ, common lineage markers of mast cells include histamine/HDC, c-Kit (CD117), tryptase and chymase. Recently, 21 more proteins were found to be selectively expressed on mast cells (Gschwandtner et al. 2017). Two candidates, namely neural adhesion molecule L1

(L1CAM/CD171) and DPP4 (CD26) were further studied and identified as novel skin mast cell markers. As L1 has been shown to be important in steady-state cell adhesion and migration of leukocytes (Maddaluno et al. 2009), it is conceivable that this protein is part of the adhesion molecule-integrin network, guiding mast cells from the bone marrow to their target tissue. In skin mast cells, L1 may also contribute to localizing mast cells in close proximity to nerves and vessels (Reinisch and Tschachler 2012). DPP4 is an aminopeptidase with broad substrate specificity. It cleaves neurotransmitters, angiogenic factors, and chemokines (Proost et al. 2000 , Lambeir et al. 2003). Cleavage by DPP4 can result in altered activity and changes in receptor binding affinity or receptor specificity. Furthermore, DPP4 is involved in matrix remodeling and fibrogenic activity (Rinkevich et al. 2015). In inflammatory skin diseases increased DPP4 expression has been observed (Van Lingen et al. 2008).

2.4. Wound healing

As a protecting shield for the body from the external environment, the skin is constantly exposed to potential injury. Thus wound healing is a fundamental process for the survival of all higher organisms (Takeo et al. 2015). It is a conserved evolutionary process among vertebrates including inflammation, blood clotting, cellular proliferation and ECM remodeling (Seifert et al. 2012, Richardson et al. 2013). However, there are species which are able to perfectly regenerate skin after injury (axolotl and xenopus), while in most mammalians, including humans, wound healing results in scar tissue that lack skin appendages (Yokoyama et al. 2011). As scar formation prevents the complete recovery of the skin function, it is favored to restore the skin to its original state. While regeneration describes renewal of specific tissue, i.e. the superficial epidermis, mucosa or fetal skin, skin repair displays an unspecific form of healing in which the wound heals by fibrosis and scar formation (Reinke and Sorg 2012).

2.4.1 Acute wound healing

Acute wound healing describes the physiological mode of wound closure and can artificially be divided into three to five phases which overlap in time and space (Fig. 5) (Reinke und Sorg 2012). The first phase of acute wound healing is the inflammatory phase, initiated by a “pre-phase”, characterized by **hemostasis and coagulation**. With an injury going deeper than epidermal layers, vessels are pervaded, leading to a flush of blood to remove antigens and microorganisms from the wound area (Strodtbeck 2001). Subsequently, a blood clotting

cascade is initiated by factors from injured skin (extrinsic) and platelets are activated for aggregation (intrinsic). This is followed by a short period of vasoconstriction (5-10 minutes) to reduce blood loss. The tissue gap is filled with a blood clot containing cytokines and growth factors as well as fibrin, fibronectin and thrombospondin, functioning as matrix for later immune cell invasion (Martin 1997). The vasoconstriction is followed by a vasodilatation, enabling thrombocytes to invade the provisional wound matrix. Platelets and leukocytes secrete growth factors and cytokines to initiate an inflammatory response (IL-1α, IL-1β, IL-6, TNF-α), stimulate collagen synthesis (FGF-2, IGF-1, TGF-β) and promote angiogenesis (FGF-2, VEGF-A, HIF-1, TGF-β) (Badiu et al. 2003). The **inflammatory phase** is characterized by infiltration of neutrophils, which are capable of phagocytosis and protease secretion, necessary for degradation of necrotic tissue as well as for elimination of local bacteria. Furthermore, they release mediators (TNF, IL-1, IL-6), enhancing the inflammatory response and stimulating VEGF and IL-8 for tissue repair (Eming et al. 2007). Macrophages enter the area of injury, supporting the process of phagocytosis of potential pathogens and cell material (Tziotzios et al. 2012). Three to ten days after injury, the phase of **proliferation and repair** is initiated. Re-epithelialization is ensured by local keratinocytes at the wound edges and by epithelial stem cells from hair follicles or sweat glands (Lau et al. 2009), activated by a set of cytokines and growth factors (EGF, KGF, IGF-1, NGF, etc.). Neovascularization and angiogenesis are stimulated by VEGF, PDGF and bFGF (Badiu et al. 2003). Activated endothelial cells secrete proteolytic enzymes like MMPs, dissolving the basal lamina and lysing surrounding tissue, so that proliferation and migration, also known as “sprouting” can take place. According to their substrate preferences MMPs can be roughly subdivided into collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11) and other MMPs (Krishnaswamy et al. 2017). In normal tissue, MMPs are expressed at basal levels, if at all. When tissue remodeling is required alike in wound healing, MMPs can be rapidly expressed and activated. Multiple different cell types express MMPs within the skin (KCs, fibroblasts, endothelial cells, and inflammatory cells such as monocytes, lymphocytes, and macrophages) (Caley et al. 2015). The major regulators of MMPs are the tissue inhibitors of metalloproteinases (TIMPs), which are specific inhibitors of MMPs. Disturbance in the balance between MMPs and TIMPs can lead to impaired wound healing, inflammation and even scar formation (Ulrich et al. 2010). Acute granulation tissue replaces the fibrin/fibronectin based provisional wound matrix. It is

characterized by a high density of fibroblasts, granulocytes, macrophages, capillaries and loosely organized collagen bundles (Enoch and Leaper 2005, Krafts 2010). **Remodeling**, as the last phase of acute wound healing occurs from day 21 up to one year after injury. The formation of granulation tissue stops as apoptosis is initiated. Collagen type III is replaced by the stronger collagen type I and angiogenic processes diminish. In contrast to fetal wound healing, certain skin components, like hair follicles or glands will never fully recover. The epidermis of the resulting scar differs from uninjured skin due to the lack of rete pegs that are normally anchored into the underlying connective tissue matrix (Robson 2001).

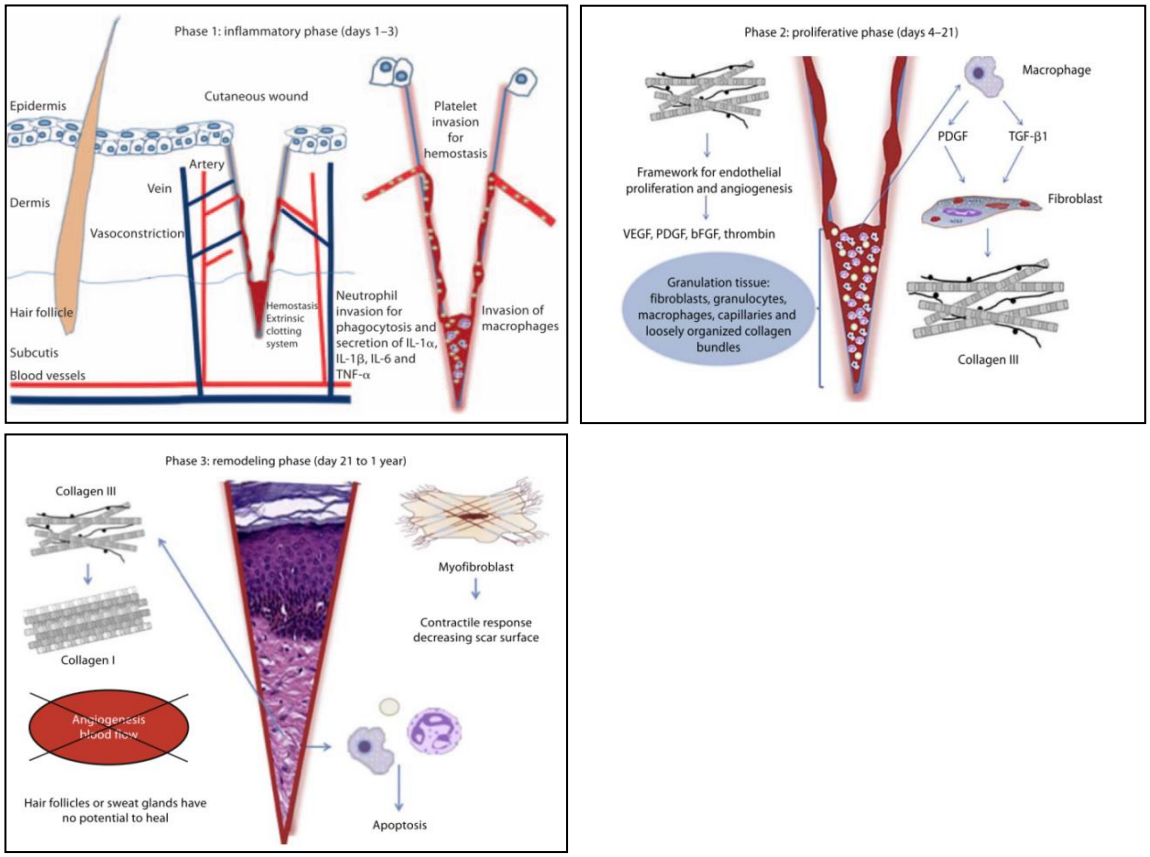

Figure 5. The three main phases of acute wound healing. Inflammatory phase (neutrophil infiltration), proliferative phase (collagen synthesis and angiogenesis), remodeling phase (reorganization processes) (adapted from Reinke and Sorg 2012).

2.4.2 Chronic wound healing and scar formation

In contrast to normal healing wounds, chronic wounds do not follow such a well-defined cascade, but are often locked in an inflammatory state (Zhao et al. 2016). The high level of mitogenic activity observed in acute wound healing is absent in chronic wounds, suppressing the transition to the proliferative phase (Stojadinovic et al. 2008). Chronic wounds are characterized by an imbalance between pro-inflammatory cytokines, chemokines, proteases, and their inhibitors as well as by an excessive infiltration of neutrophils (Schultz and Mast

1998, Diegelmann and Evans 2004). The presence of neutrophils leads to an overproduction of reactive oxygen species (ROS), which causes damage to ECM and cell membranes. Additionally, neutrophils release MMPs and other proteases, further leading to degradation of ECM and growth factors. This explains the fact that bioavailability of growth factors is quite low in chronic wounds, although their production is often increased. Pro-inflammatory cytokines (IL-1β, TNF-α), produced by activated macrophages and neutrophils, increase MMP production and simultaneously reduce their inhibitors such as TIMPs (Mast and Schultz 1996). The prolonged inflammation can also lead to forms of excessive wound healing, including keloids and hypertrophic scars, characterized by an upregulated function of fibroblasts and excessive ECM deposition (Ogawa 2017). Hypertrophic scars mainly consist of type III collagen, do normally not overgrow over the original wound boundaries and can sometimes regress with time, while keloids are strongly inflamed, consist of disorganized type I and type III collagen, extend beyond the edges of the original wound and can cause symptoms of pruritus and hyperesthesia (abnormal increase in sensitivity to stimuli of sense) (Wang et al. 2018). A study revealed that MMP2 is elevated in hypertrophic scar tissue and keloids compared to non-scarred skin from the same patient (Ulrich et al. 2010), suggesting a correlation between high abundance of MMPs in wounds and scar formation. Also fluids from chronic or very slowly healing wounds show significantly higher concentration of MMPs (MMP1, MMP2, MMP8, MMP9) and abnormal low levels of TIMPs when compared to normal healing wounds (auf dem Keller and Sabino 2015, Falanga 2004). In fetal skin, which is characterized by minimal inflammation and scarless repair, pro-inflammatory cytokines (IL-6, IL-8) were shown to be diminished (Leung et al. 2012). Also TGF-β, with its three isoforms, is involved in tissue repair (Whitby and Ferguson 1991). In adult wounds, there is a relative increase in the expression of TGF-β1 and TGF-β2 compared with TGF-β3, while fetal wounds express more TGF-β3 and decreased levels of TGF-β1 and TGF-β2. Pathologic hypertrophic scars in adults have been noted to have even higher levels of TGF-β1. Functional inhibition of TGF-β1 in adult wounds significantly reduces scarring. Conversely, addition of recombinant TGF-β1 to mid-gestation fetal wounds results in the formation of scar tissue (Lin et al. 1995).

2.5 Tape stripping (TS): an *ex vivo* wound model

Various animal models have been developed to understand the complex cellular and biochemical process of wound healing. For example, the finding that wounds heal faster when kept moist came from experiments performed in the domestic pig (Helfman et al. 1994). Although animal models have the advantage of representing a whole organ system with all components interacting with each other, there are anatomical as well as physiological differences within animal species and compared to humans (Grada et al. 2018). Thus, the development of *ex vivo* human skin wound models is a necessary step especially for re-epithelialization studies. So far, *ex vivo* human skin wounds created with (i) a scalpel (incisional wound), (ii) a small biopsy punch (partial-thickness), or (iii) pressure (suction blistering) are already established (Xu et al. 2012, Li et al. 2017, Rakita et al. 2020). TS is a mild wound model, where only the outermost layer of the epidermis, namely the *stratum corneum*, is removed. Frequently performed in order to determine the dermato-pharmacokinetics of topically applied substances (Lademann et al. 2009), TS also represents a suitable *in vivo* and *ex vivo* model for studying skin barrier repair and influx of pathogens (Berkers et al. 2019). Additionally, it can be used to remove corneocytes for further staining and/or measure protein content on the strips. Moreover, TS allows direct application of any substance on living epidermal cells, like LCs, as the outermost barrier is disrupted (Tajpara et al. 2018). Importantly, removal of the *stratum corneum* by TS does not alter the architecture of underlying epidermal or dermal cells (Fig. 6).

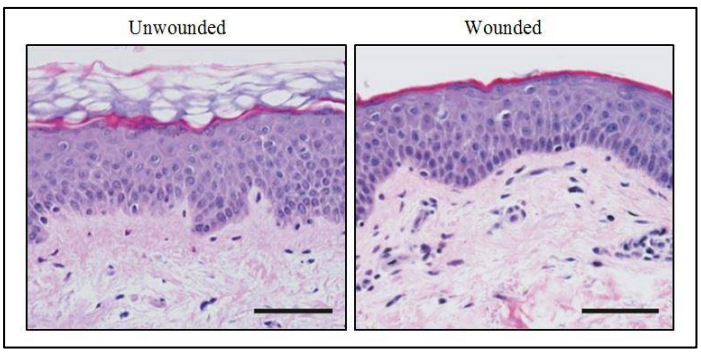

Figure 6. Comparison of unwounded and wounded human skin. Hematoxilin and eosin (H&E) staining revealed that the *stratum corneum* has been removed efficiently using a tape. Underlying epidermal and dermal cells do not show any morphological changes (right image). Scale bar: 50 µm. (adapted from Tajpara et al. 2018).

3. Aims of the thesis

OCT is a widely used antiseptic molecule shown to have antibacterial, antifungal and partially antiviral effects (Koburger et al. 2010, von Rheinbaben und Wolff 2002). Due to the broad pH range in which OCT is active, it is perfectly suitable for wound care (Metcalf et al. 2014). Additionally, OCT has the capacity to clear bacterial biofilms in patients with chronic wounds and has a comparably low toxicity (Cutting und Westgate 2012). Recent *in vivo* studies revealed that OCT is promoting skin wound healing accompanied with improved scar quality after surgical procedures (Matiasek et al. 2018).

The purpose of this study was to shed light on the unknown mechanism by which OCT might contribute to tissue regeneration and improved wound healing. To address this, a superficial wound model by TS of *ex vivo* abdominal human skin was used. Characterization of the protein content and activity of wounded skin biopsies that were topically treated with OCT and cultured for 48 hours (h) in comparison to control-treated and untreated wounded skin was achieved using three different methods:

(i) *liquid chromatography-mass spectrometry (LC-MS)* – analysis of the total protein profile

(ii) *enzyme-linked immunosorbent assay (ELISA)* – quantification of distinct cytokines and proteases in culture supernatants

(iii) *enzyme activity assay* – evaluation of protease activity in culture supernatants

4. Materials and Methods

4.1 Materials

Reagents and Buffers	**Company**
1X DMEM (Dulbecco's Modified Eagle Medium)	Gibco, USA
Pen/Strep	Gibco, USA
1X and 10X PBS	Gibco, USA
Aqua dest.	Braun Melsungen AG, Germany
Kodan forte	Schülke & Mayr GmbH, Germany
BSA	Merck KGaA, Germany
Formaldehyde	SAV Liquid Production, Germany
Tween	Merck KGaA, Germany
octenilin® wound gel	Schülke & Mayr GmbH, Germany
OCT-free gel	Schülke & Mayr GmbH, Germany
Triton-X	Merck KGaA, Germany
Na_2CO_3	Merck KGaA, Germany
NaH CO_3	Merck KGaA, Germany
2N H_2SO_4	Carl Roth GmbH + Co. KG
TMB substrate solution	BD Biosciences, USA
Paraffin	Sanova Pharma GmbH, Austria
HRP; #34028	Thermofisher Scientific, USA
Assays and Kits	**Company**
MMP1 Duo Set ELISA	R&D Systems, USA
MMP2 Duo Set ELISA	R&D Systems, USA
MMP3 Duo Set ELISA	R&D Systems, USA
TIMP-1 Duo Set ELISA	R&D Systems, USA
IL-6 Duo Set ELISA	R&D Systems, USA
MMP activity assay	AAT Bioquest, USA
Antibodies	**Company**
IL-8 Capture antibody; #M108	Thermofisher Scientific, USA
IL-8 Detection antibody; #M802B	Thermofisher Scientific, USA
Softwares and Devices	**Company/Source**
Incubator	Thermofisher, USA
Microtome HM 335 E	Microm, GMI, USA
Dermatome; Acculan® 3Ti	Aesculap, Inc. USA
Nano HPLC-system	Thermofisher Scientific, USA
Orbitrap mass spectrometer; QExactive HF	Thermofisher Scientific, USA
Tissue homogenizer; Precellys 24	Bertin Technologies SAS, France
Microplate reader; BMG FLUOstar OPTIMA	BMG Labtech Inc. USA
Microplate photometer; Multiskan™ FC	Thermofisher Scientific, USA
GraphPad Prism 5	1992-2012 GraphPad Software Inc.
Perseus	Max Planck Institute of Biochemistry
Maxquant 1.3.0.5	Max Planck Institute of Biochemistry
DAVID Bioinformatics Resources 6.8	david.ncifcrf.gov
Microsoft Excel	Microsoft Office

4.2 Methods

4.2.1. Ethics statement

Healthy skin was obtained from female adult patients (age range: 27-52 years; locations: abdomen, upper leg, back) who have undergone routine reduction surgeries. All donors signed a declaration of consent and the study was approved by the ethics committee of the Medical University of Vienna.

4.2.2 Superficial *ex vivo* human skin wound model

Freshly isolated skin (1-2 h after surgery) was disinfected using Kodan Forte (Schülke & Mayr GmbH, Germany). D101-Squame standard self-adhesive discs (CuDerm, USA) were applied onto excised skin with constant pressure for 10 seconds (sec) and removed from top down. One area was TS 50 times in a row. Subsequently, the TS skin was cut with a Dermatome (0.6 mm; Acculan® 3Ti; Aesculap, Inc. USA) and biopsies (∅ 8 mm; Kai Europe GmbH, Germany) were taken from TS and non-TS (NTS) skin (=control). For some experiments, TS full thickness skin was biopsied (∅ 8 mm), embedded in paraffin and used for H&E staining.

4.2.3 Topical application of OCT and culture conditions

Before culture, OCT (octenilin® wound gel0.05% OCT, , Schülke & Mayr GmbH, Germany), a control gel (OCT-free hydrogel, Schülke & Mayr GmbH, Germany) or Normlgel (0.9% w/w sodium chloride solution in gel form; Mölnlycke Health Care AB, Sweden) were applied topically onto TS skin biopsies (50 microliter (µl)/biopsy). Treated and untreated TS skin biopsies, were cultured in 12-well culture plates in the presence of Dulbecco´s modified eagle medium (DMEM; 500 µl/well; Gibco, Thermofisher, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, Thermofisher) and 1% penicillin-streptomycin (Gibco, Thermofisher) for 48 h at 37°C and 5% CO_2 (Heracell™ 150i; Thermofisher, USA). On the second day, biopsies were collected, frozen in liquid nitrogen and stored at -80°C for the proteomics approach (LC-MS). Medium supernatants were collected as well and stored at -80°C for ELISA and an enzyme activity assay until use.

4.2.4 H&E staining

Freshly isolated and TS as well as NTS skin samples were fixed in 7.5% paraformaldehyde. Fixed tissues were embedded in paraffin (Sanova Pharma GmbH, Austria), cut (5 µm) with a Microtome (Microm HM 335 E; GMI, USA) and stained with H&E solution according to standardized protocols.

4.2.5 Sample preparation for proteomics analysis

Cultured TS biopsies (n=5, female, abdomen, age range: 38-45 years) that were treated with OCT, control gel and left untreated, were homogenized in 100 µl sample buffer (7.5 molar (M) Urea, 1.5 M Thiourea, 0.1 M dithiothreitol (DTT), 4% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.05% sodium dodecyl sulfate (SDS)) using ultrasound. Protein concentrations were premeasured using a Bradford assay. For each sample 20 µg protein was used for the in-solution digest performed according to a variation (Zila et al. 2018) of the FASP protocol (Wiśniewski et al. 2009). Protein containing samples were loaded onto a pre-washed 10 kDa MWCO filter (molecular weight cut-off filter; Pall Nanosep Centrifugal Devices with Omega Membrane, #OD010) and centrifuged at 14,000×*g* for 15 minutes (min) to remove all particles smaller than 10 kDa. For reduction, samples were incubated with 200 µl DTT solution (5 milligram (mg)/milliliter (ml) dissolved in 8 M guanidinium hydrochloride in 50 mM ammonium bicarbonate buffer, pH 8) at 37°C (shaker, 1100 revolutions per minute (rpm)) for 30 min. After centrifugation at 14,000×*g* for 10 min, a washing step with 50 mM ammonium bicarbonate buffer was performed. For alkylation, 200 µl iodoacetamide (IAA) solution (10 mg/ml and 8 M guanidinium hydrochloride in 50 mM ammonium bicarbonate buffer) was added and samples were incubated at 30°C for 30 min in the dark (shaker, 1100 rpm). After centrifugation at 14,000×*g* for 10 min, proteins on top of the filters were washed twice with 50 mM ammonium bicarbonate buffer. Afterwards, filters were placed in a new Eppendorf tube, and 95 µl of 50 mM ammonium bicarbonate buffer as well as 5 µl of protease solution (Promega Trypsin/Lys-C Mix, Mass Spec Grade, #V5073, 0.1 microgram (µg)/µl) were added, and samples incubated at 37°C for 18 h. After digestion, peptide samples were cleaned up with C-18 spin columns (Thermo Fisher Scientific Pierce C18 spin columns, #89870). Peptides were collected with 0.5% trifluoroacetic acid (TFA) and acidified to a final concentration of 1% TFA. Peptide samples were finally dried at 40°C using a centrifugal vacuum concentrator (miVac GeneVac Duo Concentrator) and stored at -20°C until further LC-MS analyses were performed.

4.2.6 LC-MS shotgun analysis

Dried samples were reconstituted as previously described (Bileck et al. 2014, Slany et al. 2016) in formic acid (FA; 5 µl at 30%) containing 10 fmol each of 4 synthetic standard

peptides and diluted with 40 µl mobile phase A (98% H_2O, 2% ACN, 0.1 % FA). Of this solution, 5 µl were injected into the Dionex Ultimate 3000 nano HPLC-system (Thermo Fisher Scientific). Peptides were concentrated on a pre-column (2 cm × 75 µm C18 Pepmap100; Thermo Fisher Scientific) at a flow rate of 10 µl/min, using mobile phase A. Subsequently, they were separated by elution from the pre-column to an analytical column (50 cm × 75 µm Pepap100; Thermo Fisher Scientific) applying a flow rate of 300 nl/min and using a gradient of 8% to 40% mobile phase B (80% ACN, 20% H2O, 0.1% FA), over 190 min for sample analysis. The mass spectrometric analysis was performed on a QExactive HF orbitrap mass spectrometer, equipped with a nanospray ion source (Thermo Fisher Scientific), coupled to the nano HPLC system. For detection, MS scans were performed in the range from m/z 400–1400 at a resolution of 60,000 (at m/z = 200). MS/MS scans were performed choosing a top 12 method. HCD fragmentation was applied at 27% normalized collision energy and analysis in the orbitrap at a resolution of 15,000 (at m/z = 200).

4.2.7 LC-MS data analysis and interpretation

Analysis and interpretation of LC-MS data was performed essentially as described (Zila et al. 2018). Protein inference as well as label-free quantitative (LFQ) data analysis was accomplished using the open source software MaxQuant 1.3.0.5 including the Andromeda search engine and the Perseus statistical analysis package. Protein inference was achieved aligning against *homo sapiens* in the SwissProt Database, allowing a mass tolerance of 5 ppm for MS spectra, 20 ppm for MS/MS spectra and a maximum of 2 missed cleavages. In addition, carbamidomethylation on cysteine residues was included as fixed modification whereas methionine oxidation as well as N-terminal protein acetylation was included as variable modifications. Searching criteria included a minimum of two peptide identifications per protein, at least one of them unique, and the FDR calculation based on q-values (less than 0.01) performed for both, peptide identification as well as protein inference. Prior to statistical analysis, proteins were filtered for reversed sequences, contaminants and a minimum of three independent identifications per protein in order to exclude false positive protein hits. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository and the dataset identifier PXD007592. Label-free quantification revealed LFQ values for each individual protein, which were conducted for quantitative assessment of protein regulation using the Perseus statistical analysis package. A two-sided *t* test with $p < 0.05$ was performed to identify significantly up- and

down-regulated proteins with a minimum of a twofold abundance difference (log_2 fold change). With proteins meeting these criteria, subsequent gene ontology annotation enrichment analysis was performed. Fold enrichment values for individual GO terms, count, p value and FDR were calculated using DAVID (DAVID Bioinformatics Resources 6.7, National Institute of Allergy and Infectious Diseases) and protein function was determined using the Uniprot database.

4.2.8 Preparation of skin lysates

Cultured TS biopsies (OCT-treated and controls) were transferred into tissue homogenizing CK14 tubes (2 ml; Bertin Technologies SAS, France), containing ceramic beads (1.4 mm, zirconium oxide). Triton-X (300 µl, 1%; Merck KGaA, Germany) was added and tubes were put into a tissue homogenizer (Precellys 24 Tissue Homogenizer; Bertin Technologies SAS). A programme with two cycles of 45 sec was used to lyse whole biopsies. Samples were stored at -80°C for further analysis with ELISA.

4.2.9 ELISA

Culture plates (Nunc Immuno 96 well flat-bottom culture plate; Merck KGaA) were coated with appropriate capture human antibodies such as IL-8 (Thermo Fisher Scientific, #M108), IL-6, MMPs or TIMP-1 (R&D Systems) overnight (ON) at 4°C or room temperature (RT). On the next day, plates were washed with washing buffer (0.05 % Tween in PBS) and blocked with blocking buffer (4% BSA, 0.5% Tween in PBS) or reagent diluent (1% BSA in PBS). Another washing step was performed and standards and samples were pipetted on the plates in an appropriate dilution and incubated for 2 h. After that, plates were aspirated and the detection antibody was applied (Thermo Fisher Scientific, R&D Systems, #M802B) and incubated for another 2 h. Next, plates were washed again and streptavidin-horse-radish peroxidase (HRP) (Thermo Fisher Scientific #34028, R&D Systems) was applied. After an incubation time of 20-30 min, another washing step was performed and 3,3',5,5'-tetramethylbenzidine (TMB) substrate reagent A+B (BD Biosciences, USA) was applied and incubated for 20 min. Subsequently, a stop solution (2N H_2SO_4; 50 µl/well) was added and measurement was performed immediately at 450 nm with a photometer (Multiskan™ FC Microplate Photometer; Thermofisher, USA).

4.2.10 Enzyme activity assay

The activity of several MMPs (MMP1, MMP2, MMP3, MMP7, MMP8, MMP9, MMP10, MMP12, MMP13) in culture supernatants was measured using the Amplite™ Universal Fluorimetric MMP Activity Assay Kit (AAT Bioquest, USA). Supernatants were activated with 4-aminophenylmercuric acetate (2 mM; APMA) ON. Activated supernatants and controls were pipetted in duplicates into a flat-bottom culture plate (100 µl/well; Corning® 96 Well White Polystyrene Microplate; Merck KGaA) and MMP Green™ substrate (50 µl/well; AAT Bioquest) was added. Fluorescence intensity was monitored as kinetic measurement with a plate reader (BMG FLUOstar OPTIMA Microplate Reader; BMG Labtech Inc. USA) at Ex/Em = 490/525 nm.

4.2.11 Statistical analysis

Data was analyzed using GraphPad Prism 5. An unpaired t-test was used for comparing means, respectively. The results were considered significant with *P*-values smaller than 0.05.

5. Results

5.1 TS efficiency is best in the inner region of a TS area on *ex vivo* human skin

The well-established TS method (Tajpara et al. 2018), is a technique by which the outermost layer of the epidermis (= *stratum corneum*) is removed. However, to our knowledge, it was not yet defined if biopsies taken from different positions of a TS area are comparable, or show, according to potential unequally applied pressure, differences in TS efficiency. To address this, abdominal skin of four healthy female donors (age range: 33-38 years) were TS. Subsequently, biopsies were taken from five defined regions on the TS area (Fig. 7) and embedded in paraffin. H&E was performed and light microscopy was applied to evaluate the efficiency of TS. Results from three out of four donors clearly showed that TS is most efficient in the inner region (=2) compared to outer regions (=1, 3, 4, 5) (Fig. 7). Of note, H&E staining of one donor showed no differences in TS efficiency between the five different biopsy regions. Aiming to standardize further experiments as much as possible, only biopsies of the inner region were used for all following experiments.

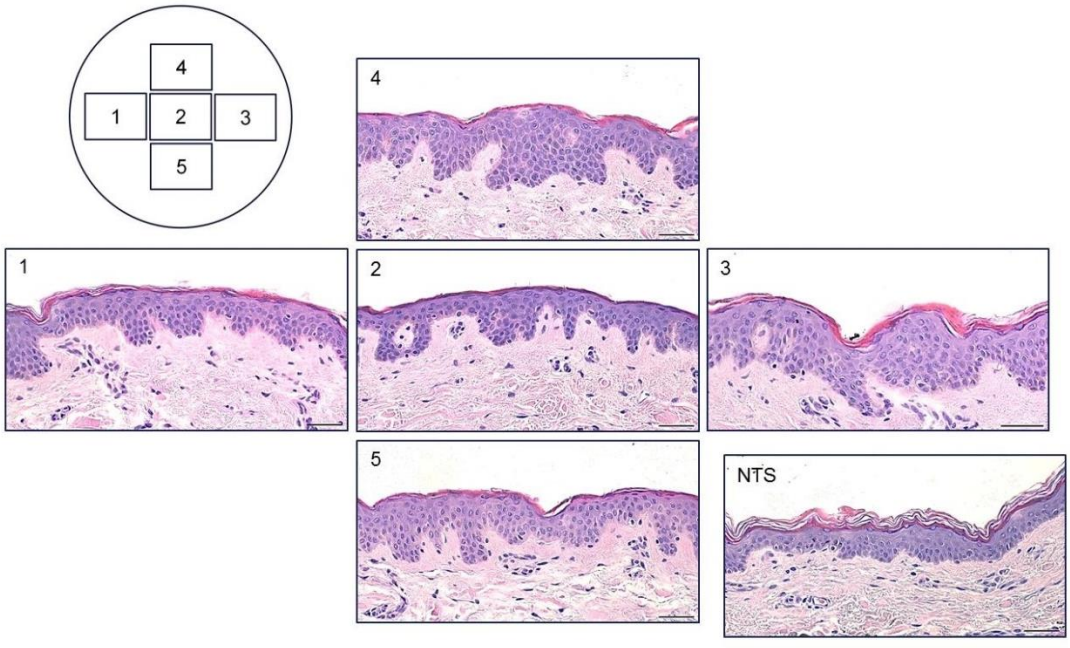

Figure 7. Complete removal of the *stratum corneum* in the inner region of the TS area. Freshly isolated abdominal skin of one female donor (37 years) was TS 50 times on the same area. Representative H&E staining (n=3) revealed that the highest efficiency in removing the *stratum corneum* is achieved in the inner region (position 2) of a TS area. Biopsy positions correspond to the numbers indicated in the circle displayed in the upper left corner. NTS = control, scale bar = 50 µm.

5.2 OCT significantly inhibits IL-8 secretion in superficially wounded *ex vivo* human skin

We have reported already that OCT significantly inhibits IL-8 secretion in a superficial human skin wound model (Nikolić et al. 2019). As an adequate control (= control gel without OCT) in the previous work has not been available, Normlgel was used and repeatedly induced higher IL-8 secretion compared to untreated TS controls. In this study, TS abdominal skin biopsies of healthy female donors (age range: 27-52 years) were treated (OCT, Normlgel, OCT-free control gel) or left untreated to comparatively assess IL-8 secretion levels in culture supernatants that were collected after 48 h of culture. Significantly lower IL-8 concentrations were identified in supernatants of OCT-treated biopsies compared to relatively similar IL-8 concentrations in supernatants of control-gel treated and untreated skin biopsies (Fig. 8), approving the anti-inflammatory potential of OCT. Of note, Normlgel induced consistently higher IL-8 levels compared to untreated and control gel-treated TS controls (Fig. 8) thus confirming our published results (Nikolić et al. 2019).

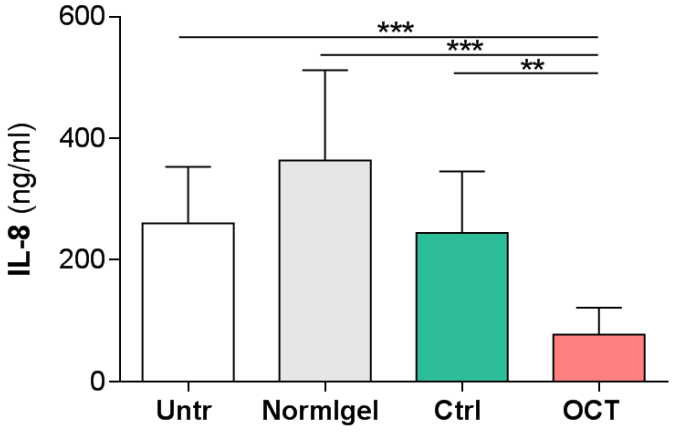

Figure 8. OCT significantly inhibits IL-8 secretion in human skin. Shown are IL-8 concentrations in culture supernatants of 48 h cultured human TS skin biopsies (n=7; females donors; age range: 27-52 years) that were topically treated with OCT (0.05% OCT in gel form), control gel (OCT-free hydrogel), Normlgel (0.9% w/w sodium chloride solution in gel form) and left untreated. IL-8 levels in supernatants were tested in triplicates with an ELISA. Data is presented as a mean±SD (n=7). An unpaired t-test was performed with GraphPad Prism. **p ≤ 0.01, ***p ≤ 0.001.

5.3. OCT-treated wounded human skin biopsies show a distinct proteomic profile compared to controls

Aiming to uncover the protein profile of human skin cells upon wounding and OCT-treatment a proteomic approach was chosen. To address this, abdominal skin biopsies of healthy female donors (age range: 38-45 years) were TS, treated with OCT, control gel or left untreated, cultivated for 48 h and subsequently prepared for LC-MS analysis. Results were

statistically analysed using the software MaxQuant 1.3.0.5 and the Perseus statistical analysis package. In total, 2622 proteins were detected. To investigate proteins significantly differentially expressed between the three groups, an unpaired t-test was utilized with LFQ values of the proteins for each group using Perseus. Proteins with a p-value lower than 0.05 and a difference lower than -1 or higher than +1, corresponding to a $\log_2$ fold change of at least 2, were considered as significant hits and were further analysed. Twenty-six proteins were found to be differentially regulated between OCT- and control gel-treated samples (Fig. 9A, Table 1). Thirty-seven proteins were found to be differentially regulated between OCT-treated and untreated samples (Fig. 9B, Table 2). Ten proteins were significantly differentially expressed between OCT-treated and untreated samples as well as between OCT treated and control treated samples (Fig. 9A, B). Additionally, sixteen proteins were differentially expressed between the control-gel group and the untreated group, showing that even applying pure hydrogel on the wound has an effect on the protein profile of skin cells. However, no protein candidate was differentially expressed between OCT-treated and untreated or OCT-treated and the control gel-treated samples, which was also found to be differentially expressed between the control gel-treated group and the untreated group, demonstrating that OCT has an effect (data not shown). Protein function was determined using the Uniprot database as well as DAVID Bioinformatics Resources. Among significant hits, several proteins are involved in the immune response as well as wound healing and tissue regeneration (Tables 1 and 2). Functional analysis was followed by literature search and potential protein candidates were validated by ELISA.

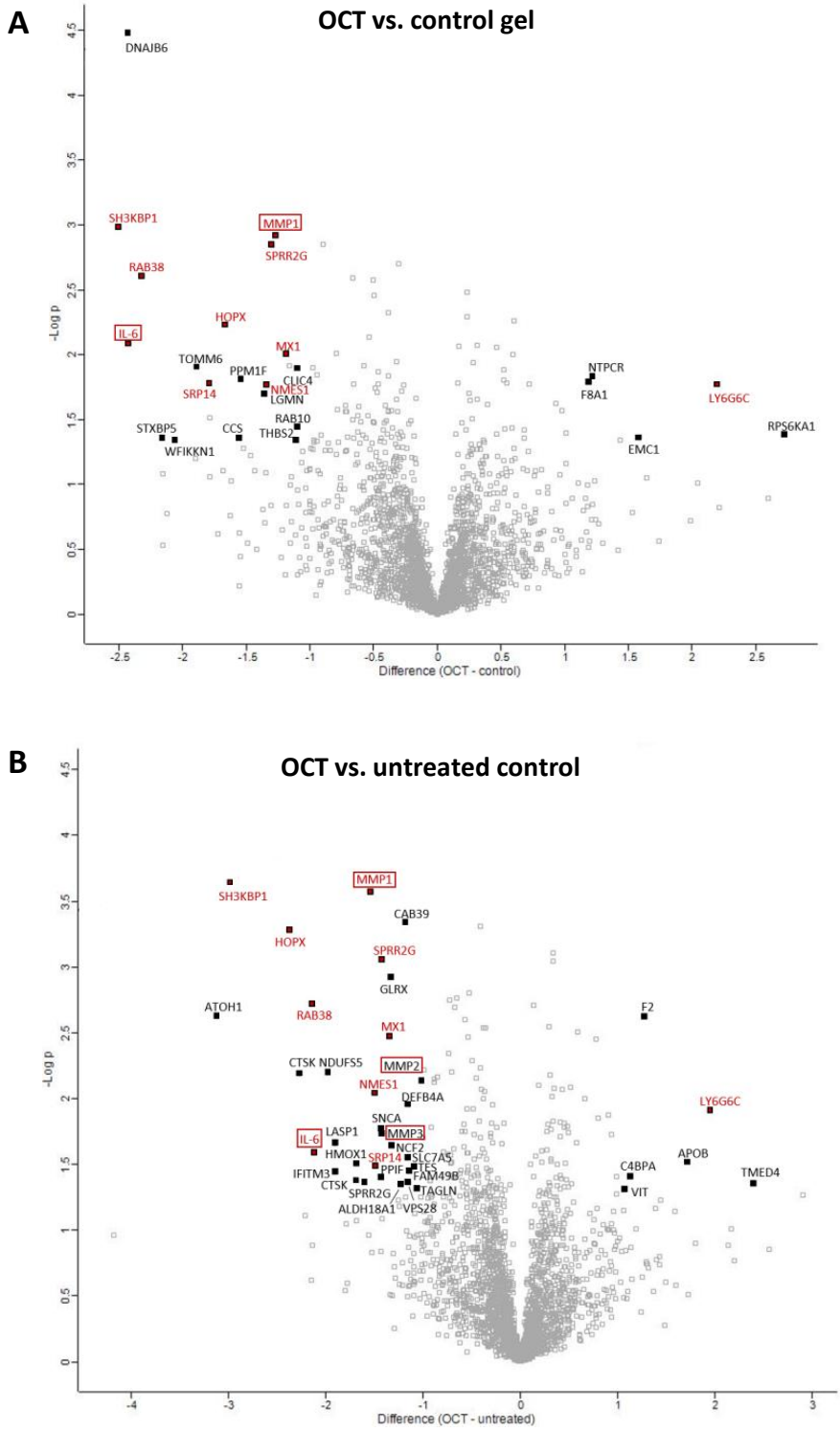

Figure 9. Differentially regulated proteins among TS OCT-treated skin and controls. Abdominal skin biopsies of healthy female donors (n=5; age range: 38-45 years) were TS, treated with OCT, control gel or left untreated, cultivated for 48 h and subsequently prepared for LC-MS analysis. Volcano plots show differences in LFQ values (fold change, logarithmic scale to the base of two) on the x-axis including their corresponding p values (logarithmic scale) on the y-axis. (**A**) Proteins with a difference lower than -1 are downregulated in OCT-treated samples, proteins with a difference higher than +1 are upregulated in OCT-treated samples, compared to control gel-treated samples. Proteins marked in red are additionally differentially regulated between OCT-treated and untreated samples. (**B**) Proteins with a difference lower than -1 are downregulated in OCT-treated samples, proteins with a difference higher than +1 are upregulated in OCT treated samples, compared to untreated samples. Proteins marked in red are additionally differentially regulated between OCT-treated and control gel-treated samples. Red boxes indicate proteins that were further analysed. Vulcano plots were generated performing a two-sided *t* test ($p < 0.05$) using Perseus. Extended information on the proteins can be found in Tables 1 and 2.

Table 1. Differentially regulated proteins in OCT- and control gel-treated skin

Acc.Nr.	Gene names	Protein names	Difference	p-value
Q15418-3	RPS6KA1	Ribosomal protein S6 kinase alpha-1	2.72936	0.0406338
P57729	RAB38	Ras-related protein Rab-38	-2.31488	0.00248603
Q5T5C0-2	STXBP5	Syntaxin-binding protein 5	-2.16046	0.0441092
Q96NZ8	WFIKKN1	WAP, Kazal, immunoglobulin, Kunitz and NTR domain-containing protein 1	-2.05995	0.0460281
Q96B49	TOMM6	Mitochondrial import receptor subunit TOM6 homolog	-1.89169	0.0123562
P37108	SRP14	Signal recognition particle 14 kDa protein	-1.79208	0.0164122
P07196	NEFL	Neurofilament light polypeptide	-1.78873	0.0307429
Q9BPY8	HOPX	Homeodomain-only protein	-1.66531	0.00577304
O14618	CCS	Copper chaperone for superoxide dismutase	-1.5529	0.0442306
P49593-2	PPM1F	Protein phosphatase 1F	-1.54092	0.015049
P35442	THBS2	Thrombospondin-2	-1.10232	0.0452981
Q8TEX9	IPO4	Importin-4	1.01008	0.0403239
P23610	F8A1	Factor VIII intron 22 protein	1.19871	0.0161568
Q9BSD7	NTPCR	Cancer-related nucleoside-triphosphatase	1.22018	0.0147584
Q8N766-4	EMC1	ER membrane protein complex subunit 1	1.58853	0.0429446
Proteins involved in tissue repair processes				
Q96B97-3	SH3KBP1	SH3 domain-containing kinase-binding protein 1	-2.50139	0.00103201
O75190-2	DNAJB6	DnaJ homolog subfamily B member 6	-2.42276	0.0000329
Q9BYE4	SPRR2G	Small proline-rich protein 2G	-1.29277	0.0014479
Q9Y696	CLIC4	Chloride intracellular channel protein 4	-1.09563	0.0126506
Proteins involved in immune response				
P05231	IL6	Interleukin-6	-2.41779	0.00811524
Q99538-3	LGMN	Legumain	2.20007	0.019888
P20591	MX1	Interferon-induced GTP-binding protein Mx1	-1.35853	0.00978011
O95867	LY6G6C	Lymphocyte antigen 6 complex locus protein G6c	-1.3394	0.0168512
Q9C002	NMES1	Normal mucosa of esophagus-specific gene 1 protein	-1.18638	0.0169618
P61026	RAB10	Ras-related protein Rab-10	-1.09958	0.0363097
Proteins involved in both, tissue repair and immune response				
P03956	MMP1	Interstitial collagenase	-1.26249	0.00119881

OCT-treated and control gel-treated cultured human TS skin biopsies (n=5; age range: 38-45 years) were tested using LC-MS. An unpaired t-test was performed with Perseus, *$p < 0.05$. Difference is displayed as $\log_2$ fold change, p-values are ordered from lowest to highest. Protein function was determined using the Uniprot database.

Table 2. Differentially regulated proteins in OCT-treated and untreated skin

Acc.Nr.	Gene names	Protein names	Difference	p-value
Q92858	ATOH1	Protein atonal homolog 1	-3.12793	0.00237
Q7Z7H5-3	TMED4	Transmembrane emp24 domain-containing protein 4	2.39738	0.044618
Q9BPY8	HOPX	Homeodomain-only protein	-2.38045	0.00053
P57729	RAB38	Ras-related protein Rab-38	-2.13996	0.001908
O43920	NDUFS5	NADH dehydrogenase [ubiquinone] iron-sulfur protein 5	-1.98058	0.006411
Q14847	LASP1	LIM and SH3 domain protein 1	-1.90196	0.021941
Q9UBR2	CTSZ	Cathepsin Z	-1.68655	0.041945
P37108	SRP14	Signal recognition particle 14 kDa protein	-1.50146	0.032403
P30405	PPIF	Peptidyl-prolyl cis-trans isomerase F, mitochondrial	-1.42846	0.040132
P35754	GLRX	Glutaredoxin-1	-1.3358	0.001207
P54886-2	ALDH18A1	Delta-1-pyrroline-5-carboxylate synthase	-1.23201	0.045378
Q9Y376	CAB39	Calcium-binding protein 39	-1.18201	0.000458
Q01650	SLC7A5	Large neutral amino acids transporter small subunit 1	-1.15907	0.028462
Q9UK41	VPS28	Vacuolar protein sorting-associated protein 28 homolog	-1.15247	0.043866
Q9UGI8-2	TES	Testin	-1.09905	0.033049
Proteins involved in tissue repair processes				
Q96B97-3	SH3KBP1	SH3 domain-containing kinase-binding protein 1	-2.99325	0.000231
P43235	CTSK	Cathepsin K	-2.27851	0.00656
P35325	SPRR2B	Small proline-rich protein 2B	-1.60542	0.043572
Q9BYE4	SPRR2G	Small proline-rich protein 2G	-1.41857	0.000889
Q01995	TAGLN	Transgelin	-1.06854	0.048312
Q6UXI7-2	VIT	Vitrin	1.05801	0.049801
proteins involved in immune response				
P05231	IL6	Interleukin-6	-2.12485	0.025918
Q01628	IFITM3	Interferon-induced transmembrane protein 3	-1.90649	0.036663
Q9C002	NMES1	Normal mucosa of esophagus-specific gene 1 protein	-1.49802	0.009124
P20591	MX1	Interferon-induced GTP-binding protein Mx1	-1.35044	0.003326
O15263	DEFB4A	Beta-defensin 4A	-1.16194	0.011058
Q9NUQ9	FAM49B	Protein FAM49B	-1.14202	0.035844
P04003	C4BPA	C4b-binding protein alpha chain	1.11854	0.038646
O95867	LY6G6C	Lymphocyte antigen 6 complex locus protein G6c	1.94051	0.012323
proteins involved in both, tissue repair and immune response				
P03956	MMP1	Interstitial collagenase	-1.54141	0.000266
P09601	HMOX1	Heme oxygenase 1	-1.68656	0.031343
P37840-2	SNCA	Alpha-synuclein	-1.43251	0.016726
P08254	MMP3	Stromelysin-1	-1.42857	0.018419
P19878-2	NCF2	Neutrophil cytosol factor 2	-1.32954	0.022825
P08253	MMP2	72 kDa type IV collagenase	-1.02733	0.007274
P00734	F2	Prothrombin	1.26839	0.002398
P04114	APOB	Apolipoprotein B-100	1.71104	0.030551

OCT-treated and untreated cultured human TS skin biopsies (n=5; age range: 38-45 years) were tested using LC-MS. An unpaired t-test was performed with Perseus, *$p < 0.05$. Difference is displayed as $\log_2$ fold change, p-values are ordered from lowest to highest. Protein function was determined using the Uniprot database.

5.4 Gene-ontology (GO) analysis uncovers proteins relevant for wound healing

For proteins found to be downregulated in OCT-treated human skin, a GO annotation was performed using DAVID Bioinformatics Resources. Of interest was primarily the function of proteins. Therefore, biological processes (BPs) were selected and displayed as bar graphs (Figs. 10 and 11). Among both groups (proteins downregulated in OCT-treated compared to control gel-treated skin, as well as proteins downregulated in OCT-treated compared to untreated skin) functions involved in wound healing were found to be represented (Figs. 10 and 11). ECM disassembly, collagen-catabolic process, proteolysis as well as angiogenesis were downregulated in OCT-treated skin compared to untreated skin (Fig. 11). Proteins involved in those functions are, among others, IL-6 and members of the MMP family (MMP1, MMP2, MMP3).

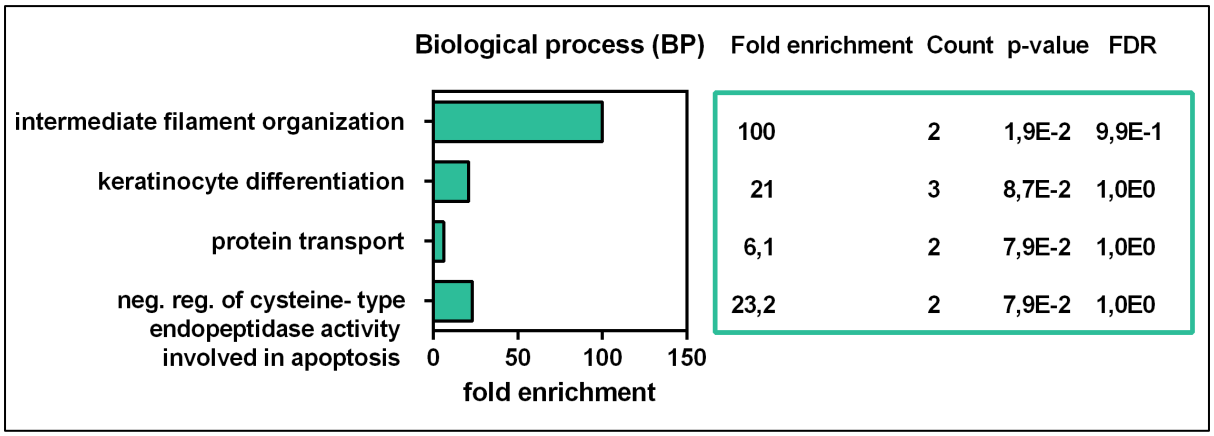

Figure 10. GO annotation enrichment analysis reveals that OCT leads to a downregulation of proteins involved in wound healing. Classification by the GO term BP shows pathways and larger processes made up of the activities of multiple proteins. Fold enrichment values for individual GO terms, count (proteins involved in the term), p value and FDR (false discovery rate, calculated using the Benjamini–Hochberg procedure), listed next to the graph, were calculated using DAVID bioinformatics resources.

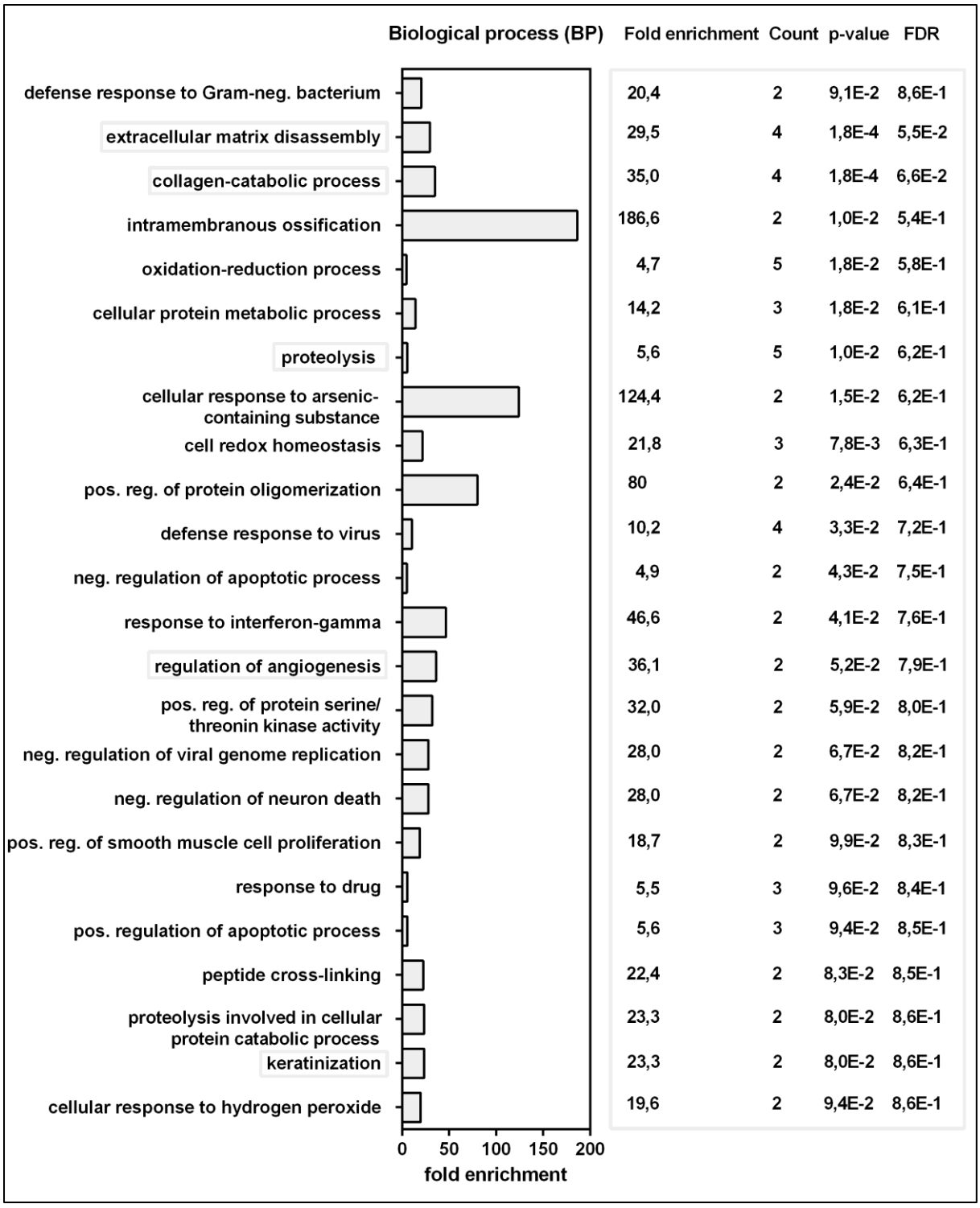

Figure 11. GO annotation enrichment analysis reveals that OCT leads to a downregulation of proteins involved in wound healing. Classification by the GO term biological process (BP) shows pathways and larger processes made up of the activities of multiple proteins. Fold enrichment values for individual GO terms, count (proteins involved in the term), p value and FDR (false discovery rate, calculated using the Benjamini–Hochberg procedure), listed next to the graph, were calculated using DAVID bioinformatics resource.

5.5 OCT significantly inhibits secretion of pro-inflammatory cytokines in wounded human skin

Potential protein candidates involved in immune response and wound healing, which are up- or downregulated in OCT-treated samples compared to controls according to LC-MS data, were tested additionally with a quantitative ELISA. We have described that OCT has anti-inflammatory potential based on significantly reduced IL-8 secretion in supernatants of cultured TS human skin biopsies *ex vivo* (Fig. 8) (Nikolić et al. 2019). IL-6 is another pro-inflammatory cytokine known to play a role in the immune response and wound healing (Barrientos et al. 2008). In this study, LC-MS data revealed that IL-6 was significantly diminished in OCT-treated biopsies compared to untreated biopsies or biopsies treated with the control gel (Fig. 12). Analysis of culture supernatants derived from the same donors similarly showed a significant reduction of IL-6 secretion in OCT treated samples compared to controls, supporting and extending our previous conclusion that OCT has an anti-inflammatory capacity (Fig. 12).

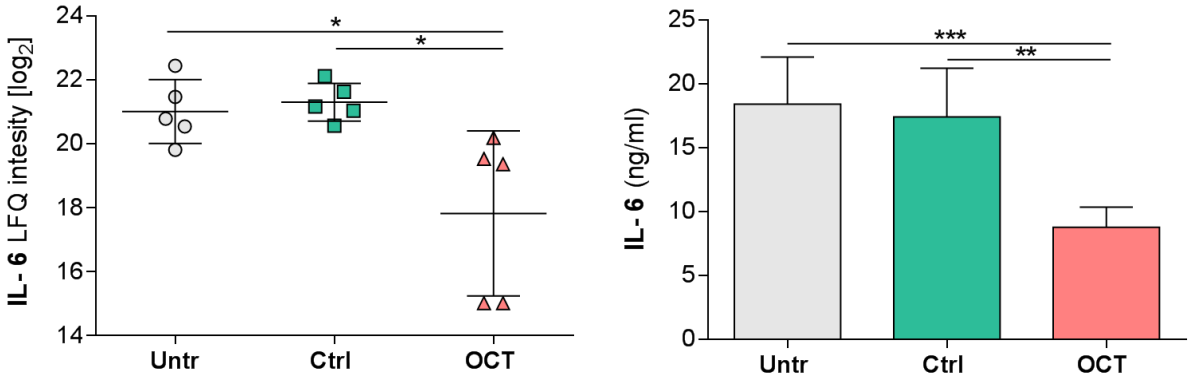

Figure 12. OCT significantly inhibits IL-6 secretion in wounded human skin. IL-6 levels were evaluated by LC-MS of TS human skin biopsies treated with OCT and were significantly lower when compared to biopsies which were untreated or treated with a control gel (left panel). Label-free quantification (LFQ) intensities in a logarithmic scale to the basis 2 are indicated. LFQ intensities for proteins not detected in a replicate were replaced by 15. IL-6 concentration in supernatants was tested in duplicates with an ELISA (right panel). Data in both graphs is presented as a mean±SD (n=5). An unpaired t-test was performed with GraphPad Prism. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

5.6 OCT inhibits secretion of proteases but not protease inhibitors in wounded human skin

MMPs and their inhibitors (TIMPs) play a crucial role in all stages of wound healing by regulating EMC degradation and deposition which allows tissue remodelling as well as cell migration (Gill und Parks 2008). Even though MMPs are essential in the process of acute wound healing, an excess can contribute to impaired wound healing and inflammation (Caley et al. 2015, Manicone und McGuire 2008). We found, that MMP1 as well as MMP2 expression (LC-MS) and secretion (ELISA) levels were significantly lower in skin biopsies treated with OCT in comparison to controls (Fig. 13). MMP3 expression was lower in OCT-

treated samples when compared to untreated samples, but not when compared to samples treated with the control gel (Fig. 13). OCT treatment of TS human skin did not influence TIMP-1 secretion..

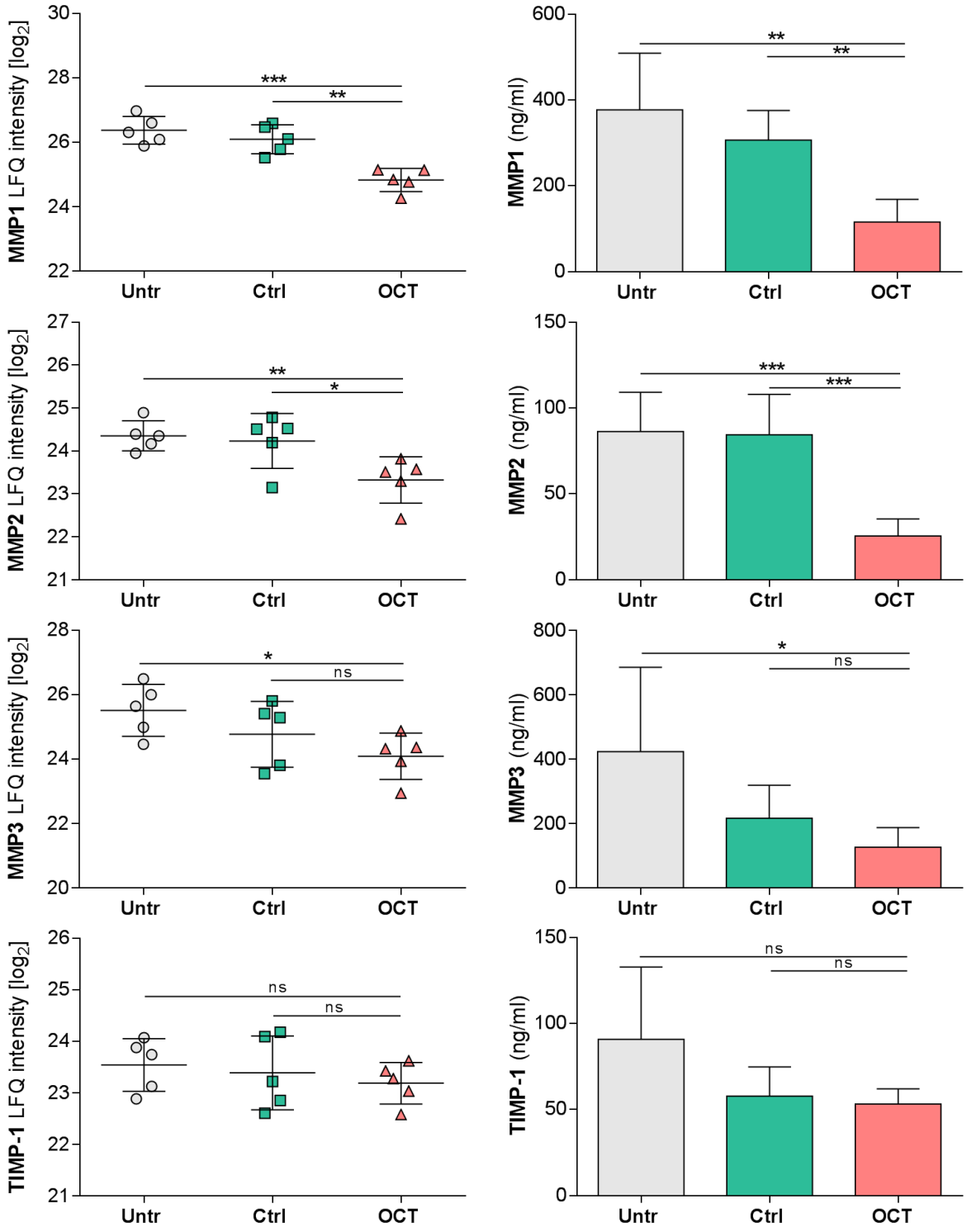

Figure 13. OCT significantly reduces secretion of proteases in wounded human skin but does not affect secretion of a protease inhibitor. Indicated MMP levels, analysed by LC-MS, were significantly lower in human skin biopsies treated with OCT compared to biopsies which were untreated or treated with a control gel, with exception of TIMP-1, which is not affected upon OCT treatment (left panel). Label-free quantification (LFQ) intensities in a logarithmic scale to the basis 2 are indicated. LFQ intensities for proteins not detected in a replicate were replaced by 15. Proteases and a typical protease inhibitor in culture supernatants were tested in duplicates with an ELISA (right panel). All data is presented as mean±SD (n=5). An unpaired t-test was performed with GraphPad Prism. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ns=not significant.

5.7 OCT inhibits protease activity in wounded human skin

Active MMPs are proteases that positively or negatively influence the process of wound healing. Therefore, not only the evaluation of their concentration in human skin is of interest, but also their enzymatic activity. Thus, an assay was employed measuring total activity of several MMPs based on the addition of an exogenous substrate. Culture supernatants of the same five donors as used for LS-MS analysis and ELISA were engaged in the activity assay. Indeed, supernatants of TS biopsies treated with OCT possessed significantly lower total MMP activity than supernatants of control groups (Fig. 14).

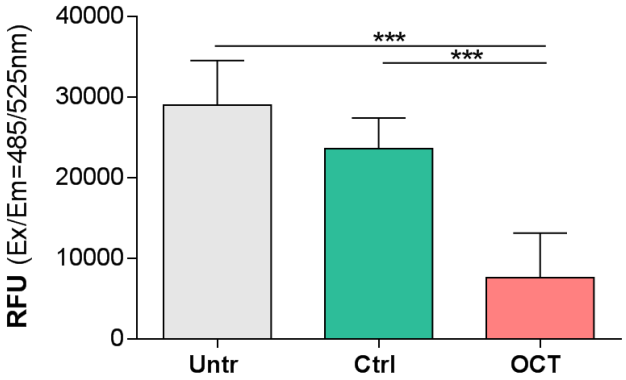

Figure 14. OCT inhibits MMP activity in wounded human skin. Abdominal skin biopsies of healthy female donors (n=5; age range: 38-45 years) were TS, treated with OCT, control gel or left untreated and cultivated for 48 h. Enzymatic activity of MMPs was significantly lower in culture supernatants of OCT-treated human skin biopsies compared to controls (untreated, control gel-treated). An unpaired t-test was performed with GraphPad Prism. ***p ≤ 0.001.

5.8 Whole skin biopsy lysates show similar results as culture supernatants

For LC-MS, lysates of whole skin biopsies were used, while ELISAs were performed with culture supernatants. To investigate if results obtained from these different sources are comparable to each other, an additional experiment was designed. Unfortunately, it was not possible to accomplish ELISAs with skin lysates as prepared for LC-MS, because chaotropic detergence was used for the proteolytic digest, potentially able to disturb fluorescence measuring but is essential to be measured by the ELISA microplate photometer. Therefore, skin from five additional female donors (age range: 39-45 years) was TS and treated with OCT, the control gel or remained untreated and was cultured for 48 h. Triton-X lysed biopsies were analysed simultaneously with culture supernatants of the same donors by ELISA. We found that in general the concentrations of the detected proteins varied between whole biopsy lysates and supernatants. For all measured proteins (IL-6, IL-8, MMP2, and

TIMP-1), higher concentrations were detected repeatedly in supernatants. Obtained data between OCT treatments and controls did not vary between supernatants and lysates, as a significant reduction of IL-6, IL-8 (Fig. 15) and MMP2 (Fig. 15) upon OCT treatment was measurable in both, lysates as well as supernatants. Also, an unaltered TIMP-1 secretion upon treatment with OCT could be confirmed for both, lysates and culture supernatants (Fig. 15).

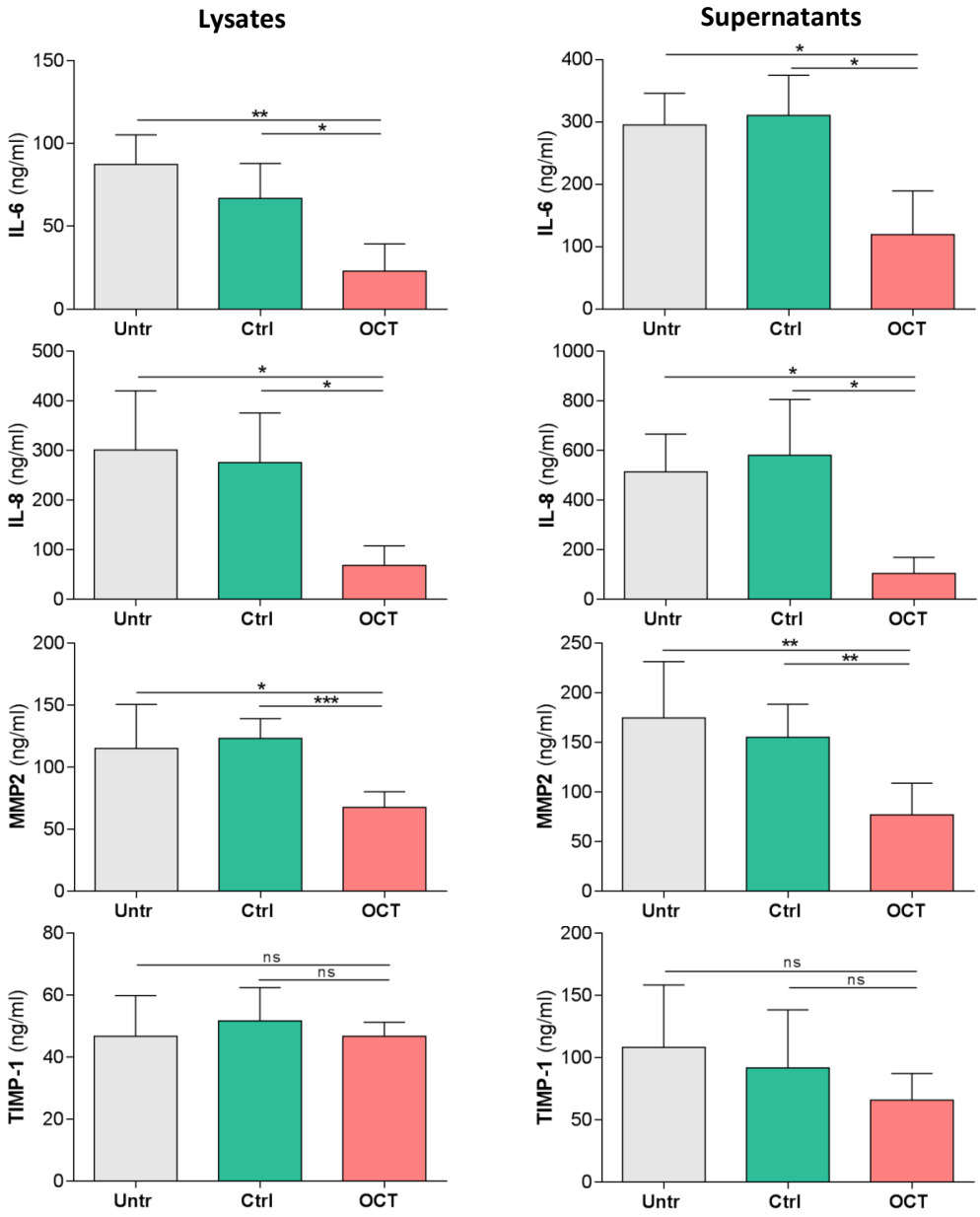

Figure 15. The anti-inflammatory and protease-inhibitory potential of OCT is evident in supernatants as well as skin biopsy lysates. Skin from five additional female donors (2 x abdomen, 2 x tights; 1 x back; age range: 39-45 years) was TS and treated with OCT, the control gel or remained untreated and was cultured for 48 h. Triton-X lysed biopsies were analysed simultaneously with culture supernatants of the same donors in duplicates by ELISA. Indicated pro-inflammatory cytokines and MMPs were significantly lower in lysates (left graph) and culture supernatants (right graph) of skin biopsies treated with OCT when compared to controls (untreated, control-gel treated). TIMP-1 levels are unaltered upon OCT treatment in both sample types, lysates (left) as well as culture supernatants (right). All data is presented as a mean±SD (IL6, IL-8: n=3; MMP2, TIMP-1: n=5). An unpaired t-test was performed with GraphPad Prism, *$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ns=not significant.

6. Discussion

OCT is a widely used antiseptic (skin, mucosa) with antibacterial, antifungal and partially antiviral effects (Koburger et al. 2010, von Rheinbaben und Wolff 2002). The broad pH range in which OCT is active, as well as the capacity to clear bacterial biofilms in patients with chronic wounds make OCT perfectly suitable for wound care (Cutting und Westgate 2012, Metcalf et al. 2014). Moreover, recent *in vivo* experiments showed that OCT also promotes wound healing associated with a lower incidence of hypertrophic scar formation when applied locally after surgical procedures (Matiasek et al. 2018). Here, we addressed the unknown mechanism by which OCT might contribute to tissue regeneration and improved wound healing using a mild, superficial wound model. We standardized as many parameters as possible when working with human tissue, including determination of the best TS area within a biopsy, evaluating optimal thickness of the skin for subsequent analysis, warranting consistent sex (female) and age range of donors within one experimental set up, and employing skin from a distinct body location (abdomen for proteomic analysis).

A detailed characterization of the protein profile of wounded skin biopsies that were topically treated with OCT in comparison to control gel-treated and untreated wounded skin was achieved using LC-MS. Out of 2622 identified proteins, 26 were found to be differentially regulated between OCT-and control gel-treated samples and 37 were differentially regulated between OCT-treated and untreated samples. In the next step we tried to identify proteins involved in wound healing and tissue regenerative processes and have therefore included a GO analysis, and database as well as literature search. Indeed, GO annotations revealed several biological processes that have been described to be involved in wound healing and in our data sets are represented among proteins downregulated in OCT treated skin. Those processes included ECM disassembly, collagen-catabolic process, proteolysis as well as angiogenesis. According to DAVID Bioinformatics Resources proteins associated with above mentioned processes were among others, IL-6 and MMP family members. IL-6 is a pro-inflammatory cytokine playing a key role in the acute phase reaction in response to immunological stress, and is further favoring transition from acute to chronic inflammation as it exerts stimulatory effects on T and B cells (Gabay 2006). In contrast to wound healing processes in adults, wound healing in fetuses is characterized by a minimal inflammation and scarless repair, which is partially attributed to a diminished production of pro-inflammatory cytokines. Significantly lower IL-6 mRNA expression and lower IL-8 mRNA

expression as well as protein concentrations were identified in fetal skin compared to adult skin in response to an incisional wound (Liechty et al. 1998, Liechty et al. 2000). Moreover, it has been reported that exogenous IL-6 leads to scar formation of human fetal skin grafts in SCID mice (Liechty et al. 2000). Further, it has been shown that IL-8 levels are significantly increased in slowly healing human burn wounds and that IL-8 decreases KC differentiation *in vitro* (Iocono et al. 2000). Our LC-MS data revealed that IL-6 levels were significantly lower in OCT-treated biopsies when compared to controls, implying that OCT has anti-inflammatory capacity. This assumption is in support to previous findings showing that OCT significantly reduces IL-8 secretion in a similar superficial human skin wound model (Nikolić et al. 2019) and was also confirmed and expanded in this study with the insertion of an additional and more appropriate control. The inhibition of IL-6 and IL-8 in OCT-treated skin biopsies is one possible explanation for the potency of OCT to enhance wound healing and prevent scar formation *in vivo*. MMPs are playing a crucial role in acute wound healing, as they dissolve the basal lamina and lyse surrounding tissue, so that proliferation and cell migration can take place. According to their substrate preferences MMPs can be divided into subgroups. MMP1 belongs to the subgroup of collagenases, MMP2 is a member of gelatinases, and MMP3 belongs to stromelysins (Krishnaswamy et al. 2017). When tissue remodeling is required, MMPs can be rapidly expressed by a variety of cells (Caley et al. 2015). Regulation of MMP levels mainly occurs via TIMPs. In chronic wounds or wounds showing delayed wound healing often an imbalance between TIMPs and MMPs has been observed (Ulrich et al. 2010, auf dem Keller und Sabino 2015). A high and long lasting expression as well as activity of MMPs might lead to increased ECM degradation, alteration of cytokine profile, and degradation of growth factors, culminating in delayed or absent wound closure (Martins et al. 2013). Moreover, it has been shown that mRNA expression of MMP2 is highly increased in different types of human scar tissue when compared to normal tissue derived from the same patient (Ulrich et al. 2010). Our proteomics analysis revealed significantly lower MMP1 and MMP2 levels in cultured wounded human skin treated with OCT, in comparison to both, untreated skin and skin treated with the control gel. For MMP3 we noted a significant reduction for OCT-treated biopsies in comparison to untreated biopsies, but not to biopsies treated with the control gel, suggesting that application of the hydrogel on the wound already leads to a decrease of MMP3 secretion. Similar results were obtained with culture supernatants of the same donors, suggesting that OCT also possesses protease-inhibitory

capability. Using an assay measuring total activity of several MMPs (MMP1, MMP2, MMP3, MMP7, MMP8, MMP9, MMP10, MMP12, MMP13), we found that indeed MMP activity was significantly reduced in OCT-treated samples compared to controls. As besides MMP1, MMP2 and MMP3, other active proteases are present in the skin, which also contribute to tissue remodelling processes, we speculate that some of them might have also been altered by OCT at an undetectable level. The fact that OCT inhibits the activity of tissue MMPs might be one further explanation of the scar preventing and wound healing promoting effects of OCT *in vivo*. TIMP-1 is one of the major MMP inhibitors, usually significantly downregulated in chronic or slowly healing wounds (auf dem Keller und Sabino 2015). This was not the case in skin biopsies treated with OCT, as revealed by LC-MS and ELISA analysis. As OCT neither upregulated nor downregulated TIMP-1, the exact mechanism of MMP inhibition in our model is not yet clear and remains to be further investigated. Of note, one protein involved in wound healing found to be upregulated in OCT-treated skin samples was prothrombin. As a thrombin precursor, prothrombin plays a key role in the coagulation cascade in the first stage of acute wound healing (Reinke und Sorg 2012). While proteomics data showed a significant upregulation of prothrombin, it could not be confirmed when samples were measured by an appropriate ELISA (data not shown), as here prothrombin levels did not significantly vary between the different treatments, neither for lysates nor for supernatants, implying that the different results are not due to usage of different sample types.

We have compared two different sample types (for the proteomics approach and ELISA such as whole biopsy lysates and culture supernatants, respectively). A comparative analysis of lysates and supernatants with an ELISA revealed that even though the concentrations of the detected proteins varied between these two sample types and different body locations were used due to limited patient material, data were comparable between OCT-treated and control groups. These data confirmed that OCT has anti-inflammatory as well as protease-inhibitory potency.

To conclude, our data provide new insights into the mode of action by which OCT might improve wound healing. As pro-inflammatory cytokines as well as MMPs are expressed and secreted by a variety of epidermal and dermal cells, we next aim to uncover the cellular target of OCT. One approach to address this will be the separation of the compartments before analysis. In addition, a bioinformatics approach will be chosen, comparing our data to previous proteomics data, which is already associated to distinct cell types.

7. Summary

Octenidine dihydrochloride (OCT) is a widely used antiseptic molecule promoting skin wound healing accompanied with diminished scar formation after surgery *in vivo*. In this study we aimed to uncover the hitherto unknown mode of action by which OCT might contribute to tissue regeneration. For this purpose, a mild superficial wound model was introduced by tape stripping (TS) of *ex vivo* human skin and subsequent topical application of OCT and the respective controls. TS treated and TS untreated skin biopsies as well as culture supernatants were collected after 48 hours of culture for analysis with liquid chromatography-mass spectrometry (LC-MS), enzyme-linked immunosorbent assay (ELISA) and an enzyme activity assay. Pro-inflammatory cytokines (interleukin (IL)-6, IL-8) were significantly inhibited in TS and OCT-treated skin samples (LC-MS) and supernatants (ELISA) compared to control gel-treated and untreated skin. Also matrix metalloproteinases (MMP1, MMP2, MMP3), playing a major role in tissue remodeling and known to be elevated in slowly healing wounds, were significantly reduced in TS and OCT-treated samples and supernatants compared to controls. An enzyme activity assay confirmed that total MMP activity in skin was indeed reduced upon OCT treatment. TIMP-1, one of the major MMP inhibitors, which is typically diminished in chronic wounds, was not significantly altered in OCT-treated skin samples compared to controls. Additionally, the concentrations of selected proteins (IL-6, IL-8, MMP2, TIMP-1) were comparatively assessed in whole biopsy lysates and culture supernatants of TS and OCT-treated samples and controls with ELISA. Results showed significant differences between OCT treated skin and controls in both sample types, supernatants and lysates. Together, our findings uncover that OCT has anti-inflammatory as well as protease-inhibitory capacities, thus providing new insights into the mode of action by which OCT might improve wound healing.

8. Zusammenfassung

Octenidin-dihydrochlorid (OCT) ist ein weit verbreitetes antiseptisches Molekül, welches bei postoperativer Anwendung *in vivo* die Wundheilung fördert sowie eine Narbenbildung vorbeugt. Allerdings ist über den wundheilungsfördernden Mechanismus von OCT noch wenig bekannt. Ziel der Studie war es daher, neue Erkenntnisse über die Wirkungsweise mit der OCT zu einer verbesserten Wundheilung beiträgt, zu gewinnen. Hierfür wurde auf humaner Haut *ex vivo* eine oberflächliche Wunde mittels „tape stripping“ gesetzt und anschließend mit OCT und entsprechenden Kontrollen behandelt. Nach einer Kultivierungszeit von 48 Stunden wurden die Hautbiopsien sowie Kulturüberstände zur Analyse mittels Liquid-Chromatographie-Massenspektometrie (LC-MS), Enzyme-linked Immunosorbenassay (ELISA) und einen Enzymaktivitätsassay herangezogen. Die Konzentration an pro-inflammatorischen Zytokinen (Interleukin (IL)-6, IL-8) war in Hautbiopsien (LC-MS) und Kulturüberständen (ELISA), die mit OCT behandelt wurden, signifikant geringer als in unbehandelter oder mit Kontrollgel behandelter Haut. Auch die Konzentration von Matrix Metalloproteinasen (MMP1, MMP2, MMP3), welche eine maßgebliche Rolle bei der Geweberegeneration spielen, jedoch in chronischen Wunden oft überexprimiert sind, war in verwundeter, OCT behandelter Haut signifikant reduziert. Mit einem Enzymaktivitätsassay konnte bestätigt werden, dass OCT tatsächlich die Gesamtaktivität von MMPs in humaner Haut inhibiert. TIMP-1, ein MMP-Inhibitor, dessen Expression in chronischen Wunden üblicherweise reduziert ist, war in OCT-behandelter Haut nicht verändert. Um zu testen, ob es zwischen Lysaten und Kulturüberständen maßgebliche Unterschiede gibt, haben wir ausgewählte Proteine (IL-6, IL-8, MMP2, TIMP-1) sowohl in Kulturüberständen als auch Lysaten von verwundeten Hautbiopsien, welche entweder mit OCT oder den entsprechenden Kontrollen behandelt wurden, mittels ELISA analysiert. Signifikante Unterschiede zwischen OCT-behandelter Haut und Kontrollen konnten sowohl in Lysaten als auch in Kulturüberständen nachgewiesen werden. Die Studie zeigt, dass OCT sowohl eine anti-inflammatorische als auch Protease-inhibitorische Wirkung hat und gewährt damit neue Erkenntnisse über die Wirkungsweise mit der OCT zu einer verbesserten Wundheilung beiträgt.

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