Paracrine factors from aggregates of fibroblasts or bone marrow stromal cells enhance skin wound healing

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Steve Jobs

Again, you can't connect the dots looking forward; you can only connect them looking backwards. So you have to trust that the dots will somehow connect in your future.

Steve Jobs

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Original publications

This thesis is based on the following original publications:

I **Peura M**, Bizik J, Salmenperä P, Noro A, Korhonen M, Pätilä T, Vento A, Vaheri A, Alitalo R, Vuola J, Harjula A, Kankuri E. Bone marrow mesenchymal stem cells undergo nemosis and induce keratinocyte wound healing utilizing the HGF/c-Met/PI3K pathway. Wound Repair Regen. 2009;17:569-77.

II **Peura M**, Siltanen A, Saarinen I, Soots A, Bizik J, Vuola J, Harjula A, Kankuri E. Paracrine factors from fibroblast aggregates in a fibrin-matrix carrier enhance keratinocyte viability and migration. J Biomed Mater Res A. 2010;95:658-64.

III **Peura M**, Kaartinen I, Suomela S, Hukkanen M, Bizik J, Harjula A, Kankuri E, Vuola J. Improved skin wound epithelialization by topical delivery of soluble factors from fibroblast aggregates. Burns. 2012;38:541-50.

IV Nuutila K*, **Peura M***, Suomela S, Hukkanen M, Siltanen A, Harjula A, Vuola J, Kankuri E. Recombinant human collagen III gel for transplantation of autologous skin cells in porcine full thickness wounds. Submitted manuscript.

* Equal contribution

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Main abbreviations

α-SMA	alpha-smooth muscle actin
BMSC	bone marrow stromal cell
c-MET	oncogene, N-methyl-N-nitro-nitrosoguanidine-transformed human osteosarcoma cell-oncogene / MNNG HOS transforming gene
CD	cluster of differentiation
CK	cytokeratin
COX-2	cyclooxygenase-2
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
EGF	epidermal growth factor
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FINECTRA	set of paracrine mediators released from fibroblast aggregate cultures
GAG	glycosaminoglycan
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage/colony-stimulating factor
H&E	hematoxylin and eosin
HGF	hepatocyte growth factor, also known as scatter factor
IGF	insulin-like growth factor
IL	interleukin
KGF	keratinocyte growth factor, also known as fibroblast growth factor 7 (FGF7)
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MMP	matrix metalloproteinase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NRG	neuregulin
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PI3K	phosphatidylinositol-3-OH kinase
PRP	platelet-rich plasma
rhCol	recombinant human collagen
TGFβ	transforming growth factor-β
TNF-α	tumor necrosis factor-α
VEGF	vascular endothelial growth factor

Abstract

Skin wound healing is a tightly regulated sequence of events involving activation and interplay of various cell types. Dermal fibroblasts guide crucial re-epithelialization events such as keratinocyte migration and proliferation. Fibroblast-keratinocyte interaction is directed by paracrine factors as well as cell-cell and cell-extracellular matrix contacts. In large-scale wounds, such as burns, the intrinsic capacity for healing is compromised due to lack of dermal support.

Fibroblasts as well as other mesenchyme-derived cells such as bone marrow stromal cells can produce a vast array of growth factors in vitro. When activated by deprivation of extracellular matrix cell adhesion sites, the cells prefer cell-to-cell contacts, form clusters, and release growth factors. This present work focused on characterizing the use of a combination of growth factors released by mesenchymal cell aggregates, a process designated as nemosis, for treatment of skin wound healing.

We used inhibitors of several intracellular signaling pathways to define the migration-inducing mechanisms of nemosis using a keratinocyte wound-healing assay. Model human keratinocytes HaCaT-cells responded to the nemosis-derived conditioned medium with enhanced migration, proliferation, and viability. A c-Met-specific small molecule tyrosine kinase inhibitor as well as an anti-hepatocyte growth factor antibody inhibited these responses. The study aimed to characterize means of improving wound healing by combining biological materials, such as fibrin or recombinant human collagen III with the growth factors to construct a transplantable cell- and growth factor-containing matrix. The effect of these active matrices on wound healing was analyzed in porcine partial-thickness and full-thickness wound models.

We observed that conditioned medium from cell aggregates could easily be trapped, transferred to the wound, and gradually released from a fibrin matrix. When we treated porcine partial-thickness wounds with the growth factor-containing fibrin matrix, we observed an increase in the proliferation and migration of hair-follicle and lateral wound-edge keratinocytes. In addition, granulation tissue formation was enhanced when compared with formation on saline-treated wounds. We found that keratinocyte transplantation in porcine full thickness wounds was feasible with a recombinant human collagen type III matrix.

The results presented here indicate that cell-cell contact-activated fibroblasts or bone marrow stromal cells improve keratinocyte transplantation with paracrine factors. Fibrin or recombinant human collagen III can serve as vehicles for delivery of these signals to the target site in acute wounds. Since these factors are also crucial for healing of chronic non-healing wounds, it is likely that our therapeutic approach will be of benefit in their treatment, too.

1. Introduction

The skin is the largest organ of the human body. It maintains body homeostasis by regulating temperature and fluid balance, produces vitamin D, and provides an essential barrier that protects us from the environment. After injury to the skin, a complex series of tightly controlled responses are initiated to restore its integrity. In extensive tissue damage, as observed in burns or other large tissue defects, the regenerative capacity of skin is compromised, causing impaired and delayed healing. Thus, to replace lost tissue, skin transplantation from uninjured areas is necessary. Skin-graft harvesting, on the other hand, creates a new wound. When the skin is extensively burned, the surgeon is faced with a lack of healthy donor sites. The time-consuming epithelialization of both donor sites, as well as the interstices of largely meshed skin grafts, leaves the body without a proper barrier function rendering it susceptible to infection and dehydration, that critically contribute to both morbidity and mortality (Church et al., 2006).

For a skin wound to heal, an adequate circulation of blood and nutrients is mandatory. Necrotic material and wound debris, a nutrient for bacteria, needs to be repeatedly removed. Additionally, wound moisture is critical, since surface dessication impairs healing, epithelialization in particular. As a conclusion, the wound microenviroment plays an essential role in healing. Systemic diseases can weaken the wound-healing response and clinicians frequently encounter hard-to-heal skin wounds. Most common are chronic non-healing wounds of the lower limb caused by diabetes or venous insufficiency, which heal slowly and require debridement and skin grafting (Briggs et al., 2004). These wounds are a major clinical problem; a substantial burden on healthcare costs.

Although a wide variety of products for the treatment of wounds exist, with new ones constantly emerging, many are expensive, complicated to use, or inefficient. A need to develop novel means of treating wounds and understanding the mechanisms of wound healing therefore remains. Growth factors and cytokines are key components in wound healing, and because their amount and activity are reduced in non-healing wounds, targeted delivery of these essential mediators to the wound bed is considered promising in promoting healing and epithelialization (Lauer et al., 2000). One method to produce an array of growth factors in large quantities is the activation of dermal fibroblasts by cell-to-cell contacts when cultivated as cell aggregates (Furukawa et al., 2001; Bizik et al., 2004; Kankuri et al., 2005). Activation of cells by their cultivation on non-adherent surfaces such as cell clusters produces a biologically effective concentration of growth factors and cytokines (Bizik et al., 2004; Kankuri et al., 2005).

In the present study, we characterized the effect of paracrine factors produced by aggregation-activated fibroblasts and bone marrow stromal cells on keratinocyte wound healing. Furthermore, we studied their transfer and effect in matrices on experimental porcine partial- and full thickness wounds.

2. Review of the literature

2.1 Skin structure and function

The skin consists of two distinct layers: the fibroblast-containing dermis and the keratinocyte-populated constantly renewing epidermis. The dermis, responsible for the skin's strength and pliability, provides support and nutrition to the epidermis and contains sweat glands, hair follicles, and associated sebaceous glands (Figure 1A). The epidermis can be divided into several separate epithelial layers based on proliferation and differentiation states. The proliferating stem and transit-amplifying cells in the basal layer differentiate layer by layer, lose their nucleus during this differentiation process, and eventually become dead corneocytes of the outermost stratum corneum (Figure 1B). The skin provides the body with a barrier against the outer world; the barrier that protects us from the environmental threats, prevents water loss, and provides thermal homeostasis (Montagna, 1962). Loss of barrier integrity, as a result of injury or disease, may lead to significant disability or even death (Singer and Clark, 1999).

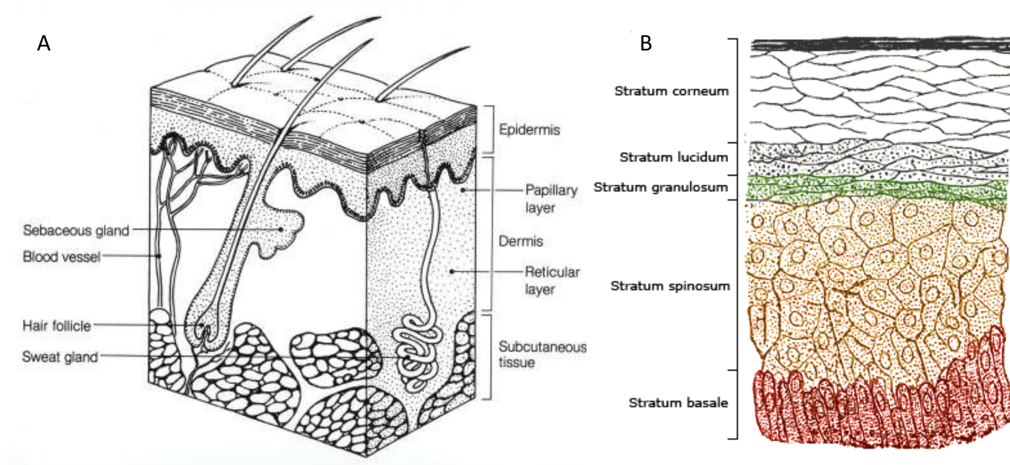

Figure 1. A schematic presentation of skin anatomy and essential skin structures (A) and layers of the epidermis (B). Adapted from The Web site of the National Cancer Institute (http://www.cancer.gov) and wikimedia commons, Files: Skin layers.png, Skinlayers.png

Recent studies indicate that there are multiple epidermal stem cell pools in distinct compartments (Watt and Jensen, 2009) (Figure 2). The hair follicle bulge reservoir is crucial for the maintainance of basal unipotent epidermal cells in the interfollicular epidermis. In uninjured skin, stem cells divide slowly. In respose to injury, however, they activate and rapidly proliferate supplying cells needed for epithelialization. In superficial wounds, restoration of the epidermal barrier can occur through proliferation and migration of

epidermal stem cells in the basal cell layer (Figure 1B and 2). Wounds extending to the dermis are first replaced by granulation tissue that provides survival time and a supporting structure for stem cells in the hair follicle, and in sebaceous and sweat glands (Figure 2) to activate, migrate, and proliferate (Martin, 1997). If the wound extends below the dermis, the skin can heal only through scarring and contraction (Singer and Clark, 1999).

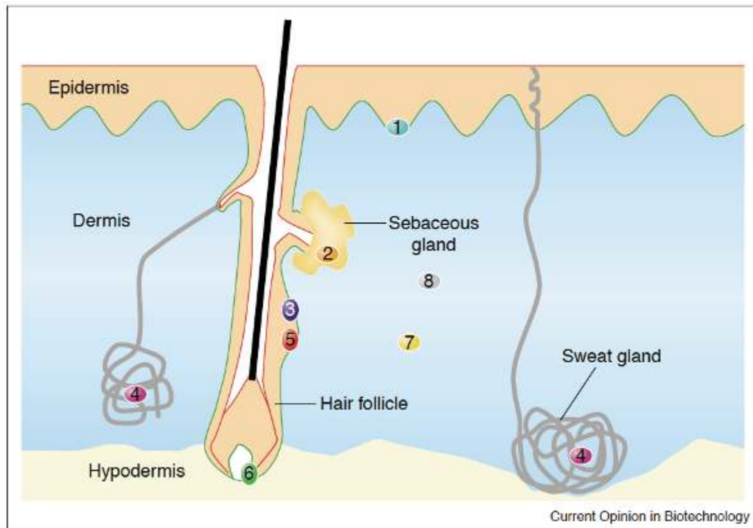

Figure 2. Skin stem cells. Epidermal stem cell (1), sebaceous stem cell (2), hair follicle bulge stem cell (3), sweat gland stem cell (4), melanocyte stem cell (5), connective tissue sheet mesenchymal stem cell (6), neural stem cell (7), and endothelial stem cell (8).

From Brouard and Barrandon, *Current Opinions in Biotechnology* 2003,15,520-252. Used with the permission of the copyright holder.

2.2 Skin wounds and wound healing

The skin is continuously exposed to the environment and is subject to mechanical stress, injury, and ultraviolet-radiation. Its well-being therefore depends on a tightly regulated healing process: a complex sequence of events in which every step is crucial for restoration of tissue integrity and function (Martin, 1997). Wound healing is a precisely orchestrated interplay between different cell types involving several mediators (Figure 3, Table I) and a multitude of intracellular signaling pathways (Singer and Clark, 1999). Immune cells (neutrophils, monocytes, lymphocytes, and dendritic cells), endothelial cells, keratinocytes, and fibroblasts undergo changes in gene expression and phenotype resulting in cell proliferation, differentiation, and migration (Schafer and Werner, 2007; Gurtner et al., 2008). Classically, three overlapping phases of wound healing can be characterized: inflammation, synthesis, and remodelling.

2.3 Stages of wound repair

2.3.1 Inflammation

The role of the acute inflammatory phase is to stop the loss of fluid and blood, to remove devitalized tissue, and to counteract infection (Gurtner et al., 2008). Immediately after injury, platelet aggregation initiates hemostasis, which is followed by the assembly of a fibrin matrix, which becomes the scaffold for migrating cells (Postlethwaite et al., 1981; Clark et al., 1982). In skin-wound healing, infiltrating leukocytes are the first-line cellular components of the inflammatory response (Eming et al., 2007). Interleukin-1 (IL-1), released by damaged keratinocytes after epidermal injury, acts as the first signal to alert surrounding and circulating cells (Kupper et al., 1986; Freedberg et al., 2001), (Figure 3). Neutrophils are also recruited to the wound by bacterial degradation products, activation of complement, degranulation of platelets, and the release of platelet-derived growth factor (PDGF) (Grose et al, 2004).

After infiltration, neutrophils produce a wide variety of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), IL-1 and IL-6, which in turn modulate the inflammation response by promoting cytokine production and leukocyte migration (Szpaderska et al., 2003). After 2 to 3 days, monocytes migrate to the wound and differentiate into macrophages. The importance of macrophages in the process of wound healing is not yet totally understood. Macrophages support wound repair by serving as antigen-presenting cells and phagocytes (Gordon et al., 2003). Macrophages also synthesize numerous potent growth factors, such as transforming growth factor beta (TGF-β), TGF-α, basic fibroblast growth factor (bFGF, also known as FGF2), PDGF, and vascular endothelial growth factor (VEGF), that support cell proliferation and synthesis of extracellular matrix (ECM) (Brancato and Albina, 2011) (Figure 3). On the other hand, macrophage deficiency can be compensated for by the redundancy of the inflammatory response (Martin et al., 2005; Gurtner et al., 2008). Interestingly, it seems that the versatile macrophages are responsible both for the induction of fibrosis and for its clearance and resolution (Martin et al., 2005).

2.3.2 Phase of synthesis

Granulation tissue formation begins 2 to 10 days after injury and overlaps with the inflammatory phase. Fibroblasts and macrophages replace the fibrin matrix with a new ECM containing collagens I and III, glycosaminoglycans (GAG), and proteoglycans (Lawrence, 1998). A number of growth factors, including PDGF, TGF-β, epidermal growth factor (EGF), (insulin-like growth factor) IGF-1, and FGF2 stimulate fibroblasts (Figure 3) (Werner and Grose, 2003; Vogler et al., 2003). PDGF functions as a chemoattractant for fibroblasts and stimulates collagen production. TGF-β promotes synthesis of collagen, fibronectin, hyaluronic acid, and other proteins in a variety of cell lines, reducing the proteolytic degradation of ECM components by reducing synthesis of protease inhibitors (Roberts et al., 1990); proteases are generally thought to activate various effects in cells. In unwounded skin and mature scars, 80

to 90% of the dermal collagen is type I, and type III collagen constitutes the remaining 10 to 20%. In early wound healing, the amount of type III collagen is increased about three-fold (Monaco and Lawrence, 2003). The phase of synthesis continues until the defect is filled with collagen that ultimately foms the bulk of the mature scar (Werner et al., 2007).

Local changes in the tissue environment (increased lactate, decreased pH, and low oxygen) activate angiogenesis, which is a central feature of granulation tissue (Remensnyder and Majno, 1968; Li et al., 2003). Matrix metalloproteinases (MMPs) degrade the ECM and thus play a central role in activating angiogenesis, leukocyte infiltration and re-epithelialization. Numerous growth factors activate angiogenesis, such as vascular endothelial growth factor A (VEGFA) and FGF2, angiopoietin, and TGF-β. During wound healing keratinocytes at the wound edge begin producing MMPs as they detach from the basement membrane and migrate across the wound bed on the newly formed provisional matrix (Clark et al., 1982; Raja et al., 2007; Schultz and Wysocki, 2009). Keratinocyte migration is stimulated by, for example, neuregulin (NRG), hepatocyte growth factor (HGF), and EGF (Werner and Grose, 2003). Keratinocyte proliferation is stimulated by factors such as: heparin binding-EGF (HB-EGF), HGF, IL-6, and granulocyte macrophage-colony stimulating factor (GM-CSF) (Werner and Grose, 2003).

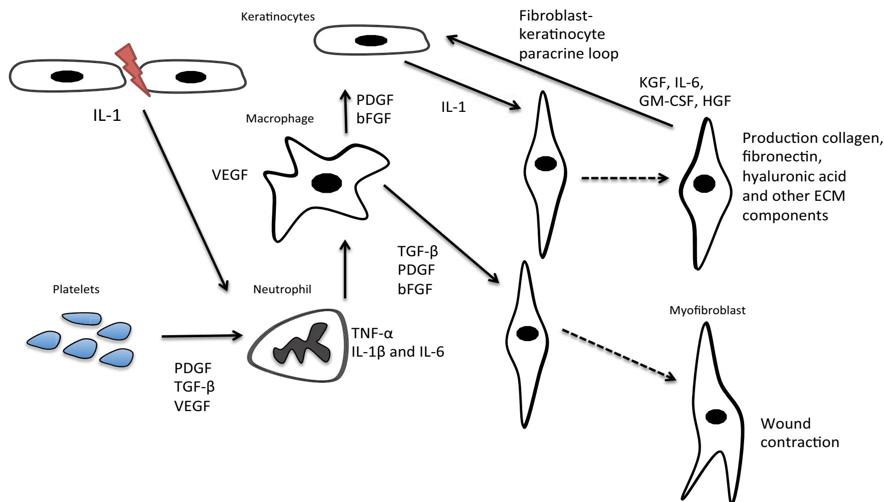

Figure 3. Paracrine stimulatory mediators and fibroblast-keratinocyte interactions in wound healing.

ECM, extracellular matrix, **bFGF**, basic fibroblast growth factor, **GM-CSF**, granulocyte macrophage-colony stimulating factor, **HGF**, hepatocyte growth factor, **IL-1**, interleukin 1, **IL-6**, interleukin 6, **KGF**, keratinocyte growth factor, **PDGF**, platelet-derived growth factor, **TGF-β**, transforming growth factor beta, **TNF-α**, tumor necrosis factor α. Arrows indicate stimulatory effects.

Figure modified from Baum and Arpey, *Dermatol Surg*. 2005;31:674-86 with the permission of the copyright holder.

2.3.3 Remodeling

The next stage of skin wound repair, remodeling, begins 2 to 3 weeks after injury and requires a year or more (Gurtner et al., 2008). During this stage, all the activated processes are gradually quenched. The majority of endothelial cells, macrophages, and fibroblasts undergo apoptosis (Kane and Greenhalgh, 2000) and the marked hypervascularity seen in granulation tissue disappears. The ECM is actively remodeled from mainly type III collagen-containing architecture into one composed of type I collagen (Lovvorn et al., 1999). The remodeling process is mainly executed by MMPs secreted by fibroblasts, macrophages, and endothelial cells (Lawrence, 1998). Although in some eukaryotic organisms the response to injury can completely regenerate the original tissue architecture; in mammals the skin never regains its uninjured properties (Levenson, 1965).

2.4 Epidermal stem cells and wound healing

In the injured skin, epidermal stem cells must also activate to provide efficient re-epithelialization (Lavker and Sun, 2000). Present knowledge underlines the fact that multiple sources of these cutaneous stem cells exist (Figure 2), and that the unipotent stem cell in the basal layer of the epidermis is a progeny of the multipotent stem cell (Taylor et al., 2000; Brouard and Barrandon, 2003; Ito et al., 2005). However, with the lack of an absolutely specific single molecular marker, the characterization of epidermal stem cells is complicated. Multipotent epidermal stem cells identified experimentally as ‘‘label-retaining cells’’ staining positive for keratin 15, cluster of differentiation 34 (CD34) and leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5), have been characterized in a specialized location within the outer root sheath of the hair follicle known as the bulge (Figure 2) (Roh and Lyle, 2006). Lgr5 is a downstream mediator of the wingless and int-pathway (WNT)-signaling pathway, found to be a marker for quiescent stem cell-like cells (Haegebarth and Clevers, 2009). Multipotent epidermal stem cells contribute to healing by migrating and producing transit-amplifying cells that can undergo several divisions (Ito et al., 2005). A subpopulation of cells lacking the bulge marker CD34 lie just below the opening of the sebaceous gland and exhibit similar properties of multipotent keratinocyte stem cells (Jensen et al., 2008). The transcription factor c-myc is a regulator of epidermal stem cell fate (Watt, 1998). c-Myc knock-out mice exhibit delayed re-epithelialization because of the inability of interfollicular epidermal cells to produce daughter keratinocytes (Schafer and Werner, 2007) stating the importance of multipotent stem cells in healing (Figure 4).

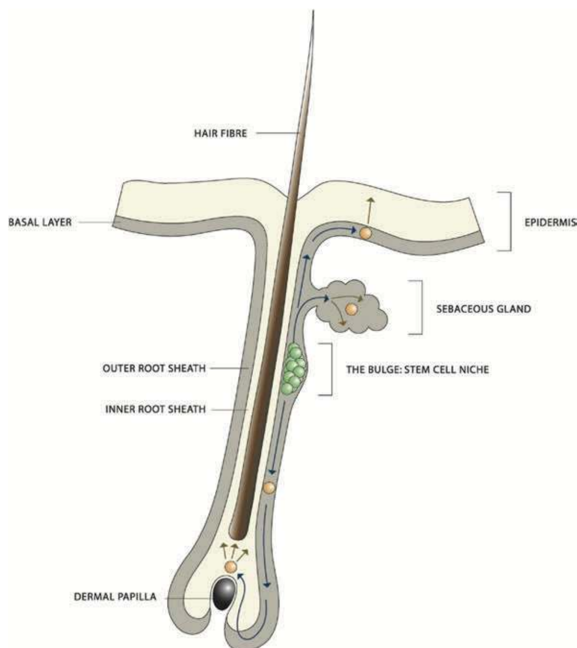

Figure 4. Location of hair follicle bulge epidermal stem cells and their migration and contribution of epithelialization in wound healing.

From Turksen *Dev Cell*. 2004;6:454-6. Used with the permission of the copyright holder.

2.5 Fibroblast populations and wound healing

Paracrine signals from the underlying dermis control keratinocyte stem cell activity (Taylor et al., 2000) fibroblasts are considered a central player in the production of the dermis-derived paracrine stimuli (Werner et al., 2007). Different pools of dermal fibroblasts are defined according to their anatomical location as papillary, reticular and hair follicle-associated dermal fibroblasts (review by Sorrell and Caplan, 2004) (Figure 1) and have various phenotypes (Harper and Grove, 1979; Azzarone and Macieira-Coelho, 1982). The hair follicle-associated dermal fibroblasts reside in the connective tissue sheet (CTS) of the hair follicle (Figure 2) (Jahoda and Reynolds, 1996). The present understanding is that local dermal fibroblasts arise from multipotent dermal mesenchymal stem cells of the CTS (Jahoda et al., 2003). These stem cells display high telomerase activity and proliferative potential (Hoogduijn et al, 2006), and posses characteristic features of mesenchymal stem cells with the capacity to differentiate into adipocytes, chondrocytes, osteoclasts, functional smooth muscle cells, and keratinocytes in vitro (Crigler et al., 2007). In a healing wound, fibroblasts transform into contractile α-smooth muscle actin (SMA)-expressing myofibroblasts (Tomasek et al., 2002; Hinz et al., 2007). Myofibroblast differentiation and wound contraction are promoted by TGF-β (Vaughan et al., 2000) (Figure 3). Apart from TGF-β signals, mechanical tension, FGF2 and PDGF regulate fibroblast to myofibroblast phenotype switching (Tomasek et al., 2002; Werner and Grose, 2003).

2.5.1 Infiltrating mesenchymal cells

Knowledge of peripheral blood-derived fibroblasts participation in wound healing dates back almost 100 years (Dunphy, 1963). Bucala et al. (1994) described a distinct population of blood-borne fibroblast-like cells, termed “fibrocytes”, that rapidly infiltrated the location of tissue injury in a wound-chamber model. Later on, what became clear is that a distinct population of blood-derived CD34+/Col I+ fibroblast-like cells contribute to wound healing by secreting the chemokines, cytokines and growth factors involved in wound repair (Mori et al., 2005; Grieb et al., 2011). Additionally, the circulating peripheral blood is considered a significant source of bone marrow mesenchymal stem cells needed for skin repair and regeneration (Stramer et al., 2007). Opalenik and Davidson showed that bone marrow stromal cells contribute to granulation tissue formation by acquiring a fibroblast phenotype (Opalenik and Davidson, 2005). Bone marrow-derived stem cells also support epidermal regeneration through paracrine stimulus (Krause et al., 2001; Aoki et al., 2004).

2.6 Paracrine interaction between dermal fibroblasts and keratinocytes

The reactions and responses of the dermis during wound healing have received extensive attention. The first report in 1961 by McLoughlin was that mesenchymal factors were required for proper epidermal differentiation. Several other key observations followed (McLoughlin, 1961, Wessels 1964; Melbye et al., 1973), culminating with the method to successfully cultivate normal human keratinocytes using fibroblast feeder cells (Rheinwald and Green, 1975, 1977). The feeder cell co-culture system revealed clearly that epidermal cell phenotype depends on interaction with mesenchymal cells. Keratinocytes seeded on the feeder cell layers retained their proliferating non-differentiated state for a longer period of time (Rheinwald and Green, 1975 and 1977). After skin wounding, dermis-derived growth factors cause changes in gene expression and activate keratinocytes to acquire a proliferative and migratory phenotype (Schafer and Werner, 2007, Werner et al., 2007). Smola et al (1993) characterized that fibroblast-keratinocyte co-cultures produce increased levels of keratinocyte growth factor (KGF or FGF7), IL-6, and GM-CSF. It was also clear that IL-1 stimulus enhanced fibroblast KGF production at both the protein and mRNA level (Brauchle et al., 1994; Chedid et al., 1994; Maas-Szabowski and Fusenig, 1996). KGF, in turn, promoted increased production of IL-1α from keratinocytes, suggesting a paracrine loop (Maas-Szabowski et al., 1999) (Figure 3).

2.6.1 Epidermal growth factor family

The epidermal growth factor (EGF) family consists of EGF, TGF-α, HB-EGF, amphiregulin, betacellulin, epiregulin, and neuregulin (NRG-1, NRG-2, NRG-3, and NRG-4) (Harris et al., 2003). Various enzymes cleave the membrane-anchored precursor molecules of EGF-family growth factors to release their active soluble forms that then act in either a paracine or an autocrine manner. Upon binding to their receptor, the ErbB-receptor (also

generally referred to as EGFR), an intracellular signaling cascade is activated. The ErbB-family is comprised of four receptors (ErbB 1-4) based on ligand affinity. EGF, TGF-α, and amphiregulin bind to ErbB1. HB-EGF, betacellulin and epiregulin bind to ErbB1 and ErbB4 (Harris et al, 2003). Neuregulins binds to ErbB2-4 receptors, which are important for the development of cardiac muscle and the central nervous system (Shirakata, 2010). Human epidermal keratinocytes express ErbB1, ErbB2, and ErbB3, but not ErbB4 (Miettinen et al., 1995; Hashimoto, 2000). In general, all EGF-family growth factors promote keratinocyte proliferation some extent. In wound healing, however, amphiregulin and EGF are considered the most potent mitogens for keratinocytes (McCawley et al., 1998; Hudson and McCawley, 1998; Schelfhout et al., 2002).

TGF-α, originally identified for its ability to induce a reversible phenotype transformation in fibroblast-like cells, is secreted by keratinocytes and promotes epithelial cell proliferation in an autocrine manner (Coffey et al., 1987). Like amphiregulin, TGF-α is over-expressed in psoriasis (Elder et al., 1989). HB-EGF was first identified in the conditioned medium of macrophages. HB-EGF binds to two receptors, ErbB1 and ErbB4, and has high affinity for heparin (Higashiyama et al., 1991). Betacellulin is expressed by suprabasal keratinocytes in particular in the granular cell layer, stimulating keratinocyte growth and controling hair follicle development (Schneider et al., 2008). Several knock-out mouse models allow characterization of the specific functions of EGF-family members as well as ErbB1. Of particular interest, are the Waved-1 mice, which have a spontaneous mutation in the *Tgfa* gene and are hence TGF-α-deficient. They are characterized by unmistakable waviness of their hair and whiskers and display delayed healing of partial- but not full-thickness wounds (Luetteke et al., 1993).

2.6.2 Hepatocyte growth factor (HGF)

Initially identified by its growth-promoting activities in hepatocytes and scattering-inducing property in epithelial cells (Gherardi et al., 1989), HGF has since proven to be a multipotent growth factor with properties promoting angiogenesis, mitosis, cell migration and morphogenesis, and inhibiting apoptosis (Stella and Comoglio, 1999). Cellular responses to HGF are mediated by a specific receptor tyrosine kinase, c-Met, and by its autophosphorylation upon ligand binding (Ma et al., 2005). HGF binding to c-Met causes receptor dimerization and transient autophosphorylation of two tyrosine residues (Y1234 and Y1235) in the receptor's intracellular b-chain (Ponzetto, 1994) (Figure 5). Mainly mesenchymal cells secrete HGF, which acts locally to stimulate epithelial cell and endothelial cell proliferation, and angiogenesis.

HGF and c-Met have been explicitly linked to skin wound healing and hair follicle morphogenesis (Lindner et al., 2000). Interestingly, c-Met is upregulated in migrating keratinocytes with MMP-9 (McCawley et al., 1998; Chmielowiec et al., 2007), and deletion of the *met*-gene strongly delays epithelialization, a finding further supporting the essential role of HGF/c-Met signaling in re-epithelialization (Chmielowiec et al., 2007). The HGF amount

in both acute and chronic wounds is increased, whereas in the latter the activation of HGF most probably is inhibited due to an increased amount of HGF-inhibitors HAI-1 and HAI-2 (Conway et al., 2007; Shirakata et al., 2007).

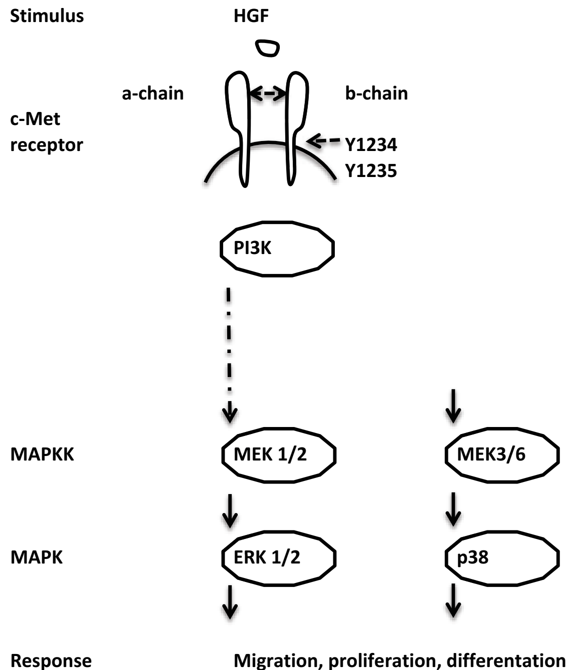

Figure 5. The main intracellular kinase cascades activated after binding of HGF to its ligand-specific c-Met receptor and subsequent receptor homodimerization. **Y1234** and **Y1235**, tyrosine residues of c-Met

PI3K, phosphatidylinositol 3-kinase; **MEK 1/2**, **MEK 3/6**, mitogen activated protein kinase kinase; **ERK1/2**, extracellular signal-regulated kinase; **p38**, protein 38 activated MAP kinase.

2.6.3 Fibroblast growth factor family

Thus far, 22 FGF members exist, and the high-affinity FGF receptor (FGFR) family consists of receptors FGFR 1 to 4. These tyrosine kinase receptors are transmembrane proteins, which work much like EGFR. KGF was one of the first growth factors involved in mesenchymal-epithelial interactions (Finch et al., 1989). It is rapidly induced in fibroblasts after wounding, exerts its paracrine effect through binding to its receptor FGFR2IIIb on keratinocytes, and promotes migration and proliferation (Brauchle et al., 1994; Chedid et al., 1994). Upon injury, KGF expression is as much as to 100-fold upregulated by factors such as IL-1, TNF-α, and PDGF in mesenchymal cells. A similar upregulation is also linked to re-epithelialization of split thickness wounds (Marchese et al., 1995). FGF2 increases in the acute wound, and plays a role in granulation tissue formation, re-epithelialization, and tissue remodeling (Powers et al., 2000). FGF2 plays a role in regulating the synthesis and deposition of various ECM components such as collagen by fibroblasts. It also increases keratinocyte motility during re-epithelialization (Sogabe et al., 2006).

2.6.4 Transforming growth factor β (TGFβ)

In wound healing TGFβ exerts a broad range of biological effects, such as keratinocyte growth inhibition, production of extracellular matrix, and synthesis of plasminogen activator and its inhibitor (Sellheyer et al., 1993). In acute wounds, it binds to a heteromeric receptor complex consisting of a combined type I and type II receptor in fibroblasts, stimulating the production of collagen (Werner and Grose, 2003). Additionally, a non-signaling type III receptor exists, which has the function of presenting TGF-β to the type II receptor (Liu et al., 2005). After autophosphorylation of serine-threonine kinases, the receptors activate downstream signaling molecules belonging to the Smad-family of transcription factors (Liu et al., 2005).

2.6.5 Platelet-derived growth factor (PDGF)

The PDGF family consists of growth factors including PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD. In addition to platelets, the macrophages, endothelial cells and keratinocytes produce PDGFs (Bennet et al., 2003). PDGFs bind to two separate transmembrane tyrosine kinase-receptors (α and β). Upon ligand binding and autophosphorylation, these receptors submit intracellular signaling through Src homology 2 (SH2) domain-containing signaling molecules (Lederle et al., 2006). PDGFs exert various actions on fibroblasts and are considered predominantly keratinocyte-derived growth factors acting in a paracrine manner on fibroblasts supporting their proliferation and ECM-production (Werner et al., 2007). Additionally, the PDGF-BB isoform is able to induce KGF in mesenchymal cells and, thus affect keratinocyte migration and proliferation (Brauchle et al., 1994).

2.6.6 Interleukin 1, interleukin 6, and interleukin 8

Inteleukins are polypeptides produced by activated cells during of inflammatory processes and have multiple targets and functions. Inflammation, trauma, and infection, among others, trigger a rapid release of pro-inflammatory cytokines IL-1, IL-6, TNFα and IL-8. Interleukins are the central effector controlling production of acute-phase proteins and participate in alerting the body to acute changes (Gabay and Kushner, 1999). IL-1 is released immediately by keratinocytes in response to barrier injury and is produced as well by neutrophils, monocytes, and macrophages. It functions in both a paracrine and in an autocrine manner to cause increased keratinocyte migration and proliferation (Raja et al., 2007). In addition, IL-1 participates in mesenchymal-epithelial interaction (Figure 3) by activating fibroblasts to produce increased amounts of KGF (Tang and Gilchrest, 1996). IL-6 is a key cytokine in the initial steps of the wound-healing response. It is produced by neutrophils and macrophages, and has both pro- and anti-inflammatory properties. IL-6 has a mitogenic and proliferative effect on keratinocytes and its expression increases significantly after wounding. Interestingly, IL-6-knock-out mice display significantly delayed healing (Gallucci et al.,

2004). Another prominent cytokine controlling keratinocyte responses is IL-8. Its primary role is to induce chemotaxis of neutrophils, but it also promotes migration and proliferation of keratinocytes (Schröder, 1992; Tuschil et al., 1992).

Growth factors	Cells	Receptor in keratinocytes
EGF-family	Fibroblasts Platelets Macrophages Keratinocyte (autocrine)	ErbB1 (EGF, TGF-α, amphiregulin HB-EGF, betacellulin and epiregulin ErbB1 (neuregulin)
Hepatocyte growth factor (HGF)	Fibroblasts	c-Met
Keratinocyte growth factor (KGF)	Fibroblasts	FGFR2IIIb
Fibroblast growth factor 2	Keratinocytes Mast Cells Fibroblasts Endothelial cells Smooth muscle cells	FGFR2IIIb
Transforming growth factor β	Keratinocytes Macrophages Lymphocytes Fibroblasts	TGF-beta receptor I and II
Platelet-derived growth factor (PDGF)	Platelets Keratinocytes Macrophages Endothelial cells	PDGF receptor α and β
Interleukin 1, 6, 8 (IL-1, -6 and -8)	Neutrophils, monocytes macrophages	Interleukin receptor

Table I Summary of central factors promoting keratinocyte functions in wound healing

2.7 Phosphoinositide-3-kinase (PI3K) signaling

Various growth factor receptors, such as EFGR, c-Met and PDGF receptor, belong to a group of tyrosine kinase receptors. Binding of the ligand to its specific receptor triggers autophosphorylation of the tyrosine kinase enzyme, which activates various intracellular downstream cascades. One of these pathways, the phosphoinositide-3-kinase (PI3K), is closely associated with wound healing. It initiates signaling leading to keratinocyte proliferation and migration, and also contributes to epidermal homeostasis (Pankow et al., 2006). In addition, PI3K-signaling is also involved in processes related to cell death and survival, and to intracellular vesicular transport (Vanhaesebroeck et al., 2012). An inhibitor of PI3K, LY294002 and its structural inactive analogue LY303511, have been useful in characterizing the effects of PI3K signaling (Arcaro and Wymann 1993; Vlahos et al., 1994).

Tyrosine kinase receptors activate intracellular programs through PI3K, such as the signaling cascade that leads to activation of the mitogen-activated protein kinases (MAPKs) (Figure 5). This cascade involves at least three levels of hierarchically organized kinases. One level of MAPKs consists for example of extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK1/2); c-Jun amino-terminal kinases (JNKs) 1, 2, and 3; p38 isoforms α, β, γ and δ, ERKs 3 and 4; and ERK5. A broad array of stimuli can activate MAPKs, but in general, ERK1 and ERK2 are preferentially activated in response to growth factors, while the JNK and p38 kinases are more responsive to stress stimuli ranging from osmotic shock and ionizing radiation to cytokine stimulation (Pearson et al., 2001). ERK1/2 signaling has been associated with cell proliferation. There occurs considerable divergence and cross-talk between pathways on multiple levels. Two structurally independent compounds, U0126 (inhibits both mitogen-activated protein kinase kinase 1 and 2: MEK1/2) and PD98059 (inhibits MEK 1) can serve to inhibit this cascade one level before MAPK (Ballif and Blenis, 2001). One of the prototype members of the MAPK-related pathway in mammalian cells is p38, which environmental stress and inflammatory cytokines activate. SB203580 is a typical p38 inhibitor, which has been very useful in describing the function of p38 in various experimental settings (Lee et al., 1994) (Figure 5).

2.8 Non-healing wounds

2.8.1 Chronic ulcers

By definition, chronic skin wounds fail to proceed through the orderly healing process over a period of 3 months according to Mustoe et al (2006) who defined four mechanisms in the pathogenesis of chronic wounds: local tissue hypoxia, repetitive ischemia-reperfusion injury, bacterial colonization of the wound, and altered cell and systemic response. Classically, three main factors account for the majority of patient cases: venous insufficiency, peripheral artery disease, and diabetes (Singer and Clark, 1999). Estimates are that in Finland 20% of men and 40% of women suffer from venous insufficiency (Laurikka et al., 1993) and

it is present in up to 75% of cases with chronic wounds. Peripheral artery disease is present in up to 22% and diabetes in 25% of such cases (Graham et al., 2003; Briggs et al., 2004). Lymphodema is becoming an even more common factor behind impaired wound healing. Damage of the lymph system, caused by surgical removal of lymph nodes or radiation therapy, impairs lymph drainage and creates massive swelling in the affected limb. This subsequently decreases skin perfusion and results in breakdown and infection (Kershner et al., 2008). Moreover, postinfectious damage of the lymph circulation can result from recurrent streptococcal erysipelas infections. Treatment of lymphodema and wounds caused by it is challenging. However, novel methods of gene therapy-induced lymphangiogenesis are promising (Cook, 2012).

In addition, conditions such as vasculitis, malignancy, and connective tissue disorders may lead to development of a chronic wound. Pressure ulcers are heterogenous chronic-wounds caused by is constant pressure of the skin causing local tissue hypoxia (Phillips, 1994). Commonly a patient has more than one etiological factor causing the wound (Phillips, 1994).

In the western world, the prevalence of chronic wounds ranges between 0.04 and 1.1% (Graham et al., 2003; Moffatt et al., 2004). It is estimated that prevalence of active venous ulcers, the largest group of wounds, in the general population of older than 18 year olds is 0.3%; incidence rate increases multiple times in the elderly population and is more common among females (Nicolaides, 2000; Laurikka et al, 1993). As chronic wounds commonly heal extremely slowly, within months or even years, they require multiple treatments and regular monitoring. Futhermore, once healed, chronic wounds frequently reappear. Based on multiple studies, it was concluded by the Royal Society of Medicine and Societas Phlebologica Scandinavica (1999) that the risk of recurrence of a venous ulcer is about 3-15%. Based on the figures presented by the wound society of Finland (Finnish Wound Treatment Society, 2008) the estimate is that in Finland each year the cost toward treatment of chronic ulcers is 190 to 270 million euros. The annual sum spent on drugs and other medical treatments per year is 2.2 billion euros, so treatment of chronic wounds is a noticeable in the total annual health care budget (Hjerppe et al., 2006; Health Expenditure and Financing 2009).

Pathogenesis of chronic wounds

Chronic wounds fail to proceed through the normal phases of wound healing, and remain in a chronic inflammatory state (Loots et al., 1998). Chronic disease, such as diabetes or venous insufficiency, disturb the normal inflammatory phase of wound healing, and causes inflammatory cells to produce proinflammatory cytokines in abundance. Futhermore, in venous insufficiency, the stasis causes pressure in the microcirculation, which exacerbates inflammatory processes; leukocyte migration into the interstitium increases, and cytokine and growth factor production enhances even more. The influx of neutrophils and macrophages is further aggravated by bacterial colonization, tissue hypoxia and decomposition. This exhausts the tissues intrinsic protective mechanisms and compromises healing (Palolahti et al., 1993;

Saarialho-Kere et al., 1998). The increased activity of MMP-2 and MMP-8 degrades growth factors and structural ECM proteins crucial for wound healing. In chronic venous stasis ulcers, the expression and activity of various MMPs, including collagenases (MMP-1, MMP-8), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), and the membrane type MMP (MT1-MMP) are upregulated (Saarialho-Kere, 1998; Gill and Parks, 2008). These observations support the idea of treating chronic wounds by replacing damaged ECM components and growth factors (Eming et al, 2002; Schultz et al, 2009).

2.8.2 Burns

Data from two federal surveys in the United States, National Hospital Ambulatory Medical Care Survey (NHAMCS) and National Ambulatory Medical Care Survey (NAMC), show that approximately 450,000 burn injuries each year receive medical treatment including 45,000 hospitalizations for burn injury (American Burn Association, 2011). In Finland, 900 patients per year require hospitalization, of whom 10% are treated in intensive care units (personal information, Dr. J.Vuola). Burns heal slowly and require several wound treatments and multiple reconstructive operations to cover tissue defects as well as post-burn scar contractures.

Complex skin wounds require long hospitalization periods and cause an ever-growing burden on healthcare costs. Moreover, the incidence rates of diabetes, obesity, venous insufficiency, and peripheral artery disease show an upward trend as the population ages. WHO estimates that now 346 million people in the world suffer from diabetes and that diabetes deaths will double between 2005 and 2030. Notably, 15% of these diabetic patients will develop a chronic wound at some point in their lives (Frykberg, 1999).

2.9 Surgical treatment of complex skin wound by skin transplantation

The gold standard treatment for skin defects is transplantation of a split-thickness skin graft (STSG). Meshing the graft by making lengthwise rows of short, interrupted cuts, enables larger defects to be covered. Meshed grafts also provide more efficient drainage of wound exudate and conform better to irregular surface contours. Meshing, however, creates holes, interstices, that must be filled by keratinocyte proliferation and migration from the surrounding graft; scarring is common when mesh-size is larger. Micrografting is an alternative method for standard STSGs (reviewed by Biswas et al., 2010). Meek (1965) developed a micrografting method, in which an STGS is sliced with a microdermatome to create small cell islands which are transplanted to the wound with or without a carrier. These cell islands have a greater surface area in relation to size, making cell migration from the margins more efficient. Moreover, MEEK-micrografting provides more accurate expansion rates compared to meshed grafts (Lumenta et al., 2011).

Full-thickness skin grafts consist of the epidermis and the entire dermis and serve for reconstruction of small wounds of the head, neck, hands, and areas of the genitals and breasts, in areas where it is crucial to avoid contracture (Grabb & Smith's Plastic Surgery, 1997). Pinch grafts are full-thickness grafts obtained by inserting a needle superficially in the skin and harvesting small pieces of skin with a scalpel. Punch/postage stamp grafts, on the other hand, are small full or split-thickness grafts cut into uniform small squares. Punch grafting became however less frequent with the improvement in meshing techniques (Biswas et al., 2010)

Harvesting a skin graft creates a donor-site wound (Lagus and Vuola, 2004). Full-thickness graft donor sites must be treated either by suturing or covering with an STSG. Donor sites of STSGs extend to the superficial dermis and heal well within 2 to 3 weeks by re-epithelialization. Donor-site wounds may be painful, and healing can be delayed in elderly patients and when the body is under systemic stress.

2.10 Tissue engineering and skin equivalents

Tissue engineering is an interdisciplinary field of science, combining biology, medicine, chemistry, and material science, among others, to develop regenerative therapies for replacing damaged or diseased tissues. It is called "a field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function". (Langer and Vacanti, 1993). These researchers characterized three general strategies for construction of artificial tissues:

1.Use of isolated cells or cell substitutes to replace damaged tissues with possible manipulation of cells before transplantion.

2. Application of appropriate tissue-inducing substances and signal molecules, such as growth factors, combined with a matrix carrying them to the damaged tissue to control and direct regeneration processes.

3. A carrier matrix (scaffold) with or without cells in **a)** closed systems, where cells are isolated from the body, allowing permeation of nutrients and wastes but preventing antibodies or immune cells from destroying the implant. In **b)** open systems, where the implanted matrix and cells are in direct contact with the recipient site and eventually become incorporated into surrounding tissues.

Attempts to create tissue-engineered skin by cell culturing with biological and synthetic materials have been made during the last decades (Table II). However, even though much is now known about the cellular interactions that guide skin regeneration, reconstruction of the anatomy and physiology of healthy skin embracing all crucial elements, is not yet possible (Metcalfe and Ferguson, 2007).

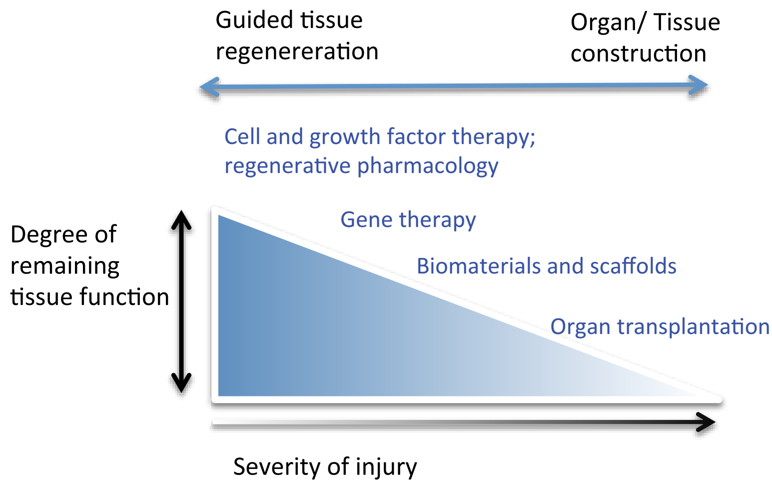

Figure 6. A schematic picture describing the basic concept for tissue engineering. Adapted from Corona et al. Regenerative medicine: basic concepts, current status, and future applications. *J Investig Med* 2010;58:849-58. Used with permission of copyright holder.

2.10.1 ECM scaffolds for tissue engineering and skin regeneration

Tissue-engineered dermal scaffolds mimic ECM structures and serve in guided tissue regeneration; a process in which the three-dimensional structure of the scaffold provides the correct milieau for host cell ingrowth and proliferation and subsequent regeneration of the tissue in situ (Figure 6). In addition to the promotion of cell migration, an ideal scaffold should provide protection againts water loss and infection. Its also must have adequate elasticity and mechanical resistance, and elicit a low immunogenic response (van der Veen et al., 2010). The scaffold structure influences various cellular differentiation processes; fibroblasts play an important role in guided tissue regeneration. Fibroblasts infiltrate into the scaffold, produce ECM proteins, and simultaneously degrade the implanted scaffold.

Dermal scaffolds are divided into natural biological, constructed biological and synthetic substitutes (review by van der Veen et al., 2010). Biological scaffolds are natural polymers constructed from such materials as collagen, fibronectin, chitosan, and fibrin. An interesting biopolymer for tissue engineering purposes is spider silk. Similarly to collagen, it can be produced in large amounts by recombinant techniques and then used for tissue engineering (Ruggiero and Koch, 2008; Fredriksson et al., 2009). In general, biopolymers elicit low toxicity and a less extensive chronic inflammatory response (Metcalfe and

Ferguson, 2007). Constructed biological scaffolds are modified with the addition of specific proteins, for example GAGs to improve rigidity. On the other hand, synthetic polymer scaffolds can be specifically produced and tailored. Additionally, they can be manufactured in large quantities inexpensively and have long shelf-life. Common synthetic absorbable materials include polylactic-co-glycolide (PLG), polyglycolic acid (PGA), polylactic acid (PLA), and polycaprolactone (PCL).

Epidermal reconstruction is based on transplantation of either autologous or allogenic cultured keratinocytes, which are transplanted as sheets or suspensions on the granulation tissue-containing neodermis that forms when the scaffold is degraded. Different scaffolds used as artificial skin substitutes will be described in the following section.

2.11 Surgical treatment of complex skin wounds by artificial skin substitutes

2.11.1 Cultured autologous skin substitutes

Cultured autologous skin grafts, frequently referred to as cultured epithelial autografts (CEA), consist of sheets of multiple layers of keratinocytes and lack a proper dermis. In the 1970s, the pioneer work of Rheinwald and Green made cell expansion techniques possible with the use of irradiated murine fibroblast feeder layers (Rheinwald and Green, 1975). Since the 1980s, this method has been clinically succesful for extensive skin defects such as burns (Green et al., 1979; Green, 1991; Wood et al., 2006). O'Connor et al. (1981) first reported the use of cultured epithelial autografts in the treatment of major burns. Various commercial products gradually emerged, but use of CEAs was limited due to mechanical fragility, lengthy cultivation periods, poor take and expensive price (Wood et al., 2006). Because CEAs lack dermal-epidermal junctions, complexes needed for connecting the epidermis to the dermis, they may adhere poorly to wound beds of variable composition (remnants of fat, muscle, fascia, dermis, or granulation tissue). Additionally these sheets are mechanically instable and prone to spontaneous blistering (Hefton et al., 1986; Hunyadi et al., 1988; Christiano and Uitto, 1996). Among the first commercial products to arise from this technology was EpiCel™. Although Epicel is made from autologous keratinocytes, it can be considered a xenotransplantation product. Manufacturing is made by co-cultivation of keratinocytes with proliferation-arrested mouse 3T3-fibroblast feeder cells. Epicel can be combined with petrolatum gauze to form transplantable keratinocyte sheets (Wright et al., 1998; Carsin et al., 2000).

The initial observation that CEAs were prone to infection and were mechanically fragile, led to development of keratinocyte suspension therapy. The hypothesis was that keratinocytes remain in a migratory non-differentiated state when seeded as suspensions and thus may be more efficient for re-epithelialization. These treatments were also cheaper, required less manual labour, and were rapidly and easily obtainable. Hunyadi et al (1988) first

described the addition of a fibrin matrix enhancing keratinocyte suspension adhesion to the wound bed. When fibroblasts were added as a supporting layer, adherence of transplanted cellular grafts onto wounded skin was even more efficient (Currie et al., 2001). Autologous keratinocyte stem cells can also be retrieved from hair follicles. EpiDex™ technology utilizes these cultured stem cells for transplantation and wound therapy (Tausche, 2003). Additional alternatives for cell delivery are, Myskin™ a polymer carrier-dressing, (Haddow, 2003) and, CellSpray™ a cell suspension spray (Navarro et al., 2000). Due to the lack of dermis, suspension treatments are associated with the same problems as are other CEAs.

Cultured skin substitute (CSS) is a CEA with an additional autologous dermal cell layer (Boyce et al., 2006). The culturing of autologous fibroblasts and keratinocytes together in a human collagen and glycosaminoglycan scaffold produces a skin substitute with an improved anatomical structure. However, the presence of melanocytes in the culture may cause uneven pigmentation (Harriger et al., 1995). Boyce et al (1995) observed an average take of 75% for the CSS in the treatment of full-thickness burns. After 1-year follow-up, however no differences occurred in the healing of autograft- or CSS-treated wounds (Boyce et al., 1995). Commercially available alternative technologies include: TissueTech™ (an esterfied hyaluronic acid matrix with autologous fibroblasts and keratinocytes), VCT01™ (an autologous fibroblast-derived ECM with overlaying keratinocytes), and LOEX skin substitute (similar to VCT01™). Autologous bilayer substitutes produce better healing but are restricted by their long culturing periods and expense.

2.11.2 Allogenic grafts

By definition, allografts are transplants between genetically nonidentical individuals of the same species. De-epithelialized dermal allografts (DED) are produced from human cadaveric donors. The acellular structure of DED serves as a scaffold for ingrowth of wound bed fibroblasts and endothelial cells. Additionally, it provides a barrier for deep partial-thickness wounds (Dalla Vecchia et al., 1999). Prior to application, the DEDs must be cryopreserved, lyophilized or glycerolized to remove all possible donor-derived cellular material, which could be infectious or antigenic. Wainwright observed that the combined use of an acellular dermal autograft AlloDerm™, with STSGs allowed use of thinner autologous grafts and improved their take (Wainwright, 1995).

Human amniotic membrane consists of a single epithelial layer, a thick basement membrane, and an avascular stroma. Underlying the epithelial layer and basement membrane is the spongy stromal compartment containing hydrated proteoglycans, glycoproteins, and collagen III that form a reticular network (Aplin et al., 1985). Epithelial cells can be cultured on a de-epithelialized amnion scaffold, a method used in reconstruction of cornea and skin (Yang et al., 2006). Additionally, amniotic membranes provide protection from evaporative loss and are effective as temporary wound dressings (reviewed by Kesting et al., 2008). Due to possible transmission of some infectious agent, only a fraction of deliveries are eligible for collection of amniotic membranes; moreover, storage conditions unfavorably modify its

properties (Niknejad et al., 2008). Due to cost-efficiency membranes are still popular in developing countries for the treatment of burn patients, and have been advocated especially for the treatment of pediatric burn patients (Ravishanker et al., 2003; Branski et al., 2008).

Addition of allogenic fibroblasts to a dermal substitute enhances its wound-healing properties through synthesis of ECM components and production of growth factors. Moreover, this results in better cosmetic results and less scarring (Purdue et al., 1997 and Lukish, 2001). Dermagraft™, a knitted biodegradable PGA/PLA mesh containing human neonatal fibroblasts releases growth factors (including FGF-1, -2 and -7, HGF, IGF, PDGF α and β, TGFβ, VEGF). It is one of the few products indicated for the treatment for chronic leg ulcers (Marston, 2004). ICX-SKN is a dermal substitute comprised of human neonatal fibroblast-produced collagen scaffold (Boyd et al., 2007). TransCyte (formerly known as Dermagraft-TC), on the other hand, uses a temporary silicone membrane and a nylon mesh covered with porcine dermal collagen incorporated with neonatal fibroblasts. Dermagraft™ and ICX-SKN are biodegradable, an obvious advantage over the nondegradable TransCyte. Storage by cryopreservation reduces cell viability and subsequently growth factor production of the Dermagraft™ scaffold and hinders its effectiveness (Mansbridge et al., 1998).

Composite allografts are the most advanced products currently commercially available. Their structure closely resembles that of normal human skin. Apligraf® uses a combination of bovine type I collagen gel with living neonatal fibroblasts as the dermal component and a cornified epidermal layer composed of human foreskin-derived neonatal keratinocytes (Trent and Kirsner, 1998). The product is approved by the FDA for the treatment of chronic wounds and has been commercially available for several years. Falanga et al (1998) published a prospective, randomized, multicenter study of 293 patients, in which the efficacy of Apligraf® was compared with compression therapy for the treatment of chronic venous ulcers. In a follow-up for 6 months, patients receiving treatment with Apligraf® were 3 times as likely to experience complete healing of chronic ulcers due to venous insufficiency. Similar results were published later by the same authors in a prospective, randomized, controlled trial of 120 patients who showed complete healing of a difficult venous ulcer with standard compression versus Apligraf® (Falanga and Sabolinski, 1999). Orcel® is an analogous bilayered cellular product with a collagen-coated bovine collagen sponge as its cell scaffold. Allogenic dermal fibroblasts are cultured within the porous sponge and allogenic keratinocytes are seeded in the collagen gel to prevent ingrowth of cells to the scaffold (Eisenberg and Llewelyn, 1998). However, less clinical data are published than for Apligraf®.

2.11.3 Advanced wound dressings

The epidermal barrier can be temporarily restored with different advanced wound dressings. Suprathel® is an acellular synthetic dressing composed of a copolymer of DL-Lactide trimethelcarbonate and α-caprolactone. It is water permeable, permits transfusion of excess moisture, and degrades gradually (Uhlig et al., 2007). It can effectively cover STSG donor sites and partial-thickness burns (Uhlig et al., 2007; Schwarze et al., 2007). Clinical

reports indicate that pain and bleeding associated with dressing changes is significantly lower but re-epithelialiazation time is unaffected (Uhlig et al., 2007; Kaartinen and Kuokkanen, 2011). Martiderm® on the other hand is composed of bovine collagen types I, III, V, and elastin. It is used to treat partial or full-thickness burns.

Bilayer substitutes function as dermal templates that promote ingrowth of host cells. In addition to the dermal component, commonly composed of animal-derived collagen, an epidermal layer helps to protect the wound from moisture loss. Developed back in the 1970s, Biobrane® and the Integra® Dermal Regeneration Template (DRT) are the most frequently used. Biobrane consists of a bi-laminate nylon mesh membrane bonded to a thin layer of silicone. The nylon mesh is coated with porcine type I to support granulation tissue formation, and the silicone layer performs the function of the lost epidermis. Although initially designed for the treatment of deep complex wounds, Biobrane is now generally regarded as a dressing for superficial burns (Greenwood et al., 2009).

Research done by Yannas and Burke, led to the development of Integra® DRT (Burke et al., 1981), an acellular scaffold composed of an outer silicone elastomer sheet and a bovine collagen and chondroitin-6-glycosaminoglycan dermal template. Integra®DRT promotes fibroblast and endothelial cell ingrowth from the host wound bed, and gradual remodeling of the matrix creates a neodermis. The dermal component is incorporated into the wound bed within 3 to 6 weeks (Stern et al., 1990). When the matrix is adequately vascularized, the upper silastic sheet is removed, and ultra-thin skin grafting is performed. Problems in these off-the-shelf products are their high price, potential for infection, and the need for secondary operations.

2.11.4 Xenografts

Xenograft, tissue taken for transplantation from another species, can serve as either temporary coverage for wounds or as templates for tissue regeneration. De-epithelialized xenografts that do not support cell ingrowth are intended mainly for temporaty coverage of clean partial-thickness wounds (Chiu and Burd, 2005). Recent modifications to porcine-derived de-epithelialized dermis include aldehyde cross-linking and impregnation of silver ions to enhance the grafts antimicrobial properties (Chiu and Burd, 2005). Commercial xenografts that support cellular ingrowth are Permacol™, an acellular porcine dermal collagen, and Oasis™, an acellular porcine small intestine submucosa-based product. Reports of Oasis suggest enhanced healing of chronic venous ulcers (Romanelli et al, 2010). Permacol, however, is mainly used as a treatment for hernias repair; the FDA considers is unsuitable for the treatment of chronic wounds (Mostow et al., 2005; Romanelli et al., 2010).

Table II History of skin substitutes

year	1895	1970	1975	1980	1985	1990	1995	2000	2005	2010
Epidermis-based skin substitute	Mangolt et al. Attempt to close wounds by epithelial seeding	Rheinwald & Green Cultivation of keratinocytes on feeder layer	Green et al. Keratinocyte transplantation **Epicel®**	O´Connor et al. Treatment of burn patient with keratinocyte suspension			**EpiDex®** Cultured hair follicle keratinocytes	autologous keratinocyte treatments: **CellSpray®** **Myskin®**		
Combined epidermal and dermal skin substitute						Boyce et al. Cultured skin substitute **Apligraf®,** Allogenous neonatal keratinocytes and fibroblasts porcine collagen I		**ICX-SKN** fibroblast-produced collagen scaffold	**VCT01™ and LOEX skin substitute** Autologous fibroblast-derived ECM with cultured keratinocytes	
Dermis/ scaffold-based skin substitutes	Cadaver skin **Alloderm®** Deepithelialized dermis		Burke et al, development of **Integra®**		**TransCyte®** Neonatal fibroblasts porcine dermal collagen with nylon mesh		**Dermagraft®** Neonatal fibroblasts lactic/ glycolic acid scaffold			

2.12 Growth factor therapy for epithelial regeneration

Various reports show that active growth factor levels drop decrease in non-healing wounds (reviewed by Barrientos et al., 2008). In extensive skin damage, substantial tissue loss compromises dermal reactivity, thus delaying reepithelialization by lack of dermal signals, and subsequently hindering final healing. Clinical experience on growth factor supplementation to accelerate wound healing, however has been discouraging (reviwed by Robson, 1997). Presently only three growth factors are effective in patient treatment: PDGF-BB, FGF, and GM-CSF. PDGF-BB is the only growth factor that has successfully completed randomized clinical trials in the United States, and is indicated for treatment of chronic wounds.

First reports in by Brown et al (1989) indicated improvement of wound healing by topical EGF treatment. Clinical trials later confirmed that wound treatment with topical EGF increases epithelialization and granulation tissue formation in venous ulcers. (Falanga et al., 1992). Moreover a randomized placebo-controlled multicenter study, Fernández-Montequín et al (2009) showed that intra-lesional injections of recombinant human EGF significantly increase granulation tissue formation and accelerate wound closure of diabetic ulcers.

A promoter of keratinocyte migration, KGF, has been studied for its possible clinical use in chronic wounds. Palifermin (rh-KGF) received approval by the FDA for treatment of oral mucositis in patients with hematologic malignancies who receive high doses of chemotherapy (Weigelt et al., 2011). Furthermore, a trial using topical application of Repifermin (rh-KGF2, FGF-10) resulted in accelerated healing of patients suffering from venous ulcers (Robson et al., 2001) and showed good results in a Phase II clinical trial.

TGF-β has been studied intensively, since various studies have shown it to be decreased due to degradation by proteolytic enzymes, particularly neutrophil elastase (Gill and Parks, 2008). Early work with TGF-β on the treatment on venous stasis ulcers was promising, but multiple trials have failed to prove efficiency for the treatment of chronic wounds (Robson, 1995).

Recombinant human PDGF-BB (becaplermin; Regranex®) gel was approved by the FDA for treatment of deep lower-extremity diabetic neuropathic ulcers that extend to the subcutaneous tissue. It has been applied with success in diabetic and pressure ulcers, being the only FDA approved drug for chronic wound treatment (Wiemann et al., 1998), and PDGF gene therapy has been applied for treatment of patients with diabetic ulcers (Margolis et al., 2004; Jinnin et al., 2005).

GM-CSF is increased in the epidermis of wounded skin, taking part in recruitment and activation of neutrophils during its inflammatory stage (Mann et al., 2001). Additionally, GM-CSF increases keratinocyte proliferation both directly and by up-regulation of IL-6 production. In patients with diabetic ulcers, because subcutaneous injections of GM-CSF

showed faster healing, the general opinion is that it could be of potential use for treatment of chronic wounds (Bianchi et al., 2001). In a randomized, blinded, controlled study by Marques et al (1997) GM-CSF proved to promote healing of leg ulcers when injected perilesionally. Wu et al (1997) studied the mechanisms by which wound healing are upregulated by GM-CSF. It seems that GM-CSF causes the generalized enhancement of activation of the tissue macrophage via upregulation of TGF-β. Recombinant human GM-CSF is sold as Leucomax (molgramostin) and Leukine (sargramostin).

2.12.3 Cell-lysate based products

Platelet-rich plasma (PRP), an autologous plasma-derived platelet suspension, has served as a wound treatment for over two decades. In addition to its function as a tissue sealant, PRP contains a broad range of growth factors, which are released by degranulation from the platelets. The composition of the product varies depending on the protocol, by which the platelet are collected, but at least PDGF-AA, BB, and AB isomers, TGF-β, platelet factor 4, IL-1, platelet-derived angiogenesis factor, VEGF, platelet-derived endothelial growth factor (PDEGF), EGF, epithelial cell growth factor (ECGF), and IGF have been found in PRP (reviewed by Lacci and Dardik, 2010). In a multicenter trial, autologous PRP was used for the treatment of diabetic foot ulcers in 72 patients with type I - or type II diabetes (Driver et al., 2006). After a 12-week treatment period with either PRP or normal saline gel, the authors observed that 68.4% of patients in the PRP-group versus 42.9% in the saline group had complete wound closure. The inconsistent methodologies in producing PRP make it difficult to directly compare different studies.

2.13 Tissue engineering of carrier matrices for topical treatment with growth factors

In addition to their use as dermal scaffolds, fibrin, collagen and gelatin can be combined with growth factors produced by recombinant technologies in yeast or bacteria to provide a slow-releasing high-concentration carrier for topical treatment of wounds (Uebersax et al., 2009).

Collagen

Collagen has been used extensively in bioengineering, and various animal-derived collagen-based skin substitutes have been introduced during recent years (Metcalfe and Ferguson, 2007). Twenty five types of collagen exist, with types-I, II, and III present most abundant in tissues. Collagens I, II, and III form rod-like right-handed triple helices consisting of three coiled subunits composed of two alpha and one beta chain, which are synthesized predominantly by fibroblasts, epithelial cells, osteoblasts, and chondrocytes (Hulmes et al., 1973). Besides providing structural and mechanical properties to tissue, collagens can interact

with growth factors and cytokines (Friess, 1998; Kanematsu et al., 2004). For controlled release, the therapeutic agent is bound into collagen structures by hydrogen bonding, covalent bonding or simple entrapment (Lee et al., 2001). Release of factors is facilitated by enzymatic cleavage of the collagen scaffold by collagenases produced by cells such as macrophages and fibroblasts. However, manipulation of collagen by chemical crosslinking makes it more resistant to this effect (Itoh et al., 2002; Uebersax et al., 2009) and concentrated collagen hydrogels seems to be more resistant to degradation (Helary et al., 2010).

Gelatin

Collagen degradation produces gelatin. Both collagen and gelatin are considered biodegradable scaffolds. In addition to biodegradability, dehydration of gelatin produces a porous sponge, which can be used for cell culture. Macroporous biodegradable gelatin spheres offer a novel alternative for dermal reconstruction. Cultivation of ecto- and mesodermal lineage cells has been successful (Huss et al., 2007). In an in vivo experiment, intradermally injected gelatin spheres stimulated dermal regeneration and degraded gradually within 26 weeks of the injection (Huss et al., 2010). Gelatin is generally considered mechanically instable, but cross-linking with GAGs has been show to improve its fragility and ease handling (Choi et al., 1999).

Fibrin

Fibrinogen is a large and complex glycoprotein consisting of three polypeptide chains linked together by 29 disulfide bonds. Thrombin cleaves serum fibrinogen to form an insoluble fibrin polymer (Mosesson, 2005). A wound-sealing fibrin clot forms when fibrils aggregate (Ferry, 1952). Fibrin is also one of the proteins taking part in the formation of a provisional matrix found in granulation tissue (Clark et al., 1982) and it is used commonly in surgery as a wound sealant and tissue adhesive. Fibrin has several advantages as a growth factor delivery matrix for clinical use due to its low host reaction and immune responses (Spiker and Mikos, 2010); a hydrogel is easily inducible by mixing fibrinogen with thrombin before application. Various growth factors such as FGF and EGF can be easily incorporated in a fibrin matrix and both fibrinogen and thrombin concentrations affect their release (Jeon et al., 2005). Pandit et al (1998) reported that FGF 1-loaded fibrin matrices significantly improved the mechanical properties of the regenerated tissue in full-thickness wounds in rabbits after 2 weeks. Moreover, Geer et al (2005) showed that application of fibrin-bound KGF to skin wounds increased epithelialization in athymic mice (Geer et al., 2005). Fibrin matrices are thus under investigation for use in growth factor therapy, cell culture, and tissue engineering (Spotnitz and Prabhu, 2005).

Electrospinning

Because the dermis is principally composed of nanoscale fibers of collagen providing structural integrity and mechanical strength, recent focus of tissue engineering has been the development and fabrication of ECM analogues composed of nanoscale fibers. The method, electrospinning, involves the application of an electrostatic force between the polymer solution and a counter metal electrode, which is kept at a certain distance. Resulting in porous nanofiber (poresize 1-10μm) matrices that mimic the structure of native ECM and can be produced simply and rather inexpensively. The use of electrospun scaffolds as a wound dressing or cellular carrier for skin substitutes has been suggested for a variety of materials including collagen, gelatin, fibrinogen, PCL and PLA (Powell et al., 2008; Kumbar et al., 2008). Even though extensive in vitro work has been made to prove the efficiency of elecrospun materials as tissue scaffolds or skin constructs for wound treatment, very limited in vivo applications have been reported and further research is needed to prove the clinical use of these products (Chong et al., 2006).

2.14 Fibroblast aggregation as an activator of growth factor production

The inflammatory response has long been considered pivotal to providing growth factor and cytokine signals orchestrating the cell and tissue movements vital for skin repair. A central link in this process is the activation of fibroblasts, characterized by vast production of proinflammatory cytokines as well as cyclooxygenase 2 (COX-2) (Buckley et al., 2001). Furukawa et al (2001) described a way to mimic fibroblast activation in vitro with the formation of human skin fibroblast aggregates using a rotational culture. These authors reported that TGF-β was upregulated in the fibroblast spheroids and suggested that combination of fibroblast spheroids in a collagen-coated PGA mesh could improve dermal wound healing (Furukawa et al., 2001). Bizik et al (2004) stated that the formation of densely packed spheroids initiates a proinflammatory, proteolytic, and growth factor response, a process that was designated as nemosis. Fibroblasts were activated by seeding them onto non-adherent surfaces, such as agarose-coated wells or to “hanging drops” depriving the cells of the possibility of adherence to the culture dish (Salmenperä et al., 2008).

A central feature of fibroblast aggregation is the massive induction of COX-2 and secretion of prostaglandins (PG): PGE2, 6-keto-PGF1α, PGF2α, and PGD2 (Kankuri et al., 2005). The cell clustering in nemosis occurs cellular fibronectin interaction with its integrin receptors (alpha5 and beta1) via Arginine-Glycine-Aspartic acid (RGD) motif, (Salmenperä et al., 2008). Characteristic to the activation of fibroblast aggregates is the production of proinflammatory cytokines (IL-1, IL-6, leukemia inhibitory factor (LIF), and GM-CSF) and chemokines (macrophage inflammatory protein- (MIP-1α), RANTES, and IL-8) as well as growth factors. One particular growth factor HGF, upregulated 1000-fold on the mRNA-level, caused mitogenic and motogenic responses of cells expressing the properly processed receptor c-Met (Kankuri et al., 2005 and 2008). In addition to upregulation of HGF, VEGF is also

evident (Enzerink et al., 2009). Fibroblast nemosis activation (measured as induction of COX-2 and IL-8) occurs at least in neonatal foreskin, fetal skin and lung, and lung-derived fibroblasts (reviewed by Vaheri et al., 2009).

2.15 In vitro model of epidermal wound healing; scratch assay

The in vitro scratch assay is a feasible and cheap method to study motility of adherent cells into cell-free space, mimicking the cell migration seen in wound healing (Todaro et al., 1965). A gap -a scratch- is created on a confluent cell culture monolayer mechanically with for example the tip of a pipette; movement of cells on the edge of the newly created gap is monitored until the empty space between the edges is filled (Haudenschild and Schwartz, 1979). Images are captured from the same location at regular intervals to determine rate of closing of the gap. An advantage of this method is that it can be combined with other techniques such as stimulation and inhibition of cell migration with pharmacological agents. Additionally, the migration path of individual cells can be monitored with the aid of time-lapse microscopy and image analysis software. Disadvantages of the scratch assay compared to other methods (Boyden chamber, single cell tracking techniques, agarose cell migration assay) include a need for large amounts of cells and lengthy cultivation periods to obtain confluent monolayers. Cell migration may be also affected by the wounding, which causes cell injury or changes in cell-to-cell adhesion, because cells adjacent to the cell-free gap become permeable due to disruption of cell-cell junctions.

2.16 In vivo porcine model of skin wound healing

Porcine skin resembles human skin anatomically and physiologically. As for skin morphology, both have a thick epidermis and a similar dermal-epidermal thickness ratio; a measurement that takes into account body site when evaluating skin properties (Vardaxis et al., 1997). Both human and pig skin have well-developed rete-ridges and dermal papillary bodies and abundant subdermal adipose tissue (Montagna and Yun, 1964.). Porcine dermal collagen is similar (Heinrich et al., 1971) and neither pigs nor humans have a panniculus carnosus, an additional striated muscle layer within the subcutaneous tissue, which is seen in small loose-skinned animals. This plays a central role in skin healing, because human and pig healing occurs largely through re-epithelialization, whereas small animals, rely mainly on the panniculus, which causes wound contraction and wound closure.

Some differences exist, however. In pig skin, a less-developed adnexal structure supporting the subepidermal plexus and lesser elastin content is apparent (Forbes et al., 1969). Additionally, pig skin contains no eccrine glands, and in unlike in humans, apocrine glands are distributed throughout the skin surface (Meyer et al., 1978). Functionally, pig and human skin are similar in terms of epidermal turnover, keratinous proteins, and lipid composition of the stratum corneum. They also share similar immunohistochemical staining for keratins 10

and 16, which is useful for describing keratinocyte function and differentiation (Wollina et al., 1991). Growth factors have mainly similar effects. EGF has been shown to accelerate partial-thickness skin-wound healing in both pigs and humans (Breuing et al., 1997; Nanney et al., 1990). Studies on IL-1α and bFGF have shown that the pig proves a superior wound-healing model compared to the rat. In pigs and humans, IL-1α significantly enhances epithelial healing (Saunder et al., 1990; Mertz et al., 1991), whereas preliminary evidence show that IL-1α may impair wound strength in rats (Maish et al., 1999). Because the physiology of wound healing is similar in pigs and humans, and the anatomical structure is closely related, the pig serves as a more reliable model for wound-healing experiments than do other animals (Sullivan et al., 2001).

2.17 In vivo human models of wound healing

Although animal models provide excellent tools for studying wound healing, differences still exist, and not all results can be directly translated to humans. However, some in vivo human models provide an alternative for animal wound-healing studies. The suction blister model, introduced by Kiistala et al. in 1964, utilizes negative pressure to create a subepidermal blister. Removal of the epidermis from the blister creates a wound, and wound healing can be followed morphometrically (Kiistala and Mustakallio, 1964; Silverman et al., 1989). Healing of a skin wound can also be measured by following water loss through the skin. This method, trans-epidermal water loss (TEWL), has been useful, particularly in studies of the effect of topical treatment and drug reactions.

3. Aims of the studies

The aim of this thesis was to investigate how paracrine mediators from aggregates of dermal fibroblasts and bone marrow stromal cells contribute to epidermal and dermal responses during skin wound healing.

The specific aims of the individual studies were to investigate

I aggregation-induced production of HGF in bone marrow stromal cells and to characterize its effect on model keratinocyte wound healing in vitro.

II use of fibrin as a controlled-release matrix for delivery of fibroblast aggregate-derived factors and keratinocytes in vitro.

III the effect of fibroblast aggregate-derived paracrine factors on epidermal healing in a porcine partial thickness wound model in vivo.

IV the biocompatibility of recombinant human collagen III for transplantation of cultured keratinocytes and dermal fibroblasts in a porcine full-thickness wound model in vivo.

4. Materials and methods

4.1 Cells and cell lines

Spontaneously immortalized HPV-negative keratinocytes (HaCaT) (Boukamp et al., 1988) were from CLS (Cell Lines Service, Eppelheim, Germany). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplied with 1% penicillin/streptomycin and 5% fetal calf serum (FCS) (Sigma-Aldrich). Human dermal fibroblasts (CRL2088, American Type Culture Collection, Manassas, VA, USA) produced keratinocyte stimulatory growth factors. The cells were cultured in DMEM supplied with 1% penicillin/streptomycin and 10% fetal calf serum (FCS) (Sigma-Aldrich, Munich, Germany). The experiments utilized passages 9 to 16.

Bone marrow aspirates of control patients participating in a clinical cell transplantation trial (Clinical-Trials.gov identifier NCT00418418) were used for this study with the patient protocols approved by the Ethics Committee of the Helsinki University Hospital, Department of Surgery. After signed informed consent and after randomization to the study protocol, bone marrow aspirates were taken under general anesthesia from the iliac crest of adult patients.

Mononuclear cells in samples from control patients were isolated with a density gradient using Ficoll-Paque (Pharmacia, Uppsala, Sweden) as described in Pittenger et al. (1999). These cells were resuspended in complete culture medium, DMEM, with low glucose, supplied with 1% penicillin/streptomycin, 10% FCS (tested for its ability to support mesenchymal stem cell expansion, StemCell Technologies, Vancouver, BC, Canada) and then plated at a density of $0.36 \times 10^6/cm^2$, and incubated under standard cell culture conditions (37C° with 5% CO^2). After 2 days, the adherent cells were washed thoroughly with phosphate-buffered saline (PBS), and were further cultured up to passages three to five in complete culture medium. The cells were assayed by flow cytometry (FACSCanto, Becton Dickinson, San Jose, CA, USA) with data analysis using FACSDiva software (Becton Dickinson). They expressed CD29, CD44, CD105, and CD166, but not CD34, CD45, or CD14 (data not shown). The multilineage differentiation potential of cultured cells was assessed through the differentiation ability of cells into adipocytes and osteoblasts. For osteogenic differentiation, cells were plated at $3x10^3/cm^2$ and cultured in DMEM with 10% FCS, 10mM β-glycerophosphate, 10^{-7} M dexamethasone, and 200 μM ascorbic acid- 2-phosphate. The medium was changed every 3 to 4 days, and after 21 days, cells were analyzed by von Kossa staining. For adipogenic differentiation, the cells were grown to confluence and cultured for 48 to 72 hours in DMEM with 10% FCS, 0.5mM isobutylmethylxanthine, 1 μM dexamethasone, 10 μM insulin, and 200 μM indomethacin. After this, the medium was changed for maintenance medium containing DMEM with 10% FCS and 10 mM insulin for 24 hours. This was repeated twice and cells were analyzed by Oil Red-O staining. Eventually, the cells showed osteogenic and adipogenic differentiation potential.

Primary keratinocyte cultures were created from surgical waste from healthy female donors undergoing breast reduction surgery. The protocols for collecting patient skin samples were approved by the Ethical Review Board of the Helsinki University Central Hospital (approval #431/E6/06). After receiving each patient's informed consent, biopsy specimens of skin were collected and kept moist at +4°C for a maximum 24 h before use. The samples were first washed with phosphate-buffered saline (PBS), then subcutaneous fat was removed, and skin pieces 1 mm wide, containing epidermis and dermis, were excised for further processing. Epidermal detachment was performed with overnight incubation in PBS containing 1.45 U/ml dispase (Dispase™, Roche, Mannheim, Germany). After incubation, the epidermis was peeled off and was incubated further at +37°C with 0.1% trypsin in PBS for 5 min. A single-cell suspension was obtained by vigorous pipetting to mechanically dissociate the epidermal sheets. Thereafter, cells were seeded in gelatin-coated flasks for further propagation. Cells in passages one to three served in the experiments. Primary keratinocytes were grown in defined keratinocyte serum-free medium (K-SFM) (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplied with 1% penicillin/streptomycin.

4.2 Reagents

HGF-neutralizing antibody was from R&D Systems (Minneapolis, MN, USA). Recombinant human HGF (rhHGF) was from PeproTech Inc. (Rocky Hill, NJ, USA). Human epidermal growth factor (EGF) was from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). PGI2 analogue, 6a-PG(PGI2), was from CaymanChemical (Cayman Chemical Company, Ann Arbor, MI, USA). Pharmacological PI3K inhibitors were LY294002, its structural inactive analogue LY303511 (Calbiochem, Nottingham, UK), and wortmannin (Calbiochem). Two selective small-molecule Met-kinase inhibitors, SU11274 (Wang et al 2003, Ma et al 2005) and PHA665752 (Calbiochem, Nottingham, UK) as well as two small molecule EGF-receptor tyrosine kinase inhibitors: PD153035 hydrochloride (Tocris Bioscience, Ellisville, MO, USA) and AG1478 (Tocris Bioscience) were used at indicated concentration to show HGF and EGF-linked keratinocyte-fibroblast responses in vitro. Inhibitors of MEK, ERK1/2, and p38 were respectively: PD98059, U0126, and SB203580 (all from Calbiochem).

4.2.1 Production of the paracrine mediators from fibroblast aggregates

Multicellular spheroids were generated as described (Bizik et al., 2004). Briefly, U-bottomed 96-well plates (Costar, Cambridge, MA, USA) were treated with low-electroendosmotic agarose (Lonza, Basel, Switzerland) prepared in sterile water to form a thin film of a nonadhesive surface. BMSCs were seeded at 8,000 cells per spheroid in 200 µl DMEM with 10% FCS. Spheroid formation was allowed to proceed for 3 days. The spheroids were collected, allowed to settle spontaneously by gravity, and were washed with serum-free medium. When spheroids were formed from dermal fibroblasts, 15 x 10^3 cells per well in 80 µL DMEM with 5% FCS were used.

4.3 Enzyme-linked immunosorbent assay (ELISA)

Conditioned medium from cell cultures was harvested to determine HGF and Aphiregulin production by BMSC as well as CRL-2088 spheroid- or monolayer cultures. Growth factor concentration was estimated by the Amphiregulin and HGF ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The absorbance of ELISA-plates was read at λ = 450 nm using a multiscan ELISA plate reader (Multiskan™, Thermo Scienfic). A standard curve was created using different concentrations of a stock human HGF and amphiregulin protein, which served as a positive control. The protein concentration of the sample was determined by regression analysis compared to the standard curve; the lab technician did the analysis.

4.4 Immunoblotting

Immunoblotting was carried out according to the standard protocol (Bizik et al., 2004). Cell samples were lysed directly in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample-loading buffer supplemented with complete miniprotease inhibitor mixture tablets (Roche, Mannheim, Germany). The samples were separated in SDS-PAGE (gradient of polyacrylamide 5-15%, 3.5% stacking gel) and then transferred nitrocellulose membranes. The bound immunocomplexes were detected by alkaline phosphatase-conjugated secondary antibodies and reagents (Promega, Madison, WI, USA). Visualization of bands was performed according to manufacturers' recommendations. The following primary antibodies served for immunoblotting: rabbit monoclonal antibody against c-Met (Neomarkers, Fremont, CA), rabbit monoclonal antiphospho-Met (Y1234, Y1235) (Upstate Biotechnology, Lake Placid, NY, USA), rabbit polyclonal antibody against COX-2 (NeoMarkers), and mouse monoclonal antibody against actin (NeoMarkers).

4.5 Immunofluorescence

Deparaffinized 4-μm sections of porcine skin served for immunohistochemical analysis in the partial-thickness wound-healing studies. PCNA was detected using the monoclonal anti-PCNA-antibody (Neomarkers) with a secondary Alexa fluor 555 anti-rabbit antibody (Invitrogen). Cytokeratin-14 (K-14) was analyzed with a fluorescein isothiocyanate isomer I (FITC) -labeled mouse anti-human cytokeratin-14 antibody (Abcam, Cambridge, UK). Analysis was done with the AxioCam Imager M2 microscope and with an AxioCam HRm digital camera (Carl Zeiss Vision GmbH, Aalen, Germany). Mouse anti-human pankeratin (diluted 1:40, Millipore, Billerica, MA, USA) antibody was used to characterize transplanted keratinocytes in the wound bed. DAB (3,3-diaminobenzidine) served as the chromogenic substrate for horseradish peroxidase (HRP) for visualization of pankeratin-positive cells.

4.6 Green fluorescent protein (gfp) transfection

HaCaT keratinocytes cells were labeled with green fluorescent protein (gfp) by transfection with a lentiviral vector carrying the gfp gene and 8 µg/mL polybrene for 24 h to create fluorescent HaCaT cells (gfp-HaCaT) for further experiments. The lentiviral vector was a kind gift from Professor Seppo Ylä-Herttuala (AIV Institute, Kuopio, Finland).

4.7 Keratinocyte assays

4.7.1 Scratch wound assay and co-culture stimulation of keratinocyte migration

Confluent HaCaT cell monolayers were serum starved after washing with PBS. After 1 day of serum starvation, the monolayers were scratch-wounded by the tip of a spatula, and washed multiple times with PBS; treatment was initiated one day after wounding. In the co-culture setting spheroids and the corresponding amount of mesenchymal cells were plated on Transwell inserts (Corning Life Sciences, Lowell, MA, USA) for physical separation of the HaCaT-cell co-culture. The experiments were continued without change of medium after placement of inserts upon the monolayer.

4.7.2 Characterization of intracellular signaling cascades

Growth factor inhibitors (SU11274, anti-HGF, LY294002, PD98059, SB203580, LY303511, U0126) were added to the wounded keratinocyte monolayer at the time of stimulation with recombinant growth factors (HGF, EGF) or with conditioned medium from BMSC spheroids (collected 3 days after initiation of spheroid formation). Use of conditioned medium prevented any effect which the inhibitors might have had on nemosis. Later on, a scratch wound assay validated the use of SU11274 as an inhibitor for both HGF- and EGF-linked migration with primary keratinocytes monolayers. Stimulation of cell migration was then initiated with rhHGF and rhEGF with or without SU11274.

For analysis of cell migration on the fibrin matrix, multicellular gfp-labelled HaCaT cell spheroids were formed in U-well plates and seeded on top of a fibrin gel which contained conditioned from spheroid or monolayer- cultures.

4.7.3 c-Met phosphorylation

Conditioned medium from BMSC spheroids stimulated HaCaT cell monolayers and samples were collected at 0 hour, 0.5 hour, 1 hour, 2 hours, and 3 hours to detect autophosphorylation of c-Met with an antiphospho-Met (Y1234, Y1235) antibody (Neomarkers).

4.7.4 MTT and gfp-fluorescence assay as measurement of cell viability

Cell respiration, an indicator of cell viability and cell growth, was assayed by the mitochondria-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan (Roche, Mannheim, Germany). At selected time points, the cultures were incubated with the substrate (5 mg/mL) for 90 min, after which the medium was aspirated, and MTT was dissolved in DMSO. The extent of MTT conversion to formazan was quantified by measurement of optical density at 550 nm with a wavelength correction of 690 nm, by a microplate reader (Multiscan MCC/340, Labsystems, Helsinki, Finland). Gfp-HaCaT cells allowed analysis of growth factor-induced keratinocyte proliferation. Viability and size of cell populations were measured and photographed with a fluorescence microscope. Before each measurement, plates were washed twice with PBS to remove unattached cells from the lattice. Eventually, fluorescence signal intensity was assessed with ImageJ 1.41 software (U.S. National Institutes of Health, Bethesda, MA, USA, http://rsb.info.nih.gov/ij).

4.8 Carriers for fibroblast-derived paracrine factors and cell transplantation

4.8.1 Fibrin matrix

The fibrin matrix was formed from commercially available fibrin sealant Tisseel Duo Quick from Baxter Healthcare, Deerfield, IL, USA. Both components of Tisseel were diluted with conditioned medium or with PBS for optimization of cell growth and proliferation. After mixing of the diluted solutions, the solid gels were further used as carrier matrices for fibroblast-derived soluble factors both in the in vitro and in vivo studies.

To demonstrate the binding and release characteristics of finectra from the fibrin matrix, the conditioned medium was collected and dialyzed with a Slide-A-Lyzer 2k dialysis cassette (Thermo Scientific, Carlsbad, CA, USA) according to the manufacturer’s protocol to remove salts, then lyophilized and labeled with a red fluorescent dye label, DyLight 594 (Thermo Scientific). A fibrin matrix was constructed with the labeled proteins as described previously. Released fluorescence was quantified with a fluorescence reader (340 nm/ 460 nm; Wallac Victor2, PerkinElmer Life Sciences, Waltham, MA, USA). For confocal imaging, DyLight 594-labeled protein and FITC (Sigma) were incorporated within the fibrin matrix during the polymerization. Confocal microscopy was carried out using a Leica TCS SP2

AOBS system (Leica Microsystems AG, Wetzlar, Germany) with argon 488 nm or DPSS 561 nm excitation lines, and an HCX PL APO CS 63x/1.40 NA objective. Image stacks were acquired through the matrices at a 240 nm z-sampling density.

4.8.2 Recombinant human collagen III (rhCol-III) matrix

RhCol-III dilute solutions (0.3% of w/w) came from FibroGen (FibroGen Inc., San Francisco, CA, USA) with a pH of approximately 2 (in 0.01 mol/l hydrochloric acid). After adjusting the pH to 7.2 with a fibrillogenesis buffer (Na-phosphate, pH 11.2 adjusted from 7.2 to 11.2 with concentrated NaOH) a collagen hydrogel was obtained with a final collagen concentration of 1.5 mg/ml. The solution was pipetted in 96-well plates, 100 µl per well, and then placed in a centrifuge at 10,000g for 1 h, whereafter excess liquid was carefully removed and a thin rhCol-III coating (final concentration, 50 mg/ml) formed in the bottom of the wells. For cell transplantation, isolated primary porcine keratinocytes and fibroblasts were cultured for one week, then detached with trypsin and seeded into rhCol-III containing syringes in separate layers of different cell amounts. Each syringe contained a total of $500 x 10^3$ cells; the keratinocyte group comprised of $500 x 10^3$ keratinocytes, and the co-culture group of $250 x 10^3$ keratinocytes and $250 x 10^3$ fibroblasts in separate layers.

4.9 Porcine wound healing assay

4.9.1 Animal care and ethical statement

All animals were maintained and treated in accordance with the Principles of Laboratory Animal Care of the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences and published by the National Institutes of Health (NIH publications No. 8623, revised 1985). This study was approved by the Operative Ethics Committee of the Helsinki University Central Hospital and by the Provincial State Office of Southern Finland (ESLH-2009-03831/Ym-23). All animals used in the studies were anesthetized with an intramuscular injection of ketamine (4-6 mg/kg s.c.) and medetomidine (0.5 mg/kg s.c.). Anesthesia was continued with intravenous infusion of propofol (4-10 mg/kg/h i.v.). The animals received daily doses of broad-range antibiotic ceftriaxone 1 g i.m post-operatively.

4.9.2 Partial- and full-thickness wound-healing assays

A simple randomization for the cranio-caudal anatomical location and paravertebral sides involved flipping a coin prior to the operation. A series of deep, mirror-image partial-thickness wounds 4 cm x 5 cm were created by the same surgeon on the lateral paravertebral skin of each pig (four wounds per side, total eight wounds per pig) with a Zimmer dermatome (Zimmer, Inc., Warsaw, IN, USA) set at 30/1000 in. The wounding procedure created a partial thickness injury that extended approximately halfway depth (50%±2%) into the

dermis, removing surface epithelium and superficial dermis, but sparing the lower dermis containing epithelial appendages such as hair follicles. Estimation of wound depth was obtained by measurement of total skin thickness prior to wounding with an 8 mm punch biopsy in the center of the wound and by comparing this with the thickness of the skin graft. Wounds were followed until the third postoperative day without dressing changes and then excised with a margin of 1 cm of unwounded surrounding skin down to the fascia.

In the full-thickness wound-healing assay, after simple randomization, 14 identical mirror-image full-thickness wounds were created by the same surgeon on the lateral paravertebral skin of each pig with an 8-mm punch biopsy tool, seven wounds per side. Treatment was initiated by application of rhCol-III hydrogel with pre-cultured fibroblasts and keratinocytes. Untreated wounds were used as controls for the treatment groups. Wounds were monitored until the fifth postoperative day without dressing changes.

4.10 Histology

Paraffin-embedded, hematoxylin and eosin (H&E)-stained 4-µm sections of each wound were analyzed and photographed (Leica IM500 and Leica QWIN software; Leica Microsystems AG, Heerbrugg, Switzerland). To assure objectivity, sections were coded during the operation, and slides were analyzed in blinded fashion for each wound. In partial-thickness wounds, the histological sections were analyzed for the amount of new epithelium, lateral epidermal cell migration, and granulation tissue formation. The new epithelium was defined as the area between the wound margins in the wound bed covered by epithelial cell islets and cells growing out of regenerating hair follicles. The area between the unwounded dermis at the bottom of the wound and newly formed epidermis was considered granulation tissue, and the underlying unwounded dermis as total dermis. Lateral migration in the border zone was measured morphometrically from the wound margin to the tip of the cells' leading edge. In full-thickness wounds, formation of granulation tissue was evaluated from the wound base, and healing of the wounds measured as the reduction in initial wound size. The Sirius Red staining–based colorimetric assay was revealed collagen type-I and III deposition in the full thickness wounds, an indicator for granulation tissue formation. Total collagen content was calculated as percent Sirius Red–stained positive area/wound area from scanned images of stained tissue sections by Image J–software (ImageJ, National Institutes of Health, http://rsb.info.nih.gov/ij).

4.11 Near-infrared spectroscopy (NIRS)

In the partial-thickness wound study, a NIRS examination involved a spectrocutometer as done previously (Kaartinen et al., 2011; Välisuo et al., 2011). The spectrocutometer consists of a single lens reflex camera (Fuji IS Pro, Fujifilm Corporation, Tokyo, Japan), an external chamber to block out ambient light, and a built-in computer controlled lighting

system that uses both visible and near-infrared light. The spectrocutometer is used to measures the estimated concentration changes (ECC) of oxyhamoglobin (oHb), deoxyhamoglobin (doHb), and melanin in the wound (Välisuo et al., 2011).

4.13 Statistical analysis

Data were analyzed by Student's paired t-test (two-tailed) where $p < 0.05$ was considered statistically significant. Case–control wounds were analyzed by the Mann–Whitney U test. In the study with NIRS-camera analysis, the correlation between the ECC of oHb and epithelial thickness was calculated from the pooled data including all wounds, regardless of treatment, with Pearson's correlation coefficient. Statistical analyses were performed with GraphPad Prism 4.0 (GraphPad Software Inc, San Diego, CA, USA).

5. Results

5.1 Activation of nemosis in bone marrow stromal cells, defined by COX-2 expression and HGF production; effect of HGF and amphiregulin on keratinocyte migration (Study I, II)

Reports state that human dermal fibroblast aggregation induces expression of the hepatocyte growth factor/scatter factor (HGF) (Bizik et al., 2004; Kankuri et al., 2005 and 2008). In the first of the studies presented in this Thesis, bone marrow stromal cells collected from four healthy donors were evaluated for analogous activation. After assessment for multilineage potential, cells were plated on nonadherent U-wells. Cluster formation occurred within 24 hours with upregulation of COX-2 and HGF (I). After 2 and 3 days of culture as spheroids, the expression of COX-2 increased 3.8 to 8.2-fold, and on the fifth day a clear induction of 62.5-fold was evident as compared with COX-2 expression levels at day 1. BMSCs from donors served then for quantification of nemosis-derived HGF. In concert with the induction of COX-2, ELISA showed an average 54.8-fold ± 22.9 (range 10.0–97.8) induction of HGF production by BMSC nemosis, whereas monolayer cultures produced only low HGF amounts (I). Interestingly, the level of HGF induction varied significantly among bone marrow samples, suggesting that although nemosis signaling proceeded in all cases, the magnitude of the effect was variable. In order to characterize that the HGF produced was active, conditioned medium from BMSC spheroids was used to stimulate HaCaT cell monolayers to analyze autophosphorylation of c-Met; the western blot revealed the phosphorylated c-Met after 30 min of stimulation (I).

The effects of paracrine factors released from activated BMSC on keratinocyte wound healing emerged from an in vitro scratch wound assay. BMSC spheroids and the corresponding amount of BMSCs were plated on cell inserts for physical separation of the co-cultures. The HGF-producing BMSC spheroids significantly enhanced keratinocyte migration from the border of the wound and accelerated scratch-wound closure compared with BMSC monolayers containing a corresponding amount of cells (I). In the wound-healing assay, nemosis stimulation showed a clear dose-dependency whereas the stimulation with the BMSC monolayer did not (I). The effect of BMSC nemosis-induced keratinocyte migration was partially inhibited by an anti-HGF antibody but nearly totally with a specific small molecule c-met kinase inhibitor SU11274 in a non-toxic concentration (I).

The inhibitory effect of SU11274 for both HGF and EGF was validated in a wound-healing assay with HaCaT cells and primary keratinocytes. SU11274 inhibited HGF completely and EGF to a lesser extent (I) in HaCaT cells, but also inhibited EGF-induced migration in primary cells (II). Furthermore, the intracellular signaling of c-met through PI3K was elucidated in an rhHGF (100 ng/ml)-induced wound-healing assay. PI3K-inhibitors were: LY294002, wortmannin (I), and LY303511-the inactive structural analogue of LY294002. Results indicated that the PI3K inhibitors LY294002 and wortmannin attenuated rhHGF-

induced wound healing, whereas LY303511 had no effect. Conversely, the effect of inhibitors of p38 (SB203580), of ERK 1/2 (U0126), and of MEK 1/2 (PD98059) all revealed inhibition of HGF-induced migration (I) (Figure 7).

Based on previous unpublished data from our group we knew that amphiregulin-gene is upregulated in the spheroid cultures. Therefore the content of amphiregulin cluster cultures was analyzed and revealed a 7-fold increase in its production (II). We designated the composition of factors deriving from mesenchymal cell nemosis as “finectra” (study II).

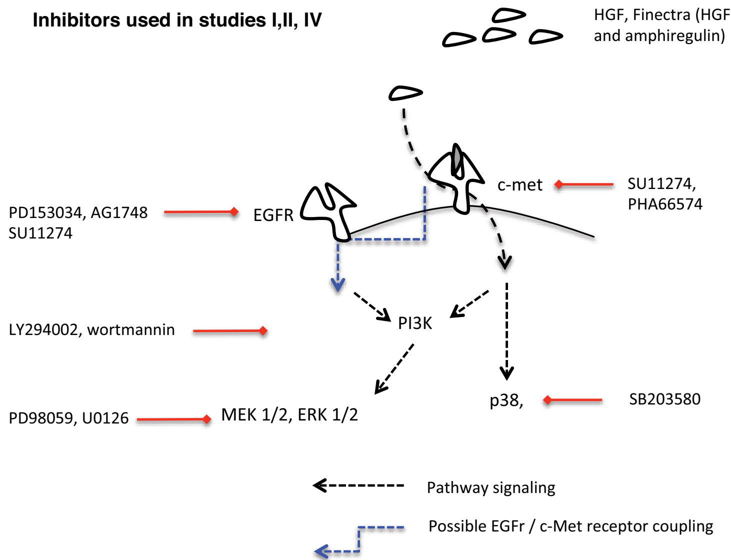

Figure 7. Validation of methodological data. Specific inhibitors of HGF-c-MET/EGF-PI3K / MEK 1/2, ERK ½, and p38 signaling-pathways are marked alongside their site of inhibition. c-Met coupling/dimerization with EGFr as a possible means of growth factor signaling. Dashed arrows indicate possible routes of HGF/finectra-induced migratory signaling. Red arrows mark inhibition.

5.2 Characterization of the finectra-fibrin matrix (II, III)

Based on results as regards fibrin as a carrier for cells and growth factors, the aim of the study was to show that a matrix formed from fibrin can incorporate finectra and that such an innovative active biological—yet acellular—matrix can support the proliferation, migration, and viability of human keratinocytes (II). Conditioned medium from dermal fibroblast spheroid cultures was collected and combined with a fibrin matrix. HaCaT keratinocyte clusters were seeded on top of the matrix, and outgrowth of cells from the spheroids was measured. Interestingly, monolayer-stimulated clusters decreased in size, whereas spheroid conditioned medium stimulated outgrowth from cell clusters (II). This

effect was inhibited with SU11274 (II). The viability of gfp-HaCaT cells and of primary keratinocytes seeded on fibrin-matrixes was measured respectively by emitted fluorescence intensity and with MTT. The fibrin matrix containing rhHGF or conditioned medium from fibroblast spheroids cultures displayed an increase in cell rate and cell survival compared to those on the monolayer-conditioned medium (II).

In order to demonstrate binding and release characteristics of the factors in the conditioned medium from the fibrin matrix the medium was labeled with a fluorescent dye (III). The release of DyLight 594 labeled finectra into PBS was measured with a fluorescence reader 2, 8 and 32 h after placing the matrix at room temperature in a shaker. Rapid release occurred already after 2 h of incubation. After 2 h of follow-up in a shaker in room temperature, 29 ± 0.87% ±SEM of the initial protein was released, at 8 h, 42 ± 3.41% and at 32 h, 56 ± 3.51%. The fluorescence of the labeled protein remained strong throughout the experiment, whereas no fluorescence was observed from the blank fibrin matrix. Confocal imaging revealed that the labeled finectra was both bound directly to the fibril structure and unbound between the fibril mesh. The data indicated that the initial 2 h-release rate is rapid (14.5% per h), but is slowed down at 2 to 8 h (2.2% per h), and remains relatively stable thereafter (0.58% per h). Extrapolating from these data, the matrix in vitro would have fully released finectra after 108 h (III). These release properties of fibrin are similar to what others have reported (reviewed by Spicer and Mikos, 2010).

5.3 Studies characterizing properties of collagen (I, III and IV) and fibrin for cell transplantation

A collagen contraction assay revealed, whether fibrin or the addition of fibrin with collagen would diminish the contractive properties of cell containing collagen matrixes. Additionally the assay defined whether a collagen/fibrin matrix could allow transplantation of fibroblast spheroids (II). Fibroblasts or fibroblast cell clusters were seeded into collagen gels containing collagen type I with or without fibrinogen, with thrombin then added to initiate fibrin polymerization. Collagen gels with fibroblast spheroids led to modest contraction of the original gel compared to that of gels containing fibroblasts as single cells. Collagen gels containing only 7% fibrin reduced contraction significantly (II).

Keratinocyte viability on rhCol-III was analyzed with gfp-HaCaT cells in co-culture setting with keratinocytes and CRL2088 fibroblasts physically separated by a porous membrane (IV). Fibroblasts stimulated gfp-HaCaT cell proliferation, which was inhibited with two EGF-receptor tyrosine kinase inhibitors; PD153035 and AG1478. When primary porcine keratinocytes were seeded on rhCol-III-coated wells, they adhered within 2 hours. However, under serum-deprived conditions fibroblast support was required for cell proliferation and migration (data not shown).

5.4 Partial thickness wound healing model (III)

The effect of finectra on healing of dermal wounds was evaluated with a porcine partial-thickness wound-healing study (III). We compared epidermal resurfacing of treatment wounds was compared saline-treated controls and monolayer-treated controls. Finectra treatment resulted in a significantly greater change in epithelium amount than for either saline or monolayer-treated wounds. The biological effect of fibroblasts was also evident in monolayer controls. The effect, however, was significantly less (30%). The area of re-epithelialization compared with that of saline-treated controls was 8.6 ± 6.6% larger in monolayer and 41.1 ± 9.0% in finectra-treated wounds (Figure 8A). Re-epithelialization originated from lateral wound edges as well as from hair follicles in which the upregulation of K-14 and PCNA was evident. The NIRS camera provided data with a statistically significant negative correlation between the ECC of oxyhemoglobin (oHb) and epidermal/epithelial thickness ($r = 0.63$, $p = 0.0013$). However, no statistically significant difference appeared in the ECC of oHb or doHb between treatment groups. Average epithelial islet-area per wound section was significantly larger in finectra-treated wounds, and cell migration from the wound edges with a leading edge was significantly improved. Both finectra and monolayer treatment stimulated granulation tissue formation more than saline controls did ($p < 0.001$ and $p < 0.05$, respectively). Finectra stimulus had, however, a significantly greater effect as compared to the monolayer controls ($p < 0.05$).

5.5 Full thickness wound healing and cell transplantation (IV)

A porcine full-thickness wound experiment characterized the biocompatibility of rhCol-III as a dermal substitute and cell carrier. Primary porcine keratinocytes and fibroblasts were isolated from a skin sample take from the animal week prior to surgery. Precultured keratinocytes and fibroblasts were centrifuged into separate layers of an rhCol-III matrix in a syringe. After overnight storage in +37°C, the bilayer gel was applied to 8-mm full thickness wounds and wounds were covered with a polyurethane dressing. On the fifth post-operative day, wound-healing was measured from H&E-stained sections with the same methods as in study III. Wound depth was significantly reduced in all treatments compared with depth in untreated controls, but no significant difference was observed between different treatment groups (data not shown). Wound contraction measured from digital images showed no difference between treatment groups. Granulation tissue thickness was significantly increased in all those treated compared with untreated controls, whereas no significant difference was observed with treatment groups measured both morphometrically and with imaging software. Transplanted pancytokeratin-positive keratinocytes were visible on the fifth postoperative day in the wound bed, indicating that rhCol-III could function as a carrier matrix for cells.

Sirius Red staining was used to quantify total collagen amount. Polarized light microscopic images revealed that collagen arrangement was disorganized, and relatively little neocollagen had formed in the healing wound (IV). However, the amount of collagen was

increased most in wounds treated with either rhCol-III alone or with rhCol-III and keratinocytes. Fibroblast-containing gel was effectively removed from the wound whereas gels without cells or with only keratinocytes remained intact. As a conclusion, changing the cell-type composition of rhCol-III matrix from keratinocytes to a 50/50 fibroblast-keratinocyte mixture dramatically altered the amount of collagen detectable by Sirius-Red staining in the wound (IV) (Figure 8B). This could be explained by the rhCol-III or keratinocyte-induced activation of collagen-degrading proteinases in the trasplanted fibroblasts.

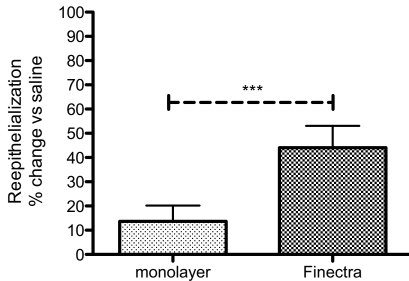

Figure 8A. Stimulation with conditioned medium from fibroblast aggregate cultures, finectra, increased amount of new epithelium in partial-thickness wounds compared with saline treated controls (*** p<0.001 as compared to monolayer controls)

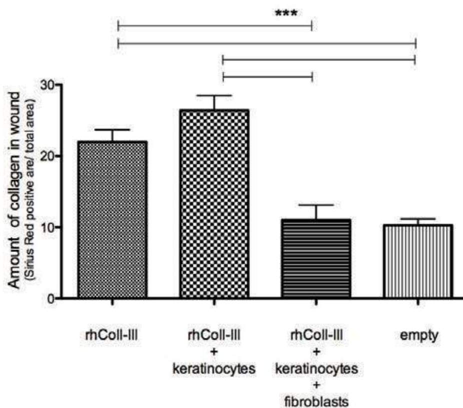

Figure 8B. Treatment of full-thickness wounds with autologous keratinocytes in an rhCol-III matrix increased amount collagen; fibroblast addition decreased detectable collagen (*** p<0.001).

6. Discussion

Novel alternatives for treatment of skin defects have emerged from recent advances in the understanding of molecular mechanisms that regulate skin physiology and wound healing (Auger et al., 2009). These include identification of such potential therapeutic molecules as HGF. Together with the progress in material sciences, tissue-engineered active artificial skin substitutes mark yet another leap forward (review by Macri and Clark, 2009). Overall, the rationale for such regenerative wound treatments controllably providing growth factors and cytokines to the wound is to provide additional spatiotemporal cell stimuli to improve the complex coordination between tissue homeostasis and regeneration (Werner et al., 2007). In order for a regenerative wound treatment to be effective, the stimulating agent that is released must reach the wound bed in its active form at the right time. Moreover, its effect should be sufficiently sustained to enable biological functionality (Macri and Clark, 2009).

Topically administered growth factor therapies have been under intensive study, Experience from clinical trials thus far have, however, clearly demonstrated that single-agent therapies such as administration of a single growth factor influence wound repair only to a mild to moderate degree. In hindsight, this should have been less unexpected because the elaborate wound repair process has considerable plasticity and several levels of control, and comprises a legion of active modulators (Gurtner et al., 2008). Thus, the synergic application of two or more carefully selected growth factors is, though as yet unexplored, a prospective alternative for skin wound treatment (Robson, 1997; Chen et al., 2010). The focus of this thesis was to characterize the role of those biological paracrine mediators that are released from mesenchymal cells such as fibroblasts or bone marrow stromal cells in wound healing. The three-dimensional aggregation step employed was to induce further growth factor and cytokine production (Bizik et al., 2004; Kankuri et al., 2005). Additionally, my aim was to characterize clinically applicable means for transporting these factors with or without cells to the wound bed in a scaffold. The hypothesis was that a mixture of factors produced by activated cells best mimics a biological paracrine pattern of stimuli able to improve wound healing.

6.1 Methodology

6.1.1 Animal models

Partial- and full-thickness wounds of young, healthy domestic pigs served as models for acute wound healing in the animal experiments (III-IV). The porcine partial-thickness wound model has several advantages. Porcine skin is superior to rodent skin in its anatomical and physiological similarity to human skin. Additionally, the structure of the epidermis is similar, and since adnexal structures participate actively in healing in both humans and pigs, the model is considered a good one for epithelial healing. Thus, the structural reconstruction and healing response can be considered relevant also in response to a human growth factor

stimulus. The pigs, however, have a powerful innate healing capacity that presents a challenge for improving and measuring wound healing. Possibilities to artificially slow the innate healing response by chemical means include streptozocin. Because these models are also not without problems (such as increased mortality) (Marshall, 1979), we chose to use healthy pigs to enable characterization of any true responses. Anatomical variation in the dermal depth of porcine skin has an effect on wound healing. Because this observable in our experiments (III), we decided to evaluate the wounds as case-controls.

6.1.2 Fibroblast and BMSC aggregate-derived factors and keratinocyte wound healing

We observed that patient-derived BMSCs responded to cell clustering by induction of COX-2 and production of HGF (I). Reports on cell aggregation-activated fibroblasts and on fibroblast nemosis indicate that the central cytokines produced after 96 h of cell clustering are IL-6, IL-8, and HGF (Kankuri et al., 2008). HGF production was induced rapidly on both protein and mRNA levels after dermal fibroblast cell clustering (Kankuri et al., 2005). In addition, we showed that activated fibroblasts also release a principal keratinocyte stimulatory growth factor, amphiregulin (II). Both HGF and amphiregulin have explicitly been linked to keratinocyte migration and epithelialization in wound healing (Toyoda et al., 1995; Schelfhout et al., 2002). Moreover, Toyoda et al (2001) report that overexpression of HGF increases vascularization and granulation tissue formation in vivo, indicating that it plays multiple roles in wound healing. Research done by Räsänen et al (2010) suggested that HaCaT-keratinocytes respond to fibroblast nemosis by increased migration in both a chemotactic and a fibronectin-mediated manner. Furhermore, they reported that TGF-β1 induced upregulation of COX-2 in migrative keratinocytes.

Upregulation of COX-2 and an imminent inflammatory response has been linked to clustering of dermal fibroblasts (Bizik et al., 2004). The induction of COX-2 was followed by production of prostaglandins (PG), notably PGI_2, PGE_2, and $PGF_{2\alpha}$ (Bizik et al., 2004). PGs, studied extensively, are principally considered to be proinflammatory mediators. In skin physiology, PGE_2 plays a role in hair follicle development and hair growth, and expression of the PGE_2 receptors EP3 and EP4 alter during the hair-growth cycle in mice (Torii et al., 2002; Lee et al., 2003). Due to observations that COX-2 is rapidly upregulated in acute wounds and that COX-2 inhibitors delay early epithelialization, it is clear that PGs also play a central role in wound healing (Futagami et al., 2002; Lee et al., 2003). Kaneko et al (1995) reported PGI_1 analogues to inhibit keratinocyte cell growth in vitro whereas PGE_1 has the opposite effect. Interestingly, in the same study the authors concluded that conditioned medium collected from fibroblasts activated by PGI_1 contained elevated amounts of IL-6, and it enhanced keratinocyte migration. Correspondingly, in our studies a PGI_2 analogue, 6α-PGI_1, used to stimulate keratinocyte migration, displayed no such effect (I).

As mentioned, both IL-6 and IL-8 act as mitogens and also promote keratinocyte chemotaxis because it is crucial for wound healing (Schröder, 1992; Gallucci et al., 2004). Grimstadt et al (2011) reported recently that in acute-wound fluid collected from reduction

mammoplasty wounds, the most abundant cytokines found were IL-6 and IL-8. This confirmed findings regarding the cytokines present in fluid of acute wounds. Interestingly, a recent findings was a that conditioned medium from MSC cultures supports keratinocyte and fibroblast migration in vitro and the main paracrine components are IL-6 and IL-8 (Walter et al., 2010).

HGF-induced keratinocyte migration

We showed that BMSCs spheroids induced keratinocyte migration utilizing, HGF and c-Met. Stimulation with conditioned medium caused rapid autophosphorylation of hepatocyte growth factor receptor (HGFR): c-Met (at the tyrosine residues Y1234/Y1235), an effect inhibitable by SU11274. The receptor phosphorylation thus indicated that HGF is one on the main factors contributing to the nemosis-induced keratinocyte responses. BMSC-conditioned medium supported keratinocyte migration, which was inhibited to a lesser extent by an anti-HGF antibody. It thus remained unclear, whether HGF is the sole factor contributing to keratinocyte migration. Additionally we know that EGFR and PG-receptors have crosstalk with c-Met. An autocrine, transcription-dependent EGFR activation after HGF stimulation has been reported, and c-Met is able to dimerize or interact with several partners on the cell membrane (Han et al., 2006).

6.2 Results

6.2.1 Partial-thickness wound-healing with fibrin-trapped growth factors from aggregate cultures

Porcine partial-thickness wounds treated with conditioned medium from cell aggregate cultures or from standard monolayer cultures revealed improved granulation tissue formation and epithelialization. However, aggregate-conditioned medium treatment was statistically significantly better (III). Studies made of partial thickness model have revealed that single growth factors indeed exert a favorable effect on epithelialization. Breuing et al (1997) reported improved re-epithelialization with topical 100 ng/ml EGF within 4-5 days, as measured by protein flux through the wound surface. Analogously, a study using the porcine partial thickness wound showed that topical 1μg/ml EGF treatment almost halved the time needed for epithelialization (Hong et al., 2006).

Nevertheless, there is limited number of reports on the use of multiple growth factors in porcine wound healing. Danilenko et al (1995) showed that a synergic effect of PDGF and KGF can enhance granulation tissue formation in a porcine burn wound model, but with no effect on epithelialization. Moreover, a clinical study by Robson et al (2000) evaluated the combined effect of GM-CSF with bFGF in the treatment of pressure ulcers with unsuccessful results. Reports also concern platelet lysate, a mixture of various growth factors released from activated platelets; Henderson et al (2003) reported that in a porcine superficial burn model,

autologous platelet gel (a concentration of platelet-lysate) stimulated collagen- and granulation-tissue formation with fibroblast proliferation, but re-epithelialization was not enhanced. Vermeulen et al (2008) later described combining PRP with a fibrin matrix and keratinocytes to enhance re-epithelialization in a porcine full-thickness model. On the other hand, the conclusion of one randomized clinical trial on the platelet-rich fibrin was that healing of neither donor sites nor autografts was significantly improved (Daniselsen et al., 2008). It is possible that donor-dependent variations of the PRP-product make comparison between different studies difficult.

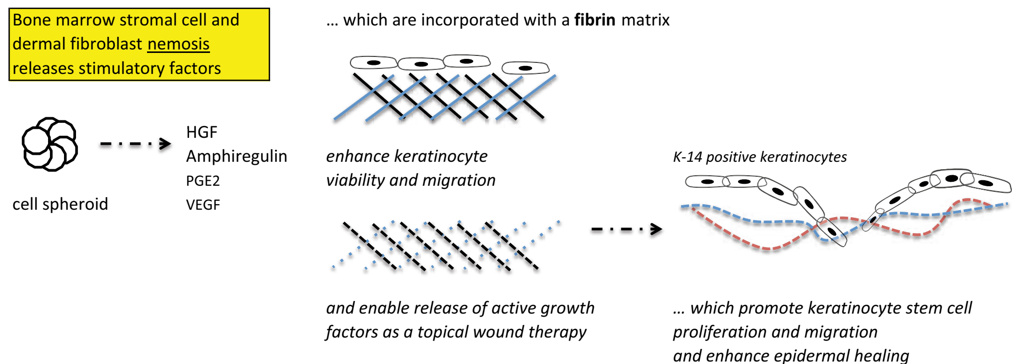

Figure 9. Conclusion of results: paracrine stimulatory factors from cell aggregate cultures stimulate keratinocyte viabilitysand migration when combined with biomatrices such as fibrin and collagen

6.2.2 Full-thickness wound treatment with keratinocyte- and fibroblast-populated recombinant human collagen III matrix

Recombinant materials provide a safer alternative for animal-derived collagens but the biocompatibility of recombinant collagens is still unclear. We sought to characterize the use of a recombinant human collagen carrier for autologous keratinocyte and fibroblast transplantation to full-thickness wounds (IV). Results showed keratinocyte transplantation with rhCol-III was successful and that addition of fibroblasts into the keratinocyte transplant dramatically altered the amount of detectable collagen in the wound. This ECM degrading effect could be caused by activation of fibroblast by either the collagen matrix or keratinocytes. This information must be taken into account in the future when designing recombinant collagen hydrogels.

6.2.3 Future prospects

Even though the clinician's repertoire includes a variety of treatments targeted at complex skin wounds, many of the present tissue-engineered products still contain foreign material,

often of xeno- or allograft origin. Viral vector transfection and gene modification can serve in production of growth factors in situ, but bear risks for viral infection and oncogenesis, thus being an unsafe therapy option (Anson, 2004). Importantly in our approach, no other added factors besides those produced by normal human cells are present. Further characterization of the conditioned medium from mesenchymal cells closely linked to promotion of wound healing may in future contribute to identification and design of the most beneficial combination of novel wound healing therapeutics.

7. Summary and conclusions

The purpose of this study was to investigate the effect of paracrine mediators from aggregation-activated or non-activated dermal fibroblasts and bone marrow stromal cells on critical aspects of wound healing: keratinocyte migration, epithelialization, and granulation-tissue formation in experimental in vitro and in vivo wound models. Furthermore, we aimed to characterize means of improving growth factor transport and keratinocyte transplantation utilizing the biological matrixes fibrin and collagen. The findings and conclusions are the following:

I Keratinocytes respond to nemosis-induced stimulatory factors in a wound healing model. Cell aggregation of patient-derived bone marrow stromal cells elicits upregulation of COX-2 and HGF and induces keratinocyte migration in model keratinocytes, HaCaT cells. The paracrine mediators stimulate keratinocytes by utilizing the c-Met/PI3K /MEK 1/2/ ERK 1/2-pathway.

II The HGF and amphiregulin-containing conditioned medium from dermal fibroblast aggregate-cultures enhances fibrin matrix cell-transplantation properties by leading to increased primary human keratinocyte migration and viability.

III Conditioned medium from dermal fibroblast-aggregate cultures improves wound healing by promoting epithelialization and granulation tissue formation in three days, when applied topically to the wound bed in an in vivo porcine partial thickness wound model.

IV Recombinant human collagen type III supports keratinocyte transplantion and improves healing of in vivo full-thickness wounds by increasing granulation tissue formation and collagen content in a porcine wound model. Keratinocyte viability and proliferation on recombinant human collagen type III is enhanced by fibroblasts via EGF stimulation.

In conclusion, when fibroblasts and bone marrow stromal cells are cultured as cell aggregates, the biological activation initiated results in release of paracrine factors including HGF and amphiregulin; this improves skin wound healing. This novel method of production of healing-inducing factors has a therapeutic potential for treatment of complex skin wounds. Futhermore, with tissue engineering, these factors can be combined to biomaterials to stimulate guided tissue regeneration in the damaged skin.

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