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Thyroxine promotes reepithelialization and angiogenesis in wounded human skin

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Abbreviations

ACTH = adrenocorticotropic hormone

ANOVA = analysis of variance

bFGF = basic fibroblast growth factor

BM = basement membrane

CCL = chemokine ligand

CK = cytokeratin

CRH = corticotropin-releasing hormone

CTGF = connective tissue growth factor

CTS = connective tissue sheath

DMEM = Dulbecco's modified Eagle medium

DP = dermal papilla

ECM = extracellular matrix

EGF= epidermal growth factor

EGFR = epidermal growth factor receptor

EMT = epithelial-mesenchymal transition

ERK = extracellular signal-regulated kinase

ET= epithelial tongue

EPC = endothelial progenitor cell

FBS = fetal bovine serum

FGF = fibroblast growth factor

FGFR = fibroblast growth factor receptor

GM CSF = granulocyte-macrophage colony-stimulating factor

HF = hair follicle

HGF = hepatocyte growth factor

HIF = hypoxia-inducible transcription factor

HPA axis = hypothalamic-pituitary-adrenal axis

HPC = haematopoietic precursor cells

HS = hair shaft

IF = immunofluorescence

IGF = insulin like growth factor

IHC = immunohistochemistry

IR = immunoreactivity

IRS = inner root sheath

KGF = keratinocyte growth factor

LCE = late cornified envelope

MAPK = mitogen-activated protein kinase

MC = mast cell

MIF = macrophage migration inhibitory factor

MMP = matrix metalloproteinases

MSC = mesenchymal stem cell

MTCO1 = mitochondrial encoded cytochrome c oxidase I

MVD = microvessel density

nM = nanomole per liter

ORS = outer root sheath

PBS = phosphate buffered saline

PDGF = platelet-derived growth factor

PECAM = platelet endothelial cell adhesion molecule

RNA = ribonucleic acid

rT3 = reverse T3

S1008A = s100 calcium binding protein A8

SCF = stem cell factor

T3 = triiodothyronine

T4 = tetraiodothyronine (L-thyroxine)

TBS = tris buffered saline

TGF = transforming growth factor

TH = thyroid hormone

THs = thyroid hormones

TNF = tumour necrosis factor-α

TR = thyroid hormone receptors

TRE = thyroid hormone response element

TRH = thyreotropin releasing hormone

TRHR = thyrotropin releasing hormone receptor

TSA = tyramide signal amplification

TSH = thyroid stimulating hormone (thyreotropin)

TUNEL = terminal dUTP nick-end labelling

VEGF = vascular endothelial growth factor

WE = William's E medium

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1 Introduction

1.1 Project overview

The management of chronic, healing-retarded wounds has become one of the major, as yet unmet challenges in current clinical medicine (Fonder et al., 2008; Sen et al., 2009; Escandon et al., 2011; Eaglstein et al., 2012; Tang et al., 2012). In particular, well-tolerated, inexpensive and effective wound healing promoters need to be urgently identified. To assist with this, simple, but predictive and clinically relevant preclinical test systems are needed that allow one to search for such wound healing-promoting agents, ideally substances that are already in widespread clinical use.

The current thesis project explores in a newly developed full-thickness organ culture assays of experimentally wounded human skin whether one such candidate agent, thyroxine (T4), holds promise as a wound healing promoter.

Subsequently, basic background information for this project on human skin biology and wound healing is presented, followed by an analysis of the major challenges that clinically applied wound healing research faces today.

1.2 Human skin

1.2.1 Structure and function

Skin (**Figure 1**) is the largest organ of human's body. The integumentary system not only protects the body from dehydration and the underlying muscles and bone from environmental damage (Bangert et al., 2011), but wards off multiple other

injuries from the body, including infectious organisms, UV light, thermal, mechanical and chemical stressors. Skin also serves as an important sensory organ that provides sense of touch, pressure, temperature, vibration, pain, and itch. Moreover, the skin and its appendages are important for social and sexual communication; metabolize hormones and neuropeptides, e.g. Vitamin D, etc.(Holt, 1978; Stucker et al., 2002; Madison, 2003; Sterry et al., 2006; Proksch et al., 2008; Krieg and Aumailley, 2011).

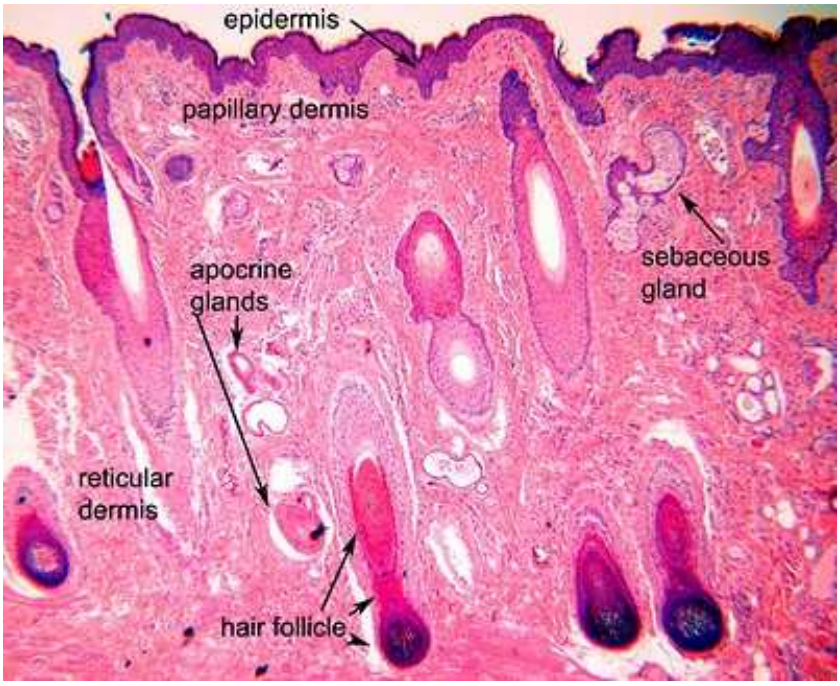

Figure 1. Histology of hair-bearing human skin

The human skin has a total area of 1.5-2.0 square meters and 12-15% of body weight (around 5 kilograms) in the adult human. However, it always varies not only from one individual to another one, but also from one region of the body to another with respect to texture, colour, thickness, and appendage structures (hair follicles, sebaceous glands, sweat glands, vessels, nerves, etc) (Gaboriau and Murakami, 2001)

(From http://eyepathology.blogspot.de/2010/10/tissue-types-epithelium-blood-muscle.html)

The skin is subdivided into three distinct anatomical compartments: the epidermis, the superficial epithelial skin layer which serves as the biological, chemical and physical barrier between the body and its environment; the dermis, i.e. the adjacent mesenchymal layer which provides crucial structural support, perfusion, innervation,

access to the general immune system and multiple secreted signals to the epidermis and holds most skin appendages; and the subcutis (also called hypodermis), the well-perfused and innervated adipose layer of skin (**Figure 1 and 2**). This composite structure connects with the underlying muscle tissue via a fibrous fascia (Stenn et al., 2006). In the context of this thesis project, we shall mainly deal with the epidermal and dermal compartments of experimentally wounded human skin.

1.2.2 Epidermis

The normal thin epidermis is a stratified, terminally differentiated epithelium and normally composed by four layers (from the superficial to the deepest) (**Figure 2a and 2b**): stratum corneum (horny layer [stratum corneum]); stratum granulosum (granular cell layer); stratum spinosum (spinous or prickle cell layer); stratum basale (basal or germinativum cell layer) which also contains epidermal stem cells, including some cytokeratin 15 (CK15)-positive epithelial progenitor cells (Moll et al., 1993; Kloepper et al., 2008; Fortunel et al., 2010; da Silva-Diz et al., 2012; de Souza et al., 2012).

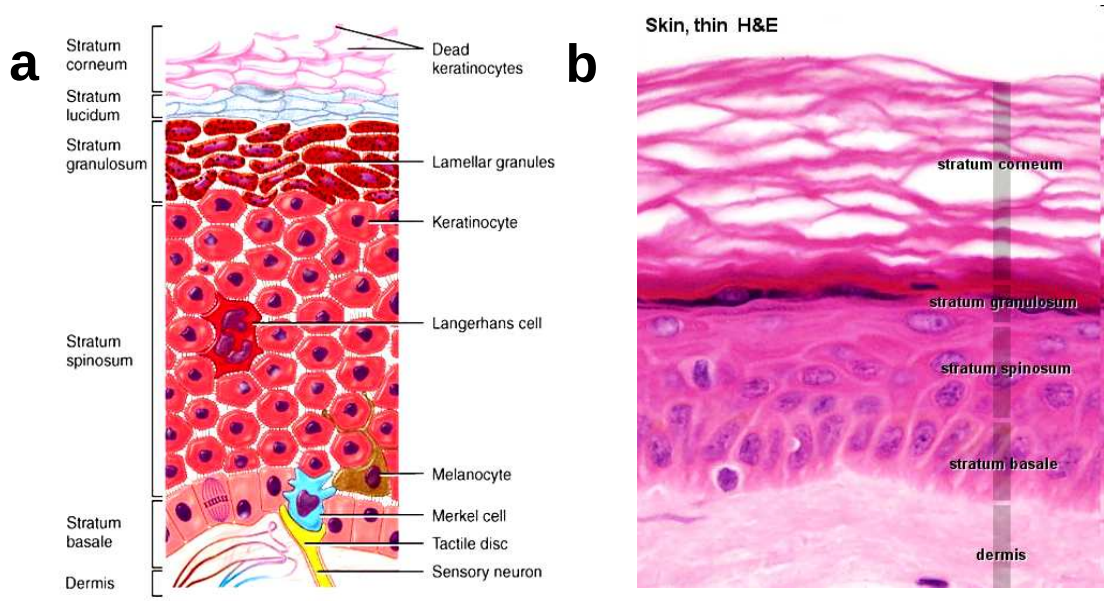

Figure 2. Schematic (a) and histological images (b) detailing the different layers and cell

types of human epidermis

(From http://www.imperial.edu/~thomas.morrell/cha_5_tortora_integument.htm [**a**] and http://en.wikipedia.org/wiki/Epidermis_%28skin%29 [**b**])

While the epidermis is constituted mainly by keratinocytes, there are several other cell populations-melanocytes (**Figure 3a)**, Langerhans cells (**Figure 3b)**, Merkel cells (**Figure 3c**), and intraepithelial T cells – while macrophages, B cells and mast cells are not found in healthy epidermis. While the epidermis contains no blood and lymphatic vessels, it is densely innervated (Sterry et al., 2006; Murphy, 2012; Reinke and Sorg, 2012) .

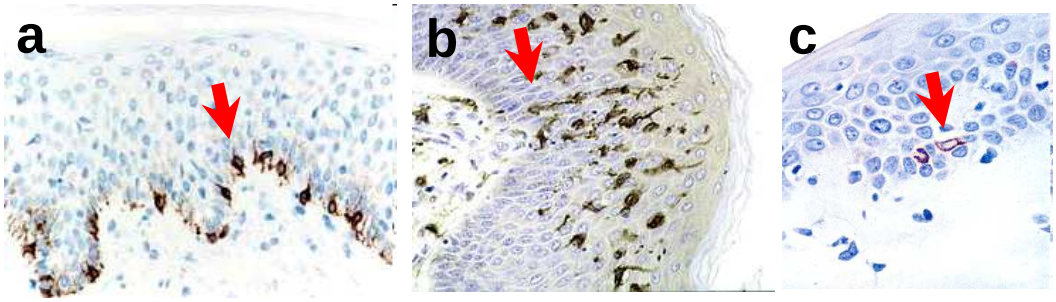

Figure 3. Immunohistochemical staining of melanocytes (a, Melan-A stain), dendritic cells (b, S100 stain, Langerhans cells), and Merkel cells (c, Cytokeratin-20 stain) in basal layer
(From: http://www.nordiqc.org/Run-7/Assessment/assessment-MLA.htm [**a**];
http://en.wikipedia.org/wiki/Langerhans_cell [**b**];
http://www.studyblue.com/notes/note/n/skinhairbreast-pics/deck/3615859 [**c**])

Melanocytes (**Figure 2a and 3a)**, a small population in the basal cell, are pigmentary cells of neural crest origin which not only are responsible for melanin synthesis and transfer, presumably to protect the nuclei of epidermal keratinocytes from ultraviolet radiation (UV), but may also exert multiple additional functions in epidermal physiology (Slominski et al., 1993; Plonka et al., 2009). It is as yet unclear whether any of the non-epithelial cell populations of human epidermis plays a functional important role in human skin wound healing.

The stratum basale is a continuous, innermost layer of undifferentiated, proliferating keratinocytes which lies next to the dermis comprise as a single cell

layer that are attached to the basement membrane (BM) by hemidesmosomes (Tsuruta et al., 2011). Located directly above the stratum basale, the keratinocytes of the stratum spinosum switch-on a program of terminal differentiation whose product, intracellular keratohyalin granules, are most prominently visible in the next layer, the stratum granulosum (**Figure 2**). Finally, in the stratum corneum, the outermost cornified layer of skin, keratinocytes have extruded their nuclei and contains densely packed intermediate keratin filaments that are bundled together in a manner that makes these non-viable cells highly resistant to environmental stressors, for example, temperature, pH, chemical insults and enzymatic digestion (Wysocki, 1999; Visscher et al., 2010; Wato et al., 2012) .

1.2.3 Dermis

The dermis is mainly composed by extracellular matrix (ECM)-embedded fibroblasts and consists of two layers, the papillary and reticular dermis. These are interspersed by blood and lymphatic vessels by autonomic and sensory nerve fibers of different type and caliber, as well as by mast cells (**Figure 4c)**, macrophages and other dendritic cells, and a few lymphocytes (**Figure 4b)**, while granulocytes are normally absent (**Figure 1 and 2)** (Sterry et al., 2006; Murphy, 2012). The fibroblasts, which also may become a contractile cell (myofibroblast) during wound contraction, play a key role in wound healing, e.g. by the production of ECM such as collagen I and IV (Vedrenne et al., 2012) (**Figure 4a)**.

Collagen is an exceedingly tough, long-lived, water-absorbing fibrous protein, and the collagen fibers mainly are responsible for the mechanical strength and extensibility of the dermis (**Figure 4a-d)** (Gaboriau and Murakami, 2001). Interspersed elastin fibers are responsible for elastic and recoil properties of the skin (**Figure 4a)** (Liu et al., 2004). The dermis also contains the bulk of skin appendages, i.e. hair follicles, eccrine and apocrine sweat glands, and sebaceous

glands, as well as mechanoreceptors that provide the sense of touch, vibration and heat (**Figure 4d)**.

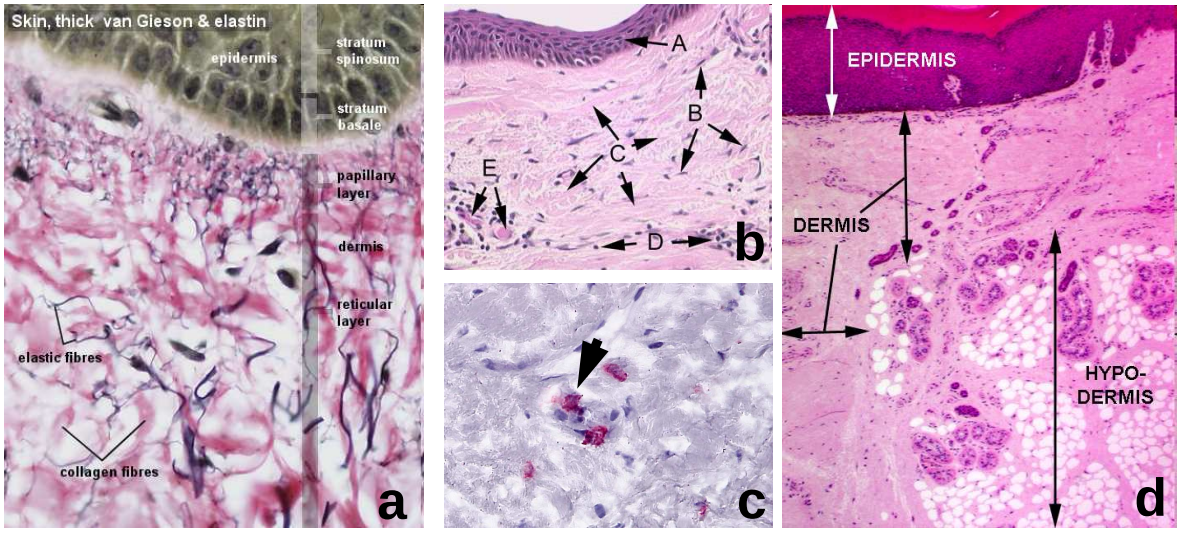

Figure 4. Structure of skin: Dermis
(**a**) Gieson & elastin staining of thick skin. (**b**) Hematoxylin and eosin staining (H&E*)* of dermis. A: Epidermis; B: Fibroblasts; C: Collagen fibre bundles; D: Lympocytes; E: Blood vessels. (**c**) Visualisation of mast cells in human dermis by Leder esterase histochemistry (black arrow, red cells). (**d**) Hematoxylin and eosin staining (H&E). ”Hypodermis” = subcutis.
(From: http://www.lab.anhb.uwa.edu.au/mb140/corepages/integumentary/integum.htm [**a**]; https://courses.stu.qmul.ac.uk/SMD/kb/microanatomy/connective/cheatlink4.htm [**b**]; http://www.vetmed.vt.edu/education/curriculum/vm8054/labs/lab14/lab14.htm [**d**]).

1.2.4 Subcutis

The subcutis (hypodermis), which mainly consists of adipocytes and endothelial cells, is a loose connective tissue layer with major regional differences in the amount and arrangement of adipose tissue (**Figure 1 and 4d)** and multiple physiological functions, which range from energy storage and thermoregulation via hormone and neuropeptide synthesis and metabolism, to the regulation of food uptake via leptin (Klein et al., 2007; Cerman et al., 2008). Subcutaneous adipocytes also operate as niche cells that provide important paracrine growth-regulatory signals, notably to the hair follicle (Schmidt and Horsley, 2012), and may promote wound healing by the release of leptin (Klein et al., 2007; Negrao et al., 2012). Moreover, adipose tissue-derived stem cells are now widely recognized as one of

the most pluripotent adult stem cell populations of the human body, with multiple potential uses in regenerative medicine (Beeson et al., 2011; Gir et al., 2012).

1.2.5 Skin appendages

The appendages of the skin – hair, nails and glands – originate from the stratum basale and grow downward into the dermis and subcutis. The hair follicle (HF) (**Figure 5**) is a characteristic feature of mammals and the only organ that shows a lifelong cyclic remodelling activity. In its cycle, the HF undergoes autonomous, cyclic transformations from a stage of growth (anagen), via regression (catagen) to relative quiescence (telogen), which have also been demonstrated that HF itself and its cycling exerted wound healing promoting effects (Paus and Cotsarelis, 1999; Jahoda and Reynolds, 2001; Stenn and Paus, 2001; Paus and Foitzik, 2004; Ito et al., 2005; Ito and Cotsarelis, 2008; Schneider et al., 2009; Ansell et al., 2010; Al-Nuaimi et al., 2012).

In the context of wound healing, the HF is of special interest in that its activities directly impact on the efficiency and quality of wound healing as well as on intracutaneous angiogenesis, both of which are significantly increased in murine skin with terminal HFs in anagen, compared to catagen or telogen skin (Mecklenburg et al., 2000; Yano et al., 2001; Lau et al., 2009; Ansell et al., 2010). The sweat glands of human skin may also play a more prominent role in human skin wound healing than previously thought, as their stroma is rich in nestin+ stem cells, which can be utilized to promote wound healing in murine skin *in vivo* (Danner et al., 2012) and wounded organ-cultured human skin (Liao. et al., 2012).

The HF consists of mesenchymal part the dermal papilla (DP) and the connective tissue sheath (CTS) that surrounds the entire HF and forms the infundibulum, isthmus, bulge and hair bulb (Paus and Cotsarelis, 1999; Schneider et al., 2009)

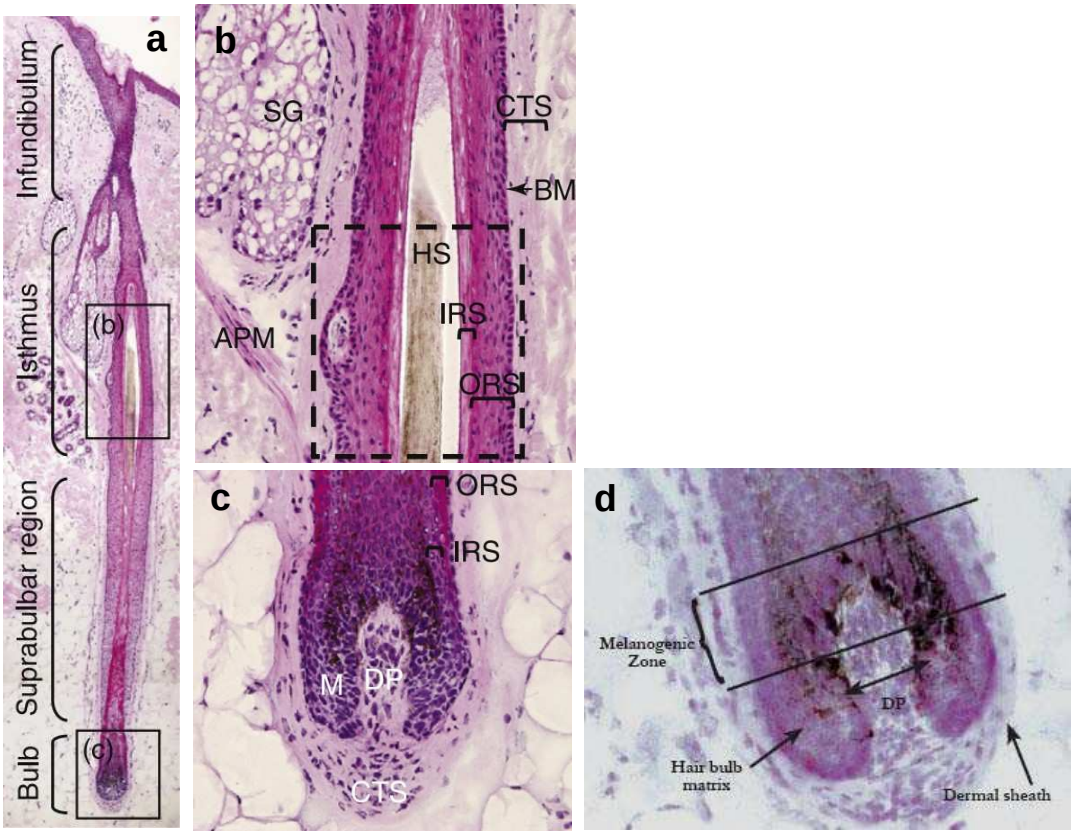

Figure 5. Histomorphology of human scalp hair follicle

(**a**) H&E section showing infundibulum, isthmus and anagen-associated (suprabulbar and bulbar area) components of the hair follicle. (**b**) High magnification image of the isthmus. The dashed square indicates the approximate location of the bulge; (**c**) High magnification image of the bulb. (**d**) Melanin granules. Graying of hair results from gradual dysfunction of the hair bulb's pigmentary unit (including decreased tyrosinase activity of HF melanocytes) and gradual loss of the regenerative potential of HF melanocyte stem cells in the bulge (Paus, 2011).

(BM: basal membrane; APM: arrector pili muscle; CTS: connective tissue sheath; DP: dermal papilla; M: matrix; HS: hair shaft, IRS: inner root sheath; ORS: outer root sheath; SG: sebaceous gland).

(From Schneider et al., 2009 Image No 1 [**a-c**] and http://www.pgbeautygroomingscience.com/whats-next.php [**d**]).

(**Figure 5**). HFs display a very dense innervation system, especially of the bulge and isthmus region. The perifollicular neural plexus release neurotransmitters, neuropeptides and neurotrophins and may thus fulfill important trophic and regulatory functions in hair biology (Botchkarev et al., 1997a; Botchkarev et al., 1997b; Paus et al., 1997; Botchkarev et al., 1998; Peters et al., 2001; Botchkarev et al., 2004; Botchkarev et al., 2006; Anderson et al., 2010).

1.3 Wound healing

Human adult skin wound healing is a complex multi-stage, dynamic event, which is modulated not only by local wound healing-regulatory factors, the state of tissue perfusion and innervation, and the functionality of relevant stem cell pools, but also by systemic mediators (such as steroid hormones), the nutritional and metabolic status as well as the absence/presence of deficiencies (e.g., vitamin C, Zn^{++} and Fe^{++} ions) (Behm et al., 2012; Medlin, 2012; Reinke and Sorg, 2012; Valacchi et al., 2012). Acute wounds generally show five distinct, but overlapping phases. These are customarily summarized under the headings 1) “haemostasis”, 2) “inflammation”, 3) “proliferation”, 4) “remodeling”, and 5) “scarring” (**Figure 6**) (Singer and Clark, 1999; Werner and Grose, 2003; Stuart Enoch, 2008).

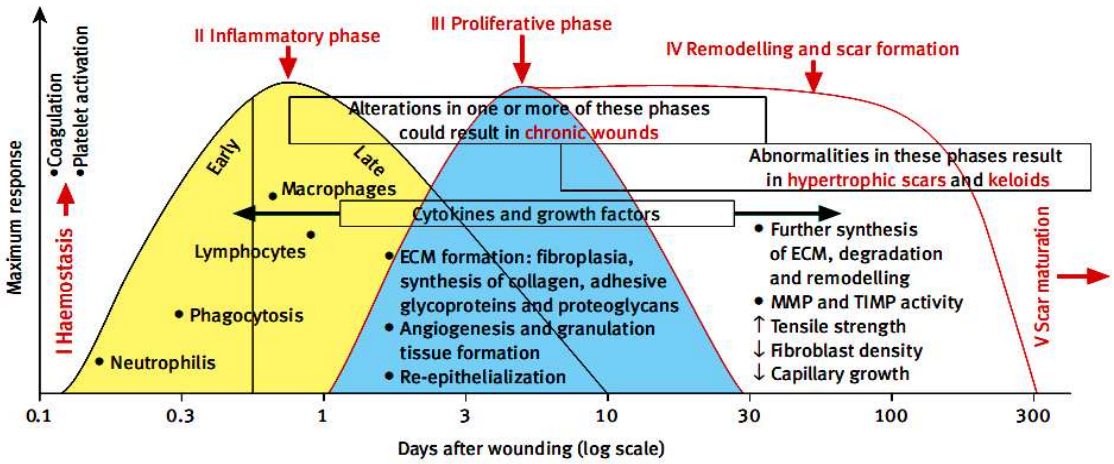

Figure 6. Phases of wound healing
ECM: Extracellular matrix; MMP: Metalloproteinase; TIMP: Tissue inhibitors of metalloproteinase (From Stuart Enoch, 2008).

1.3.1 Wound healing phase 1: haemostasis

The first phase of wound healing, haemostasis, is characterized by micro vascular and extravasation of blood into the wound and occurs immediately after the injury within seconds to minutes. This not only prevents blood loss, but also provides a

matrix for immigrating cells that is necessary for the subsequent wound healing phase to progress successfully (Velnar et al., 2009; Arwert et al., 2012).

Platelets are integral into this phase, which are essential for the entire healing process, since they not only provide hemostasis but also de-granulate and release their alpha granules, which secrete critical growth factors, including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF β), epidermal growth factor (EGF), platelet factor-IV, insulin-like growth factor-1 (IGF). These initiate not only the extrinsic and intrinsic coagulation cascades, but also are important wound healing promoters (Lawrence, 1998; Gurtner et al., 2008; Lucas et al., 2010; Gantwerker and Hom, 2011b, a) (**Table 1**). Fibrin polymerization supports clot formation and provides the scaffolding matrix for the infiltrating cells (fibroblasts, leukocytes, macrophages and keratinocytes) in the subsequent phases of wound healing. The platelets also release vasoactive substances such as catecholamines and serotonin act via specialized receptors on the endothelium, thus increasing microvascular permeability, which leads to fluid exudation into the extravascular space (Findikcioglu et al., 2012).

1.3.2 Wound healing phase 2: Inflammation

Clinically, the inflammatory phase (**Figure 7**) is characterized by redness, heat, swelling and pain, which is caused by vasodilatation and increased capillary permeability. In the early stage of this phase, neutrophils are the first key leukocytes that are attracted to the wound site (within 1-2 days after injury) by a number of chemoattractive agents, including fragments of ECM protein, TGF-β, complement components (e.g. C3a, C5a) and formyl-methionyl peptide products from bacteria and platelet (Singer and Clark, 1999; Stuart Enoch, 2008; Velnar et al., 2009; Gantwerker and Hom, 2011a). In a short time, neutrophils become sticky and, through a process of margination, start to adhere to the endothelial cells in the adjacent blood vessels and begin to intensely move through the vessel wall

(diapedesis) (Singer and Clark, 1999; Stuart Enoch, 2008).

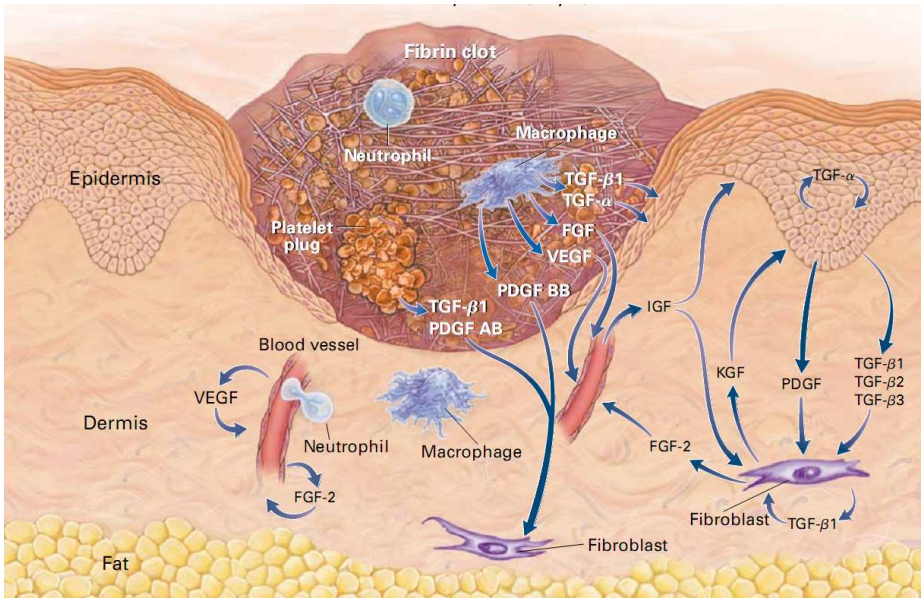

Figure 7. Inflammatory phase of wound healing

A selection of growth factors thought to be necessary for directing and regulating cell movement into the wound is shown here. TGF-a: transforming growth factor a; FGF: fibroblast growth factor; VEGF: vascular endothelial growth factor; PDGF: platelet-derived growth factor; IGF insulin-like growth factor and KGF: keratinocyte growth factor

(From Singer and Clark, 1999 Image No 2. For a more comprehensive list of wound healing modulators, see **Table 1**)

In the late stage of the inflammatory phase, phagocytic cells such as macrophages and other lymphocytes appear in the wound and continue to clear debris and bacteria (Eming et al., 2007; Stuart Enoch, 2008; Velnar et al., 2009). Macrophages have long been thought to be the most important cells in the late stage of the inflammatory process and seem to act as the key modulator cells in the repair process (Rodero and Khosrotehrani, 2010; Gantwerker and Hom, 2011b; Arwert et al., 2012).

Though likely less crucial than macrophages (Mahdavian Delavary et al., 2011), mast cells also are functionally important in the early stage of wound healing, namely in murine skin wounds *in vivo* (Weller et al., 2006; Shiota et al., 2009). Besides contributing to antimicrobial defense (Metz et al., 2008; Murphy, 2012),

they may also limit tissue damage (e.g., rescue implantation defects) (Noli and Miolo, 2001; Woidacki et al., 2013), promote angiogenesis (Trabucchi et al., 1988; Maurer and Metz, 2005; Ammendola et al., 2012; Genovese et al., 2012), tumor biology (Gotlib et al., 2013; Jiang et al., 2013) and even exhibit selective uptake of IgE based on an special imaging techniques in perivascular (Cheng et al., 2013). After activation by tissue injury, mast cells located at the wound margin degranulate to release various important mediators essential to initiate the inflammatory response of injured tissue and to activate regional endothelial cells such as histamine, different proteases, tumor necrosis factor-alpha (TNFa) (Ng, 2009; Noli and Miolo, 2010; Killick et al., 2011). In turn, the endothelial cells also influence mast cell function by releasing e.g. stem cell factor (SCF), interleukin-3 (IL-3) and thrombin, which enhance migration, proliferation and local differentiation of mast cells (Baghestanian et al., 1997; Huang et al., 1998; Noli and Miolo, 2001, 2010).

Intriguingly, during the final stage of wound healing, mast cells may enhance scar formation, and they interfere with scarless repair in fetal skin; this suggests that mast cells may mediate the transition from scarless to fibrotic healing during fetal development (Wulff et al., 2011). In fact, excessive mast cell activities have long been suspected to play an important role in the formation of hypertrophic scars and keloids (Smith et al., 1987; Akaishi et al., 2008; Ammendola et al., 2012; Bagabir et al., 2012).

1.3.3 Wound healing phase 3: Proliferation

This wound healing stage occurs 2-10 days after injury and is characterized by reepithelialization, angiogenesis and extensive ECM synthesis and remodelling (Gurtner et al., 2008).

1.3.3.1 Role of reepithelialization in skin wound healing

The first event in this stage is reepithelialization (**Figure 8**), which is marked by the

proliferation and migration of keratinocytes near the leading edge of the wound (Gurtner et al., 2008; Gantwerker and Hom, 2011b). Epidermal cells migrate as a sheet, prolonging lamellipodia alongside the progressing edge and then loosen their attachments to the underlying dermis; this allows them to migrate in a 'leapfrog' manner across the temporary matrix (Stuart Enoch, 2008). Once these keratinocytes encounter the ECM of the wound mesenchyme, they attach near the inner wound edge and begin to lay down a new basement membrane (Gantwerker and Hom, 2011a). During this process, the stimulation of integrin receptors expressed by these keratinocytes with ECM proteins such as fibronectin and vitronectin provides important guiding and regulatory signals (Singer and Clark, 1999; Margadant et al., 2010; Schultz et al., 2011). Reepithelialization is an energy-consuming process, requires an appropriate, moist environment, and is regulated and adjusted by several growth factors such as keratinocyte growth factor (KGF), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) (Gurtner et al., 2008; Stuart Enoch, 2008).

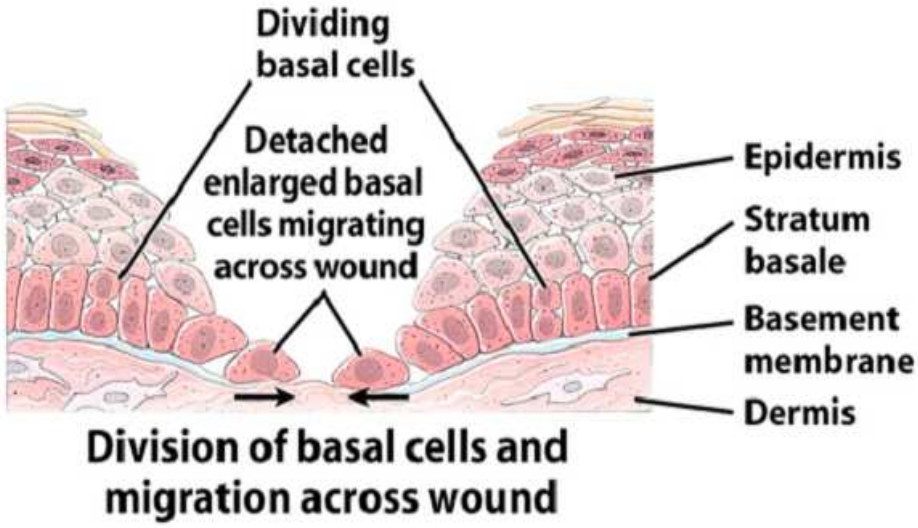

Figure 8. Reepithelialization in skin wound healing

Reepithelialization takes place as keratinocytes differentiate from the stem cells in the basal stratum and migrate over the wound edge to fill in the defect. Migration stops, signaled by contact inhibition as the wound defect fills in.

(From Gantwerker and Hom, 2011b Image No 1).

1.3.3.2 Role of angiogenesis in skin wound healing

Angiogenesis is a critical step in wound healing and takes place concurrently during all post-haemostasis phases of wound healing (Ahluwalia and Tarnawski, 2012; Reinke and Sorg, 2012). Proteolytic enzymes released into the connective tissue degrade ECM proteins. Some angiogenesis-promoting factors, such as bFGF, induce endothelial cells to release plasminogen activator (which converts plasminogen to plasmin) and procollagenase (which is converted to active collagenase by plasminogen activator, and possibly via tryptase released by degranulating mast cells) (Krejci-Papa and Paus, 1998). Collagen fragments from the injured basement membrane also exert pro-angiogenic properties and thus promote the formation of new blood vessels at the injured skin site (Singer and Clark, 1999; Aikio et al., 2012).

Modelling and establishment of new blood vessels are critical in wound healing so as to re-establish tissue perfusion and takes place during all phases of the reparative process. In addition to attracting neutrophils and macrophages, numerous angiogenic factors secreted during the haemostatic phase promote angiogenesis (Pierce et al., 1991; Servold, 1991; Takeshita et al., 1994; Velnar et al., 2009). Two particularly important ones are vascular endothelial growth factor A (VEGFA) and fibroblast growth factor 2 (FGF2; also known as bFGF) (**Table 1**). For example, local application of VEGF to wounds in an animal model of diabetes can normalize wound healing (Gurtner et al., 2008). Furthermore, angiogenesis can also result from the recruitment of bone marrow-derived endothelial progenitor cells (EPCs), although this event is probably not very prominent, at least in non-ischemic wounds (Gurtner et al., 2008).

Angiogenesis ceases once the wound is filled with new granulation tissue and many of the new formed blood vessels collapses as a result of the endothelial cell apoptosis (Singer and Clark, 1999). Any medications that interfere with new blood

vessel formation (i.e., the antiangiogenic drug bevacizumab: Avastins) can result in delayed wound healing (Gurtner et al., 2008; Gantwerker and Hom, 2011b), while excessive, unchecked angiogenesis is thought to significantly contribute to hypertrophic scar or keloid formation (Yang et al., 2003; Ammendola et al., 2012; Mogili et al., 2012).

1.3.3.3 Role of fibroblasts and extracellular matrix (ECM)

In the later stage of proliferation phase (i.e. 2-4 days after wounding), fibroblasts are gathered from the edge of the wound or via bone marrow stimulation by a number of factors, e.g. PDGF and TGF-beta, which are secreted for example by macrophages (Gurtner et al., 2008; Behm et al., 2012). Some of these fibroblasts differentiate into myofibroblasts, which are responsible for wound contraction so as to bring the wound edges together for faster wound closure. Fibroblasts and myofibroblasts affect each other and produce ECM (e.g., fibronectin, hyaluronan, type I and III collagen, proteoglycans) which support further cell immigration into the wound and are essential for the repair process and subsequent remodelling (Gurtner et al., 2008; Stuart Enoch, 2008; Gantwerker and Hom, 2011b). Unwounded dermis contains 80% type I and 25% type III collagen, whereas wound granulation tissue is characterized by an increase of type III collagen to 40% (Robson et al., 2001; Velnar et al., 2009).

By day 3-5 after wounding, granulation tissue usually is well established. Histologically, this becomes visible by proliferating fibroblasts and the presence of capillary loops embedded into a loose ECM, as a morphological indicator of angiogenesis or formation of new blood vessels from pre-existing vasculature at the locate of wound (neovascularization) (Singer and Clark, 1999; Broughton et al., 2006; Stuart Enoch, 2008). With progressing collagen accumulation, the density of the blood vessels gradually declines and the granulation tissue subsequently

matures to form a scar (see below) (Velnar et al., 2009; Dulmovits and Herman, 2012).

1.3.4 Wound healing phase 4: Maturation and remodeling

The remodeling phase begins 2-3 weeks after injury and lasts for 1 year or more as the provisional ECM and type III collagen is replaced with type I collagen and the remaining cell types (e.g. endothelial cells, macrophages and myofibroblasts) of the previous phases undergo apoptosis (Gurtner et al., 2008; Gantwerker and Hom, 2011a). Some authors consider scar formation as a separate, fifth phase of wound healing (see **Figure 6**) (Clark, 1991). Furthermore, over a period (6–12 months), the ECM is reconstructed that type I collagen dominates again (Lovvorn et al., 1999; Gurtner et al., 2008). This process is performed by matrix metalloproteinase that are secreted by fibroblasts, macrophages and endothelial cells at the wound locate and modulated by growth factors, cytokines, and phagocytic stimuli (Broughton et al., 2006; Gurtner et al., 2008; Stuart Enoch, 2008).

Epithelial–mesenchymal interactions probably continuously adjust skin integrity and homeostasis during this final wound healing stage (Szabowski et al., 2000; Gurtner et al., 2008). Also, there must be regulatory feedback loops that control the presence, proliferation/differentiation/apoptosis, retainment and number of various cell types within the repaired skin (Gurtner et al., 2008). Excessive, unchecked fibrosis at this stage causes hypertrophic scar (with the scar limited to the wound area) or keloid formation (with the scar extending beyond a wound edge) (Broughton et al., 2006; Carantino et al., 2010; Zhang et al., 2011a; Zhang et al., 2011b; Schwartzfarb and Kirsner, 2012).

However, the injured skin never fully regains the complete functionality of uninjured skin (Levenson et al., 1965; Gurtner et al., 2008). Interestingly, scarless wound healing does occur in fetal skin (Ferguson et al., 1996; Aller et al., 2012; Lo et al.,

2012) and complete skin regeneration is seen in adult lower vertebrates, such as salamanders (Brockes et al., 2001). Thus, fetal and amphibian wound healing may provide important pointers as to how complete repair of adult human skin may be achieved in the future, and how excessive scar formation may be avoided (Ferguson et al., 1996; Satish and Kathju, 2010; Aller et al., 2012; Kathju et al., 2012; Lo et al., 2012).

1.3.5 Key molecular controls of human skin wound healing

Multiple cytokines and molecules regulate these 4-5 different, but overlapping stages of skin wound healing (**Table 1**) (Schafer and Werner, 2008a; Behm et al., 2012). The mechanisms they modulate the process are intricate and proceed interactionally during the whole repair procedure (Werner and Grose, 2003; Schafer and Werner, 2008a). From this multitude of wound healing-modulatory agents, only a few shall be briefly discussed.

Table 1. **Growth factors, chemokines and cytokines in wound healing**

Modified and extended after Behm et al. 2012 and Barrientos et al. 2008 (Barrientos et al., 2008; Behm et al., 2012).

Molecule	**Main cellular source(s)**	**Wound healing- related function**	**References**
Activin	Keratinocytes, fibroblasts	Granulation tissue formation, keratinocyte differentiation, re-epithelialization,	Werner and Grose, 2003; Sulyok et al., 2004; Bamberger et al., 2005; Antsiferova et al., 2009
Adiponectin	Keratinocytes, fibroblasts	Keratinocytes proliferation, differentiation, angiogenesis	Mahadev et al., 2008; Salathia et al., 2012; Shibata et al., 2012
Angiopoietin-1/2	Fibroblasts	Angiogenesis	Pola et al., 2001; Le et al., 2008
Betacellulin	Keratinocytes	Epidermal homeostasis, hair follicle morphogenesis and cycling, and wound	Schneider et al., 2008

		angiogenesis	
CCL17	Keratinocytes	fibroblast migration, increase NGF(+) lymphocytes and mast cells	Kato et al., 2011; Jang et al., 2012
CTGF	Fibroblasts	Fibroblast proliferation, synthesis of collagen	Alfaro et al., 2010; Shah et al., 2012
CX3CL1	Macrophages, endothelial cells	Inflammation, angiogenesis, collagen deposition	Ishida et al., 2006; Ishida et al., 2008
CXCL10, CXCL11	Keratinocytes, endothelial cells	Re-epithelialization, tissue remodeling	Satish et al., 2003; Yates et al., 2007
EGF	Keratinocytes, macrophages, fibroblasts	Re-epithelialization	Jiang et al., 1993; Schneider et al., 2008; Melchionna et al., 2012
FGF-2 (bFGF)	Keratinocytes, fibroblasts, endothelial cells	Angiogenesis, granulation tissue formation	Pierce et al., 1991; Sogabe et al., 2006; Tiede et al., 2009a
FGF-7, FGF-10	Fibroblasts, keratinocytes	Re-epithelialization, detoxification of ROS	Steiling and Werner, 2003; Braun et al., 2004
Follistatin	Keratinocytes	Re-epithelialization, enhanced keratinocyte proliferation	Antsiferova et al., 2009
HGF	Fibroblasts	Suppression of inflammation, granulation tissue formation, angiogenesis, re-epithelialization	Yoshida et al., 2003; Min et al., 2005; Buchstein et al., 2009
IFNs	Fibroblasts, lymphocytes	Inhibition of fibroblasts proliferation and collagen synthesis	Shen et al., 2004; Ganapathy et al., 2012
IGF-1	Fibroblasts, keratinocytes	Fibroblast proliferation, synthesis of collagen	Semenova et al., 2008; Emmerson et al., 2012
IL-1	Macrophages, leukocytes, keratinocytes, fibroblasts	Inflammation, angiogenesis, re-epithelialization, tissue remodelling	Wood et al., 1996; Wiegand et al., 2009
IL-4	Leukocytes	Collagen synthesis	Ruckerl et al., 2006; Mosser and Edwards, 2008
IL-6	Fibroblasts, endothelial cells, macrophages, keratinocytes	Inflammation, angiogenesis, re-epithelialization, collagen deposition, tissue remodelling	Lin et al., 2003; McFarland-Mancini et al., 2010; Ebihara et al., 2011

IL-17	Macrophages	Inflammation, re-epithelialization	Rodero et al., 2012
IL-27	Macrophages	Suppression of inflammation, collagen synthesis	Ruckerl et al., 2006
KGF-1	Fibroblasts	Epithelial cell proliferation	Lin et al., 2006; Marti et al., 2008
Leptin	Keratinocytes	Angiogenesis, fibroblast proliferation, collagen synthesis	Larcher et al., 2001; Poeggeler et al., 2009
MIF	Endothelial cells, melanocytes, keratinocytes, luminal sweat gland cell	re-epithelialization, inflammatory responses and ECM homoeostasis	Gilliver et al., 2010
PDGF	Platelets	Inflammation, re-epithelialization, collagen deposition, tissue remodelling	Greenhalgh et al., 1990; Pierce et al., 1991; Pierce et al., 1992
TGF-α	Keratinocytes, macrophages	Re-epithelialization	Brem et al., 2007; Behm et al., 2012
TGF-β	Fibroblasts, keratinocytes, macrophages, platelets, mast cells	Inflammation, angiogenesis, granulation tissue formation, collagen synthesis, tissue remodelling, leukocyte chemotactic function	Penn et al., 2012; Ryu et al., 2012
TNF-α	Monocytes macrophages keratinocytes, neutrophils	Inflammation, reepithelialization	Nemenoff, 2012
VEGF	Keratinocytes, fibroblasts, macrophages, endothelial cells	Inflammation, angiogenesis	Jang et al., 2012; Slusarz et al., 2012; Wilgus and DiPietro, 2012

CCL17: Chemokine ligand 17; CTGF: Connective tissue growth factor; CX3CL1: Chemokine (C-X3-C motif) ligand 1; CXCL10/11: Cysteine-X amino acid-cysteine ligand 10/11; ECM: Extracellular matrix; EGF: Epidermal growth factor; ERK: Extracellular signal-regulated kinase; FGF: Fibroblast growth factor; HGF: Hepatocyte growth factor; IFNs: Interferons; IGF-1: Insulin-like growth factor-1; IL: Interleukin; KGF: Keratinocyte growth factor; MIF: Macrophage migration inhibitory factor; PDGF: Platelet-derived growth factor; ROS: Reactive oxygen species; TGF: Tumor growth factor; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor.

1.3.5.1 Selected proinflammatory cytokines

Proinflammatory cytokines, particularly interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), are sharply up-regulated during the inflammatory phase of wound healing, stimulate the synthesis of other wound healing-promoting agents (e.g. platelet-activating factors) and facilitate the migration of leukocytes from peripheral blood to the wound (Lau et al., 2009).

Immediately after injury, IL-1 is released by keratinocytes due to the epidermal barrier disruption (Graves et al., 2001; Menon et al., 2012). In inflammatory phage, neutrophils, monocytes, and macrophages also produce large amounts of IL-1. Through autocrine signaling, IL-1 increases keratinocyte migration and proliferation (Raja et al., 2007), e.g. IL-1 induces keratin 6 and 16 expression to promote reepithelialization (Komine et al., 2000; Freedberg et al., 2001). IL-1β stimulates wound healing via mitogen-activated protein kinase (MAPK) pathways, the nuclear factor (NF)-κB pathway, and prostaglandin E_2 (Arai et al., 2011).

In addition to its autocrine effects, IL-1 stimulates fibroblasts to secrete important cytokines and growth factor required for wound repair via paracrine signaling, such as keratinocyte growth factor (KGF), fibroblast growth factor (FGF)-7, IL-6, granulocyte-macrophage colony-stimulating factor (GM CSF) and hepatocyte growth factor (HGF); these, in turn, further promote keratinocyte proliferation and migration (Lau et al., 2009; Aden et al., 2010; Menon et al., 2012).

Interestingly, TNF-α, another key proinflammatory cytokine released in large quantities during the early phases of wound healing, can even induce keratinocytes to undergo epithelial-mesenchymal transition (EMT) in human skin wound healing via induction of bone morphogenetic protein (BMP) (Yan et al., 2010). This may contribute to granulation tissue formation and may impact on the subsequent scarring process. Owing to its key role in wound healing, TNF-α has been therefore

been singled out as an important therapeutic target, namely during impaired cutaneous wound healing (Ashcroft et al., 2011).

1.3.5.2 Selected growth factors

Upon injury the platelets secrete numerous growth factors and cytokines, such as platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), and epidermal growth factor (EGF), which promote and attract inflammatory cells into the wound site. PDGF plays a significant role in each stage of wound healing. It is synthesized and released from platelets, smooth muscle cells, activated macrophages, fibroblasts and endothelial cells also express PDGF (Heldin and Westermark, 1999). PDGF induces activated macrophages to produce growth factors and cytokines that drive wound repair towards the proliferative phase (Shah et al., 2012). PDGF also stimulates myofibroblasts differentiation and kicks off the proliferation phase by initiating fibroblast migration into the wound site (Heldin and Westermark, 1999; Shah et al., 2012). PDGF also greatly promotes angiogenesis during wound healing (Uutela et al., 2004; Sun et al., 2007; Lin et al., 2009a).

TGF-β is another key growth factor throughout wound healing, which promotes the proliferation of macrophages, fibroblasts and endothelial cells and just greatly stimulates granulation tissue formation (Valluru et al., 2011; Honardoust et al., 2012; Lee et al., 2012). Although it is overall an immunoinhibitory growth factor (Murphy, 2012), TGF-β facilitates the immigration of inflammatory cells into the wound and amplifies the granulation tissue-promoting effects of macrophages (Barrientos et al., 2008; Wi et al., 2012; Yang et al., 2012b). It also up-regulates VEGF secretion by keratinocytes and thus stimulates angiogenesis *in vitro* (Riedel et al., 2007). .

EGF is released by platelets, macrophages, and fibroblasts and works on keratinocytes via a paracrine signal (Franz et al., 2007). *In vivo,* EGF improved

wound healing as evidenced by accelerated reepithelialization, granulation tissue formation and neovascularisation (Dogan et al., 2009). This has encouraged the use of exogenous EGF in clinical wound management as early as 1973 (Savage and Cohen, 1973). However, its effect in chronic wounds was limited by the substantial degradation of exogenous EGF and the imbalance between matrix metalloproteinase (MMP) and MMP inhibitors (Hardwicke et al., 2008). Nevertheless, if these obstacles can be overcome, EGF may still be of benefit in the management of chronic wounds, e.g. by the induction of endogenous EGF production via gene therapy, or by EGF administration in appropriate polymers or electrospun nanofibers (Hong et al., 2006; Choi et al., 2008).

1.3.5.3 Selected other wound healing modulators

Many additional agents impact on wound healing. For example, endothelial insulin/IGF signaling is recognized to be essential for vascularization in diabetic mice, and reducing endothelial insulin/IGF signaling directly contributes to diabetes-associated impaired wound healing (Aghdam et al., 2012). The chemoattractant protein-1/C-C chemokine receptor type 2 (CCL2/CCR2) pathways is now appreciated as important in mediator that regulates the influx of inflammatory cells into skin wounds (Lu et al., 2010; Saederup et al., 2010). CCR2 stimulation by appropriate chemokine ligands recruits the blood monocytes/macrophages into skin wound sites and initiates vascularization of wound healing *in vivo* (Willenborg et al., 2012).

Two other emerging wound healing research frontiers are the role of hydrogen peroxide (H_2O_2) and of Toll-like receptors (TLRs), key innate immune receptors. Distinct H_2O_2 gradients are established during different wound healing stages and impact on the progression of wound healing (Schafer and Werner, 2008b; Schreml et al., 2011). TLRs stimulation, e.g. by keratinocytes, also regulate wound

inflammation, and promote tissue repair in many settings (Chen et al., 2012b; Dasu and Rivkah Isseroff, 2012; Huebener and Schwabe, 2012).

1.3.6 Role of stem cells in wound healing

Stem cells are found in all multi-cellular organisms and have long been appreciated to be of critical relevance in tissue regeneration and regenerative medicine. Stem cells are self-renewing and can differentiate into diverse specialized cell types, depending on their degree of commitment to tissue lineages (Korbling and Estrov, 2003; Sasaki et al., 2008; Furusawa and Kaneko, 2012; Sancho-Martinez et al., 2012). Their paramount importance as a cell pool for tissue regeneration during wound healing, namely in the skin, is well-established (**Figure 9**) (Lau et al., 2009; Falanga, 2012).

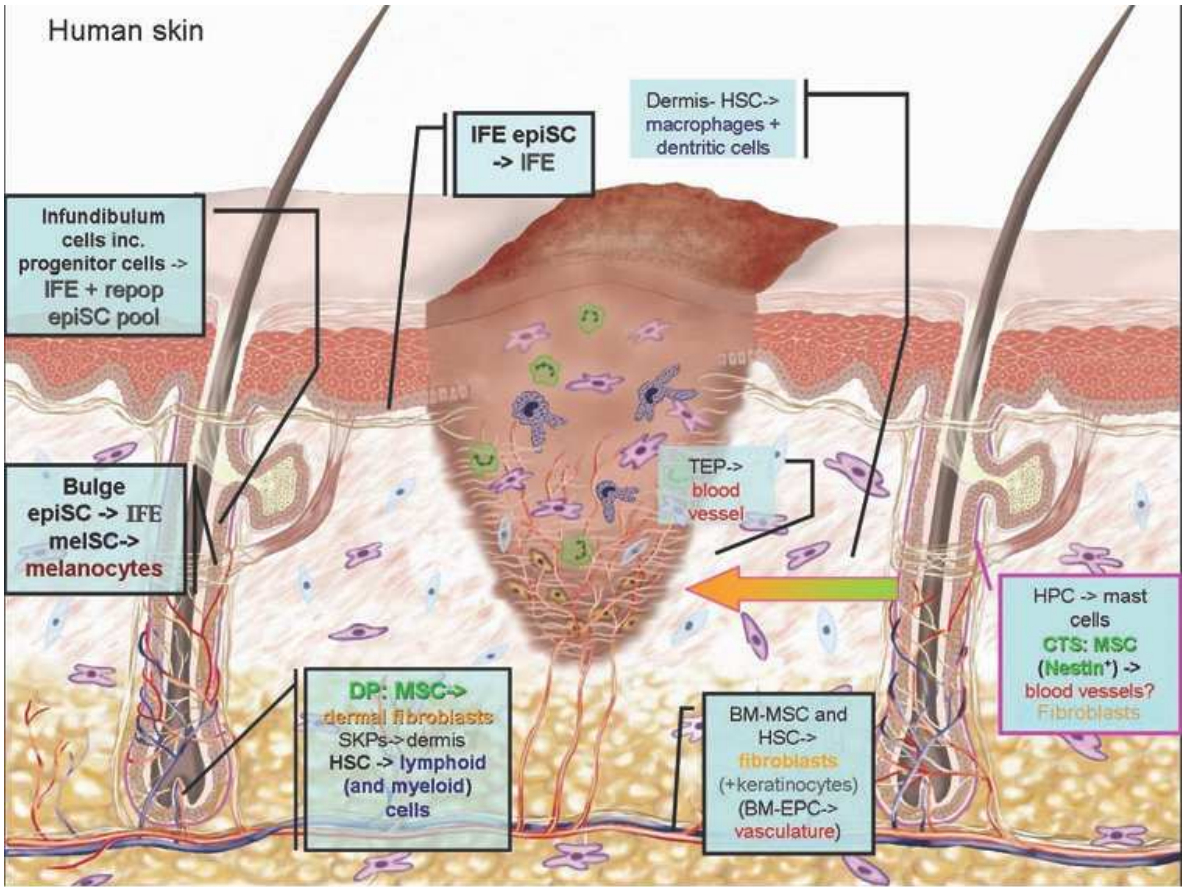

Figure 9. Role of stem/progenitor cell populations in cutaneous wound healing
(BM, bone marrow; CTS: connective tissue sheaths; DP: dermal papilla; EC, endothelial cell; EPC, endothelial precursor cells; epiSC, epithelial stem cell; HPC, haematopoietic precursor cells; HSC,

haematopoietic stem cell; IFE, interfollicular epidermis; melSC, melanocyte stem cell; MSC, mesenchymal stem cell; SKPs, skin progenitor cells; TEP, tissue endothelial progenitors).
(From Lau et al., 2009 Image No 1).

1.3.6.1 Epithelial stem cells

There are various stem cells population resident in different location within skin epithelium. For example, HF stem cells located in the bulge region of the outer root sheath (**Figure 9**) sustain the cyclic regeneration of the hair follicle, but also play an important role in the regeneration of sebaceous glands and the epidermis upon injury (Ito et al., 2005; Tiede et al., 2007; Ito and Cotsarelis, 2008). Lgr6+ epidermal stem cells can establish the hair follicle, sebaceous gland, and interfollicular epidermis and contribute to long-term wound repair (Snippert et al., 2010). HF derived-keratin 15+ epithelial stem cells migrate from the bulge into the epidermis of wound edges and contribute to the reepithelialization of wound healing (Mardaryev et al., 2011). Lhx2 mediates Sox9, Tcf4 and Lgr5 in the bulge epithelial stem cells of hair follicle to promote wound reepithelialization after injury (Mardaryev et al., 2011). These studies suggest that epidermis stem cells serve as a key player in the formation of different skin appendages during various processes of wound healing (Arwert et al., 2012).

Nestin+ pluripotent stem cells reportedly located in the bulge region of murine HFs can form new blood vessels during skin angiogenesis *in vivo* (Amoh et al., 2004). However, in human skin, nestin+ cells appear to be exclusively located outside of the skin epithelium, e.g. in the stroma of HFs and sweat glands (Tiede et al., 2009c) so that they are best considered as intra-mesenchymal stem cells.

1.3.6.2 Mesenchymal stem cells

Besides epithelial stem cells, whose presence in sufficient quantity and functionality is a key determinant of successful reepithelialization (Harris et al.,

2012; Plikus et al., 2012), mesenchymal stem cells are also critical for cutaneous wound healing. *In vivo* evidence exists that that GFP+ bone marrow-derived mesenchymal stem cells (BM-MSCs) accelerate wound closure and differentiate into keratinocytes to enhance reepithelialization (Sasaki et al., 2008). Application of allogeneic GFP+ BM-MSCs to the wound bed also increases tube formation by endothelial cells and thus promotes angiogenesis (Wu et al., 2007). One of the underlying mechanisms by which BM-MSCs enhance wound healing appears to be the secretion of cytokines and chemokines via paracrine signaling, such as VEGF, IGF, EGF, keratinocyte growth factor, angiopoietin-1, macrophage inflammatory protein-1 – important wound healing promoters (**Table 1**). Therefore, BM-MSCs can recruit macrophage, keratinocytes and endothelial cells into the wound site to promote wound healing (Chen et al., 2008).

Human sweat glands have recently been recognized as a rich source of nestin+ progenitor/stem cells (Petschnik et al., 2009), whose transplantation to wounded mouse skin can improve vascularization during dermal regeneration *in vivo* (Danner et al., 2012).

1.3.6.3 Other stem cells

Haematopoietic stem cells (HSCs) are widely thought to be the main source for the leukocytes that migrate into the wound site during the inflammatory phase of wound healing (Lau et al., 2009; Lu et al., 2011; Sel et al., 2012). HSCs have been demonstrated to improve dermal wound healing by promoting angiogenesis as well as migration and proliferation of fibroblasts via secreting monocyte chemoattractant protein-1 and GM-CSF (Templin et al., 2009).

However, inflammatory cells such as mast cells may also arise locally from resident intracutaneous progenitor cells in adult skin mesenchyme. The connective tissue

sheath (CTS) of HFs has been documented to serve as a potent local reservoir of connective tissue-type mast cell precursors, both in mice (Kumamoto et al., 2003) and in humans (Ito et al., 2010; Sugawara et al., 2012). The mast cell precursors may involve in cutaneous wound healing and mature within the CTS via stimulation with stem cell factor (SCF), a key mast cell differentiation- and proliferation-promoting factor, into mature skin mast cells (Lau et al., 2009). Interestingly, both peptide neurohormones released from the skin epithelium, such as corticotropin-releasing hormone (CRH) (Ito et al., 2010) and endocannabinoids that stimulate cannabinoid receptor type 1 (Sugawara et al., 2012), may play a central role in controlling the degranulation and the maturation of mast cells from resident progenitors within the CTS. Therefore, the CTS, which is routinely injured during skin wounding, may well be an important participant in wound healing (Judl et al., 2011).

Revascularization, a main challenge during wound healing, partially occurs through angiogenesis in the presence of endothelial progenitor cells (Bonello et al., 2012; Kang et al., 2012). Topical application of *ex vivo* expanded EPCs (dilute in PBS) by subsequently semi-permeable transparent dressing cover in diabetic mice promotes neovascularisation of a full-thickness excisional wound via increasing the expression of VEGF and bFGF (Asai et al., 2012). One underlying molecular mechanism may be CCL5/CCR5 interaction, since the absence of CCR5 can reduce EPC accumulation, while CCL5 induces CCR5-dependent EPCs migration into the wound site (Ishida et al., 2012).

1.4 Why more effective wound healing promoters are urgently needed

Since in our aging societies the incidence of skin ulcers as a result of retarded

wound healing is steadily rising and constitutes an ever-larger health care burden, this phenomenon may well be called an “ulcer epidemic”. This makes it an urgent challenge for both, clinical medicine and health care providers, to rapidly develop better strategies for more effective, safe, and affordable wound healing management (Sen et al., 2009; Eaglstein et al., 2012; Markova and Mostow, 2012).

1.4.1 Ulcer epidemiology and health care burden

Skin ulcers have become a major public health concern worldwide and consume enormous health care investments; moreover, skin ulcers are a growing cause of patient morbidity (Sen et al., 2009; Eaglstein et al., 2012; Markova and Mostow, 2012). Skin ulcer incidence and prevalence vary a lot between different populations, as do patient compliance in terms of follow-up visits, which makes epidemiological studies challenging.

However, it has been estimated that 1-2 % of the world population will experience a chronic wound healing condition during their lifetime (Sen et al., 2009; Eaglstein et al., 2012; Markova and Mostow, 2012). The incidence of skin ulcers has been reported to be 0.35% in the UK (Vowden et al., 2009), 4.5% in India (Shukla et al., 2005), 1.5%–20.3% in China (Fu, 2005), 8%–12% in Mexico (Gillespie et al., 2012), and there are more than 200,000 skin ulcer-afflicted Australians at any one time (Gillespie et al., 2012).

The estimate average cost of curing a single ulcer is $1,100 to $2,800, that of an infected one is $17,000 (Kalker et al., 1982; O'Meara et al., 2009). These costs are steadily rising. In the USA, chronic wounds affect about 6.5 million patients and an estimated $ 25 billion annually are spent on the management of chronic wounds (Sen et al., 2009). Already in 2009, the market for ulcer care and wound treatment-products had been projected to reach more than U.S.$ 15 billion one

year later (Sen et al., 2009). Thus, the overall health care burden of chronic skin ulcers is enormous, above and beyond the personal burden suffered by ulcer patients and their ulcer-associated morbidity and loss of quality of life.

1.4.2 Current chronic ulcer therapy

Retarded wound healing, leading to ulcer formation, results from the deficiency of essential wound healing requirements, such as an sufficient supply of oxygen, nutrients, proteins, vitamins, co-factors, an effective immune system, a normal, sequential release of growth factors and the avoidance of excessive and protracted inflammation (Gunter and Machens, 2012; Markova and Mostow, 2012; Valacchi et al., 2012). Wherever this is defective and substitution therapy is insufficient, a number of ulcer wound treatment strategies are available.

These include e.g. wound cleansing and debridement (incl. therapeutic application of maggots), wound care strategies that keep the ulcer moist, pressure offloading, negative-pressure wound therapy, application of growth factors and proteolytic enzymes, electrostimulation, and hyperbaric oxygen therapy (Gunter and Machens, 2012; Markova and Mostow, 2012; Sebastian et al., 2012; Ud-Din et al., 2012; Valacchi et al., 2012; Wong and Gurtner, 2012; White-Chu and Reddy, 2013). To enhance therapeutic efficiency, the topical application of wound management gents through lipid colloidal carriers, such as vesicular systems (traditional liposomes and ethosomes) and solid lipid nanoparticles, is being explored (Chen et al., 2012c; Gokce et al., 2012; Nam et al., 2012; Valacchi et al., 2012). These new carrier systems have a number of advantages, such as high penetration and absorption within a natural skin-moisture environment (Valacchi et al., 2012).

However, very frequently, these therapeutic modalities are ineffective non-applicable, or unavailable in a given chronic ulcer patient, or superinfection

and persistent perfusion/innervation deficits destroy the wound healing progress that may have been achieved. Therefore, in numerous patients ulcer management painfully often remains unsatisfactory, since all these therapies are only symptomatic and since most patients are not only old, but also suffer from associated metabolic disorders (diabetes, obesity), hypertension, venous insufficiency and/or atherosclerosis, resulting in severe ischemia (Valacchi *et al.*, 2012). Thus, more effective, cost-efficient, safe, and evidence-based management strategies for skin ulcer therapy must urgently be developed (Brolmann et al., 2012).

1.5 Why simple, clinically relevant *in vitro*-wound healing assays are needed

To better understand the complexity of wound healing, its many distinct stages, and its main determinants and regulatory elements, the use of appropriate clinical and preclinical wound healing models is essential. *In vivo* animal models have been indispensable for understanding the significant differences in wound healing fetal versus adult skin and between different vertebrate species (Amadeu et al., 2003; Ramelet et al., 2009; Satish and Kathju, 2010). However, such *in vivo* models are complicated and costly, and rarely can claim to fully reproduce clinically relevant human wound healing conditions the same condition in humans. Hence, it is extremely important to study wound healing not only in animal models (Ansell et al., 2012; Deshmukh and Gupta, 2013; Wang et al., 2013), but also in human skin itself.

1.5.1 Existing *in vitro* wound healing assays

In vitro models are generally simple, fast, and less costly, and permit the study of cell behaviour in a controlled environment, using human cells or tissue, and raise minimal ethical concerns compared to *in vivo* wound healing models (Gottrup et al., 2000). They also facilitate investigation of the mechanisms of action of pharmacological agents or defined wound healing-regulatory factor.

Table 2. *In vitro* models for wound healing investigation

Modified and extended after Gottrup et al. 2000 (Gottrup et al., 2000)

Model type	Available research read out parameters
Single cell systems	
Adherent cells	
Monolayer (Yu et al., 1993)	Migration, proliferation, protein syntheses
Typical model: **scratch assays** (Yu et al., 1993)	Migration, proliferation, protein syntheses
Three-dimensional (Grinnell, 2003)	Cell-matrix interactions, migration, proliferation, protein syntheses, wound contraction
Non-Adherent cells (Postlethwaite et al., 1976)	Chemotaxis
Multi-cellular systems	
Co-cultures (Kamalati et al., 1989; Werner and Grose, 2003; Radtke et al., 2013)	Cell-cell interactions
Three-dimensional (Bell et al., 1983)	Cell-matrix interactions, cell-cell interactions migration, proliferation, protein syntheses, wound contraction
Organ Cultures	
Intact skin (Garlick and Taichman, 1994; Lu et al., 2007)	Epithelialisation, tensile strength, morphology
Punch in a punch model (Moll et al., 1998)	Epithelialisation, migration, proliferation, angiogenesis

However, it must always be kept in mind that *in vitro* experiments cannot fully represent the *in vivo* situation. In the current context, they routine lack skin perfusion and innervation, do not permit wound entry of circulating immunocytes and stem cells, and lack important molecular or cellular players of physiological

skin wound healing (Gottrup et al., 2000; Miao et al., 2012). With these caveats in mind, some frequently used *in vitro* wound healing assays are briefly discussed (**Table 2**).

1.5.1.1 Scratch assay

A simple, well-developed method is the “scratch assay”, which was originally developed for studying astroctye injury (Yu et al., 1993). Here the repairing cells proliferate around the central, scratched area and move into the core of this surrogate ‘wound’. The migration of keratinocytes can also be determined by this model. By mechanical foreign force, e.g. with a plastic pipette tip (Cha et al., 1996), a needle (Calderon et al., 1996), a stub adapter (Kheradmand et al., 1994), or a rotating silicone tip and razor blades (Yu et al., 1993; Gottrup et al., 2000), a confluent keratinocyte monolayer is injured, thus creating a wide gap. The experimentally induced loss of cell-cell contact stimulates migration (**Figure 10**).

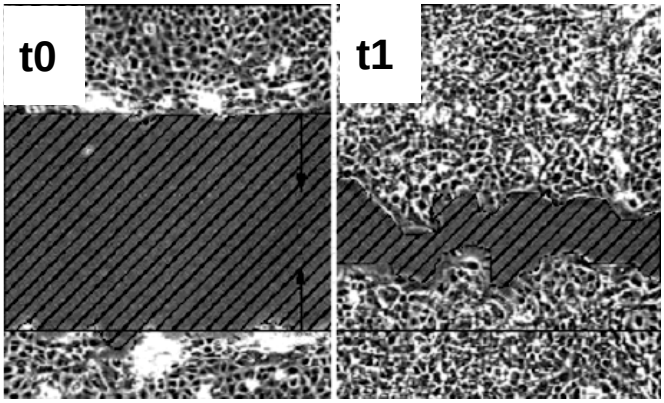

Figure 10. Scratch assay model
Migration ability of cells is evaluated in a confluent monolayer of cells at time 0 (t0) and at conditional time (t1) by fragmenting the surface area occupied by the cells from the background (cf. hatched areas).
(Modified from Olivier Debeir, 2008 Image No 2)

This – highly artificial – model allows one to study cellular wounding responses in a well-controlled culture environment, which excludes interactions with other cells and with all agents not contained in the medium (Miao et al., 2012). The disadvantage of this model is that this model cannot really be compared with

physiological wound healing of human skin: this 2-dimensional culture system that investigates only one cell type does not reproduce the *in vivo* status, while the complex multidirectional cell-cell and cell-matrix interactions in the epidermis, the dermis and the immune system as well as multiple other elements essential to normal skin wound healing are all missing (Koschwanez and Broadbent, 2011).

1.5.1.2 Three dimensional models

To overcome the problem that two dimensional cell monoculture assays lack proper interactions between different cell populations as well as important cell-ECM interaction (Miao et al., 2012), wound healing models have been developed, where different cell types are co-cultured under three-dimensional conditions (Auxenfans et al., 2009; Janin, 2011; Lynch and Ahsan, 2013).

The classical one is a fibroblast-populated collagen matrix (usually type I collagen), which permits the observation of cell contractility and matrix deposit. In this model, fibroblasts are seeded into a disc-shaped collagen matrix which contracts as the cells contract and migrate over time (Greenhalgh, 2005). Cell morphology and motility (Friedl and Brocker, 2000), response to pharmacological agents, protein production (Asselineau and Bell, 1984), and fibroblast proliferation can easily be studied in this 3D culture model (Amadeu et al., 2003; Miller et al., 2003). Another advantage of this model is that it allows getting an analogous environment that fibroblasts and the matrix can expose to the mechanical forces *in vivo* situation, which, to some extent, simulates the early granulation tissue formation during early wound repair stage (Wong et al., 2011).

Such a fibroblast-populated collagen “pseudodermis” or, alternatively, human acellular dermis (Lee et al., 2000; McDonald et al., 2011; Hirokawa et al., 2012) can also be populated by additional cell populations such as endothelial cells and

macrophages (Callegari et al., 2007; Stevenson et al., 2010) and can be used as support structure for a “pseudoepidermis” constructed from isolated epidermal or HF keratinocytes that have been seeded onto it (e.g. (Boehncke and Schon, 2007)). Such so-called “skin equivalents”, which may survive for several weeks or even months under appropriate culture conditions, can then be experimentally wounded to observe aspects of both epidermal and dermal regeneration under highly controlled *in vitro* conditions (Pena et al., 2012; Shimoda et al., 2012; Yang et al., 2012a).

1.5.2 Available punch-in-a-punch wound human skin assays

However, these 3D systems usually do not faithfully reproduce human skin (e.g. absence of skin appendages, melanocytes, Merkel cells, intracutaneous immunocytes and the full range of skin stem cell populations). Thus intact fragments of partial or full-thickness human skin have been developed (Lu et al., 2007; Xu et al., 2012).

Moll et al. pioneered a simple punch-in-a-punch wound healing model , for which a 6 mm punch biopsy of partial thickness human skin is cultured after having been further wounded by a central, small punch (3mm) (Moll et al., 1998). This is placed dermis-side down on gauze in a culture dish containing Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS incubated at 37℃ at the air-liquid interphase so that the medium is only in contact with the bottom side of skin fragment, while the epidermis surface remains constantly exposed to the air (Moll et al., 1998). Most recently, during the execution of the current thesis project, Xu et al. published another optimized partial-thickness human *ex vivo* skin culture model (Xu et al., 2012): After removing the subcutis integral skin fragment with intact epidermis and partial-thickness dermis is placed on the bottom of a nylon mesh cell strainer with the epidermis facing up, is fixed by sutures, and cultured in a 6-well

plate with DMEM supplemented with 10% FBS at 37℃ with 5% CO_2 (**Figure 11**).

However, these assays operate in the presence of high concentrations of serum from a non-human species (10% FBS), with the exact composition unknown. Moreover, the skin used in these models excludes the subcutis, and thus its wound healing-promoting pluripotent stem cells and adipokines (Kim et al., 2007; Poeggeler et al., 2009). Also, skin appendages are missing. The latter defect of these models is severe since HFs and sweat glands, and possibly even sebaceous glands, as well as their associated epithelial and mesenchymal progenitor cells are increasingly appreciated as major players in mammalian skin wound healing (Jahoda and Reynolds, 2001; Ito et al., 2005; Lu et al., 2007; Lau et al., 2009; Ansell et al., 2010; Danner et al., 2012; Lu et al., 2012a).

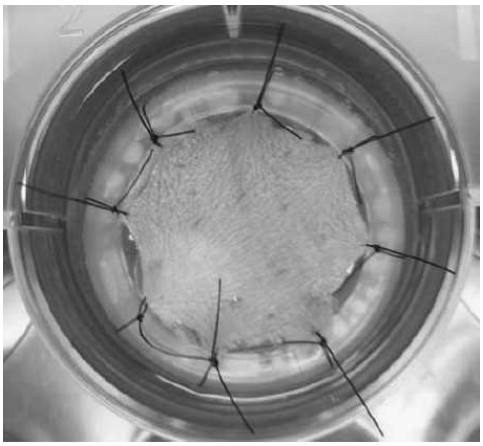

Figure 11. Partial-thickness human *ex vivo* skin culture
(From Xu et al., 2012 Image No 2)

To overcome these limitations, we have developed a highly standardized human skin wound healing assay in the current study, which is based on a full-thickness, serum-free human skin organ culture assay previously developed in our lab (Lu et al., 2007) and on the “punch-within-a-punch”-design (Moll et al., 1998) (for details, see Materials & Methods).

1.6 Hormones as modulators of wound healing

While it has long been known that hormones impact on wound healing, endocrine controls of tissue regeneration have become a recent focus of research interest (Dioufa et al., 2010; Sosne et al., 2010; Kiaris et al., 2011; Novotny et al., 2011; Emmerson and Hardman, 2012; Oh et al., 2012; Tarameshloo et al., 2012; Wang et al., 2012) (**Figure 12**). Since aging impairs wound healing, this has suggested that namely sex steroid hormones play a fundamental role in wound healing (Yanai et al., 2011; Zhang et al., 2011c; Sgonc and Gruber, 2012). However, the exact role of specific hormones in human skin wound healing and the underlying mechanisms of action remain largely obscure.

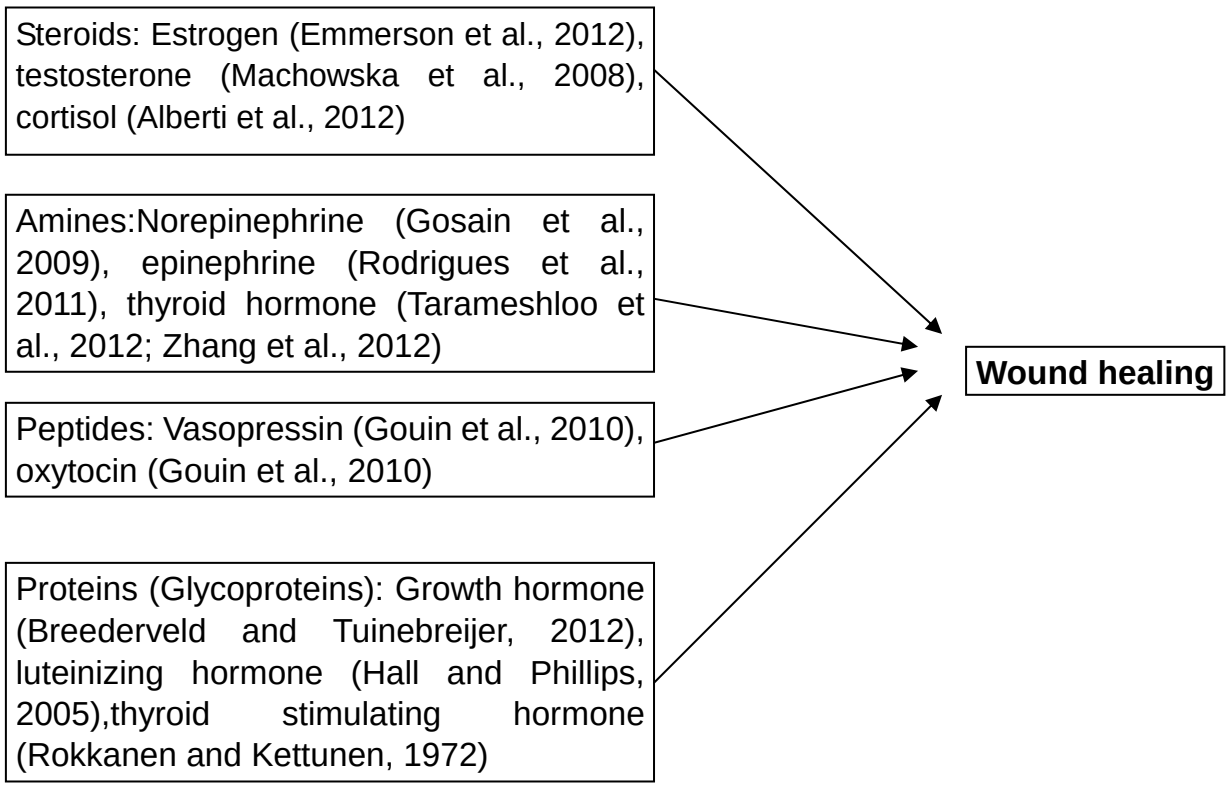

Figure 12. Selected wound healing associated hormones

Sex steroids such as estrogens and androgens are mostly synthesized in the adrenal cortex, ovary, and/or testis and are then released systemically to fulfill their multiple physiological tasks. However, we now know that many other tissues, including human skin, human HFs, and human sebaceous glands, are major

peripheral sites of steroid hormone synthesis and metabolism (Rogoff et al., 2001; Slominski et al., 2008; Makrantonaki and Zouboulis, 2009) and even have fully functional equivalents of the central hypothalamic-pituitary-adrenal axis (CRH→ ACTH→ cortisol→ CRH) established (Slominski et al., 2002; Slominski et al., 2005; Slominski et al., 2007; Slominski et al., 2008; van Beek et al., 2008; Ito et al., 2010) (**Figure 15**).

In generally, estrogen can promote wound healing process in men and elderly women, by adjusting cytokine and growth factor expression, inflammation process, matrix deposition, improving reepithelialization, stimulating angiogenesis and wound contraction and has similar effects on mice wound healing to that observed in human (Gilliver et al., 2007; Emmerson et al., 2012) (**Figure 13**). The sex steroid precursor dehydroepiandrosterone (DHEA) exerts similar effects as estrogen, which suggests that it may stimulate wound healing via DHEA transformation to estrogen (Mills et al., 2005; Gilliver et al., 2007). Androgens, in contrast, significantly retard wound repair in aged humans, in part by increasing the inflammatory response (Gilliver et al., 2007; Emmerson et al., 2012) and by modulating cytokine expression and inducing excessive collagen deposition (in mice) (Ashcroft and Mills, 2002) (**Figure 13**).

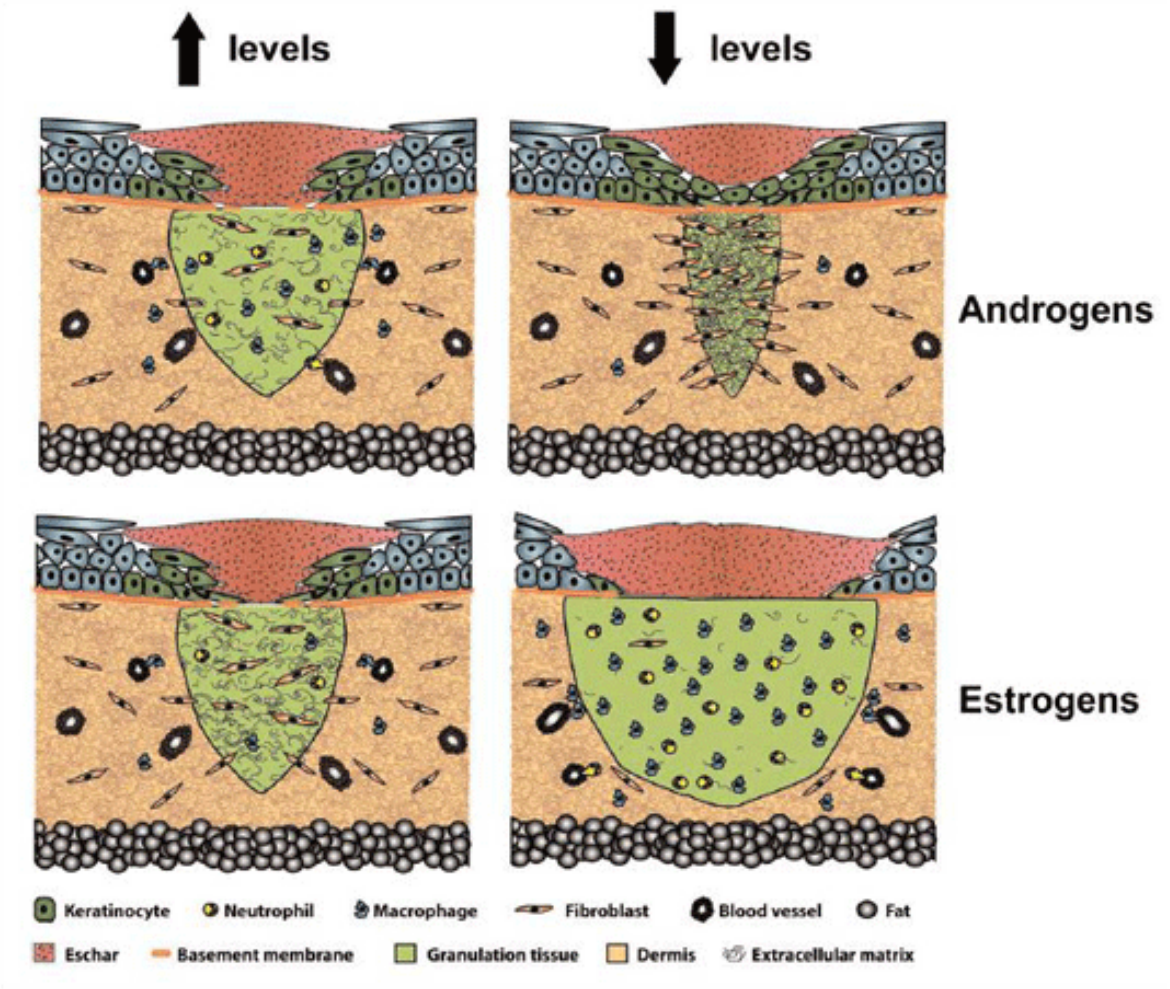

Figure 13. The effects of sex hormone levels on wound healing
Androgens play an inhibitory role in wound healing (compare top left with top right), whereas estrogens exert diametrically opposed effects to those of androgens (compare bottom right with top right).
(From Hardman, 2005 Image No 2).

Psychological stress also substantially delays wound healing in both human and animals, presumably via upregulating glucocorticoid (GC) and catecholamine levels, by altering immune response and inducing hyperglycemia (**Figure 14**) (Guo and Dipietro, 2010). This is thought to inhibit cell proliferation and differentiation as well as collagen production, the expression of cell adhesion molecules that direct immune cell trafficking, and to reduce the expression of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α at the wound site, which are necessary for the early inflammatory stage of wound healing (Gouin et al., 2010; Guo and Dipietro, 2010;

Broadbent and Koschwanez, 2011). In addition increased skin and serum GC levels may increase the risk of wound site infection (Guo and Dipietro, 2010). However, topical low-dose GC administration can even accelerate wound healing while systemic high-dose, long use of GC has a big increased risk of wound infection.

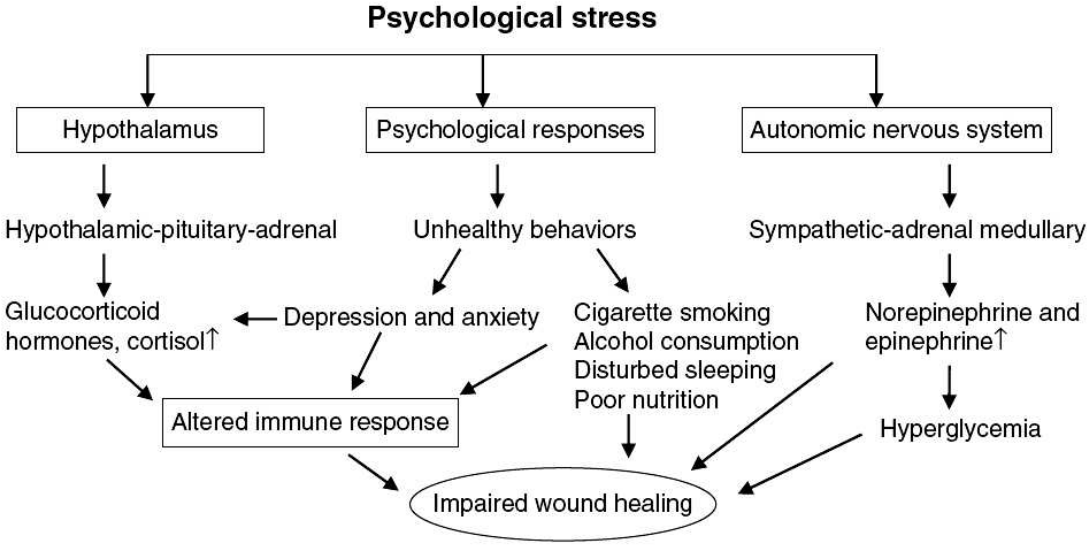

Figure 14. The effects of stress on wound healing
(From Guo and Dipietro, 2010 Image No 1).

Besides increased GC and catecholamine levels, abnormal neuroendocrine signaling along the central and intracutaneous HPA axes that is associated with psychological stress (e.g., increased CRH and ACTH levels) may negatively impact on wound healing via the recognized epithelial growth-inhibitory and mast cell-activating properties of CRH and ACTH, which could further promote excessive intracutaneous inflammation (Paus et al., 2006; Joachim et al., 2008; Ito et al., 2010). Thus, the - as yet insufficiently explored - (neuro)endocrine controls of cutaneous wound healing are likely to be of major clinical relevance.

1.7 Existing evidence that thyroid hormones may promote wound healing

Accumulating evidence suggests that, besides the above-mentioned hormones, thyroid hormones (THs) are particularly interesting, yet only poorly studied and ill-understood endocrine signals in the context of human skin wound healing. Human skin and its hair follicles (HFs) are classical THs target organs (Freinkel and Freinkel, 1972; Kaplan et al., 1988; Messenger, 2000; Safer et al., 2001; van Beek et al., 2008) and thyroid diseases are well-known to affect human skin structure and function on multiple levels (Holt and Marks, 1977; Holt, 1978; Doshi et al., 2008). Yet, even though thyroxine (T4) is one of the most frequently administered hormones in clinical medicine, it is not used in dermatological therapy.

However, T4 promotes murine hair growth *in vivo* (Safer et al., 2001) and human hair growth *in vitro* (van Beek et al., 2008), operates as the chief endocrine control of amphibian metamorphosis, which entails major changes in skin function (Kress et al., 2009). Most importantly, T4 has already been reported to stimulate wound healing in both rats (Erdogan et al., 1999) and mice (Safer et al., 2005) *in vivo*. Likewise, topical T3 enhances wound healing in guinea pigs, presumably by promoting wound contraction (Kassem et al., 2012). Together with the fact that T4 has long been administered in clinical medicine, which is very cost-efficient and reasonably stable, and has a fully defined toxicolgical profile upon clinical application (Brenta et al., 2007; Moreno et al., 2008; Goldsmit et al., 2010; Biondi and Wartofsky, 2012), this makes THs (see Section **1.9**) particularly intriguing candidate wound healing promoters.

1.8 The emerging role of the hypothalamic–pituitary–thyroid (HPT) axis in skin biology

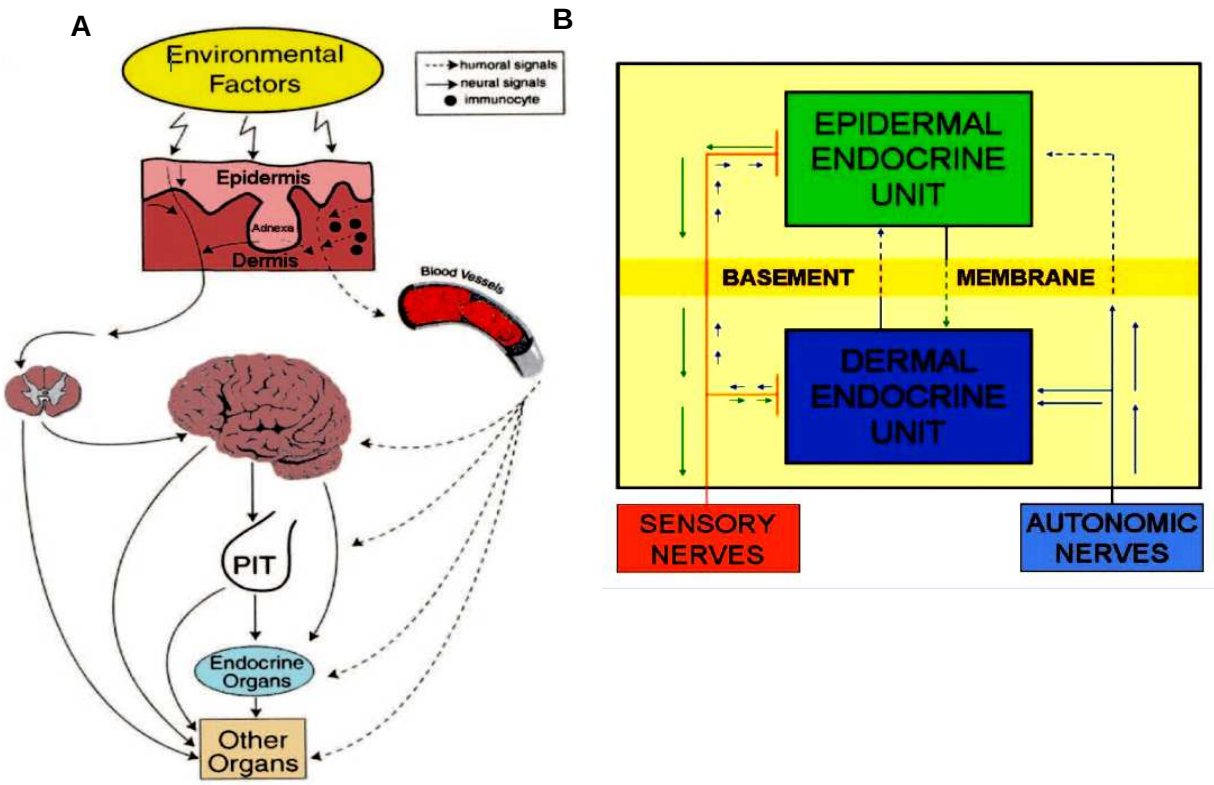

Figure 15. Skin neuroendocrine system regulates systemic (A) and local (B) homeostasis
Human skin is now recognized as an important peripheral endocrine organ, which is closely cross-linked to central neuroimmunological system and enable fast and selective responses to the environment for local and systemic homeostasis (Zmijewski and Slominski, 2011; Peters et al., 2012; Valdes-Rodriguez et al., 2012). Essentially all skin cell populations and skin appendages are not only important (neuro-)hormone and neuropeptide targets, but also synthesize and metabolize these. For example, the skin not only modulates cutaneous Vitamin D production, but also exerts a few neuropeptides functions and synthesis, which of including elements of hypothalamic-pituitary-adrenal (HPA), and hypothalamic-pituitary-thyroid (HPT) axes (Slominski et al., 2002; Bodo et al., 2010; Cianfarani et al., 2010; Gaspar et al., 2010). These newly synthesised hormones and neuropeptides primarily exert paracrine or autocrine activities *in situ* and are regulated by a number of environmental and intrinsic factors, such as solar radiation, humidity, and temperature. (PIT, pituitary)
(From Zmijewski and Slominski, 2011 Image No 1).

Besides the prominent cutaneous involvement in thyroid disease, there is growing evidence that several key players in the hypothalamic-pituitary-thyroid (HPT) axis (TRH→TSH→THs) (**Figure 15**) are expressed on the gene and/or protein level in human skin (**Table 3**) and that TRH and TSH are major novel modulators of human skin biology. These range from the stimulation of human hair growth and

pigmentation by TRH via the regulation of keratin expression by TSH to the promotion of keratinocyte energy metabolism and mitochondrial biogenesis by both TSH and TRH (Slominski et al., 2002; Gaspar et al., 2009; Bodo et al., 2010; Cianfarani et al., 2010; Gaspar et al., 2010; Paus, 2010; Poeggeler et al., 2010; Gaspar et al., 2011; Knuever et al., 2012).

Table 3. **Elements of the HPT axis are expressed in human skin *in situ* and/or cultured human skin cells**

HPT axis gene/gene product	Comments	Reference	Associated endocrine effect and/or function in skin biology	Reference
TRH	Restrictedly expressed in dermal and follicular papilla fibroblasts, neonatal but not adult keratinocytes and melanoma cells *in vitro*. In addition, human scalp hair follicles express TRH mRNA and protein *in situ*.	Slominski et al., 2002	TRH treatment significantly stimulates hair shaft formation and hair matrix keratinocyte proliferation, suppresses apoptosis of hair matrix keratinocytes, and prolongs active hair growth (anagen) in human hair follicle organ culture. TRH stimulate TSH expression epidermis *in situ*.	Gaspar et al., 2009; Bodo et al., 2010; Gaspar et al., 2010
TRH Receptor (TRHR)	Human scalp hair follicles express TRH-R mRNA and protein *in situ*	Slominski et al., 2002	Thyroid-releasing hormone probably exerts effects on both classical and non-classical receptor, e.g. MC-1R	Slominski, 2005
TSH	Human epidermis expresses TSH on the gene and protein levels *in situ*.	Gaspar et al., 2010	Both systemic and intracutaneous−generated TSH modulate human skin epithelial biological functions (e.g., keratin expression).	Bodo et al., 2010; Paus, 2010; Ramot et al., 2012

TSH Receptor	TSH-R mRNA is expressed in cultured keratinocytes, epidermal melanocytes and melanoma cells *in vitro*. However, regarding the TSH-R expression *in situ*, contradictory results are shown. In Bodo et al study, TSH-R was expressed in normal human skin at the gene and protein level only within mesenchyme. However, Cianfarani et al. found that TSH-R expressed in the epidermis in non-scalp human skin.	Slominski et al., 2002; Bodo et al., 2009; Cianfarani et al., 2010	1. TSH-R-expressing cells also expressed the sodium iodide symporter and thyroglobulin genes. 2. TSH-R expression in the skin by autoantibodies may play very important physiological and pathological role in skin autoimmune disease. 3. TSH-R stimulation by systemic TSH promotes the proliferation of human epidermal keratinocytes and dermal fibroblasts.	Slominski et al., 2002; Bodo et al., 2009; Paus, 2010
Deiodinases D_2 and D_3	Gene expression in skin biopsy and in the majority of human epidermal and dermal cells cultured *in vitro* Human HFs transcribes deiodinase genes (D2 and D3). After T4 treatment with hair follicle, a significantly higher fT3 level, when compared to vehicle group, was measured by electrochemiluminescent immunoassay in organ culture medium.	Slominski et al., 2002; van Beek et al., 2008	Intraceutaneous transcribed deiodinases may convert T4 to T3 in human skin.	van Beek et al., 2008; Tiede et al., 2009b
TH receptor	Thyroid hormone receptor beta 1 is expressed in the human hair follicle	Billoni et al., 2000	THs can modulate the selected keratins expression; even stimulate keratin 15 + progenitor cells, apoptosis and differentiation. Moreover, THs can also prolong the duration of hair growth (anagen phase) and wound healing (mice, rat, pig). THs inhibit TSH expression in human epidermis *in situ*.	Erdogan et al., 1999; Safer et al., 2005; Ramot et al., 2009b; Bodo et al., 2010; Tiede et al., 2010

1.9 The biology of thyroid hormones

This leads us to a brief consideration of TH biology, i.e. essentials of TH synthesis, TH receptor (TR)-mediated signalling, and non-classical TH actions.

1.9.1 TH synthesis

The thyroid hormones (THs), triiodothyronine (T3) and thyroxine (T4), are widely thought to operate as chief regulators of energy metabolism (Boelen et al., 2012; Johannsen et al., 2012; Kafi et al., 2012). They are synthesized by a fundamentally different mechanism than all other endocrine hormones (**Figure 16**). T4 is the major secreted form (90%) in blood, which include 4 iodine atoms and long half-life (7 days) than T_3 (1 day), whereas active T3 is converted from T4 within cells by deiodinases (5'-iodinase) mainly in liver and kidney (Molina, 2006; Gu et al., 2007; van Beek, 2009). However, T4 exerts a 100 times lower affinity to the thyroid hormone receptor (TRs) than T3. The majority of THs (>95%) binds to carrier proteins: thyroid-binding globulin (TBG), transthyretin (TTR, or prealbumin), and albumin (Gu et al., 2007). Even only 0.3% of T3 and 0.03% of T4 are unbound, they metabolically active at the tissue and cellular level (Molina, 2006; Gu et al., 2007; van Beek, 2009). However, inactive T3, reverse T3 (rT) which synthesized by metabolism of T4, affect as an antagonist to T4 activity (van Beek, 2009). Generally, there are three types of deiodinases: I, II, III (D1, D2, D3, respectively), which charge the activity of thyroid hormones by removing of specific iodine moieties from the precursor molecule T4 (**Figure 17**) (Bianco and Kim, 2006; van Beek, 2009). In the tissues, these enzymes can play a role in not only activate, but also inactivate thyroid hormones, which depends on whether they act on the phenolic or tyrosyl rings of the iodothyronines. D2 deiodinate the active form of thyroid hormone T3 via removal of an iodine atom on the outer ring of T4, whereas D3 inactivates T3 and exerts as the major inactivating enzyme. D1 is a kinetically inefficient enzyme that

activates or inactivates T4 on both rings, but its role in health remains to be further investigated (Bianco and Kim, 2006; van Beek, 2009).

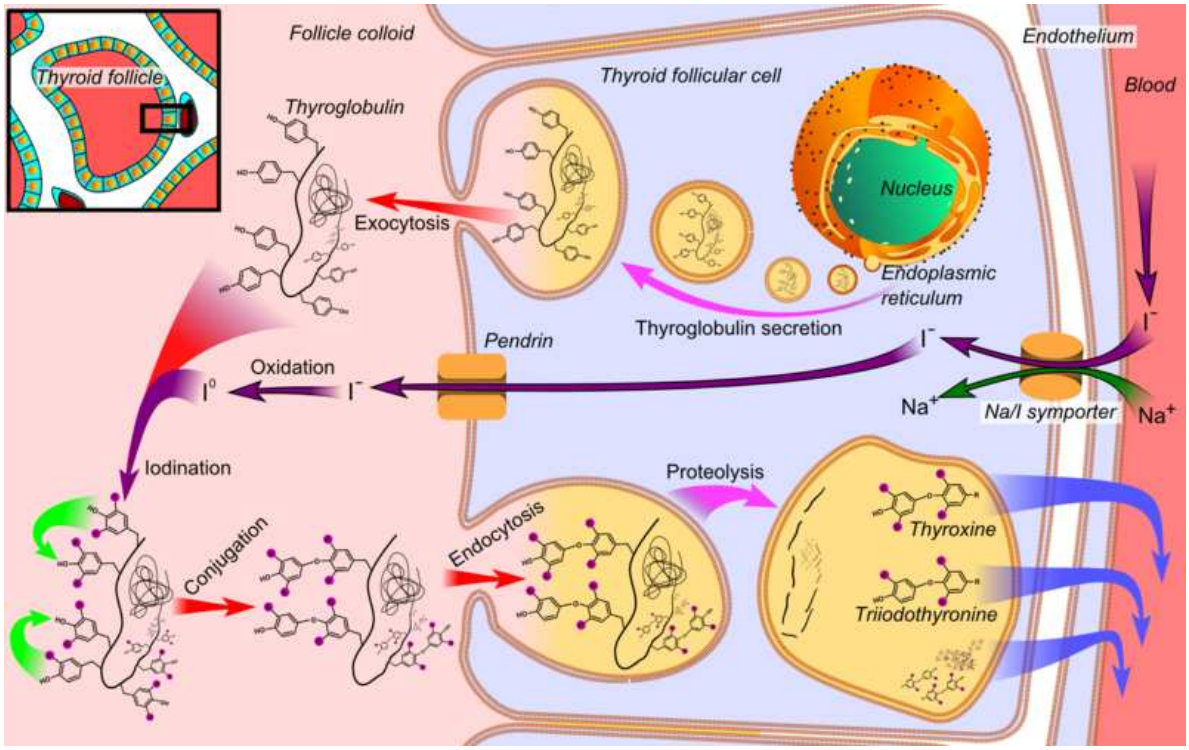

Figure 16. Thyroid hormones synthesis

THs are synthesized in thyroid gland follicular (epithelial) cells. The entire synthesis process calls for two essential raw materials (tyrosines and Iodine) and includes three main steps: accumulation raw materials, manufacture of hormone by the enzyme thyroid peroxidase (TPO), and release of the free hormones into the blood.

(From http://en.wikipedia.org/wiki/File:Thyroid_hormone_synthesis.png)

For thyroid hormone synthesis a sodium-iodide symporter (NIS), which located in the basolateral membrane of follicular epithelial cells, exerts an energy-dependent iodide uptake effort (van Beek, 2009). During the uptake process, the glycoprotein thyroglobulin (TG), a large complex protein which contains several tyrosine residues and released by the endothelial cells in a TSH-dependent manner, functions as producing matrix for TH-generation and account for the main part of the colloid at the same time (van Beek, 2009; Brent, 2012; Warner and Mittag, 2012). In the lumen, iodide is oxydidated to iodine by thyroid peroxidase (TPO), an

enzyme called thyroid peroxidase which also made with the thyroid epithelial cells released into the colloid within the follicle. Hydrogen Peroxide (H_2O_2), which regenerated in active follicular cells by thyroid oxidase type 1 and 2 contingent on calcium availability and hydrogenated nitrogen amino-adenine-dinucleotide phosphate (NADPH), play a very role in this chemical transformation (van Beek, 2009; Brent, 2012). Then with the catalytic effect of TPO, binding with one or two of the transformed iodide atom produces monoiodotyrosine (MIT, T1) or diiodotyrosine (DIT, T2), which are combined to create the finally hormones forms: triiodothyronine = T3 (DIT + MIT) or tetraiodothyrosine = T4 (DIT + DIT). Finally, the thyroid hormones are stored in the colloid and remain as a part of thyroglobulin until the thyroid is ready to secrete them into blood from thyroglobulin under the stimulation of TSH (van Beek, 2009; Brent, 2012). The THs synthesis process is strictly regulated by the HPT-axis (**Figure 18**).

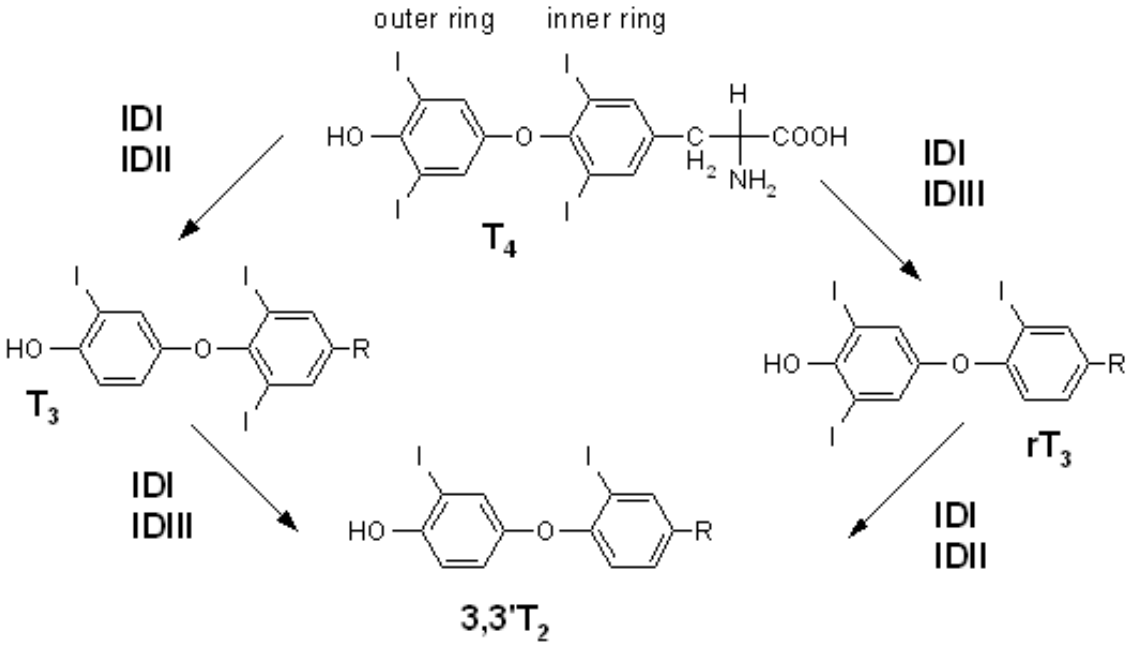

Figure 17. Reactions catalyzed by specific deiodinase isoforms
(From http://en.wikipedia.org/wiki/Iodothyronine_deiodinase)

Thyroid hormones primarily exert effects to all cells in our body. They mainly play a very essential role in stimulating whole body metabolism, which appears to lead, at

least partly, increase oxygen consumption and ATP hydrolysis rate. In addition, THs are essential for several tissue growths, differentiation and development, such as, bone, subcutaneous tissues. Moreover, except effects mentioned above, THs on cardiovascular, central nervous system, reproductive system has also been well known.

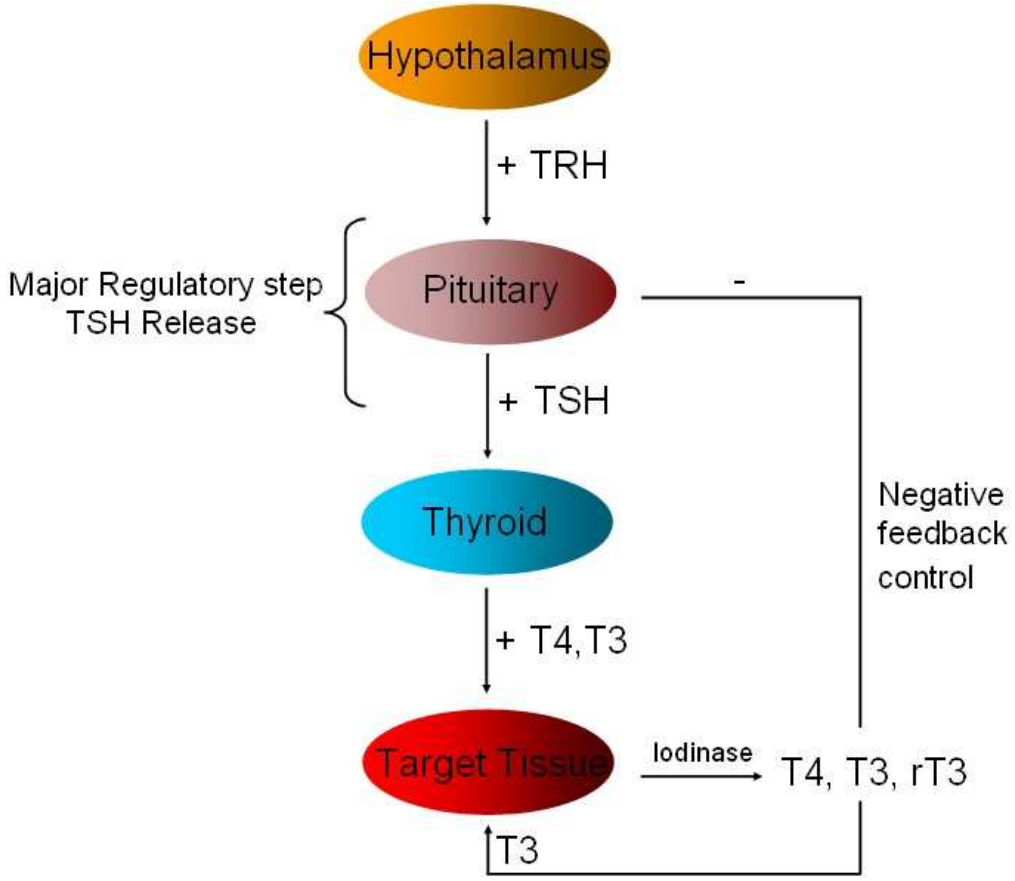

Figure 18. Regulation of thyroid hormones synthesis process

Thyroid stimulating hormone (TSH) is the chief stimulator of T3 and T4 synthesis (see **Figure 15**) via by interacting with thyroid epithelial cells through specific membrane receptors (TSH-R). Instead, both pituitary and intracutaneous TSH production is regulated by TRH, which is synthesized in the paraventricular nucleus of the hypothalamus, and also in human skin epithelium (Bodo et al., 2010; Gaspar et al., 2010; Paus, 2010). In turn, both T3 and T4 negatively reduce both TSH and TRH secretion. T3/T4 also inhibits TSH expression in human skin (Bodo et al., 2010). If T3 and T4 synthesis is reduced, TSH and TRH synthesis will increase, which in turn is inhibited by increasing TH serum level, thus creating effective feedback loops.

(TRH: thyreotropin releasing hormone, TSH: thyreotropin or thyroid stimulating hormone, T3: triiodothyronine, T4 = thyroxine)

1.9.2 Classical and non-classical signalling of thyroid hormones

Thyroid hormones primarily exert not only genomic effects but also non-genomic action (Davis et al., 2008). For their genomic action, THs enter cells through membrane transfer proteins (or transporter) and then further move inside the nucleus to bind to thyroid hormone receptors (TRs) (**Figure 19A**), thus creating an active TH-TR complex with high affinity and specificity (85% T3, 15% T4) in a dimeric form with the retinoid-x-receptor (van Beek, 2009). There are four isoforms of thyroid hormones receptor: TR-α1, TR-α2, TR-β1 and TR-β2. However, only three of them (TR-α1, TR-β1 and TR-β2) are able to bind thyroid hormones, while TR-α2 may have TH-inhibitory functions by binding T3/T4 without transmitting a signal (Cheng et al., 2010; Boelen et al., 2012; Brent, 2012; Burris et al., 2012). TRs belong to the large family of nuclear hormone receptor that effect as specific consequence of DNA associated with a couple of co-promoters and act by stimulating downstream specific gene transcription (thyroid hormone response elements, TRE) (Moore and Guy, 2005; Dittrich et al., 2011) (**Figure 19**). In the absence of THs, TR bind DNA usually result in inhibiting gene transcription.

In all tissues these transcription-modulatory effects exert function in cellular metabolism: oxygen consumption, heat generation, protein synthesis and degradation. Furthermore, THs are essential in several developmental processes, especially in amphibian metamorphosis (Paris et al., 2008; van Beek, 2009). Moreover, THs might play an important role in evolution, since many of the thyroid-associated gene encoding proteins, such as TR, TPO, TRH, TSH, sodium-iodide symporter (NIS) and deiodinases, were detected in amphibians, therefore were evolutionary preserved (Paris et al., 2008; van Beek, 2009).

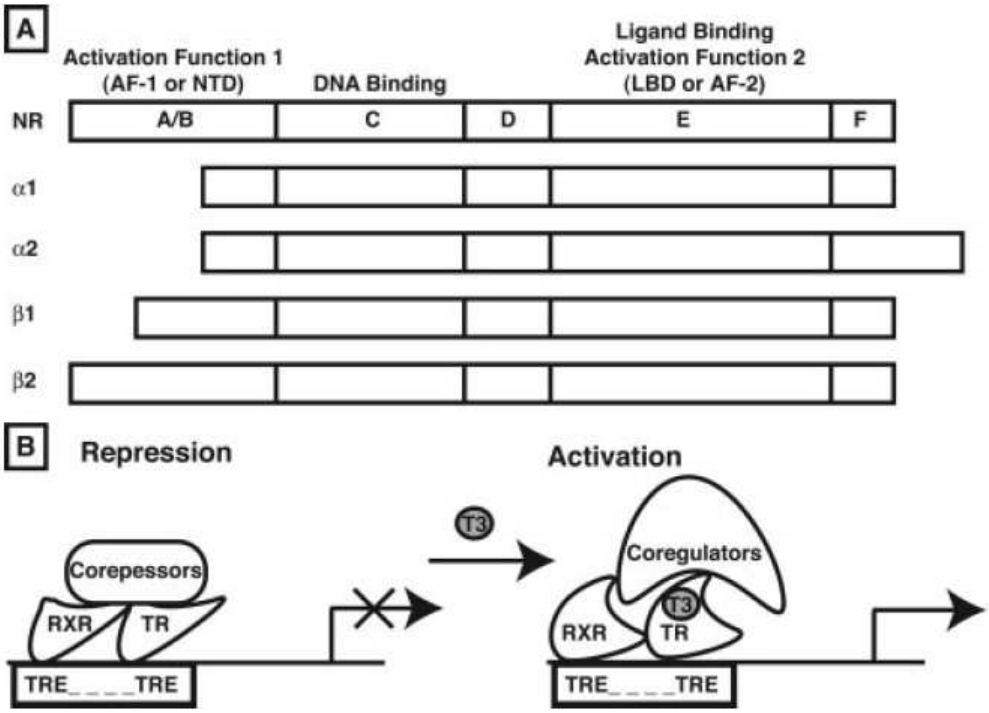

Figure 19. Thyroid hormone receptor (TR) structure and nuclear action

(**A**) Diagram of the primary structure of the TR subtypes; (**B**) mechanism of TR transcriptional control. TR forms a heterodimer complex with RXR that recognizes specific TREs.

(AF: Activation function; LBD: Ligand binding domain; NTD: Amino-terminal transactivation domain; TRE: Thyroid hormone response elements)

(From Moore and Guy, 2005 Image No 2).

Besides the classical ("genomic") actions of TH described above, T3/T4 also exert non-genomic actions in many cell types during the multiple physiological processes by TR-independent, membrane-associated pathway (**Figure 20**). The non-genomic action of T3/T4 mainly include membrane Ca^{2+}-ATPase activity (Davis et al., 1983), Na, K-ATPase activity (Lei et al., 2003), angiogenesis (Luidens et al., 2009), cancer cell proliferation (Davis et al., 2006), initiation of transcription of hypoxia-inducible transcription factor-1 gene (HIF-1 gene) (Bhargava et al., 2009; Cheng et al., 2010; Moeller and Broecker-Preuss, 2011; Vicinanza et al., 2012).

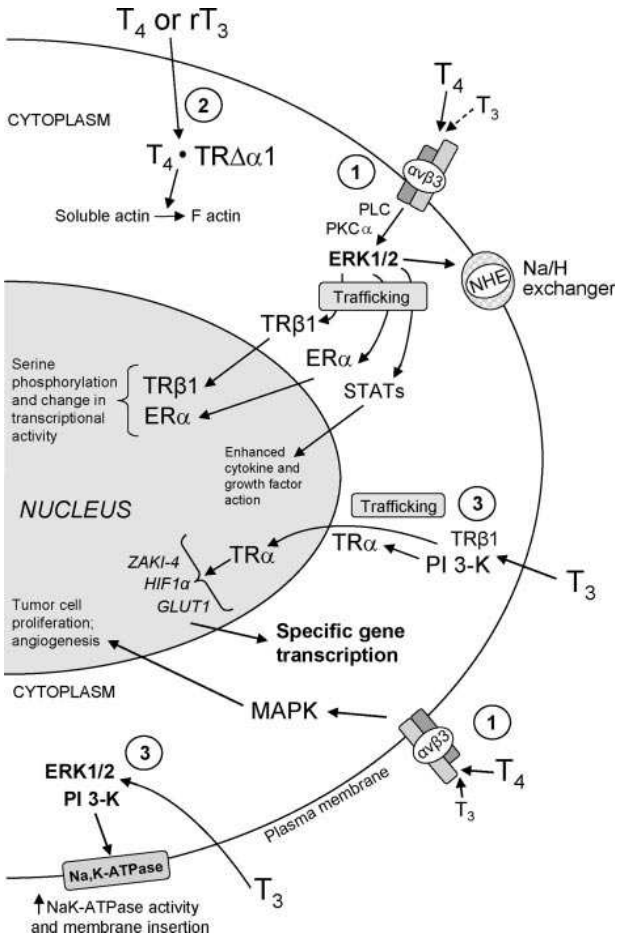

Figure 20. Nongenomic actions of thyroid hormones

(avß3: Integrin avß3; ER: Estrogen receptor; ER: Estrogen receptor; ERK 1/2: Extracellular-signal-regulated kinases 1/2; GLUT1: Glucose transporter-1; MAPK: Mitogen-activated protein kinases; NHE: Na/H exchanger; PI3K: phosphatidylinositide 3-kinases; PKC: Protein kinase C; PLC: Phospholipase C; STAT: Signal transducer and activator of transcription; TR: thyroid hormone receptor)

(From Cheng et al., 2010 Image No 5)

1.10 Current use of T4 in clinical medicine

T4 is one of the most widely used hormones in clinical medicine, especially for treating TH deficiency (hypothyroidism). In addition, it is also available for treatment or prevention of euthyroid goiter, congenital hypothyroidism, myxedema coma or stupor and TSH suppression in well-differentiated thyroid cancers and thyroid nodules (Grozinsky-Glasberg et al., 2006; Shoemaker et al., 2012). Since T4 can

easily be digested and absorbed by gut and is metabolised more slowly than T3, it has normally given by oral and just only once daily administration (75-200 microgram Levothyroxine) (De Leo et al., 2000; Pollock et al., 2001; Vaisman et al., 2001).

Levothyroxine is a pharmaceutical preparation of physiological thyroxine (T_4), which is metabolised more slowly than T3 and therefore normally only requires once-daily administration, and typically used for treat hypothyroidism (Grozinsky-Glasberg et al., 2006; Vaidya and Pearce, 2008). Even though some patients feel better on desiccated, natural THs than when biosynthetic THs are used. Unfortunately, there is no convincing objective evidence yet to support this claim (Grozinsky-Glasberg et al., 2006).

Since T4 is the clinically most-used TH and would thus be available for immediate clinical testing in a wound healing trial, and had already been shown by us to modulate human skin, skin appendage, and HF epithelial stem cell functions in organ culture (van Beek et al., 2008; Tiede et al., 2010), this thesis project entirely focused on testing the effects of T4 on human skin wound healing *in vitro*.

1.11 Working hypothesis

Specifically, we hypothesized that T4 may promote human skin wound healing and that this hormone would be particularly interesting for wound healing management, since it is not only FDA-approved and inexpensive, but also very well-known in its toxicology and can be applied both systemically and topically (Safer et al., 2001; Bach-Huynh et al., 2009; Hennessey et al., 2010; Colucci et al., 2011).

1.12 Questions addressed

To test this working hypothesis, we have studied in a newly developed full-thickness wounded human skin organ culture assay (see below, **Figure 21**) whether and how T4 impacts under serum-free conditions on reepithelialization, angiogenesis, and mast cells in experimentally wounded human skin *in situ*.

2 Materials and Methods

2.1 Materials

2.1.1 Human skin samples and ethics approval

Human scalp or corporal skin samples were obtained anonymously from patients undergoing plastic or reconstructive surgery with informed consent and Institutional Research Ethics Committee permission (University of Luebeck, license Aktenzeichen: 06-109) and according to Helsinki Declaration principles.

Our study included skin samples from 6 patients aged 26-67 years (average 52.7 years). The profile of each patient is listed in **Table 4**. Since, under the applicable ethics license, sample collection had to be done in anonymized form, only age, sex and skin sample location were known (thyroid status and medication unknown). In addition, we also visually examined the skin for macroscopic indications of tissue decomposition. Only good quality skin samples were chosen for organ culture.

Table 4. Characteristics of patients included in this study

Patient Number	Age (years)	Sex	Location
Patient 1[a]	67	Female	Temporal
Patient 2[a]	42	Female	Breast
Patient 3[a]	59	Female	Forearm
Patient 4[b]	61	Female	Middle face
Patient 5[b]	61	Female	Temporal
Patient 6[b]	26	Male	Buttock

a: used for T4 experiment analysis; b: used for inhibitory bFGF antibody treatment experiments.

2.1.2 Antibodies, chemicals, buffers, kits, equipment

Table 5 and **6** lists the primary and secondary antibodies that were used for immunohistochemistry/immunofluoresence studies (see below). **Table 7** summarizes the reagents employed and the respective vendor, while **Table 8** lists the buffers that were used, **Table 9** the utilized kits, **Table 10** organ culture

reagents, and **Table 11** the equipment that was employed

Table 5. Primary antibodies used for immunohistology in this study

Name	Host	Dilution	Method	Source	Positive control	Clone
MTCO1	Mouse	1:50	DAB	Mitosciences, Eugene, OR, USA	Skin epidermis (Lu et al., 2007; Poeggeler et al., 2010; Knuever et al., 2012)	1D6E1A8
Keratin 6	Mouse	1:10	Indirect IF	PROGEN, Heidelberg, Germany	Suprabasal layers of the ORS; suprabasal layers of wounded skin (van Beek et al., 2008)	Ks6.KA12
Keratin 15	Mouse	1:100	TSA	Chemicon, MA, USA	Scalp skin (Tiede et al., 2010)	LNK15
VEGF	Rabbit	1:500	Indirect IF	Abcam, Cambridge, UK	Skin epidermis (Brenner et al., 2009)	ab46154
bFGF	Mouse	1:50	Indirect IF	Abcam, Cambridge, UK	Skin epidermis (Brenner et al., 2009)	ab181
PCAM(CD31)	Mouse	1:30	Indirect IF	Dako, Glosturp, Denmark	Dermal microvessel (Mecklenburg et al., 2000)	M0823
FGFR1	Mouse	1:100	Indirect IF	Abcam, Cambridge, UK	Skin epidermis (Brenner et al., 2009)	ab829
Cortactin	Mouse	1:100	Indirect IF	Millipore, Temecula, CA	lamellopodia of migrating keratinocytes (Ceccarelli et al., 2007; Gendronneau et al., 2008)	4F11
C-kit (CD117)	Rabbit	1:1000	TSA	Cell Marque, Rocklin, CA	Hair follicle CTS (Sugawara et al., 2012)	YR145
Ki67	Mouse	1:100	Indirect IF	Dako	proliferating hair matrix and epidermal keratinocytes of normal human skin (Bodo et al., 2007)	MIB-1

MTCO1: Cytochrome c oxidase 1; DAB: Diaminobenzidine; IF: Immunofluorescence; TSA: Tyramide Signal Amplification; VEGF: Vascular endothelial growth factor; bFGF: Basic fibroblast growth factor; PECAM: Platelet endothelial cell adhesion molecule; FGFR1: Fibroblast growth factor receptor1; VEGFR: Vascular endothelial growth factor receptor.

Table 6. Secondary antibodies used for immunohistology in this study

Name	Conjugated with	Dilution	Company
Goat anti-mouse	biotin	1:200	Beckmann Coulter
Goat anti-mouse	FITC	1:200	Jackson ImmunoResearch
Goat anti-mouse	Rhodamin	1:200	Jackson ImmunoResearch
Goat anti-rabbit	FITC	1:200	Jackson ImmunoResearch

Table 7. Chemicals

Name	Company
Aceton	Roth
Alcohol	Merck
Antibody diluent	Dako
Antibody diluent	DCS
DAPI (4',6-diamidino-2-phenylindole)	Roche
Diaminobenzidine (DAB)	Vector
Eosin E	Sigma
Endogenous peroxidase	Merck
Eukitt	Sigma
Ethanol (100 %)	Roth
Foetal calf serum Gold	PAA Laboratories GmbH
Fluoromount-G	Southern Biotech
Glacial ethanoic acid	Roth
Goat normal serum	Dako
Hydrogenperoxide	Merck
KH_2PO4	Merck

Light green	Fluka
Mayer’s Haemalaun	Merck
Mounting medium Faramount	Dako
Mounting medium Flouromount-G	Southern Biotec
Na_2HPO4	Roth
N,N-dimethylformamide	Sigma
Naphtol-ASD chloroacetate	Sigma
Paraformaldehyde	Merck
Pararosaniline	Merck
Potassium dihydrogen phosphate	Merck
Shandon Cryomatrix	Thermo
Sodium chloride (NaCl)	J.T. Bakker
Sodium dihydrogen phosphate	Merck
Sodium dihydrogen phosphate monohydrate	Merck
Sodium nitrit	Merck
Sörensen A	Roth
Sörensen B	Merck
TdT(Terminal dioxynucleotidyltransferase)-Enzym	Qbiogene
Toluidine blue O	Sigma
Tris-HCl	Roth
Triton X	Roth
Trizma Base	Sigma
Trypsin/EDTA	Gibco™(Invitrogen)
Tween 20	Merck
Xylol	Merck

Table 8. Buffers #

PBS (pH = 7.2) Phosphate buffered saline	8.0g sodium chloride
	1.8g sodium dihydrogen phosphate monohydrate
	Aqua dest. ad 1000ml
TBS (pH = 7.6) Tris buffered saline	6.1g Trizma Base
	8.8g sodium chloride
	Aqua dest. ad 1000ml
TNT (pH = 7.5) Tris buffered saline triton-x added	15.76g Tris-HCl
	8.766g sodium chloride
	500µl Tween 20
	Aqua dest. ad 1000ml

Sodium hydroxid (NaOH) and hydrogen chloride (HCl) were used to adjust the pH.

Table 9. Staining Kits

Name	**Company**
Alkaline Phosphatase (AP)	Vectastain
Apop Tag® Fluorescein In Situ Apoptosis Detection Kit	Millipore
Avidin/Biotin Blocking Kit	Vector
Fast Red Tablets	Sigma
TSA™ Fluorescein System	Perkin Elmer LAS, Inc.
TSA™ Tetramethylrhodamine System	Perkin Elmer LAS, Inc.

Table 10. Organ culture reagents

Name	**Company**
bFGF inhibitory antibody	R&D systems
Fetal bovine serum (FBS)	PAA Laboratories
Hydrocortisone	Sigma

Insulin	Sigma
L-glutamine	Gibco™ (Invitrogen) Corporation
PBS (sterile)	PAA Laboratories GmbH
Penicillin G/ streptomycin	Gibco™ (Invitrogen) Corporation
T3	Sigma
T4	Sigma
William´s E Medium (2,2 g/l NaHCO3)	Biochrom KG
William's E culture medium	William´s E Medium (2.2g/l NaHCO3) Biochrom KG, **supplemented with:**
	L-glutamine (200mM)
	Penicillin G/ streptomycin (1 %)
	insulin (10mg/ml)
	hydrocortisone (0.05 mg/ml)

2.1.5 Table 11. Equipment

Name	**Company**
Bright field microscope	Krüss
Cell culture incubator Autoflow	Nuaire™
Centrifuge 5810	Eppendorf
Cryostat	Leika
Fluorescence microscope (8000)	Biozero Keyence
Laminar airflow	ScanLaf
Neubauer counting chamber	Brand
pH meter	Knick
Pipettes	Eppendorf
Safety cabinet Clean Air	Thermo
Scale	Kern EW
Vortexer	IKA®MS3 basic

2.2 Methods

2.2.1 Human skin wound healing organ culture model

The human skin wound healing assay modified the previously published “punch-in-a-punch” design of Moll (Moll et al., 1998) and our established full-thickness human skin organ culture assay (Lu et al., 2007; Bodo et al., 2010; Gaspar et al., 2010; Poeggeler et al., 2010). The notable difference to the Moll et al. assay was that hair-bearing, full-thickness adult human skin (including subcutaneous fat) was used, which cultured under serum-free conditions in William’s E medium (instead of DMEM) supplemented with 2mmol/liter, L-glutamine, 10 ng/ml hydrocortisone, 10 µg/ml insulin and antibiotics (Philpott et al., 1990; Lu et al., 2007). Also, a number of (immuno-)histomorphometric read-out parameters for the quantitative assessment of defined wound healing aspects was newly established (see below).

In brief, first, 2 mm punches were made in the obtained skin samples. Then, a wider (4 mm) punch was set in the surrounding skin so as to obtain “punch-within.a punch” skin fragments (**Figure 21**). Samples were frozen immediately for analysis (day 0) or transferred to six-well plates containing supplemented William’s E medium (Lu et al., 2007).

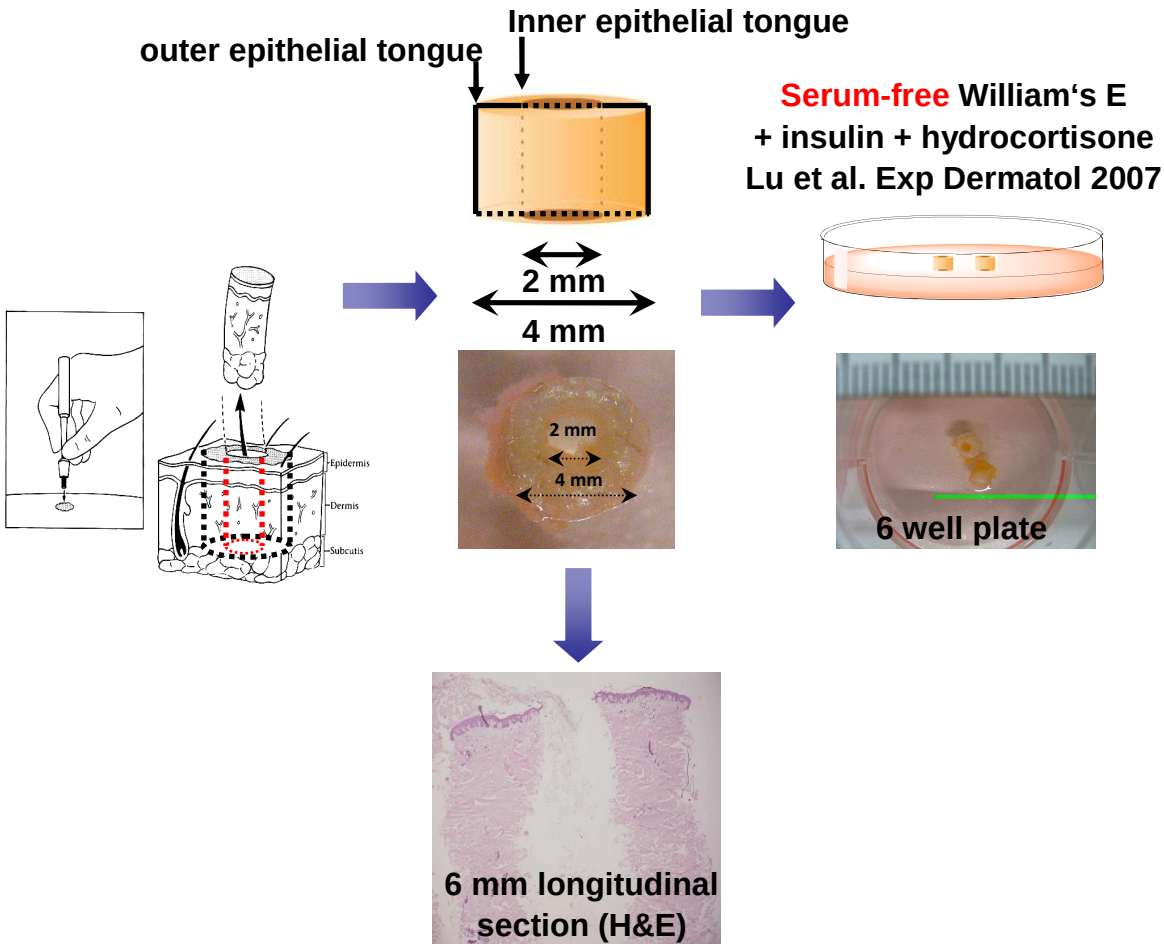

Figure 21. Schematic diagram of wounded human skin organ culture model (details, see Section 3.1 **Figure 25)**
(The black cartoon (lefthand) was modified from http://www.answers.com/topic/skin-biopsy).

Each well contained 2 skin punches in 3 ml medium and the punch surface continuously faced into the air while floating in medium (air-liquid interphase design). After 24 hours (day 1), William's E culture medium (used as a vehicle control) or the test agents T4 (10nM, 100nM or 1000nM) were added (**Figure 22**). These concentrations were selected based on our previous *in vitro* studies, which had shown that under the different concentration (10nM, 100nM, 1000nM), T4 modulate multiple hair biology parameters: up-regulates hair matrix keratinocytes proliferation, prolongs the duration of the hair growth phase (anagen) *in vitro*, increase thyroid hormone-responsive keratins cytokeratin 6 (CK6) and 14 (CK14)

expression and stimulate intrafollicular melanin synthesis (van Beek et al., 2008).

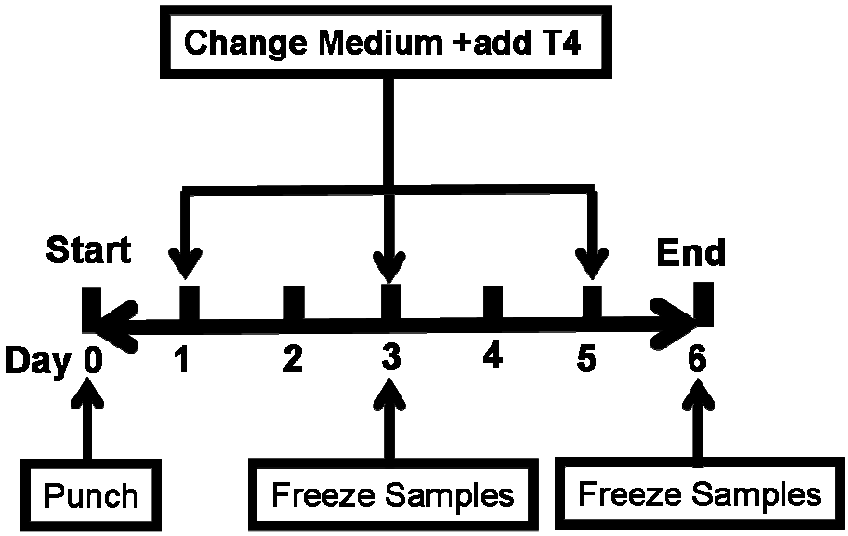

Figure 22. Organ culture protocol of this study

The incubation medium change and sample freeze was performed following the culture protocol and incubation medium was changed and randomly selected skin punches from each experimental condition were frozen at day 3, 6 as summarized in **Figure 22**. Human skin fragments were embedded in Shandon cryomatrix (Thermo Fisher Scientific; Waltham, MA, U.S.A.) before cutting longitudinal 6 µm cryoslides for further analyses. Normally, we cut all the samples under -21℃ to -23℃, whose temperature choose dependent on the amount of fat tissue in the wound healing punches, and afterwards the slides stored at -80℃ refrigerator again until staining procedures. Organ culture and snap freezing were performed under standardized, sterile conditions in order to minimize any confounding influences on the samples.

2.2.2 Histochemistry

2.2.2.1 Haematoxylin and eosin staining

Routine histology was performed by staining with Mayer's Haemalaun (Merck, Darmstadt, Germany) and 0.1 % eosin E (Sigma-Aldrich, St Louis, MO, U.S.A.).

This H&E staining was used for the general structure of wound tongue, evaluation the reepithelialization of the area and the length of the wound tongue.

Firstly, all the slides were dried for 10 minutes at room temperature and then fixed them in Acton for 10 minutes at -20 ℃. After fixat ion, the slides washed in distilled water 1 to 3 minutes and then directly stained with Haematoxylin for 10-15 minutes. Next steps wash the stained slide in running tap water for around 15 minutes and stained in eosin in 1 minute. Next, the slides were differentiated in sequential solutions (70% alcohol-96% alcohol-96% alcohol-100% alcohol -100% alcohol-Xylol I-Xylol II) for a few times and Xylol II for 10 minutes. Finally, the slides were very carefully mounted with Eukitt® solution and then dried them until ready to use (Note all the procedures were done under a laminar airflow).

2.2.2.2 Mast cell histochemistry 1: Toluidine blue

For detection of MCs and their characteristic metachromatic granules, one histochemical staining method, toluidine blue was performed on 6 µm cryoslides as previously described (Ito et al., 2010; Sugawara et al., 2012).

Toluidine blue stock solution (1 g Toluidine blue O and 100ml 70% alcohol, and mix well to dissolve) and sodium chloride (1%: 0.5 g sodium chloride dissolves in 50 ml distilled water, and adjusted ph value to 2-2.5) were prepared firstly and then diluted to working solution (5 ml Toluidine blue stock solution and 45 ml 1% Sodium chloride dissolved and then adjust pH value around 2.3). The toluidine blue working solution should be made freshly before use. Cryoslides were dried firstly 10 minutes at room temperature, washed in distilled water 2 minutes and then stained in toluidine blue working solution 1 minute. Next, slides were rinsed in distilled water 3 to 4 times and dehydrate quickly through (70% alcohol-96% alcohol-96% alcohol-100% alcohol -100% alcohol-Xylol I-Xylol II) and finally mounted with

Eukitt® medium.

2.2.2.3 Mast cell histochemistry 2: Leder esterase

Leder esterase histochemistry, also known as chloroacetate esterase reaction, depends on the activity of an enzyme found in mast cells and granulocytes that is capable of hydrolyzing aliphatic and aromatic bonds (Leder, 1979). Leder esterase mast cell histochemistry is an excellent, very sensitive marker for staining human skin mast cells (Ito et al., 2010; Sugawara et al., 2012).

For Leder esterase staining, cryoslides were dried 10 minutes at room temperature and then fixed in 1% paraformaldehyde for 10 minutes at room temperate. After the fixation, the slides washed in distilled water around three times, 5 minutes per time and then stained in the incubating medium (more detail see below) for 40 minutes and washed them in running tap water 5 minutes. Next, slides were counterstained with hematoxylin for 30 seconds to 1.5 minutes and then washed in running tap water 5 minutes again, differentiated in ascending sequential solutions (70% alcohol-96% alcohol-96% alcohol-100% alcohol -100% alcohol-Xylol I-Xylol II) for a few times and Xylol II for 10 minutes. Finally, all the slides were mounted with Eukitt. Incubating medium was prepared immediately before use by our previously protocol: naphthol-ASD-chloroacetate (10 mg; Sigma Aldrich), *N, N*-dimethylformamide (1 ml; Sigma Aldrich), Sörensen working buffer (35ml, Sörensen A: Na_2HPO4; water free, Roth, Karlsruhe, Germany; Sörensen B: KH_2PO4; Merck, Darmstadt, Germany), and nitrosylated pararosaniline (pararosaniline and sodium nitrite; Merck, Darmstadt, Germany) (Ito et al., 2010). All the staining procedure should be mandatorily done under the bench, since the above materials used for this staining are very harmful to our health.

2.2.3 Immunohistochemistry (IHC)

MTCO I: An indirect marker for screening energy metabolism

For the immunodetection of MTCO1 by IHC, as the previously described peroxidase-based avidin-biotin complex immunostaining method with a monoclonal antibody that specifically recognizes MTCO1 (mitochondrial encoded cytochrome c oxidase I), a multi-chain transmembrane protein located in the inner membrane of mitochondria and an indirect marker for screening energy metabolism, was employed (Bodo et al., 2009; Bodo et al., 2010; Poeggeler et al., 2010; Knuever et al., 2012). After fixation in acetone, blocking of endogenous peroxidase (3% H_2O_2) and pre-incubation with goat serum [10% in Tris-buffered saline; Dako], cryoslides were incubated with anti-MTCO1 antibody (**Table 5**). Next, cryoslides were stained with biotinylated goat anti-mouse IgG (1:200 for 45 minutes at room temperature; Beckmann Coulter, Merseille, France) as secondary antibody and then performed with an avidin-biotin kit (Vector), followed by DAB substrate-chromogen system (Vector) (van Beek et al., 2008). Counter-staining was performed with Mayer's hematoxylin.

2.2.4 Immunofluorescence (IF)

For the detection of indirect immunofluorescence, the previously described methods were used (Lu et al., 2007; Bodo et al., 2009; Bodo et al., 2010). Cryoslides were fixed in acetone for 10 minutes at -20 ℃, washed in Tris buffered saline (TBS), pH 7.6 and incubated at 4℃ over nigh t with primary antibody (**Table 5**) diluted in DAKO Antibody Diluents (DAKO North America, Carpinteria, CA). Slides were washed 3 times in TBS, then incubated at 4 ℃ for overnight with secondary antibody (Which secondary antibody chooses was depended on the primary antibody host and availability in our secondary antibody list, normally FITC (fluorescein isothiocyanate) or Rhodamine conjugated secondary antibody were used) (**Table 6**) diluted in DAKO Antibody Diluent, then washed 3 times in TBS and incubated in 0.1 µg/ml 4′, 6-Diamidino-2-phenylindol (DAPI) (Roche Diagnostics,

Risch, Switzerland) for 5 minutes. After washing 3 times in TBS, slides were embedded in Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Negative controls were performed by following the above protocol, but incubating samples without primary antibody. Absence and presence of immunoreactive cells in well-described human skin locations served as additional internal negative or positive controls. All samples were photographed for analysis with a Keyence Biozero-8000 Microscope (Keyence Corporation, Higashi-Nakajima, Osaka, Japan), and the excitation light channel chooses to depend on which secondary antibody used. Since all immunostaining experiments were conducted with appropriate positive and negative controls for each investigated parameter, the only specific IR (beyond background) was evaluated in the current study.

2.2.4.1 Ki67/TUNEL: Markers for proliferating and apoptotic cells

For the detection of proliferating and apoptotic cells in this system, Ki67/TUNEL double-immunofluorescence was performed as described previously (van Beek et al., 2008; Bodo et al., 2009). For the quantitative evaluation and comparison of the double-staining, DAPI-, Ki-67-, or TUNEL-positive cells were counted in clearly defined area indicated by reference (the newly formed epidermal tissue was addressed as a new “epithelial tongue” [ET], for details see **Figure 23**) (Ramot et al., 2011). The number of DAPI-positive cells served as the “total number of cells,” from which the percentage of Ki-67-positive and/or TUNEL-positive cells in the ET was calculated to enable comparison between vehicle and test groups (Ramot et al., 2011).

2.2.4.2 Cortactin: A marker for keratinocyte migration

Cell migration is achieved through dynamic reorganization of the actin cytoskeleton (Mitchison and Cramer, 1996; Pollard and Borisy, 2003; Ceccarelli et al., 2007). Cortactin is a diffusely expressed actin-binding protein originally demonstrated as a

substrate for Src kinase (Ceccarelli et al., 2007). Cortactin, distributed in lamellipodia of migrating keratinocytes, contains an actin-binding domain, which have been developed as a marker of migration keratinocytes (Ceccarelli et al., 2007; Gendronneau et al., 2008). Therefore, in this study, cortactin is used as a marker for migration, using the described general immunofluorescence protocol (**see also Table 5**) (Ceccarelli et al., 2007; Gendronneau et al., 2008).

2.2.4.3 Cytokeratin 6: A marker for wounded and hyperproliferating epithelium

Cytoeratin 6 (CK6) is a member of the type II keratin family, which is usually expressed together with CK16 and/or CK17, is the inducible expression in response to stressful stimuli such as wounding (Ramot et al., 2009b; Windoffer et al., 2011). Also, CK6 is induced early on in wound-proximal keratinocytes and maintained during reepithelialization (Wojcik et al., 2000; Rotty and Coulombe, 2012). CK6 is also constitutively expressed in the HF's ORS (van Beek et al., 2008; Ramot et al., 2009b). It has recently been shown that genetic ablation of CK6 results in enhanced keratinocyte migration, which is functionally important in wound repair (Wojcik et al., 2000; Rotty and Coulombe, 2012). CK6 negatively modulates Src kinase activity and the migratory potential of skin keratinocytes during wound repair process by a cultured skin explants model (Wojcik et al., 2000; Rotty and Coulombe, 2012). Here, CK6 was used as a marker for wound healing-associated epithelial differentiation, using our established immunofluorescence protocol (van Beek et al., 2008) (see also **Table 5**).

2.2.4.4 CD31: A marker for endothelial cells

CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), is found on the surface of many cell populations, e.g. endothelial cell, which have been widely accepted as a marker of angiogenesis (Mecklenburg et al., 2000;

Baluk and McDonald, 2008). Therefore, we used CD31 as a marker of angiogenesis during the wound repair, and the immunostaining followed the immunofluorescence protocol described above (see also **Table 5**).

2.2.4.5 bFGF/VEGF: Growth factors for promoting angiogenesis

During wound healing of normal tissue, both bFGF and VEGF resulted in the angiogenesis process: it mediated the formation of the new vessel (Przybylski, 2009). Moreover, they have been demonstrated that they, at least in part, are involved in TH-induced angiogenesis (Davis et al., 2009). Here, we immunostained these two antigens to obtain first mechanistic insights into how T4 may modulate angiogenesis in our model system. The immunostaining protocol was run as described above (see also **Table 5**).

2.2.4.6 FGFR1: bFGF receptor

FGFR1 is a receptor tyrosine kinase whose ligands are specific members of the fibroblast growth factor family such as bFGF (FGF-2). It has been demonstrated that the proangiogenic actions of T4 may be transduced by mitogen-activated protein kinase, specifically, extracellular regulated kinase 1/2 (ERK1/2) and this effect mediated at least in part via up regulating FGFR1 expression (Davis et al., 2009; Luidens et al., 2009). Thus, to further investigate the mechanism underlying the proangiogenic activity of T4, FGFR1 immunostaining was also performed, following the protocol described above (see also **Table 5**).

2.2.5 Tyramide Signal Amplification (TSA)-Immunofluorescence

To investigate keratin 15 (CK15) and C-Kit expression, we used the highly sensitive tyramide signal amplification (TSA) staining method (TSA kit, Perkin Elmer, Boston, USA) (Ito et al., 2010; Sugawara et al., 2012). Firstly, the slides were dried 10 minutes at room temperature and then fixed in acetone 10 minutes at -20 ℃. After

fixation, slides were washed with TNT buffer and blocked endogenous peroxidase by (3% in PBS, 15 minutes, room temperature), which followed by treatment with avidin and biotin (each for 15 minutes). After pre-incubated for 30 minutes with 5% goat normal serum in TNT buffer, the slides were incubated with the primary antibody at indicated diluents concentration (more detail see **Table 5**) in TNB (Tris HCl+NaCl+Casein) supplemented with 2% normal rabbit serum, overnight at 4 ℃. On the following day, a secondary biotinylated antibody was incubated to the slides at a dilution of 1:200 in TNB with 2% normal rabbit serum for 45 minutes at room temperature, and then incubated with the streptavidin conjugated horseradish peroxidase (TSA kit, Perkin Elmer, Boston, USA) diluted 1:100 in TNB for 30 minutes at room temperature. Next, FITC/Rhodamine-tyramide amplification reagent 1:50 in amplification diluent was applied and then counterstained with DAPI for 1 minute and mounted with Fluoromount®.

2.2.5.1 Cytokeratin 15: A marker for epithelial stem cells and their immediate progeny

Cytokeratin 15 (CK15), a member of type I keratin family group is restricted to immature epithelial progenitor cells in the epidermis and HF, and is prominently expressed by human HF epithelial stem cells in the bulge and their immediate progeny (transit amplifying cells (Cotsarelis, 2006; Kloepper et al., 2008; Tiede et al., 2010). Here CK15 was investigated to analyse whether in wound tongue area, the number of CK15 positive cells was increased or not in T4 treated groups, and the staining protocol used was described above (see also **Table 5**).

2.2.5.2 C-kit (CD117): An intra-mesenchymal marker for mast cell progenitor cells

C-Kit, also known as CD117 and mast/stem cell growth factor receptor, is expressed not only mast cells, but also on hematopoietic progenitor cells,

melanocytes, cardiac stem cells and many sarcomas and carcinomas (Ito et al., 2010; Sugawara et al., 2012). In human skin mesenchyme, C-Kit is an excellent marker for identifying both immature and differentiated connective tissue type mast cells, which visualizes many more mast cells than can be detected by mast cell histochemistry (Weller et al., 2006; Fuehrer et al., 2009; Ito et al., 2010; Rodewald and Feyerabend, 2012; Sugawara et al., 2012). The TSA-IF technique described above (More details: see **Table 5**) and previously reported by our lab (Ito et al., 2010; Sugawara et al., 2012) was used.

2.3. Microscopy

All skin sections were photographed for analysis with a Keyence Biozero-8000 Microscope (Keyence Corporation, Higashi-Nakajima, Osaka, Japan), which was used for both fluorescence and light microscopy. This portable fluorescence microscope permits to generate high-quality photos without any other auxiliary equipment and does not require a dark room.

2.4 Quantitative (immuno-) histomorphometry

2.4.1 Assessment of reepithelialization

For quantitative of reepithelialization, the H&E staining of epithelial tongue were analyzed, both sides (outer and inner epithelial tongue), defined, reference indicated reference areas were analyzed, which based on the visible edge of the stratum corneum where the punch had been placed and the corresponding region in the basal layer of the epithelial tongue [ET] (See **Figure 23** for details). ImageJ software (National Institutes of Health, Bethesda, MD) was used for evaluation.

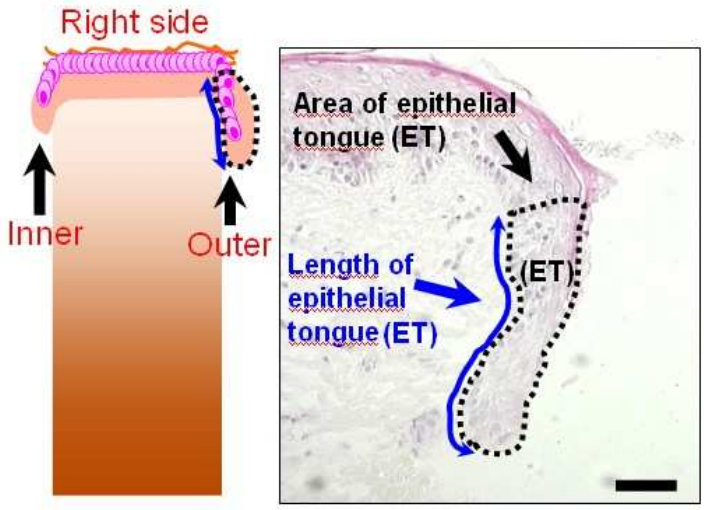

Figure 23. Analysis of reepithelialization in this model
Epithelial tongue (ET) area was measured in the reference area marked with blacked dotted line and epithelial tongue (ET) length was measured marked with the blue line.

2.4.2 Assessment of apoptotic/proliferating cells: Ki-67/TUNEL

Based on our previously published quantitative immunomorphometrical techniques (Gaspar et al., 2010; Holub et al., 2012), the number of apoptotic (TUNEL) and proliferating (Ki-67) cells could be analyzed in the new generated human wound epithelial tongue *in situ*. The numbers of Ki-67 and TUNEL positive cells were counted in the ET and then calculated as the percentage to the total number of DAPI positive cells in the same ET area.

2.4.3 Assessment of immunoreactivity

All photos in each read-out parameter were taken at the same exposure time and magnification. The staining immunoreactivity (IR) in wound ET area was analysed by using the NIH/Image software (Bethesda, Maryland, USA), as described previously (Knuever et al., 2012; Sugawara et al., 2012), and then all the values were normalised to control as 100%.

2.4.4 Assessment of angiogenesis

To analyze the angiogenesis process, firstly, we taken 3 visual field (x400) photos

per wound punch side as below the dotted-line marked reference area: two visual fields next to the wound tongue, one visual field under that two fields far from epidermis (200µm) (**Figure 24**). Next, there were three read-out parameters investigated in this study, as our previously described methods (Mecklenburg et al., 2000).

1. Count the number of CD31+ nucleus/visual field;
2. Calculate CD31 staining immunoreactivity per visual field followed as above (**2.4.3**);
3. Count the number of CD31+ lumina/visual field.

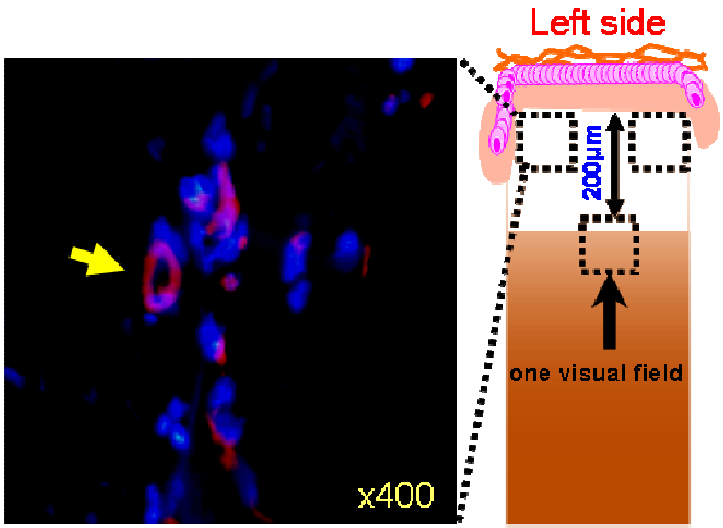

Figure 24. Analysis of angiogenesis in this model
Yellow arrow: Cross-section of CD31+ blood vessel, recognizable by its central lumen. Whenever possible, photos were taken from the reference areas indicated above on both the right and the left side of the tissue section.

2.4.5 Assessment of mast cell number and degranulation

By taking photos with a 400 fold magnification, mast cells under the wound tongue of wound healing model *in situ* were counted and classified into two categories: not degranulated (lower than five granules) and degranulated (five or more granules), as described previously (Ito et al., 2010; Sugawara et al., 2012).

2.5 Statistical analysis

All the data are given as means±SEM (standard error of the mean). For the immunohistochemistry, immunofluorescence, and quantitative imunohistomorphometry of new ‘tongue’, both sides of area and immunoreactivity (IR) were measured in the outer and/or inner tongue (the values obtained for the inner and outer wound edges were combined). For each test parameter, 3 to 6 non-consecutive sections from 3 to 6 different organ-cultured skin fragments derived from 3 different patients were analyzed. Data were pooled, since the results trends from all three patients were highly comparable.

One-Way ANOVA by appropriate post hoc comparison was used at single time points. If the values did not follow a Gaussian distribution, Kruskal-Wallis ANOVA test was employed. Statistical analysis was carried out by Graphpad prism 5.01 (Graph Pad software, Inc., San Diego, CA, USA), and p value <0.05 were regarded as significant.

3. Results

3.1 Reepithelialization of wounded adult human skin can be studied and quantified under serum-free long-term organ culture conditions

In this new serum-free organ culture assay of full-thickness wounded adult human skin, the expected phenomenon of epithelial sheet movement over a floating skin fragment (epiboly) (Stenn, 1981; Brown et al., 1991) was observed already after 1 day of organ culture: As shown in **Figure 25**, a compact rim of epidermal keratinocytes formed an “epithelial tongue” (ET) that covered the exposed dermis at the inner and outer wound edges. This suggests that serum and its epiboly-promoting key ingredient, vitronectin (Brown et al., 1991), are not indispensable for epithelial sheet movement, or that wounded human epidermal keratinocytes produce this spreading factor themselves (the fact that ET enlargement progressed after day 1, renders it unlikely that sufficient vitronectin was retained in the tissue after surgery, microdissection and tissue handling).

With progressing culture duration, typical signs of tissue degeneration also became apparent, heralded by detachment of the stratum corneum and increasingly compacted epidermal keratinocyte nuclei. These degenerative phenomena became routinely visible by day 6 (**Figure 25**). Interestingly, however, despite these parallel degeneration events in the same skin fragment, our pilot experiment shown that the newly formed “epithelial tongue” retained its morphology until day 9 suggesting a higher viability and more autonomous growth of these cells than the epidermal tissue from which it originated.

To quantify the extent of reepithelialization, we used two complementary methods: a) measurement of the length of the ET; b) planimetric measurement of the entire

ET area of the ET (More details see **Figure 23 and 25**).

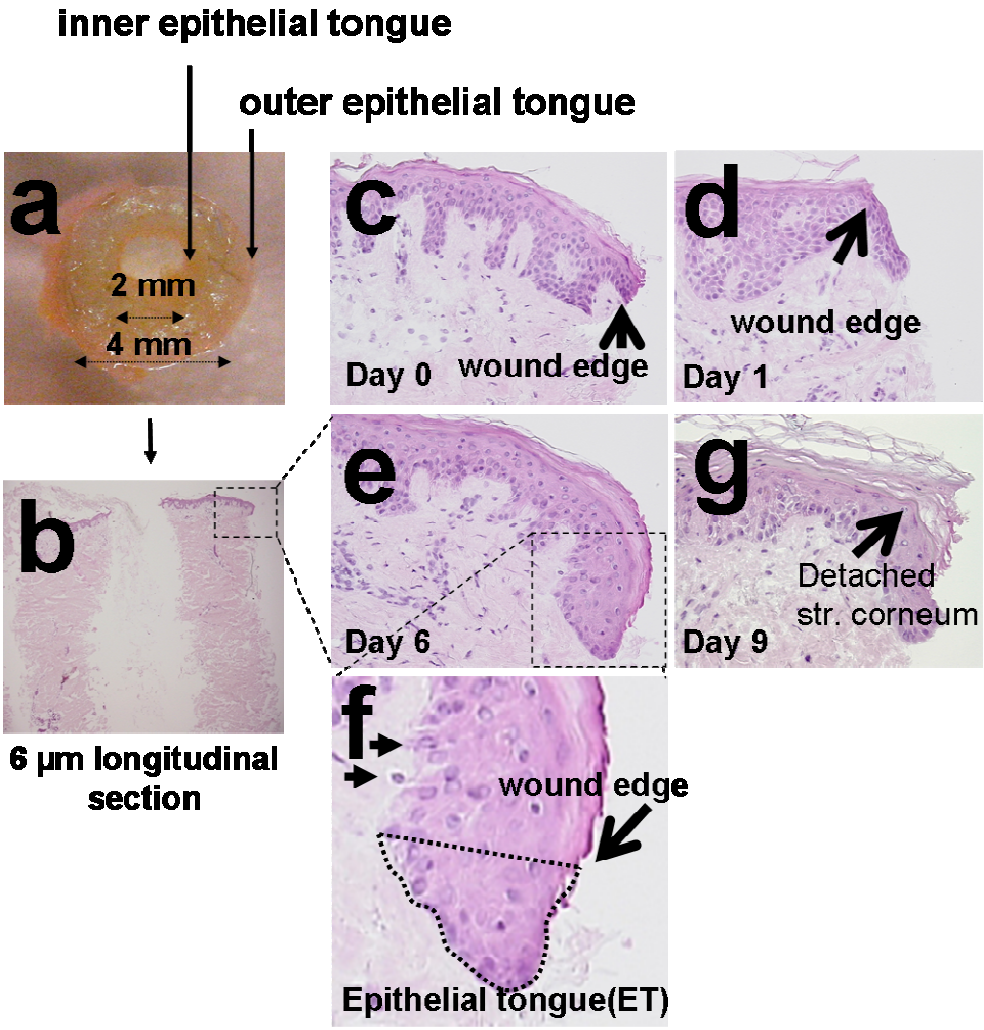

Figure 25. Example of wounded full-thickness human skin fragment, punch-in-a-punch design

(**a**): View from above: (**b**): longitudinal section. (**c-g**): Longitudinal sections through outer wound edge (0 and 9 days, H & E). Large arrows in **c**, **d** and **f** indicate the border between native and regenerated epithelium (based on the visible edge of the stratum corneum where the punch had been placed and the corresponding region in the basal layer of the epithelial tongue [ET]). (**f**) The ET area is demarcated by a dotted black line (**e**). Small arrows indicate increasingly compacted epidermal keratinocyte nuclei. (**g**) Large arrows indicate detachment of the stratum corneum.

Generally, reepithelialization (ET formation) progressed faster and was more pronounced in the outer edge of the wounded skin fragment compared to the inner wound edge (**Figure 26**). This suggests that reepithelialization/epiboly conditions in freely floating wounded human skin fragments under serum-free conditions are

more favorable at the outer wound edge and that ET measurements performed in this region are more likely to detect even subtle reepithelialization-promoting or -inhibitory effects of test agents than in the inner edge. However, human skin ulcers reepithelialization in a centripetal fashion as ulcers typically only has an inner wound edge. Therefore, it should be kept in mind that ET formation at the inner wound edge of organ-cultured "punch-within-a-punch" human skin fragments probably reflects the clinical reality of ulcer reepithelialization more closely than ET formation in the skin fragment periphery and is therefore translationally more relevant. Thus, if a test agent promotes both inner and outer ET formation, this enhances confidence that it may also do so *in vivo*. Therefore, where not indicated otherwise, ET measurements were subsequently performed at **both inner and outer wound edges**.

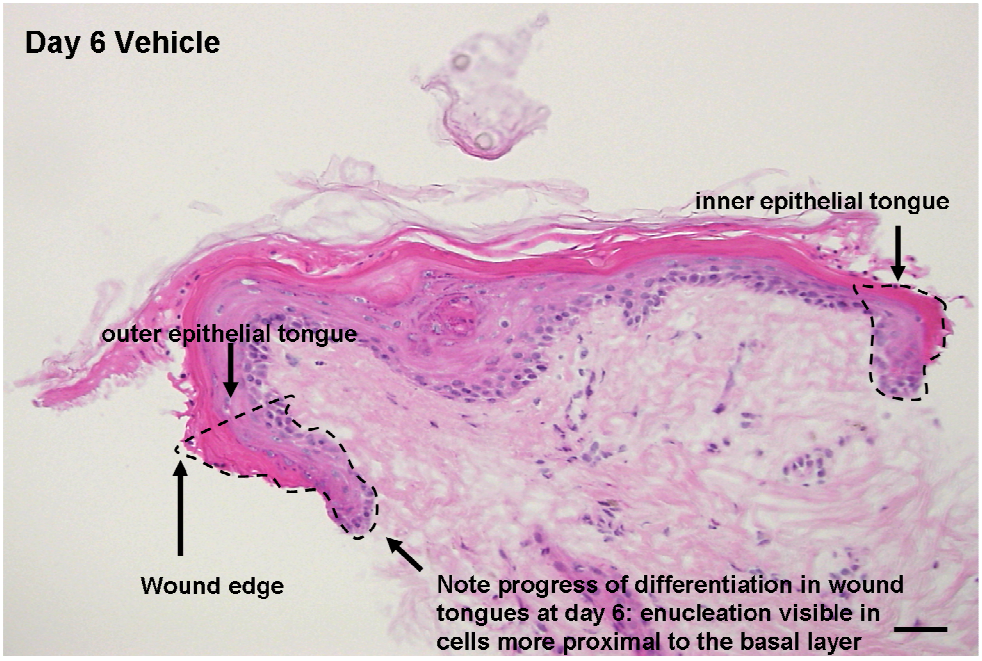

Figure 26. Representative picture of the inner and outer wound edge epithelial tongue
Scale bars=50µm.

3.2 Thyroxine (T4) promotes reepithelialization and angiogenesis in wounded human skin

Next, we asked whether T4, in concentrations that we had previously shown to profoundly modulate human HF growth in organ culture (van Beek et al., 2008) and that correspond to the T4 dose range that has previously been used in animal wound healing studies *in vivo* (Safer et al., 2005), stimulates reepithelialisation in organ-cultured wounded aging human skin.

3.2.1 T4 promotes human skin reepithelialization *in situ*

Compared to the vehicle control, T4 administration to the serum-free culture medium (William's E medium supplemented with insulin, hydrocortisone, and glutamine) significantly stimulated reepithelialization of human skin by day 3 after skin wounding (**Figure 27 a-d**). Thereafter (day 6), T4 further promoted ET elongation (which reflects keratinocyte migration) slightly, even though significance was not reached. Instead, the total ET area (i.e. the total mass of regenerated epidermis) did not get significantly increase in the T4-treated group while compared to vehicle-treated skin fragments (**Figure 27 c, d**).

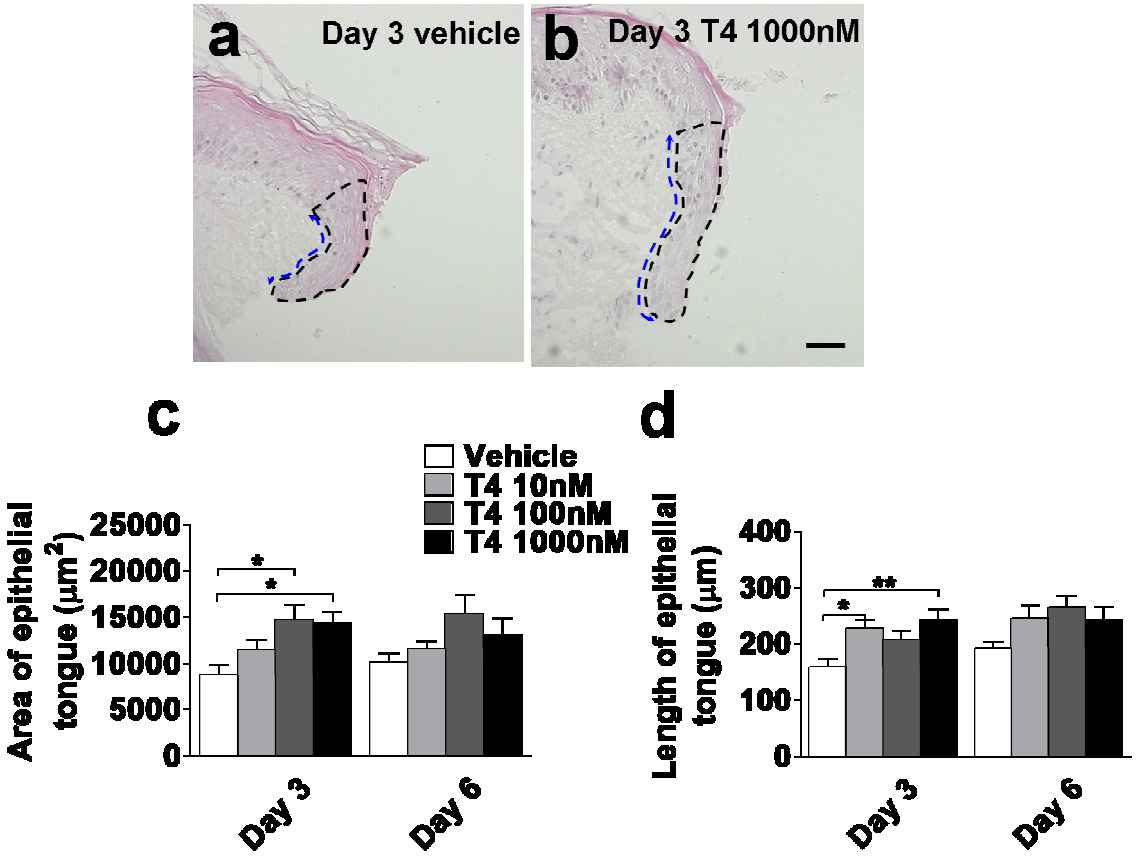

Figure 27. T4 promotes human skin reepithelialization *in situ*

(**a, b**) Haematoxylin and eosin histochemistry overview of wounded human skin fragment. After 3 days, the regenerated epithelial tongue (ET) areas (blacked dotted line) and length (blue dotted line) were significantly greater after treatment with T4 compared to vehicle alone (**a-d**). Note that ET length is taken as an indicator of keratinocyte migration during reepithelialization, while the ET area indicates the total mass of regenerated epithelium. Number of independent experiments: n=3-6 (i.e. 3-6 skin fragments derived from three distinct individuals were analyzed per test/control group). ET area and length were measured as indicated (see also **Figure 23**). Data were pooled, since the results trends from all three patients were highly comparable. One-Way ANOVA by appropriate post hoc comparisons was used and data are represented as Mean±SEM. *p<0.05; **p<0.01. Scale bars=50µm.

3.2.2 T4 promotes migration of wounded human keratinocytes *in situ*

It is well-recognized that efficient and well-controlled keratinocyte migration is a key factor during reepithelialization (Brown et al., 1991; Raja et al., 2007). The observed ET length-promoting effects of T4 (**Figure 27**) suggested that T4

stimulates keratinocyte migration. Therefore, we attempted to support this concept by assessing cortactin immunoreactivity (IR), a sensitive marker of migrating keratinocytes (Ceccarelli et al., 2007; Gendronneau et al., 2008). In fact, in a pilot experiment (1 patient), T4 up-regulated cortactin IR, demonstrating ectopic cortactin-positive domains in keratinocytes at the leading edge of the spreading ET (**Figure 28**). Together with the ET length data, this strongly suggests that, initially, T4 stimulates keratinocytes migration over the wound edge.

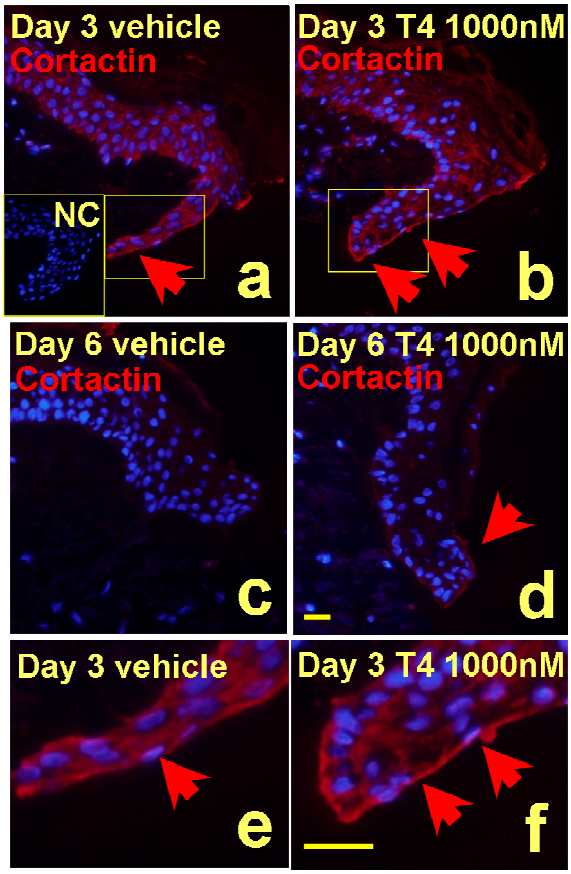

Figure 28. T4 promotes wounded human skin keratinocytes migration *in situ*
Immunofluorescence microscopy for anti-cortactin (red), counterstained with DAPI (blue) of the wound margins after 3 days (**a, b**) and 6 days (**c, d**) T4 treatment (Qualitative data from a single organ culture, one patient). Red large arrows indicate the ectopic cortactin-positive domains in keratinocytes at the leading edge (see also high-magnification: **e, f**). NC: negative control. Scale bars in **a-b** =50µm, **e and f** =100µm.

3.2.3 T4 promotes human skin epidermal keratinocyte proliferation and apoptosis *in situ*

To further assess the role of keratinocyte migration versus proliferation/apoptosis during ET formation, and the effects of T4 on these parameters, quantitative immunohistomorphometry of Ki67+ or terminal deoxynucleotidyl transferase dUTP nick end labeling-positive (TUNEL+) cells was performed. This showed a significant up-regulation of Ki67+ cells in the ETs of wounded skin fragments that had been treated with high dose T4 (100nM, 1000nM) (**Figure 29 a, b and c**) at day 3.

However, after this early epithelial repair phase, this stimulatory effect of T4 on keratinocyte proliferation in the ET tended to get lost (day 6). During the early repair phase, apoptosis in the ET was slightly, but not significantly up-regulated by high-dose T4 (**Figure 29 a, b and d**), possibly as a result of enhanced tissue remodeling during the 3rd phase of wound healing ("proliferation") (see **Figure 6**). These findings may reflect the recognized complexity of T4's effects on tissue remodeling events, namely on the balance between epithelial cell proliferation and differentiation (Kress et al., 2009), within the newly regenerated epithelium, and further support the concept that the stimulation of keratinocyte migration is an important component of the reepithelialization-promoting effects of T4.

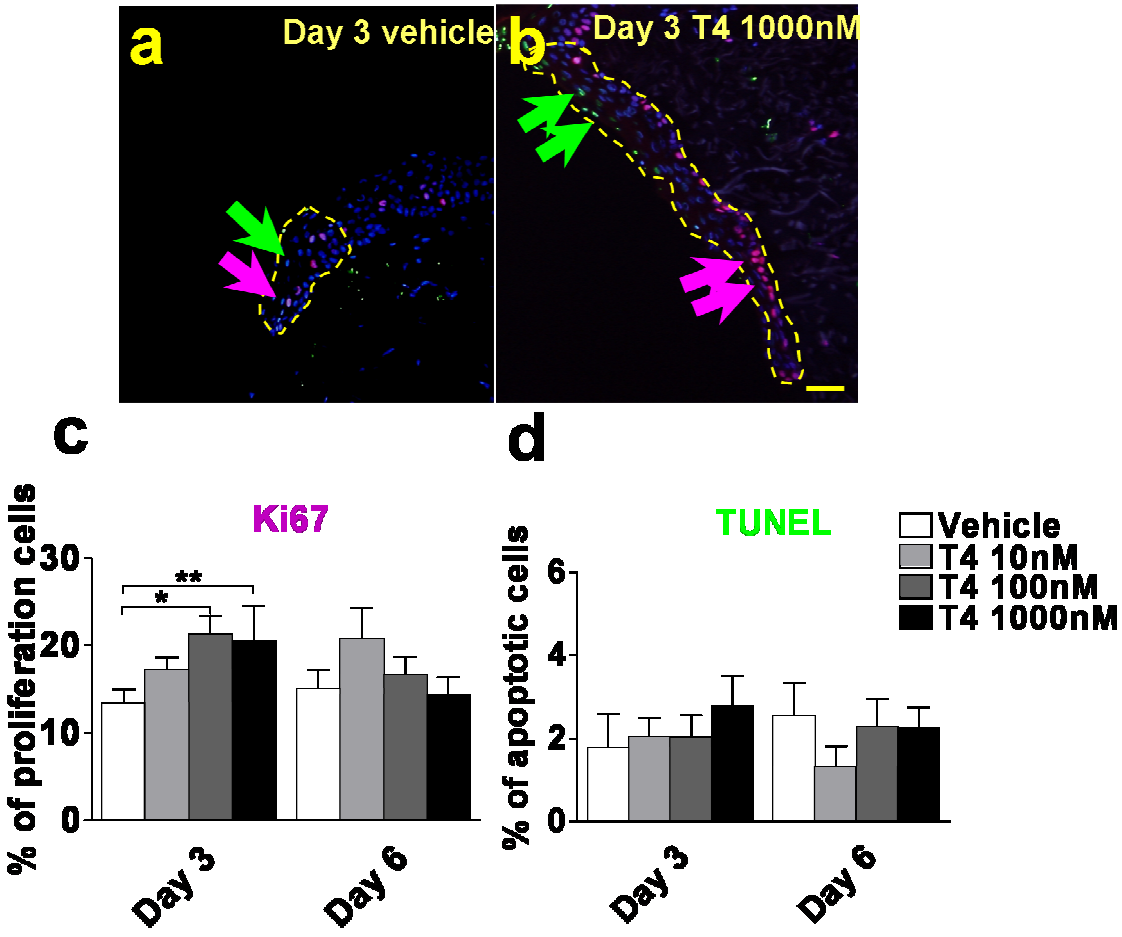

Figure 29. T4 stimulates human epidermal keratinocytes proliferation *in situ* after wounding
(**a-d**) Quantitative double-immunohistomorphometry for proliferating (Ki-67+, red arrows) and apoptotic (TUNEL+, green arrows) within the ET (dotted line: reference area=ET). Significantly more Ki-67+ cells were seen after 3 days of culture with 100nM or 1000nM T4 treatment, and after 6 days with 10 nM T4 (**c**). A tendency towards more TUNEL+ cells in the ET was seen after 3 days of T4 treatment (**d**). Number of independent experiments: n=3 (3-6 skin fragments derived from three distinct individuals were analyzed per test/control group; pooled data; one-way ANOVA; Mean±SEM). *p<0.05; **p<0.01. DAPI, 4'-6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; Scale bar=50µm.

3.2.4 T4 up-regulates expression of the wound healing-associated keratin, CK 6

Next, we assessed whether T4 also impacts on the major wound healing-related keratin, CK6 (Ramot et al., 2009a; Ramot et al., 2009b; Windoffer et al., 2011; Rotty

and Coulombe, 2012). CK6 is expressed in human epidermis primarily upon wounding and under conditions of hyperproliferation, while scattered foci of CK6 expression can be detected also on normal human epidermis (Ramot et al., 2009a). CK6 deletion even functionally impacts on murine skin wound healing *in vivo* (Wojcik et al., 2000) and on murine keratinocyte migration *in vitro* (scratch assay) (Rotty and Coulombe, 2012). Moreover, the promoter region of the CK6 gene carries a thyroid hormone-response element (TRE) (Radoja et al., 1997; Wojcik et al., 2000), and topical T3 reportedly increases CK6 protein expression in murine HFs *in vivo* (Safer et al., 2005). CK6 IR in the ET was assessed by quantitative immunohistophometry.

As shown in (**Figure 30a and 30b**), T4 significantly increased CK 6 expression in the newly formed ET of wounded human skin. Interestingly, the peak of T4-stimulated CK6 IR occurred several days after the proliferation peak (**Figure 30**, compare with **Figure 29**). Note that even in wounded control skin, epidermal CK6 expression was strong and widespread.

These findings provide the first evidence that T4 stimulates CK6 protein expression in human epidermis *in situ*, are in line with previous reports that THs stimulate CK6 expression (Radoja et al., 1997; Safer et al., 2005), and confirm that CK6 is a sensitive, proliferation-independent, marker protein for regenerating human skin epithelium.

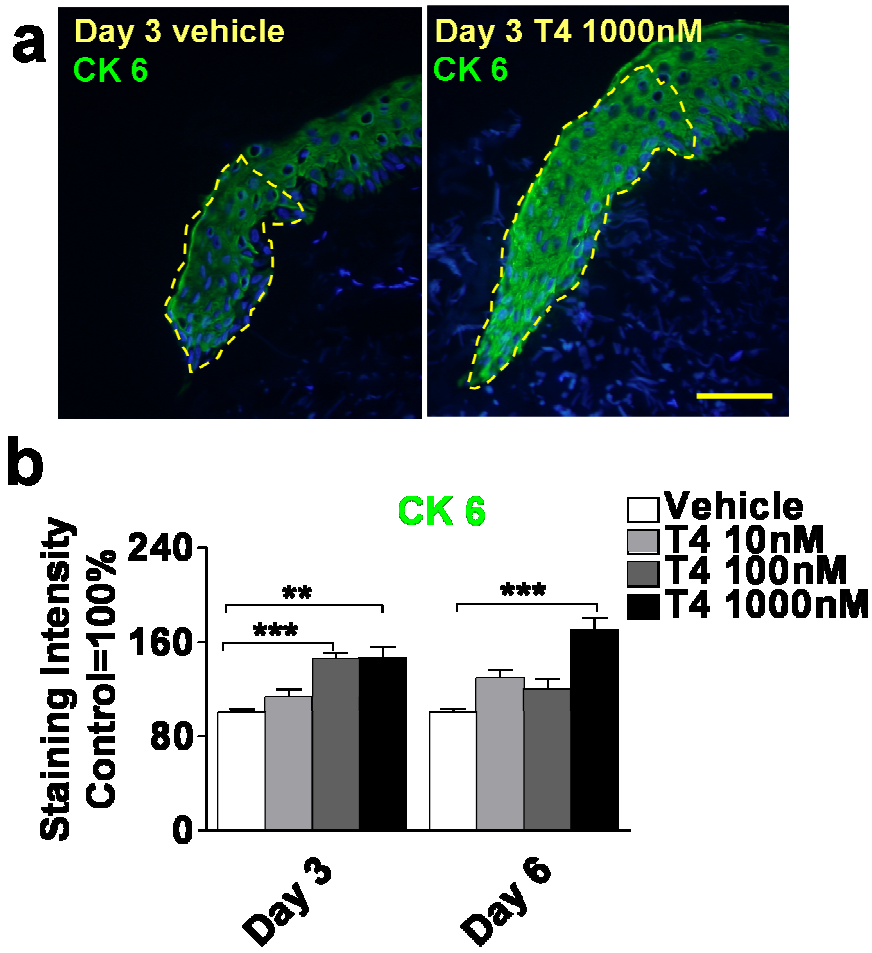

Figure 30. T4 increases expression of the wound healing-associated keratin, CK 6
Cytokeratin 6 (CK6) protein expression within the newly regenerated ET was significantly upregulated by high-dose T4 treatment compared to vehicle. Staining intensity was measured in dotted line area and normalized vehicle as 100%. Number of independent experiments: n=3 (3-6 skin fragments derived from three distinct individuals were analyzed per test/control group; pooled data; one-way ANOVA; Mean±SEM). ***p*<0.01; ****p*<0.001. CK: Cytokeratin; Scale bars=50µm.

3.2.5 T4 up-regulates expression of the epithelial stem cell-associated keratin, CK15

Successful epithelial repair in the skin depends on the availability and functionality of epithelial progenitor cells from which more differentiated epithelium can be regenerated (see Introduction: **1.3.6.1**). Therefore, we next assessed whether T4 also stimulated the *in situ*-protein expression of CK15, which demarcates both epithelial stem cells and their immediate progeny (transit amplifying cells), mainly in

the HF, but also in the epidermis, namely under wound healing conditions (Ito et al., 2005; Cotsarelis, 2006; Tiede et al., 2007; Tiede et al., 2009b). This was further encouraged by the prior demonstration that THs stimulate CK15 expression in human HF epithelial stem cells *in situ* and *in vitro* (Tiede et al., 2010).

CK15 IR in the new formed ET was analyzed by quantitative immunohistomorphometry. As shown in **Figure 31**, this showed that CK15 IR in the basal layer of the ET was significantly higher than in the control group at day 3. This striking stimulatory effect was lost by day 6 (**Figure 31**).This finding provides the first evidence that T4 stimulates epithelial stem/progenitor cells in human wounded skin *in situ*, and is in line with our previous report that T4 also enhances CK15 gene and protein expression in organ-cultured human HFs and in isolated HF epithelial progenitor cells (Tiede et al., 2010).

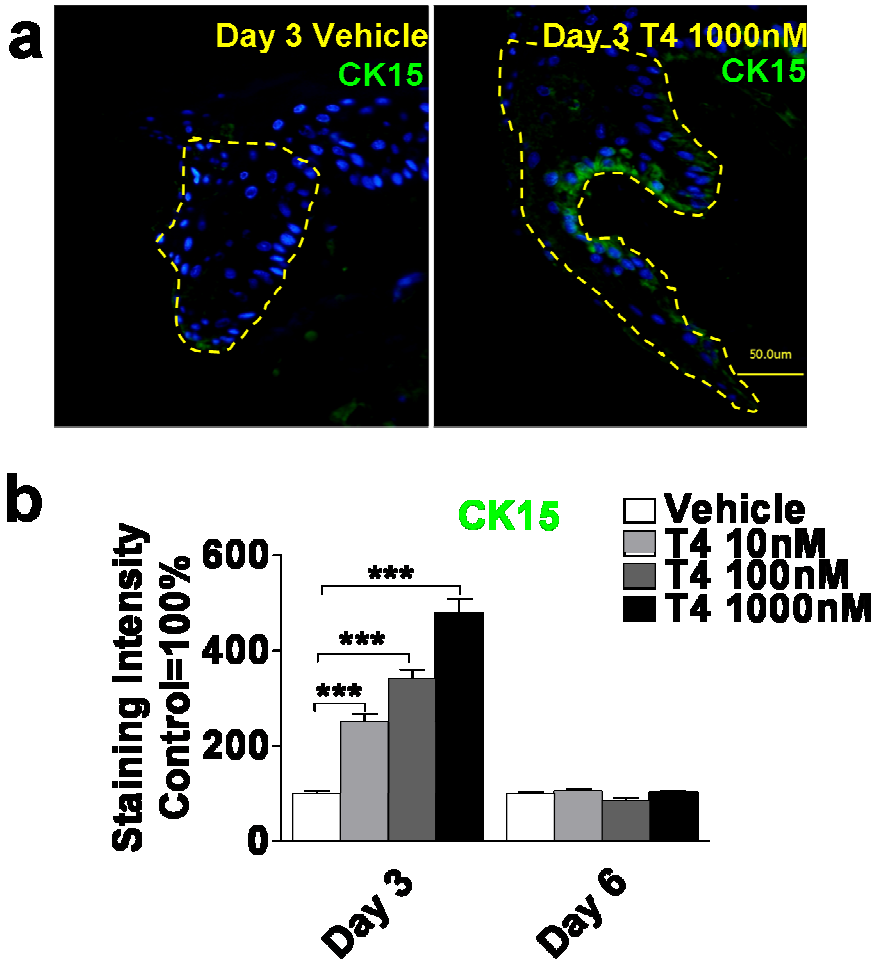

Figure 31. T4 up-regulates expression of epithelial stem cell-associated keratin, CK15
CK15 expression is significantly up-regulated by T4. Green fluorescence staining represents Keratin 15 immunoreactivity in the new wound tongue. Staining intensity was measured in dotted line area and normalized vehicle as 100%. Number of independent experiments: n=3 (3-6 skin fragments derived from three distinct individuals were analyzed per test/control group; pooled data; one-way ANOVA; Mean±SEM). ****p*<0.001. CK: Cytokeratin; Scale bars=50µm.

3.2.6 T4 stimulates energy metabolism during human skin reepithelialisation

Since epithelial regeneration is a highly energy-consuming process (Li et al., 2004; Almaca et al., 2009; Clerici and Planes, 2009) and since THs are the best-studied endocrine stimulators of mitochondrial activity (Harper and Seifert, 2008), we next asked whether T4 affects the intraepithelial protein expression of mitochondrial cytochrome c oxidase subunit I (MTCO1), a mitochondria-specific key enzyme in

the electron transport chain (Hebert et al., 2003; Clerici and Planes, 2009). Previously, we had shown that, in organ-cultured human skin, epidermal MTCO1 IR is an excellent mitochondrial biology screening marker, since it correlates well with mitochondrial cytochrome c oxidase activity and MTCO1 transcription, and even points towards mitochondrial biogenesis (Poeggeler et al., 2010; Knuever et al., 2012). Again, IR was assessed by quantitative immunohistomorphometry.

Indeed, T4 significantly up-regulated MTCO1 IR in the new ET after wounding. This effect was most pronounced on day 3, but still present on day 6 (**Figure 32a and 32b**). This represents the first documentation of such an effect in human skin, and fits well with the recognized stimulation of mitochondrial function by THs (Harper and Seifert, 2008). Since increased MTCO1 IR in human epidermis is an excellent *in situ*-indicator of enhanced mitochondrial energy metabolism (Poeggeler et al., 2010; Knuever et al., 2012), this finding invites the hypothesis that T4 also stimulates epidermal energy metabolism during wound healing.

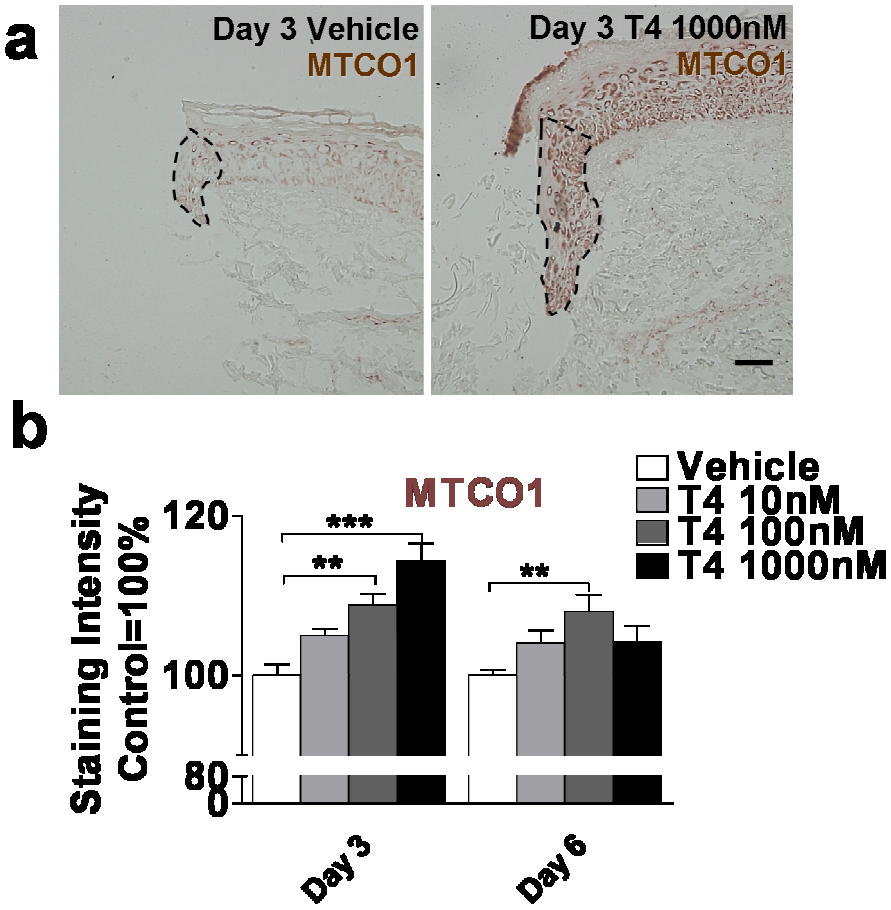

Figure 32. MTCO1 expression is significantly up-regulated by T4

Brown staining represents MTCO1 immunoreactivity in the regenerated ET. Note that MTCO1 immunoreactivity in the non-wounded epidermis was also up-regulated by T4 (not quantified). Staining intensity was measured in dotted line area and normalized vehicle as 100%. Number of independent experiments: n=3 (3-6 skin fragments derived from three distinct individuals were analyzed per test/control group; pooled data; one-way ANOVA; Mean±SEM). **p<0.01; ***p<0.001. MTCO1: Mitochondrial cytochrome c. Scale bars=50µm.

3.2.7 T4 stimulates intracutaneous angiogenesis in wounded human skin

Since angiogenesis is a critical determinant of cutaneous wound healing (Ahluwalia and Tarnawski, 2012; Park et al., 2012; Roy and Sen, 2012; Dipietro, 2013), we also asked whether angiogenesis can be studied at all in the current organ culture assay, despite the fact that skin perfusion is abrogated under assay conditions, with the likely consequence of blood vessel collapse and rapid degeneration of the skin

microvasculature. This was studied by quantitative CD31 immunoreactivity, with emphasis on the measurement of CD31+ vessel lumina (an indicator of microvessel density [MVD]), since this provides an optimal *in situ* marker for angiogenesis (Mecklenburg et al., 2000). This showed that, even after 6 days of organ culture, prominent CD31 IR (**Figure 33**) and even (very few) CD31+ blood vessel cross-sections with a lumen can (**Figure 34**) still be detected, both in control and test wounded human skin *in vitro*. Therefore, this simple preclinical wound healing assay is well-suited to study angiogenesis *in situ*.

Next, we asked whether T4 treatment impacts on CD31 protein expression and/or angiogenesis in wounded human skin, since T4 is a recognized pro-angiogenic hormone (Mousa et al., 2005; Mousa et al., 2006; Davis et al., 2009; Pinto et al., 2011; Chen et al., 2012a; Chen and Thibeault, 2012). This showed that T4 significantly increased angiogenesis (**Figure 33-34**): Both the total CD31 immunoreactivity in defined reference areas (**Figure 33a, 33b and 33c**) and the number of CD31+ positive endothelial cells (**Figure 33a, 33b and 33d**) were significantly up-regulated in T4-treated wounded skin fragments. Most importantly, T4 (100 nM) also significantly increased the microvessel density compared to vehicle control, measured as number of cross-sections of CD31+ vessel lumina (Mecklenburg et al., 2000) (**Figure 34**).

This suggest that T4 enhances angiogenesis in wounded human skin *in vitro*, even in the absence of pro-angiogenic serum components and functional blood vessel perfusion. Thus, T4 promotes human skin wound healing at both the epithelial regeneration and angiogenesis level.

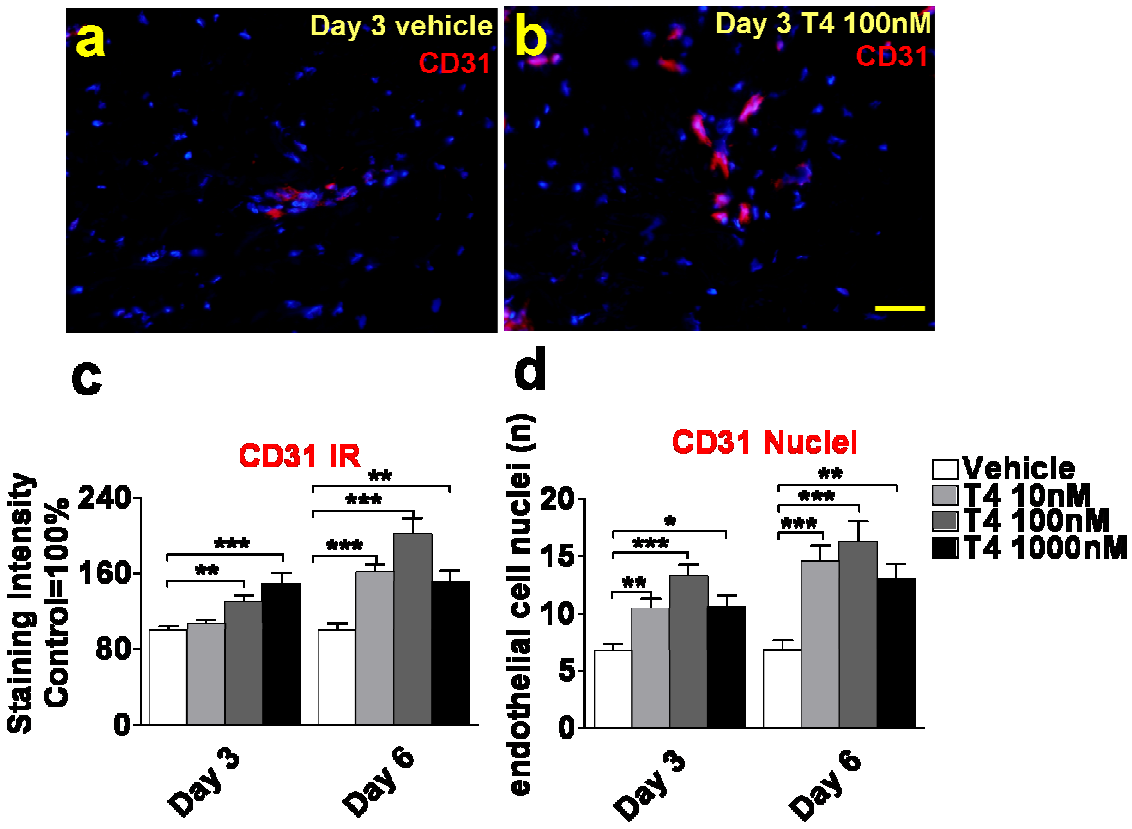

Figure 33. T4 stimulates angiogenesis in wounded human skin *in situ*

(**a-b**) To analyze angiogenesis of the wound, the number of CD31+ cells (red, **a**), total CD31 immunoreactivity under the ET (**a**), per visual field were counted in defined reference areas (see also Materials & Methods: **Figure 24**) by quantitative immunohistomorphometry. (**c**) General CD31 immunoreactivity was significantly up-regulated by T4 at days 3 and 6. Staining intensity was measured in dotted line area and normalized to vehicle control results (=100%). (**d**) T4 also increased the number of (CD31+/DAPI+) endothelial cell nuclei. Number of independent experiments: n=3 (3-6 skin fragments derived from three distinct individuals were analyzed per test/control group; pooled data; one-way ANOVA; Mean±SEM).*p<0.05; **p<0.01; ***p<0.001. IR: Immunoreactivity. Scale bars = 50µm.

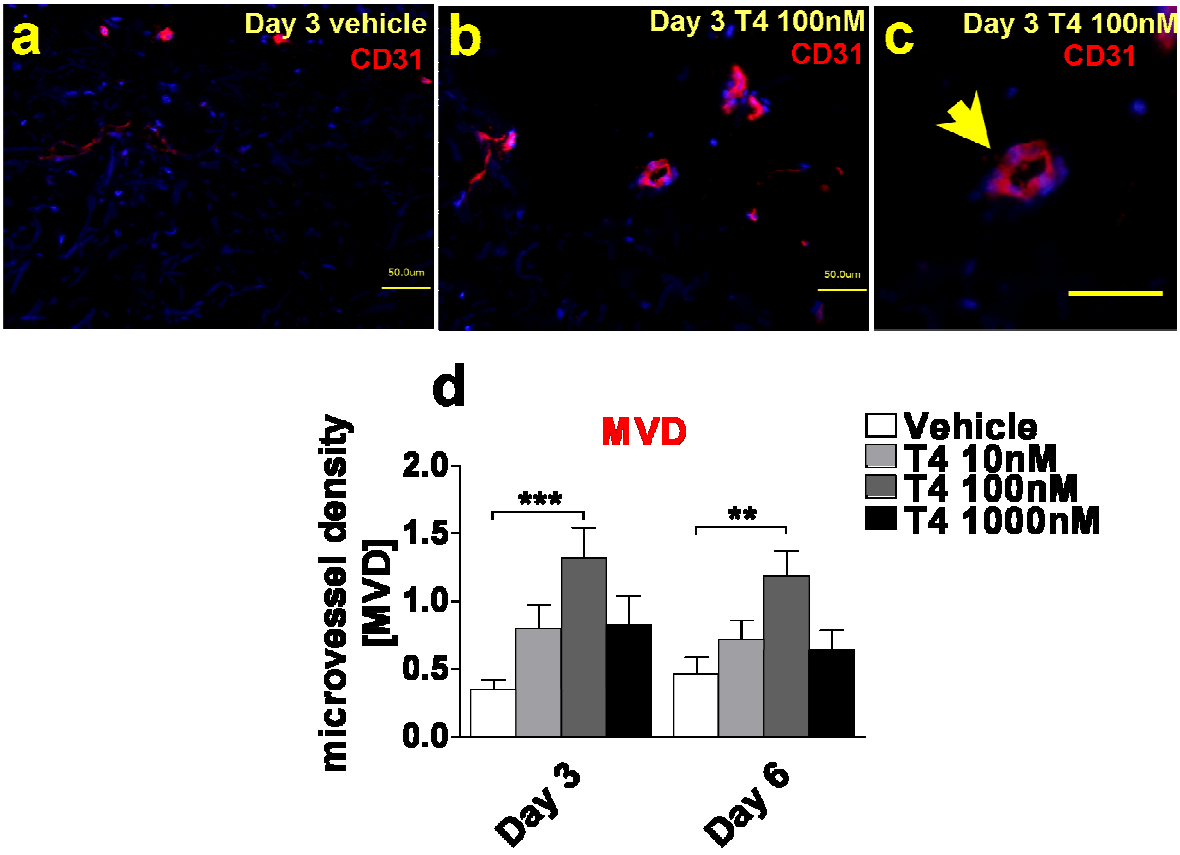

Figure 34. T4 stimulates microvessel density in wounded human skin *in situ*

To analyze angiogenesis of the wound, the number of CD31+ blood vessel cross-sections (lumina) (yellow arrow, **c**) per visual field was counted by quantitative immunohistomorphometry. Number of independent experiments: n=3 (3-6 skin fragments derived from three distinct individuals were analyzed per test/control group; pooled data; one-way ANOVA; Mean±SEM). ***p*<0.01; ****p*<0.001. MVD: Microvessel density (=number of CD31+ blood vessel cross sections with visible central lumen (see also Materials & Methods: **Figure 24**). Scale bars in **a, b** =50µm, **c**=200µm.

3.2.8 T4 up-regulates bFGF and FGFR1 expression

To obtain first indications as to a potential mechanism of action for the pro-angiogenic activity of T4 in our assay, the protein expression of bFGF and its receptor, FGFR1, in the newly formed ET was investigated since bFGF is upregulated by T4 (Davis et al., 2004) and since increased bFGF secretion is a key pro-angiogenic factor (Cao et al., 2011) (VEGF was not further examined due to problems with IF/IHC specificity). FGFR1 IR was assessed, since the

proangiogenic actions of T4 may be mediated at least in part via up regulating FGFR1 expression (Luidens et al., 2009).

Quantitative immunohistomorphometry revealed that high-dose T4 significantly increased bFGF (**Figure 35**) immunoreactivity in the ETs, as measured by quantitative immunohistomorphometry.

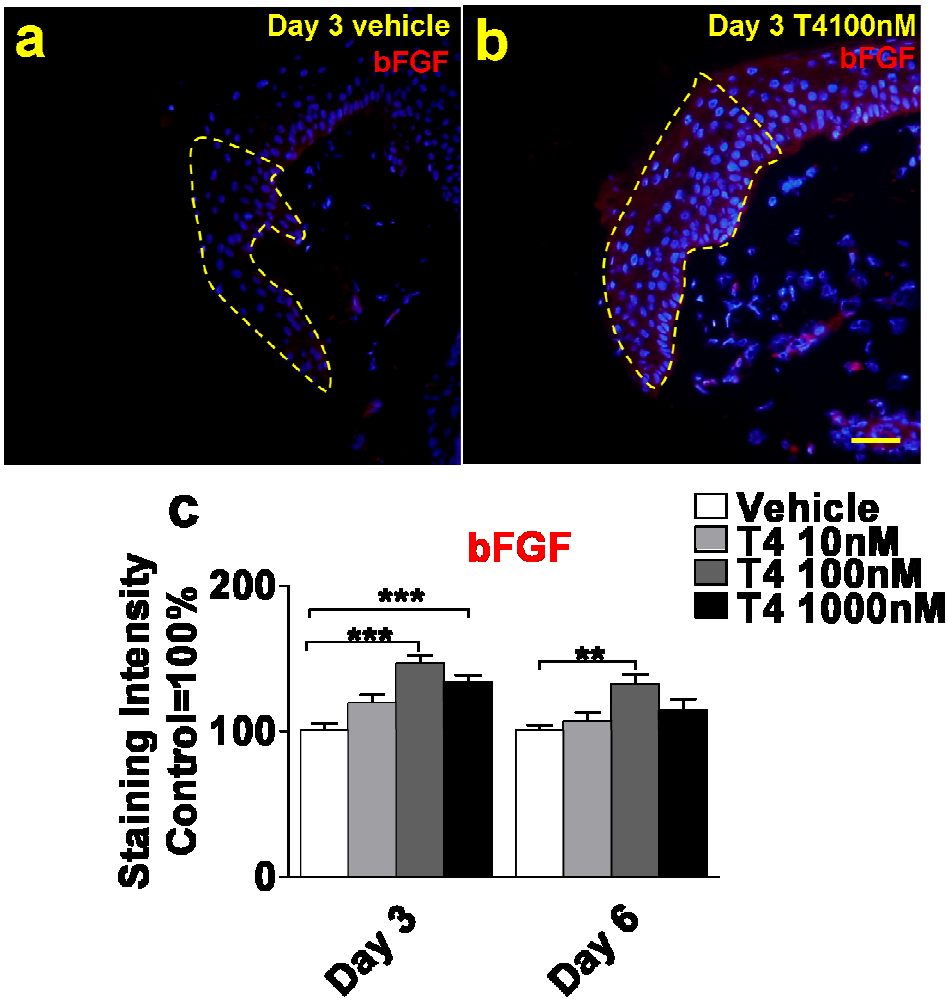

Figure 35. T4 stimulates bFGF immunoreactivity in wounded human skin *in situ*

bFGF expression (red fluorescence) were significantly up-regulated by T4 in the regenerated ET (staining intensity was measured in the indicated reference area, dotted line) at day 3 and day 6 after high-dose T4 treatment. Staining intensity was measured in dotted line area and normalized vehicle as 100%. Number of independent experiments: n=3 (3-6 skin fragments derived from three distinct individuals were analyzed per test/control group; pooled data; one-way ANOVA; Mean±SEM). ***p*<0.01; ****p*<0.001. bFGF: Basic fibroblast growth factor. Scale bars=50µm.

As shown in **Figure 36**, FGFR1 immunoreactivity in ETs was also significantly increased by T4 (100 nM, 1000 nM) compared to vehicle. This supports the hypothesis that bFGF/FGFR1-mediated signaling may underlie at least some of the pro-angiogenic effects of T4 in organ-cultured human skin.

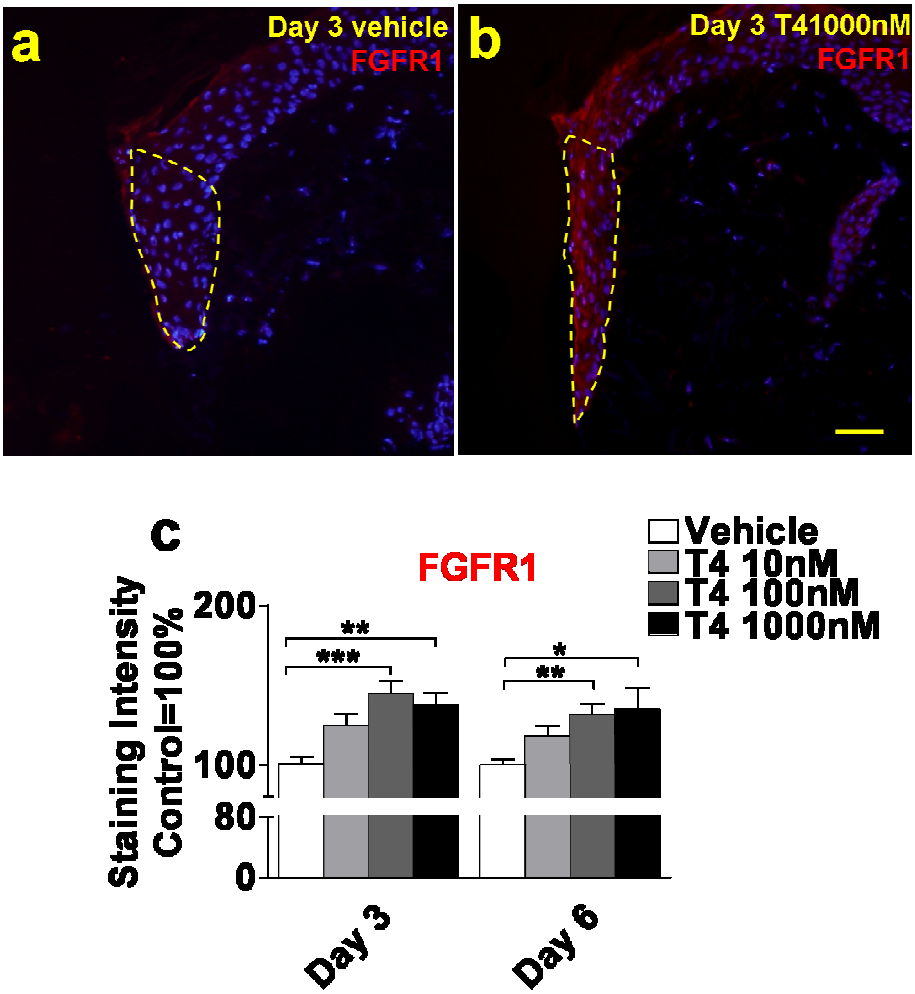

Figure 36. T4 stimulates FGF receptor 1 immunoreactivity in wounded human skin *in situ*
FGFR1 expression (red fluorescence) were significantly up-regulated by T4 in the regenerated ET (staining intensity was measured in the indicated reference area, dotted line) at day 3 and day 6 after high-dose T4 treatment. Staining intensity was measured in dotted line area and normalized vehicle as 100%. Number of independent experiments: n=3 (3-6 skin fragments derived from three distinct individuals were analyzed per test/control group; pooled data; one-way ANOVA; Mean±SEM). *p<0.05; **p<0.01; ***p<0.001. FGFR1: Fibroblast growth factor receptor 1. Scale bar=50µm.

3.2.9 T4-induced angiogenesis and re-epithelisation effects are, at least in part, bFGF-dependent

To probe this hypothesis, bFGF-neutralizing antibody (8 µg/ml) (Davis et al., 2004) was co-administered with T4 for 3 days. Importantly, this counteracted the stimulatory effects of T4 on all three angiogenesis parameters: CD31 protein immunoreactivity (**Figure 37a**), the number of CD31+ endothelial cells (**Figure 37b**), and–most importantly–on microvessel density (**Figure 37c**). bFGF expression was also reduced by administration of this inhibitory antibody (**Figure 37d**). Unexpectedly, bFGF-neutralizing antibody almost abolished the stimulatory effects of T4 on human skin reepithelialization (**Figure 37e and 37f**), suggesting that even the T4-induced promotion of reepithelialization depends on bFGF/FGFR1-mediated signaling.

Taken together, this suggests that the wound healing-promoting effects of T4 in organ-cultured human skin, namely its promotion of epidermal regeneration and dermal angiogenesis in organ culture depend, at least in part, on bFGF/FGFR1-mediated signaling (Time constraints prevented to also study the role of VEGF and PDGF in the current thesis project).

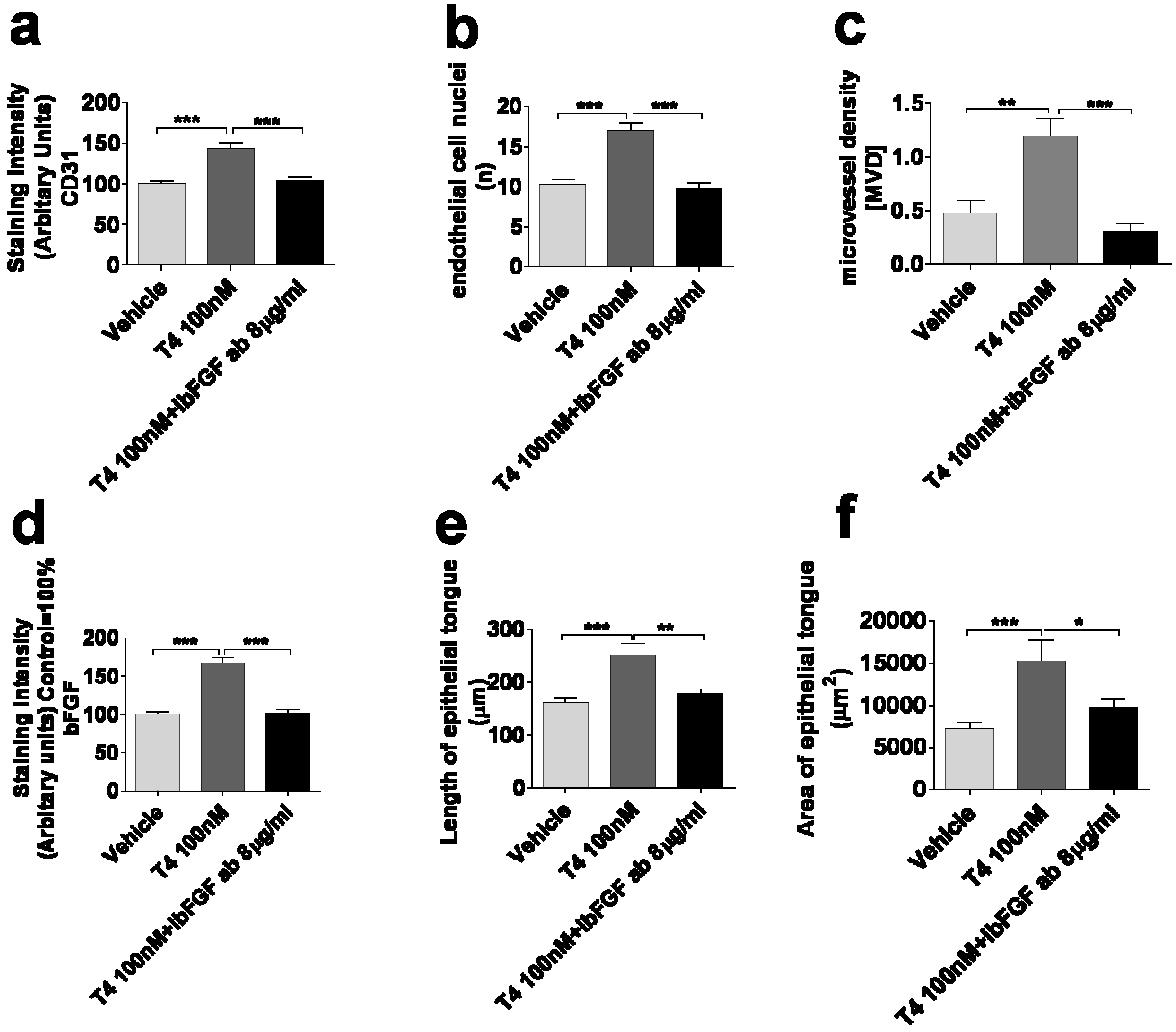

Figure 37. T4-induced angiogenesis and re-epithelisation effects are, at least in part, bFGF-dependent

Intensity of the immunoreactivity of CD31 (**a**), number of CD31+ cells (**b**), number of CD31+ lumina (**c**), and intensity of bFGF immunoreactivity in the regenerated ET (**d**), area and length of epithelial tongue in new ET (**e, f**), were all significantly enhanced under T4 stimulation compared to the control groups. Co-treatment of T4 (100 nM) with inhibitory bFGF antibody (8 µg/ml) for 3 days largely abolished these T4 effects (**a-f**). Number of independent experiments: n=3 (in total, 6 skin fragments derived from three distinct individuals were analyzed per test/control group; pooled data; one-way ANOVA; Mean±SEM).*p<0.05; **p<0.01; ***p<0.001. DAPI, 4'-6-diamidino-2-phenylindole; MVD: Microvessel density; ibFGF ab: Inhibitory bFGF antibody.

3.2.10 T4 increases the number of interfollicular MCs, and induces their activation and increase the C-Kit positive MCs

Given the importance of mast cells (MCs) in wound healing and angiogenesis (Weller et al., 2006; Ammendola et al., 2012; de Souza et al., 2012; Jung et al.,

2012) and that histamine promotes bFGF-induced experimental angiogenesis (Lu et al., 2012b), we also studied the impact of T4 on histochemically detectable, mature mast cells in human skin as well as on c-Kit+ intradermal cells (which primarily demarcates both immature mast cell progenitors and differentiated mast cells (Ito et al., 2010; Sugawara et al., 2012).

Interestingly, T4 (100 nM) significantly increased the number of both histochemically detectable, toluidine blue+ **(Figure 38)** and c-Kit+ human skin MCs *in situ* (**Figure 39**). T4 also stimulated mast cell degranulation (**Figure 38b**). One pilot assay in which by Leder esterase staining was used for mast cell histochemistry independently confirmed these results (**Figure 40**).

This raises the question whether T4 may stimulate not only mast cell activation, but also mast cell proliferation and/or maturation from resident progenitor cells in human skin (note the T4-induced increase in the number of c-Kit+ cells: **Figure 36**), just as we have previously shown for corticotropin-releasing hormone (CRH) (Ito et al., 2010) and cannabinoid receptor 1 (CB1) antagonists (Sugawara et al., 2012).

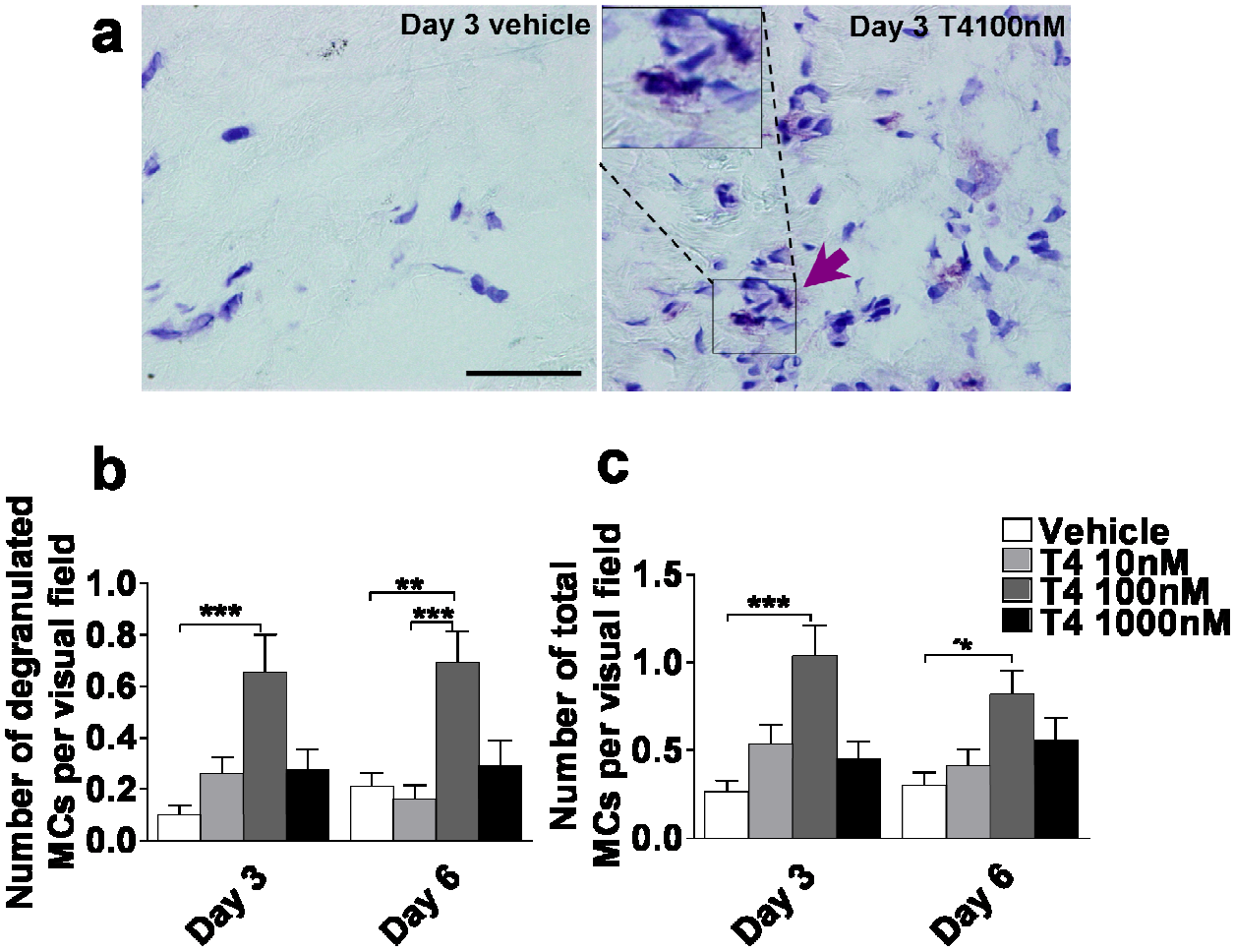

Figure 38. T4 treatment increases the number of mast cells and stimulates their degranulation

(**a**) Intradermal MCs under the ET were visualized by toluidine blue histochemistry. The number of degranulated (red arrow) (**b**), total MCs(**c**) per visual field were counted after culture for 3 and 6 days. In total, 6 skin fragments derived from three distinct individuals were analyzed per test/control group; pooled data; one-way ANOVA; Mean±SEM) . *p<0.05; **p<0.01; ***p<0.001. Scale bars=50µm.

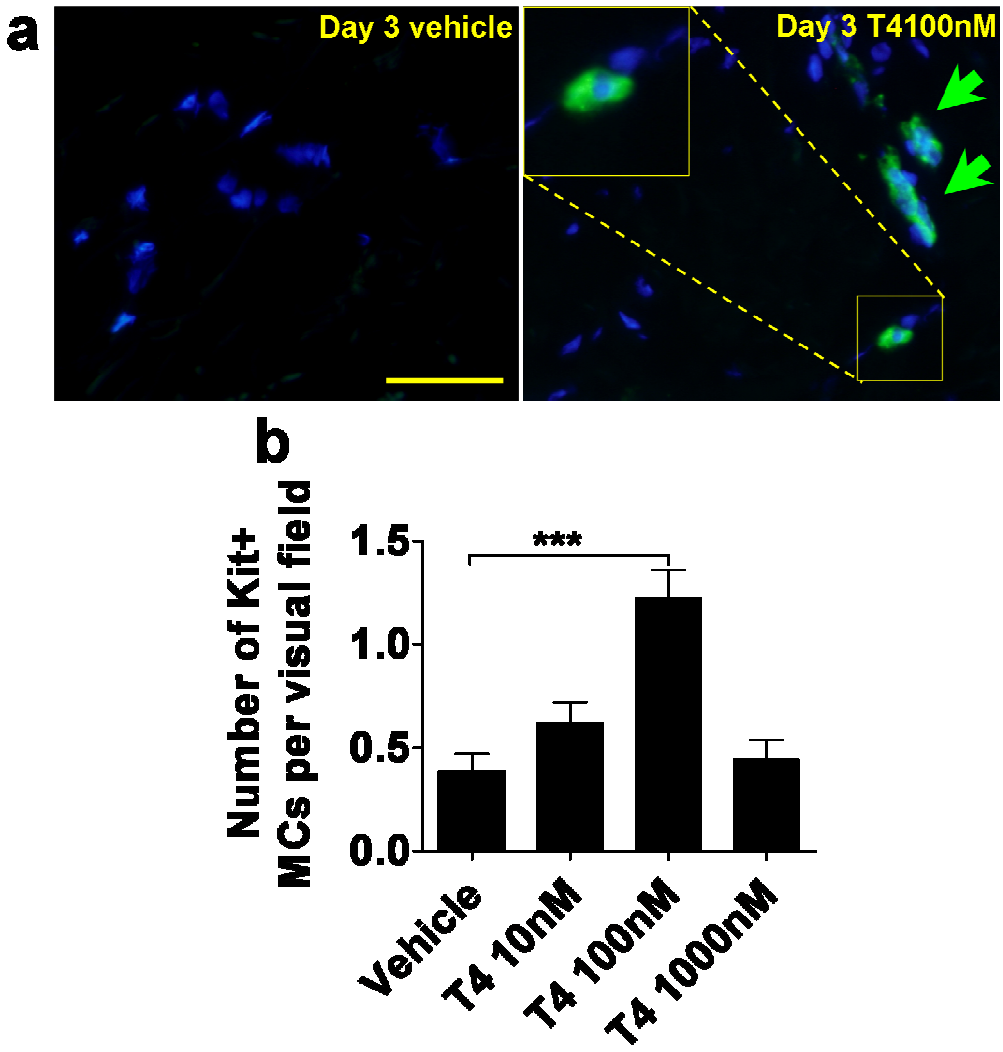

Figure 39. T4 treatment increases C-kit+ mast cells

(**a**) Intradermal MCs under the ET were visualized by c-Kit immunofluoresence (green arrow). The number of c-Kit+ cells (**b**) per visual field was counted after 3 days. In total, 6 skin fragments derived from three distinct individuals were analyzed per test/control group; pooled data; one-way ANOVA; Mean±SEM). ***p<0.01; Scale bars=50µm.

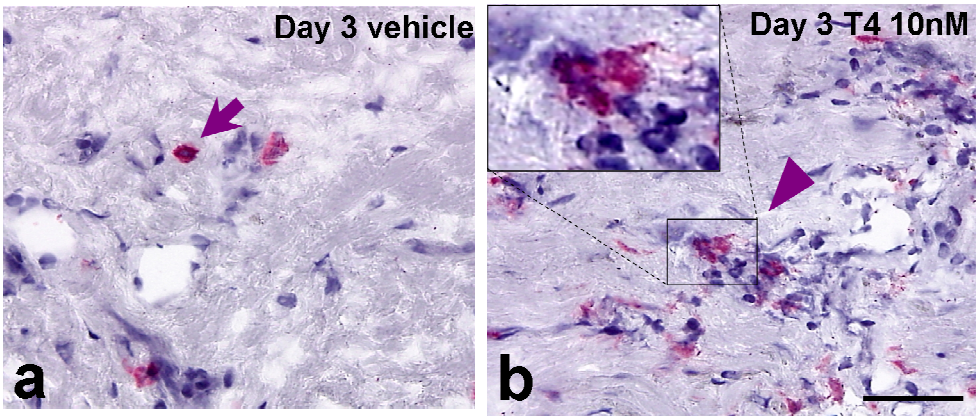

Figure 40. T4 stimulates mast cell degranulation by Leder esterase staining
One whole pilot culture was performed and intradermal mast cells under the epithelial tongue were visualized by Leder esterase. Representative picture shown in day 3 vehicle (**a**) and day 3 T 10 nM (**b**). 'Degranulated' (arrowhead) and 'nondegranulated' (arrow) MCs were detected by Leder esterase histochemistry. Scale bars=50µm.

4. Discussion

This thesis project reports the successful development of a human skin wound healing *in vitro*-assay that provides a unique tool to examine long-standing questions and molecular mechanism underlying wound healing. Following in the footsteps of Safer et al (Safer et al., 2003; Safer et al., 2004, 2005), who had demonstrated that the topical application of T4 accelerated wound healing in mice, the first evidence is provided that T4 exerts wound healing-promoting effects also in human skin wound healing *in vitro.* Namely, T4 promotes both reepithelialization, and angiogenesis, up-regulates the protein expression *in situ* of two key keratins in wound healing (CK6, CK15), stimulates both keratinocyte migration and epithelial progenitor cells (i.e. increased CK15 IR), and up-regulates the number and activity of dermal mast cells in organ-cultured, wounded human skin. Given its immediate availability for clinical application and testing, this makes T4 a highly interesting, inexpensive, and toxicologically well-defined novel candidate wound healing promoter in the future management of human skin ulcers.

This current study also provides novel insights into the possible mechanisms of the endocrine regulation of human skin wound healing by showing that bFGF/FGFR-mediated signaling plays an important role in the wound healing-promoting properties of T4. However, the mechanism by which T4 stimulates reepithelialization and angiogenesis remains largely unknown. Yet, the initial pointers to novel T4 target candidate genes in human skin that were obtained in the current study provide a valuable basis for mechanistic follow-up studies.

In the current study, sample donors were included only just based on normal euthyroid status in order to avoid skin changes by altering thyroid hormone status. Since our available ethics permit did not allow for the collection of patient details beyond age, gender and skin location (e.g., euthyroid versus hypothyroid status,

presence of thyroid-peroxidase (TPO) or TSHR autoantibodies), one limitation of the current study is that we cannot correlate the responses seen in the skin fragments from the three investigated individuals with the thyroid status of the donors.

However, it should be noted that serum-free organ culture of human skin always occurred under severely “hypothyroid” assay conditions, i.e. only the culture medium of test groups contained T4, while control groups contained only those traces of intracutaneous THs that the tissue may have retained after surgery. Therefore, it is reasonable to propose that the wound healing-promoting properties of T4 seen in the current assays system most accurately reflect the response of hypothyroid wounded human skin to T4 administration. Viewed from this perspective, the stimulatory effects of T4 fit perfectly to the well-recognized phenomenon of generally retarded wound healing in hypothyroid patients (Talmi et al., 1989; Feitosa Dda et al., 2008; Jaul, 2010) and rodents *in vivo* (Ozawa et al., 2003; Ekmektzoglou and Zografos, 2006; Tha Nassif et al., 2009; Zimmermann et al., 2009). Generally, at least 3 different individual’s antigens normally were checked to minimize inter individual difference.

Another potential limiting factor of the current study is that skin sample transport and processing times after surgery varied substantially. Though all skin samples were continuously kept in William’s E medium at 4 °C from surgical removal until the onset of organ culture, and even though the time between surgery and organ culture never exceeded 24 hours, different degrees of tissue damage suffered before, during, and after transport as well as difference in the time from surgery to organ culture set-up could account for substantial interindividual variations in the response of human skin to T4 stimulation. However, as suggested by the relatively low SEM values, even though data from all three patients were pooled, and by the

fact that the results trends seen in all three patients were nicely comparable, the response of wounded human skin to T4 stimulations is consistent and fairly robust to the potential variations mentioned above.

Besides maintaining a stable temperature, it is critical to also maintain the correct pH during tissue transport and dissection for organ culture to preserve and standardize the quality of human skin, since e.g. even small, prolonged milieu deviations into the alkaline pH range during tissue preparations can severely damage human skin vitality (Paus Lab, unpublished observations). Though the colour indicator present in William's E medium (phenol red) provides pragmatic guidance in this respect, in the future, a sprayable luminescent pH imaging technology that has been recently been developed for use on human skin *in vivo* (Schreml et al., 2012), might perhaps be employed before human skin samples are set-up for organ culture so as to further standardize and refine this wound healing organ culture assay.

Two critical questions are whether the T4 concentration range we tested was well-chosen, and whether we should also have tested T3. Given the painfully limited availability of human skin for organ culture, we were forced to make educated choices. Here, we selected the range of 10nM-1000nM T4, since range included one order of magnitude above and below the physiological T4 serum concentration (100 nM) (Lin et al., 1999). Instead, the typical range of T4 serum concentration in hypothyroid patients is 4 nM to 30 nM (Avalos et al., 1986), while hyperthyroid patients show T4 serum levels between 181 nM and 321 nM (Avalos et al., 1986). Also, the above dose range was best comparable to the ones previously explored in two human HF organ culture studies (Billoni et al., 2000), which had demonstrated prominent responses of human skin tissue to these doses *in vitro*. The supraphysiological test dose (1000 nM) was selected in order to

guarantee ample T4 uptake into (non-perfused!) human skin punches and sufficient intracutaneous enzymatic conversion into T3 (see Introduction: **1.8**).

In fact, with most of the assessed read-out parameters, the obtained results, suggest that the above dose range was well-chosen since all tested doses tended to elicit a response compared to vehicle controls, and since a tendency towards “dose-dependent” effects of T4 was evident. However, a full dose-response study would, ideally, have required the testing of 5 different concentrations (this was impossible due to insufficient tissue availability) so that no stringent claims on the “dose-dependency” of the observed T4 effects on the investigated wound healing read-out parameters can be made. However, the current study facilitates the design of follow-up studies with an extended dose range that is informed by the current results.

The quantitatively predominant endogenous TH in the blood is T4, while the biologically most active form (T3) is converted from T4 within cells by deiodinases (Slominski et al., 2002). Deiodinase gene expression in human skin biopsies and in the majority of human epidermal and dermal cells *in vitro* has been demonstrated, and human HFs were shown to transcribe deiodinase genes (D2, D3) (Slominski et al., 2002; van Beek et al., 2008). Furthermore, after treatment of human HFs with T4, a significant increase in the fT3 level was measured by electrochemiluminescent immunoassay in the supernatant, compared to vehicle-treated control HFs (van Beek et al., 2008). Thus, there is no doubt that human skin tissue can convert T4 to T3 so that it is sufficient to test T4.

However, it needs to be emphasized that even non-converted T4 may exert biological effects on its own, e.g. via quick-acting membrane mediated signalling (see **Figure 20**) (Cheng et al., 2010; Boelen et al., 2012). In some read-out

parameters, e.g. hair follicle proliferation and cytokeratin 6, T4 (10nM-1000nM) actually exerted greater modulatory effects than T3 (10^{-3} nM-10nM) (van Beek, 2009). Also, unexpectedly, in preliminary screening experiments not reported in the current thesis, we had noted that only 10-1000 nM T4, but not 10^{-3} nM to 10nM of T3 exerted wound healing-promoting effects in human skin organ culture (data not shown).

Taken together, this makes it particularly intriguing to ask whether the observed wound healing effects of T4 were mainly mediated via non-classical TH signalling pathways (cf. **Figure 20**). In fact, a growing body of evidence suggests that, besides the many direct TH target genes that show a TRE in their promoter region, there are many additional, TRE-negative genes which nevertheless are regulated by THs (Shen et al., 2004; van Beek et al., 2008) or by non-classical, receptor-independent TH activities (Davis et al., 2004; Mousa et al., 2008; van Beek et al., 2008; De Vito et al., 2012). Our gene profiling data support this concept further (data not shown). Thus, the current study also provides important new pointers to further investigate the molecular targets and mechanisms that underlie the wound healing-promoting effects of T4 on wounded human skin in the absence of serum and other extracutaneously generated steroid hormones.

The main reason for focusing on T4, however, was translational one: Since T4, but not T3, is licensed for use in clinical medicine and has been extensively administered to patients already for many decades, we argued that it is most important to investigate the effects of T4 on human skin wound healing, as this TH is clinically and pharmaceutically most relevant.

Bioengineered human skin “equivalents” have been advocated as suitable wound healing models (Falanga et al., 2002; MacNeil, 2007; Wigger-Alberti et al., 2009).

However, the generation of such skin “equivalents” is costly, time-consuming, labor-intensive, and requires substantial cell culture expertise. Moreover, these reconstitution assays can not claim to fully reflect native human skin, and employ cultured cells whose properties likely have been altered during isolation, culture, and reconstitution. Furthermore, these assays typically lack several skin cell populations appreciated as important wound healing protagonists (e.g., endothelial cells, macrophages, and mast cells) (Wulff et al., 2011; Korybalska et al., 2012) as well as skin appendages. Given the increasingly appreciated role of hair follicles in wound healing (Ito et al., 2005; Lau et al., 2009; Ansell et al., 2012; Martinez-Martinez et al., 2012), this further questions whether skin “equivalents” are satisfactory *in vitro*-wound healing models.

Instead, the human skin wound healing assay reported here has multiple advantages over previously reported *in vitro*-models:

1) It is very simple and cost-efficient, and can be performed with minimal tissue culture know-how and limited laboratory equipment, and offers an instructive tool to screen drugs or pharmacological substances in preclinical wound healing studies. Preliminary evidence from our laboratory suggests that other hormones, such as estrogen, prolactin, thymosin beta-4, and TRH, also exert profound wound healing-promoting effects in this assay system. Thus, the current assay is well-suited for testing the wound healing-modulatory properties of a wide range of steroids and peptides.

2) In contrast to its predecessor assay (Moll et al., 1998) and the concomitantly developed assay of Xu et al (Xu et al., 2012), the current assay operates under precisely defined, serum-free conditions, and uses a metabolically optimized medium (Philpott et al., 1991) that we had previously shown to sustain the long-term organ culture of human skin (Lu et al., 2007).

3) In contrast to Moll et al. (Moll *et al.*, 1998) and Xu et al. (Xu et al., 2012), our

assay also uses adult, full-thickness human skin which includes the subcutis, with its wound healing-promoting pluripotent stem cells and adipokines (Kim et al., 2007; Poeggeler et al., 2010), and its skin appendages. The latter may be quite relevant since hair follicles and their associated epithelial and mesenchymal progenitor cells likely exert important functions in cutaneous wound healing (Ito et al., 2005; Lau et al., 2009; Ansell et al., 2012; Martinez-Martinez et al., 2012).

4) The current assay system encompasses a number of sensitive, quantitative morphometric and molecular read-out parameters that are not routinely used in previous *in vitro*-wound healing assays, such as H&E, Ki67/Tunel, CK6 and CD31. These read-out parameters facilitate highly standardized, biologically instructive, and easily reproducible *in vitro*-wound healing research in the human system. Furthermore, some other interesting read-out parameters, e.g. electrical stimulation which has been demonstrated that it accelerated wound healing via increasing blood flow and haemoglobin levels in acute cutaneous wounds without affecting wound closure time (Ud-Din et al., 2012), might also be studied or developed in the future in our current organ culture assay.

Thus, this simple preclinical assay cannot only be set-up easily and relatively inexpensively, and allows to the quantitative evaluation of numerous instructive *in situ*-read out-parameters, but is also closer to the clinical wound healing reality than any other current *in vitro*-assay.

By measuring longitudinal reepithelialization (length of the new wound ET) and cortactin IR, we provide direct evidence that T4 promotes the migration of human epidermal keratinocytes during wound healing *in situ*. Though the underlying mechanisms require further investigation, this is line with growing evidence that T4 can stimulate the migration of several different cell populations (Matrisian, 1990; Bohnsack and Kahana, 2013; Peeters et al., 2013).

For example, the migration of neuronal and glial cells has been shown to be regulated by T4 (Farwell et al., 1990; Farwell et al., 2006). The mechanisms underlying this phenomenon were subsequently investigated by Lenoard and coworkers, who demonstrated that T4 plays a role in the conversion of soluble actin into F-actin, which is responsible for cell migration. This is a novel, non-genomic effect of T4 that could affect polymerization and accordingly, the physical state of a key component of the cytoarchitecture of migrating cells (Farwell et al., 1990; Siegrist-Kaiser et al., 1990; Leonard and Farwell, 1997). Our *in vitro* data are well in line with these findings (**Figure 28**), since T4 upregulated cortactin IR at the migratory edge of the newly formed ETs. Cortactin is a widely expressed actin-binding protein which is most prominently expressed in the lamellopodia of migrating keratinocytes and which had been originally identified as a substrate for Src kinase (Ceccarelli et al., 2007; Gendronneau et al., 2008). Double staining with F-actin and cortactin has been developed as a very useful marker to show the migrating cells (Gendronneau et al., 2008). Thus, the results from the current study suggest that T4 may regulate the formation and recognition of critical extracellular migration guidance actin-binding proteins, thereby controlling accelerated keratinocyte migration and reepithelization after human skin wound healing. This hypothesis deserves to be followed-up by cortactin/F-actin double-staining and quantitative immunohistomorphometry as well as by functionally interfering e.g. with Src kinase activity and/or actin-binding.

In line with our previous findings on the impact of THs on human HF matrix keratinocyte proliferation (van Beek et al., 2008), the current study shows that T4 increases human epidermal keratinocyte proliferation *in situ.* This fits well to other experimental studies which have shown that THs, in physiological concentrations, promote the proliferation not only of epidermal keratinocytes, but also of fibroblasts (Holt and Marks, 1977; Safer et al., 2001; Safer et al., 2003; Safer, 2012), human

thyroid cells (Lin et al., 2007), breast cancer cells (Tang et al., 2004) and glioma cells (Davis et al., 2006), granulosa cells (Verga Falzacappa et al., 2009), cardiac fibroblasts (Yao and Eghbali, 1992) *in vitro* and endothelial progenitor cells *in vivo* (Shakoor et al., 2010). One of the underlying mechanisms identified so far is that cell surface receptors may mediate these proliferative effects, since tetrac, a naturally-occurring deaminated derivative of T4 (tetraiodothyroacetic acid) (Mousa et al., 2008), opposes the trophic effect of agonistic thyroid hormone analogs (Mousa et al., 2008). Subsequently additional studies showed that the proliferative effect of iodothyronines can be blocked by RGD peptides that bind to an Arg-Gly-Asp recognition site on integrin molecules which are critical for TH membrane binding, and by antibody to integrin αvβ3 (Cheng et al., 2010).

However, the results obtained by systemic versus topical administration of THs to the skin were contradictory (Kress et al., 2009). *In vivo*, skin proliferation effect regulated by T3 may be counteracted by inhibitory factors, dependent on the systemic level of T3 (Safer, 2012). In fact, the cell's response to THs appears to be very much dependent on the cell type in question, its developmental state (progenitor or differentiated cell), its pathophysiological state (normal or tumor cell) and, eventually, the cellular context and the interaction of THs and TRs (Kress et al., 2009).

The keratin genes encode epithelial-specific intermediate filaments, making up about 30% of the protein of the epidermis (Safer et al., 2005; Kress et al., 2009; Ramot et al., 2009b; Coulombe and Lee, 2012). While keratins 1 and 10 are associated with terminal epidermal differentiation, keratins 5 and 14 are expressed in the basal skin layer; their expression decreased as the skin cells differentiate. Expression of, keratins 6 (investigated here), 16, and 17 is associated with both, epidermal (hyper-)proliferation and epithelial repair (Safer et al., 2005; Ramot et al.,

2009b; Coulombe and Lee, 2012). In keratin 6 knockout mice, superficial wound healing was reduced, compared to normal genotype mice (Wojcik et al., 2000). In addition, proliferation-associated cytokeratin 6 gene expression is decreased in hypothyroid mice and significantly stimulated in these by supraphysiologic doses of thyroid hormone (Safer et al., 2004, 2005). This is persuasive since the CK6 promoter is well-known to exhibit a TRE (Tomic et al., 1990; Radoja et al., 1997).

The CK6 IR results from the current study, which showed up-regulated CK6 protein expression *in situ* in wounded human skin epithelium after application of supraphysiological T4 doses, perfectly correspond to the previous *in vivo* work by Safer et al. in mice (Safer et al., 2004, 2005). Moreover, our laboratory has previously demonstrated that both T3 and T4 up-regulate CK6 expression in human HF epithelium (van Beek et al., 2008).

However, the exact mechanism underlying this phenomenon *in situ* and *in vivo* remains unclear. In fact, proliferation-associated cytokeratin genes reportedly contain inhibitory TREs in their promoter regions (Tomic et al., 1990; Ohtsuki et al., 1992; Tomic-Canic et al., 1996; Radoja et al., 1997), suggesting that their expression should be reduced in the presence of THs. Furthermore, while normal CK6 expression levels may be essential for physiological wound healing (Wojcik et al., 2000; Rotty and Coulombe, 2012), supraphysiological T4 doses may promote wound healing via TR- and TRE-independent mechanisms of action. Certainly, the comprehensively interpret the current CK6 protein *in situ* expression data, the obvious limitations of quantitative immunohistomorphometry make it desirable to complement this method by both qRT-PCR and fully quantitative Western blot analyses (the latter could not be run because this would have required much larger amounts of human skin than were available for the current study).

That T4 also stimulates protein expression of the prototypic epithelial stem/progenitor cell-marker keratin, CK15 (Cotsarelis, 2006; Tiede et al., 2007; Ramot et al., 2011), in human wounded skin *in situ*, is in line with our previous report that T4 also enhances CK15 gene and protein expression in organ-cultured human HFs *in situ* and in isolated HF epithelial progenitor cells (Tiede et al., 2010). In any case, since the CK15 promoter has not been reported to exhibit a TRE, the fact that T4 does modulate CK15 protein expression further encourages one to systematically investigate non-genomic, non-classical T4 signaling pathways in human skin.

One critical question here is whether continued stimulation of these epithelial progenitor cells by T4 may have undesired long-term consequences. In fact, T4 may just stimulate CK15 expression (and epitehlia, cell activity) only initially, while it may subsequently promote epithelial stem cell differentiation or even apoptosis (in human HFs, prolonged T4 stimulation inhibited proliferation and apoptosis of CK15+ cells at day 6) (Tiede et al., 2010). Thus, in theory, T4 stimulation could also exert a counter-regulatory effect through which excessive epithelial regeneration is down-regulated.

The current study provides the first evidence that T4 exerts pro-angiogeneic effects in human skin, which may play a role in the accelerating reepthelialization seen *in vitro*. This observation is well in line with the fact that THs are now well-documented to promote angiogenesis in many tissues and/or cells under physiological and pathological circumstances and in different experimental models (Luidens et al., 2009; Cheng et al., 2010), such as, chick chorioallantoic membrane (CAM) system (Davis et al., 2004; Bergh et al., 2005), the human dermal microvascular endothelial cell (HDMEC) microtubule assay (Mousa et al., 2005), etc., and the underlying molecular mechanism of the proangiogenic actions of thyroid hormones were both non-genomic and genomic (Fonder et al., 2008;

Luidens *et al.*, 2009).

Most of the proangiogenic actions of THs on endothelial cells and vascular smooth cells seem to initiate at target cell surface receptors, e.g. integrin avβ3, and are then transduced into downstream intracellular signals (e.g. via the ERK1/2 pathway) (Cheng et al., 2010). This, in turn, modulates the transcription of pro-angiogenic targets genes such as bFGF and VEGF; moreover, the integrin receptor for thyroid hormone engages in crosstalk with vascular growth factor receptors, such as bFGF, VEGF and EGF receptors (Davis et al., 2009; Luidens et al., 2009; Cheng et al., 2010). In addition, T4 can induce treated cells to release bFGF in an autocrine manner in CAM model, which promotes cell proliferation. Interestingly, when co-cultured with T4 and bFGF inhibitory antibody, the release action was blocked, suggesting that the proangiogenic effects of T4 depend, at least in part, on the release of one or more vascular growth factors (Luidens et al., 2009; Cheng et al., 2010).

In line with previous research, the pro-angiogenic effects of T4 in our model also are mediated, at least in part, by increased bFGF/FGFR expression and signaling, based on the observation that inhibitory bFGF antibody blocks of the angiogenesis effects of T4. However, the contribution of other important pro-angiogenic signaling pathways known to respond to TH stimulation, such as VEGF and PDGF (Sterry et al., 2006; Tang et al., 2012), remain to be systematically evaluated in wounded human skin.

HIF1α provides another interesting potential connection between THs and angiogenesis: HIF1α is a potent modulator of the tissue response to hypoxia, which serves as an important stimulus for angiogenesis during wound healing (Andrikopoulou et al., 2011) and tumor growth (Escandon et al., 2011) and is constitutively expressed in human epidermis, mainly in the basal layer (Rezvani et

al., 2011). T4 can induce HIF1α expressions via activation of phosphatidylinositol 3-kinase (PI3K), rather than ERK1/2 (Moeller et al., 2005; Moeller et al., 2006). Moreover, the activation of PI3K, resulting in HIF1α gene expression, may involve the integrin surface receptor for THs (Lin et al., 2009b). Intriguingly, emerging evidence suggests that HIF 1α also playd a role in reepithelialization of the wound bed via promoting keratinocyte migration. In addition, expression and activity of HIF1α are diminished under retarded wound healing conditions such as aged and diabetic skin (Andrikopoulou et al., 2011). Thereby, in addition to T4's bFGF/FGFR-mediated effects on angiogenesis, T4 may also accelerate both angiogenesis and wound healing via up-regulating HIF 1α expression and activity in wounded human skin.

Mast cells have been shown to play an important role in early inflammatory phase of wound healing and also to profoundly modulate proliferation and tissue remodeling in skin (Weller et al., 2006; Kennelly et al., 2011) as well as angiogenesis (Norlen, 2003; Rodewald and Feyerabend, 2012) (see Introduction: **1.3.6.3**). However, no stimulatory effect of T4 on human skin mast cells has previously been reported, especially not *in situ*. The current study, therefore, provides first evidence that T4 prominently impacts on human dermal mast cells within their natural tissue habitat. The available (immune-)histomorphometric mast cell data provide no insight into whether the observed mast cell effects (i.e. increase in the number of mast cells, triggering of human skin mast cell activation/degranulation *in situ*) occur directly (e.g. via stimulation of mast cell TR) or indirectly (e.g. via stimulating the release of mast cell-regulatory factors such as SCF or nerve growth factor). The specifically mast cell identification method based on KIT(+)CD49b(-) staining would be interesting in the further investigation (Liu et al., 2013).

Yet, T4 now has to be counted among the increasing number of hormones that, besides cortisol and ACTH (Arck et al., 2006; Paus et al., 2006; Murphy, 2012) as well as CRH (Ito et al., 2010) and cannabinoid receptor 1 (CB1) antagonists (Sugawara et al., 2012), regulates dermal mast cells activities in normal human skin. Also, if endogenous THs really do promote human skin mast cell activation and maturation from resident intracutaneous progenitor cells, as is suggested by the current findings (just like CRH and CB1 antagonists (Ito et al., 2010; Sugawara et al., 2012)), the application of newly developed TR antagonists such as Amio and Dron (Hara and Verkman, 2003) might become clinically interesting in may common diseases, in whose pathogenesis excessive mast cell activities are critically involved (e.g. atopic eczema, urticaria, allergic asthma, allergic rhinoconjunctivitis, prurigo).

The current mast cell data are also interesting in the context of inflammation (**Figure 38-40**), since, on the one hand, T4-induced increased mast cell degranulation may positively contribute to the inflammatory phase of wound healing. On the other hand, excessive mast cell activation and increase in the number of mast cells may have long-term deleterious effects on wound healing by preventing the timely down-regulation of inflammation that is required for optimal wound healing (Proksch et al., 2008; Kennelly et al., 2011; Wulff et al., 2011). So far, conflicting reports on the effects of THs on inflammation have been published. In rat brain, treatment with THs up to 5 days of age reportedly resulted in a decrease of histamine levels and mast cell numbers (Sabria et al., 1987). Instead, treatment of thyroiditis with suppressive TH doses improved chronic urticaria, which results from excessive skin mast cell degranulation and histamine release (Schocket, 2006). Thus, T4 effects on human skin mast cells may well be context-, dose-, and time-dependent. Clearly, this requires further investigation.

In summary, the simple, but clinically relevant human skin wound healing-assay used here has generated the first definitive preclinical evidence that T4 promotes reepithelialization and angiogenesis of experimentally wounded human skin *in vitro*. Moreover, our study suggests that this occur at least in part via up-regulation of bFGF/FGFR1-mediated signalling. In view of the overall medical importance of wound healing disorders and the urgency to develop more effective, inexpensive, and reasonably safe wound healing-promoting agents for the treatment of chronic skin ulcers (Markova and Mostow, 2012), our data encourage one to now systematically explore T4 as wound healing promoter in appropriate clinical trials. Like other steroid hormones, this FDA-approved agent is a stable, inexpensive and both topically and systemically applicable, has a well-known toxicological profile, and is routinely employed in clinical practice (Biondi and Wartofsky, 2012; Cooper and Biondi, 2012). Therefore, together with the existing *in vivo*-evidence from animal models (Safer et al., 2001; Safer et al., 2005; Kassem et al., 2012; Tarameshloo et al., 2012), the current preclinical human data render it timely to clinically explore T4 as a candidate promoter of human skin wound healing, namely as a topically applied agent (so as to minimize the risk of inducing a hyperthyroid state). Readily available and inexpensive drugs (e.g. L-thyroxine) could rapidly be repositioned for ulcer management as novel indication for the therapeutic administration of T4.

5. Summary

Despite growing evidence that thyroid hormones (TH) promote wound healing in animals, their effects in human skin wound healing are almost unexplored. Therefore, we wished to evaluate whether thyroxine (T4), which could easily be applied topically under clinical conditions, promotes reepithelialisation and angiogenesis in experimentally wounded human skin *in vitro*.

Wounded full-thickness human skin was organ-cultured in serum-free medium, using a 'punch-within-a-punch' design. The newly formed epithelial tongues at the inner and outer edges were analyzed by quantitative (imuno-)histomorphometry, using a set of instructive reepithelialisation and angiogenesis markers. Reepithelialisation was significantly promoted by 100 and 1000 nM T4, compared to vehicle controls. T4 also significantly up regulated the proliferation of epidermal keratinocytes as well as expression of the wound healing-associated keratin, CK6, and of the epithelial stem cell-associated keratin, CK15. Furthermore, expression of the endothelial cell marker, CD31, as well as the number of CD31+ vessel cross sections were up-regulated, indicating that T4 stimulates intracutaneous angiogenesis during skin wound healing. Both reepithelialisation and angiogenesis appear to be mediated, at least in part, via up-regulation of bFGF/FGF receptor1-mediated signalling. Given the importance of mast cells in wound healing and angiogenesis, it is interesting to note that T4 also increased the number and degranulation of dermal mast cells in T4-treated human skin fragments.

Taken together, these findings provide the first definitive preclinical evidence that T4 promotes reepithelialisation and angiogenesis in wounded human skin. This strongly encourages one to further explore topical T4 in the management of chronic skin ulcers.

6. Zusammenfassung

In der klinischen Medizin ist das Management von chronischen, verzögert heilenden Wunden zu einer der größten und bislang unbewältigten Herausforderungen geworden. Insbesondere müssen dringend gut verträgliche, kostengünstige und wirksame Wundheilungspromotoren identifiziert werden. Um dies zu unterstützen, sind einfache, wohl aber prognostisch und klinisch relevante vorklinische Prüfsysteme erforderlich, welche die Suche nach solchen wundheilungsfördernden Wirkstoffen erleichtern. Im Idealfall handelt es sich dabei um Substanzen, die sich bereits weitläufig im klinischen Einsatz befinden.

Thyroxin (T4) ist eines der gebräuchlisten Hormone in der klinischen Medizin und wird vor allem für die Behandlung der Schilddrüsenfunktionsstörungen verwendet . Üblicherweise wird synthetisches T4 nur einmal täglich oral verabreicht. Nach intestinaler Aufnahme wird T4 auf zellulärer Ebene dann zu T3 umgewandelt.

T4 ist also das klinisch gebräuchlichste Schilddrüsenhormon und würde deswegen für sofortige klinische Wundheilungs-Untersuchungen zur Verfügung stehen. Außerdem konnten wir bereits zeigen, dass T4 das Wachstum von menschlichen Haarfollikeln sowie epitheliale Haarfollikelstammzellenfunktionen in Organkultur moduliert. Daher konzentriert sich die vorliegende Dissertation gänzlich auf die Wirkungen von T4 in Bezug auf die Wundheilung menschlicher Haut *in vitro*.

Die Dissertation stellt zunächst grundlegende Projekt-Eckdaten in Bezug auf die Biologie der menschlichen Haut und der Wundheilung vor und analysiert dann die erheblichen Herausforderungen, mit denen die klinisch angewandte Wundheilungsforschung konfrontiert ist. Besonders wichtig ist es, neue

Medikamente zu enwickelen, welche die Reepithelialisation, Granulationsgewebebildung und Angiogenese stimulieren. Dabei ird auch auf die besondere Bedeutung von Mastzellen und Makrophagen hingewiesen, deren kontrollierte Aktivierung für eine optimale Wundheilung wichtig ist.Es wird die Hypothese begründet, warum T4 die Wundheilung menschlicher Haut fördern könnte. Diese Hypothese stützt sich zum einen auf bereits publizierte Tierversuche, in denen topisch appliziertes T4 die Wundheilung (z.B. in der Maushaut) beschleunigte und zum anderen darauf, dass T4 humanes Haarwachstum (in vitro) stimuliert. Da erhebliche biologische Parallelelen zwischen der Haarwuchsregulation und der Wundheilungskontrolle bestehen und Haarwuchsstimulatoren grundsätzlich auch Kandidaten dafür sind, Wunheilung beschleunigen zu können, macht dies die Testung vonm T4 bsonders interessant. Hinzu kommt, dass T4 schon seit Jahrzehnten ein kostengünstiges klinisches Standardtherapeutikum , dessen Wirkungs- und Nebenwirkungssepktrum gut bekannt ist und das sowohl systemisch als auch topisch appliziert werden kann.

Um diese Arbeitshypothese zu testen, wurde in einem vom Doktoranden mitentwickelten neuen Wundheilungs-Organkulturmodell anhand von auf standardisierte Weise verwundeter humaner Vollhaut untersucht, ob und wie T4 unter serumfreien Bedingungen Reepithelialisation, Angiogenese und Mastzellaktivitäten beeinflusst. Dazu wurde ein "Doppel-Loch-Design" („punch-in-a-punch“) verwendet. Die Wirkungen von T4 auf Reepithelialisation, Angiogenese und Mastzellaktivitäten wurden v.a. durch quantitative (Immun-)Histomorphometrie untersucht, wobei verschiedene Marker herangezogen wurden.

Zunächstz konnte das der o.g. in vitro-Wundheilung-Assay mit menschlicher

Vollhaut weiter charakterisiert und standardisiert werden, wodurch ein wertvolles, klinisch besomders interessantes Instrument für die präklinische Wundheilungsforschung entwickelt wurde.

Ferner konnte erstmal gezeigt werden, T4 die Wundheilung der menschlichen Haut *in vitro* stimuliert. Und zwar fördert T4 sowohl die Reepithelialisierung der vedrwundeten Epidermis also auch Angiogenese im Hautmesenchym, T4 hochreguliert ferner die Protein-Expression *in situ* zweier Schlüssel-Keratine (CK6, CK15) bei der Wundheilung und stimuliert sowohl die Keratinozyt-Migration (größte Länge der epithelialen „Wundzungen“; gesteigerte Cortactin-Expression) als auch epitheliale Progenitorzellen (d.h. vermehrte CK15 Immunreaktivitä). Ferner regelt T4die Anzahl und Aktivität der dermalen Mastzellen in organkultivierter, verletzter Humanhaut hoch.

Zieht man seine sofortige Verfügbarkeit in Bezug auf klinische Verwendung und Testung in Betracht, so legen diese präklinischen Untersuchungen in einem einfachen, aber klinisch sehr relevanten in vitro-Modell nahe, dass T4 ein hochinteressanter, kostengünstiger und toxikologisch wohldefinierter neuer Kandidat als Wundheilungspromotor für die zukünftige Behandlung von Wunden, insbesondere von chronischen Wunden ist.

Die vorliegende Studie bietet zudem auch neue Einsichten in Bezug auf mögliche endokrine Regulations-Mechanismen der Wundheilung der menschlichen Haut, indem sie aufzeigt, dass bFGF/FGFR-vermittelte Signalwege eine wichtige Rolle bei den wundheilungsfördernden Eigenschaften von T4 spielen. Jedoch bleibt der Mechanismus, bei dem T4 die Reepithelialisation und Angiogenese stimuliert, größtenteils unbekannt. Aus einer Microarray-Analye haben sich aber bereits ersten Indikatoren für neue T4 Kandidaten-Zielgene in der menschlichen Haut

ergeben. Dies liefert eine wertvolle Basis für mechanistische Anschlussstudien.

Selbstverständlich bleibt jetzt zu prüfen, ob T4 seine Wundheilungs-fördernden Wirkungen auch unter erschwerten Bedingungen (z.B. chronische Gewebshypoxie und Entzünung, Diabetes mellitus, periphere Neuropathie) entfaltet. An einem Surrogatassay, der solche chronischen Wundheilungsbedingungen zumindest teilweise nachzustellen versucht, wird aktuell gearbeitet (auch unter Beteiligung des Doktoranden).

Eine Einschränkung erfährt diese vorliegende Studie auch dadurch, dass der Schilddrüsenstatus der Hautspender unbekannt war (die vorliegende Ethikgenehmigung erlaubte nur anonymisierte Angaben überAlter, Geschlecht und Lokalisation der Haut).

Es ist jedoch darauf hinzuweisen, dass serumfreie Organkultur menschlicher Haut immer unter ausgeprägt "hypothyreotischen" Untersuchungsbedingungen erfolgt und allenfallsSpuren von intrakutanem T4 enthält, die das Gewebe intra-und postoperativ retiniert hat. Die beobachteten Hautreaktionen auf T4 könnten also gut die Wundheilungantwort von hypothyreotischer Humanhaut widerspiegeln. Unter diesem Gesichtspunkt passen die beobachteten Wundheilungs-stimulierenden Wirkungen von T4 gut zu der klinischen Beobachtung, dass die Wundheilung bei hypothyreotischen Patienten *in vivo* grundsätzlich verzögert abläuft.

Zusammengenommen liefern diese Forschungsergebnisse den ersten definitiven präklinischen Beweis dafür, dass T4 die Reepithelialisation und Angiogenese in experimentell verletzter, menschlicher Vollhaut *in vitro* Haut fördert. Dies ermutigt stark dazu, topisches T4 als Nächstes in der Behandlung von chronischen Hautulzera weiterführend zu untersuchen.

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I dedicate this thesis to my forever lovely father.

9. Curriculum Vitae

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Education:

Sep.1997-Jul.2000 Senior High School, Anhui Province, P.R.China

Sep.2000-Jul.2005 **Bachelor Degree** in Medicine (= **Medical School final exam**) from Bengbu Medical College, Bengbu, P.R.China

Sep.2005-Jul.2008 **Master Degree** in Surgery from Guangxi Medical University, Nanning, P.R.China

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Aug. 2008-Sep. 2009 Resident, Department of Hand and Plastic Surgery in the Second Affiliated Hospital of Wenzhou Medical College, Wenzhou, P.R.China

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10. Publications/Abstracts

Parts of this thesis were published or presented as follows:

Published abstracts:

1. **Guoyou Zhang**, Natalia T Meier, Wolfgang Funk, Markus Geisen, Sebastian Debus, Ralf Paus: Thyroxine (T4) promotes reepithelialization and angiogenesis in wounded human skin *in vitro*. *J Invest Dermatol* 131, S1 (2011)
2. N. T. Meier, **G. Zhang**, T. Schafer, M. Geissen, V. Emelianov, E. Kleinspehn, M. Augustin, S. Debus, R. Paus. An *in vitro* test system to study human epidermal wound healing *in situ*. *Exp Dermatol* 20(2):168 (2011)

Oral and poster presentation:

1. **Guoyou Zhang**, Natalia T Meier, Wolfgang Funk, Markus Geisen, Sebastian Debus, Ralf Paus. Thyroxine (T4) promotes reepithelialization and angiogenesis in wounded human skin *in vitro*. 41st European Society for Dermatological Research, Barcelona, Spain, 2011

The core results of this thesis project are currently being written-up for publication: **Guo-You Zhang,** Natalia T. Meier, Tian Liao, Markus Geisen, Wolfgang Funk, Sebastian Debus, Frank Siemers, Ewan A. Langan, Ralf Paus. Thyroxine (T4) promotes reepithelialization and angiogenesis in wounded human skin *in vitro.* (Target journal: *J Invest Dermatol*, **under preparation)**

Some of the *methods* used in the current thesis project, to whose establishment/standardization the doctoral candidate has contributed, have already been submitted for publication:

1. Natalia Meier, David Pattwell, **Guo-You Zhang**, Vladimir Emelianov, Roberto Paredes, Sebastien Debus, Matthias Augustin, Wolfgang Funk, Enrique Amaya, Jennifer Kloepper, Matthew Hardman, Iain Haslam, and Ralf Paus. Thyrotropin-releasing hormone (TRH) promotes wound reepithelialization in frog and human skin. *Plos One* (**under review**)
2. Tian Liao, Janin Lehmann, Sabine Nagel, Arzu Yay, **Guo-You Zhang**, Anna Emilia Matthieβen, Sandra Danner, Frank Siemers, Charli Kruse, Jennifer E. Kloepper, Ewan A. Langan, Stephan Tiede, Ralf Paus. Nestin+ progenitor cells isolated from adult human sweat gland stroma stimulate reepithelialisation and angiogenesis in human skin organ culture. *J Invest Dermatol*. (Decision: “Revise and Resubmit”; revised manuscript submitted Dec 2012).

3. T Liao, A Yay, **G Zhang**, S Nagel, AE Petschnik, S Danner, C Kruse, S Tiede, R Paus. Cell-based regenerative medicine by nestin-positive stem cells: Promotion of human skin wound healing in organ culture by pluripotent adult human progenitor cells isolated from human sweat gland stroma. *J Invest Dermatol* 132(S2): S7 (2012)

Co-authored articles unrelated to the data presented in the current thesis project:

1. Ramot Y, **Zhang G**, Bíró T, Lisztes E, Funk W, Ingber A, Langbein L, Paus R. TSH is a novel neuroendocrine regulator of selected keratins in the human hair follicle. *J Dermatol Sci*. 2011;64(1):67-70.
2. Y. Ramot*, **G. Zhang***, J. Hardman, I. S. Haslam, T. Bíró, L. Langbein, R. Paus Thyrotropin-releasing hormone modulates keratin expression in human skin. *Br J Dermatol*. (**Accepted**) (*: joint first authors)
3. **Zhang GY**, Li QF, Cai JL, Fu XB, Gao WY. Reconstructive surgery after female genital mutilation. *Lancet*. 2012;380(9852):1468-9
4. **Zhang GY**, Liao T, Gao WY. MUC5B promoter polymorphism and pulmonary fibrosis. *N Engl J Med*. 2011;365(2):178
5. **Zhang GY**, Li QF, Cai JL, Cao YL, Fu XB. Allogeneic fibroblasts and keratinocytes for venous leg ulcers. *Lancet*. 2013;381(9864):372.
6. **Zhang GY**, Li QF, Fu XB. Allogeneic and autologous mesenchymal stem cells for chronic heart disease. *JAMA*. (**In press**).