Regulation of fibroblast activity by keratinocytes, TGF-β and IL-1α
Dedicated to my daughters Astrid and Matilda
Regulation of fibroblast activity by keratinocytes, 
TGF-β and IL-1α 
-studies in two- and three dimensional in vitro models
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Abstract

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Dysregulated wound healing is commonly associated with excessive fibrosis. Connective tissue growth factor (CTGF/CCN2) is characteristically overexpressed in fibrotic diseases and stimulated by transforming growth factor-β (TGF-β) in dermal fibroblasts. Reepithelialisation and epidermal wound coverage counteract excessive scar formation. We have previously shown that interleukin-1α (IL-1α) derived from keratinocytes counteracts TGF-β-stimulated CTGF-expression. The aim of this thesis was to further explore the effects of keratinocytes and IL-1α on gene and protein expression, as well as pathways, in TGF-β stimulated fibroblasts. Fibroblasts were studied in vitro by conventional two dimensional cell culture models and in a three dimensional keratinocyte-fibroblast organotypic skin culture model.

The results showed that IL-1 suppresses basal and TGF-β-induced CTGF mRNA and protein, involving a possible TAK1 mechanism. Keratinocytes regulate the expression of fibroblast genes important for the turnover of the extracellular matrix. Most of the genes analysed (11/13) were regulated by TGF-β and counter regulated by keratinocytes. The overall results support a view that keratinocytes regulate fibroblasts to act catabolically (anti-fibrotic) on the extracellular matrix.

Transcriptional microarray and gene set enrichment analysis showed that antagonizing effects of IL-1α on TGF-β were much more prominent than the synergistic effects. The most confident of these pathways was the interferon signaling, which were inhibited by TGF-β and activated by IL-1α. A proteomics study confirmed that IL-1α preferentially counteracts TGF-β effects. Six new fibroblast proteins involved in synthesis/regulation were identified, being regulated by TGF-β and antagonized by IL-1α. Pathway analysis confirmed counter-regulation of interferon signaling by the two cytokines. These findings have implications for understanding the role of fibroblasts for inflammatory responses and development of fibrosis in the skin.

Keywords: Fibroblast, Keratinocyte, TGF-β, IL-1α, coculture, fibrosis CTGF/CNN 2, dermal, organotypic culture.

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Fibroblaster är de vanligaste cellerna i dermis och ansvarig för uppbyggnad och reparation av vävnad, t.ex. efter sårskada. Funktionellt är den beroende av signaler från andra celler, framför allt från inflammatoriska celler och keratinocyter, men även extracellulär matrix har en reglerande funktion. Fibroblaster kan differentiera till så kallade myofibroblaster, som är en mer kontraherande och prolifererande fenotyp. Vid fibrotiska tillstånd ansamlas myofibroblaster i vävnaden, vilket ger en ökad matrixsyntes och vävnadskontraktion.

Fibros ses ofta som en komplikation efter långvarig inflammation i vävnaden. I fibrotisk vävnad förekommer regelmässigt uttryck av connective tissue growth factor (CTGF/CCN2), ett ”matricellulärt” protein med betydelse för bl.a. vävnadssuppsyntes och kärnybildning. CTGF uttrycks generellt inte i normal hud, utan induceras vid t.ex. en sårskada.

Transforming growth factor-β (TGF-β) är en pro-fibrotisk faktor som inducerar uttryck av CTGF genom Smad signaleringsvägen. Årrvävnad utvecklas under sårläkningens senare del. Vanprérande är klagomål med begränsas av en tidig reepitelisering, t.ex. genom att täcka såren med autolog hud eller andra substitut. Under åter-epiteliseringen är keratinocyter och fibroblaster beroende av kommunikation med varandra, för att återupprätta epidermis och begränsa fibros. En fibros-dämpande mekanism kan vara keratinocyt-medierad nedreglering av CTGF i fibroblaster. I en sammodlingsmodell har vår grupp tidigare visat att keratinocyter, via interleukin-1α (IL-1α), nedreglerar uttrycket av CTGF. Detta skulle, åtminstone delvis, kunna förklara den gynnsamma effekt som hudepitelet har på fibroblasternas aktivitet och därmed ärerbildningen.

I första arbetet går vi in i detalj på intracellulära mekanismer för IL-1α och βs effekt på uttrycket av TGF-β stimulerat CTGF. Vi beskriver hur IL-1 påverkar signal-transduktionen från TGF-β som leder till minskad syntes av CTGF. Vi fann att IL-1 minskade intracellulär Smad 3 fosforylering efter TGF-β-stimulering. Detta kan i sin tur förklaras av ett observerat förhöjt uttryck av Smad 7, som hämmar Smad interaktion med TGF-β receptorn. Detta leder sedan till en minskad aktivitet av en transfekterad promotor innehållande Smad 3-bindande sekvenser. Vidare såg vi att om man slår ut mRNA för det intracellulära proteinet TAK1, med RNA interferens teknik, minskas effekten av IL-1.

Sammantaget identifierades Smad 7 och TAK1 som två faktorer som medierar hämningen av signaltransduktion från TGF-β receptorn till minskat CTGF uttryck, i huvudsak reglerat på promotor nivå.
I det andra arbetet, etableras en in vivo-lik organotypisk odlingsmodell där vi på ett förbättrat sätt kan studera interaktionen mellan keratinocyter och fibroblaster. Den organotypiska modellen har använts till att studera fibroblasternas uttryck från gener som är viktiga för remodellering av bindväv, fr.a. extracellulära matrixproteiner, proteaser och dess hämmare. Sammanfattningen av våra resultat visar att keratinocytorna påverkar fibroblasterna att agera katabolt på extracellulära matrix, i enlighet med kliniska observationer att reepitelialisering och epidermal täckning av sårområden minskar ärrbildningen.

I tredje arbetet genomförs en transkriptions microarray med efterföljande analys av signaleringsvägar, för att göra en mer omfattande analys av hur IL-1 kan hämma effekter av TGF-β. Vald koncentration av cytokiner och tidpunkt för analysen är baserade på uttrycket av CTGF genen. Resultatet visade att IL-1 mestadels hade en antagonistisk effekt på TGF-β reglerande gener. Genom att analysera funktionellt grupperade gener, gav studien stöd för att balansen mellan TGF-β och IL-1 har betydelse för cellens reglering av cell cykeln (celldelning), samt känslighet för apoptos (celldöd). Med samma analys visar vi att interferon signaleringsvägen hämmads av TGF-β och inducerades av IL-1. Interferon inducerande gener (IFIT1, IFIT3 och IFIH1) verifierades med qPCR.

Det fjärde arbetet är en uppföljning av det tredje men på protein nivå; med kvantitativ proteomik undersöktes IL-1s påverkan på TGF-β effekter. Uttryck av CTGF och ett antal andra proteiner (SEMA7A, COL5A1, NRP1, DCN and LUM) identificerades som reglerades av TGF-β och med antagonistisk effekt av IL-1. IL-1s förmåga att inducera interferon signalering bekräftades på protein nivå, vilket vi också visade sker även utan TGF-β stimulering. Ett antal protein uttryck verifierades dessutom på mRNA nivå. Analyser av funktionellt grupperade gener och proteiner gjorda i tredje och fjärde arbetet visade också att TGF-β påverkar negativt IL-1 signalering.

Sammantaget ger avhandlingen nya ledtrådar om samspelet mellan keratinocyter och fibroblaster, samt viktiga cytokiner aktiva vid inflammation och sårläkning. Ökad kunskap kring dessa processer kan bidra till förbättrad behandling av dysfunktionella tillstånd relaterade till vävnadsnybildning.
LIST OF PAPERS

The following papers and manuscript in present thesis, referred to in the text by their roman numerals.


Published papers have been reprinted with permission from the Publisher.
Additional studies not included in this thesis:


# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Cq</td>
<td>Quantification cycle value</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehydhyde-3-phosphate</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1α</td>
<td>Interleukin-1α</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin-1 receptor</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>ISGs</td>
<td>Interferon stimulated genes</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>KGF/FGF7</td>
<td>Keratinocyte growth factors/fibroblast growth factor-7</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency associated peptide</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasmoinogen activator inhibitor-1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>QMS</td>
<td>Quantitative Mass Spectrometry</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SSc</td>
<td>Systemic sclerosis</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGF-β activated kinase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper cytokine response type 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper cytokine response type 2</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitors of matrix metalloproteinase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TYK</td>
<td>Tyrosine kinases</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-Smooth muscle actin</td>
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INTRODUCTION

Structure and function of the skin

The skin is composed of three integrated layers; the outer epidermis, the dermis and the inner hypodermis or subcutis (figure 1). It provides protection against ultraviolet light, mechanical and chemical insult. Moreover, it prevents dehydration and acts as a physical barrier to prevent invasion of microorganisms. The skin is also an energy store, the major organ for thermoregulation and synthesize vitamin-D. It is the largest sensory organ in the body and contains a variety of receptors for touch, pressure, pain and temperature. Skin appendages (or adnexa) such as sweat glands, seba-ceous glands, hair follicles (with arrector pili) and nails are skin-associated structures, that extends from the epidermal surface into the dermis. Adnexa serve a various functions including sensation, lubrication and heat preservation/loss.

Epidermis

The epidermis is a keratinizing stratified epithelium, forming a protective barrier covering the body’s surface. The keratinized layer is shed continuously and replaced by the progressive movement and maturation of cells from the germinal layer. During cornification, the process whereby living keratinocytes are transformed into non-living corneocytes, the plasma membrane is replaced by layers of ceramides which become linked to an envelope of structural proteins (the cornified envelope). Keratinocytes are the main cells in epidermis and the process of maturation of basal cell to desquamation takes from 5-6 weeks [1]. This dynamic process is represented by generation of four morphological distinct layers separated from the dermis by a basement membrane. The basal layer or stratum basale is the germinal layer with a high mitotic activity providing a constant supply of new keratinocytes. The stratum spinosum contains cells that are in the process of growth and early keratin synthesis. The filamentous protein cytokeratin, the predominant synthetic product of these cells, aggregate to form tonofibrils which converge upon the desmosomes of the plasma membrane forming characteristic “prickles”, naming this layer. The stratum granulosum is characterized by intracellular granules which contribute to the process of keratinization. The process of keratinization is through to involve the combination of tonofibril and keratinohyalin elements to form the mature keratin complex. Cell death occurs in the outer
most aspects of the stratum, releasing lysosomal enzymes. Stratum corneum consists of flattened dead cells, devoid of nuclei and other organelles and filled with mature keratin. The stratum corneum is composed of three lipid components: ceramides, cholesterol and fatty acids, and represents the major barrier for microorganisms, environmental substances and water loss. Epidermis also contains Langerhans cells (antigen presenting cells), melanocytes (melanin producing cells), and Merkel cells (sensory cells).

**Dermis**

Dermis is the middle layer of the skin, composed of mostly connective tissue and few cells. The connective tissue provides structural support for the skin and other organs. The fibroblasts are the most abundant cell responsible for the production of fibers, ground substance and extracellular matrix (ECM)-regulating enzymes in the skin. Collagen and elastin fibers provide the skin with strength and elasticity. There are several collagen types that constitute about 70% of the dry weight of the skin. Nerves (with mechano- thermo- and pain receptors), blood vessels and lymphatic vessels are also distributed in dermis. Other cellular infiltrations of dermis are lymphocytes, mast cells and tissue macrophages involved in nonspecific defence and immune surveillance [2]. The dermis can further be divided into a papillary region just adjacent to the epidermis and a deep thick area of known as reticular dermis [3].

**Subcutis**

Subcutis (or hypodermis) is the deepest layer, consisting of a network of connective tissue and adipocytes. The dermis and subcutis are integrated with each other through nerve, lymphatic and vascular networks, as well as epidermal appendages. It helps the body conserve heat and protects the body from trauma.
Figure 1. The skin. Reprinted with permission from Wikimedia commons, Author Daniel de Souza Telles, 24 Jan 2010, https://commons.wikimedia.org/wiki/File%3AHumanSkinDiagram.jpg
Epithelial-mesenchymal interactions

Interactions between epithelial and mesenchymal cells are necessary during organogenesis in embryonic life, and for maintenance of tissue homeostasis in the adult. Adult epithelial tissue continuously renews their structure by proliferation, migration and differentiation, processes that are similar to those occurring during development.

Interactions during embryogenesis and adult life

During embryogenesis, the histogenesis and normal development of epithelia in many organs are dependent on epithelial-mesenchymal interactions. Interactions typically are reciprocal with respect to epithelium and mesenchyme, or alternatively influence each other as development proceeds. In the adult skin, the connective tissue influences are also essential for regular epithelial growth and differentiation. The underlying mesenchyme has both instructive and permissive effects on morphogenesis and in situ differentiation of adult epithelia, demonstrated by transplantation experiments with cross-recombinants, using epithelial and connective tissue components of different organs. Absence of mesenchymal influence, as studied with isolated cultured keratinocytes, showed deficient manifestation of epithelial growth and differentiation. Such studies, clearly demonstrate that epidermal homeostasis and differentiation are regulated by diffusible factors provided by the mesenchyme. Tissue interactions also seem to be committed to a specific pathway of differentiation; cultures of epithelial cells do not lose their intrinsic potential and respond to appropriate extrinsic regulator stimuli. These discoveries were of importance for the development and use of culture model systems to study epithelial-mesenchymal interactions in vitro [4].

Studies of interactions in vitro

Epithelial-mesenchymal interactions are difficult to study under in vitro conditions, due to the many variables involved in the experimental conditions that cannot be properly controlled. The role of epithelial-mesenchymal interactions for the keratinocyte stem cell phenotype was demonstrated by Reinwald and Green by using irradiated mesenchymal cells as feeder cells [5]. The feeder cell-culture clearly showed that the epidermal stem cell phenotype depends on interactions with mesenchymal cells, providing a microenvironment which supports the stem cell phenotype and dramatically favored epidermal proliferation. Growth factors play a critical role for supporting epidermal proliferation, but addition of
even complex mixes of such factors cannot replace the net effect of mesenchymal cell, indicating a more complex interplay. Epithelial growth and differential could be studied in models resembling more the in vivo situation. All lining epithelial tissue, including the epidermis, are surface epithelia: there upper cell sheets are exposed to the outer environment and nursed from the basal side from the mesenchyme. Therefore, a dermal equivalent model have been developed and predominantly been used since then [6, 7]. In this model, mesenchymal cells are incorporated into a collagen gel and epithelial cells are plated on top of the subsequently retracted gels, and the whole unit is lifted to the air-medium interface. The collagen gel is contracted by the mesenchymal cells, provided the gel is detached from the surface and free floating in the medium. The extent of contraction is proportional to number of cells and inverse proportional to the collagen concentration [8]. Keratinocytes cultured in monolayers never achieve the state of terminal differentiation, only in advanced three dimensional in vitro systems keratinocytes develop into a well-ordered epithelial structure. This kind of model offers an opportunity to analyse the cellular mechanisms of tissue formation, such as cell-cell interactions, the regulation of proliferation and differentiation as well as the reepithelialization process after wounding [9]. The usage of dermal equivalents makes the complex composition of the skin easier to study, and for analytical purposes epithelial and mesenchymal cells from different species have also been utilized [8]. Based on these studies, various commercially available skin substitute models (SkinEthic, Epiderm, EpiSkin) have been developed for pharmaceutical and chemical compound testing. Various skin substitutes are available for clinically treatment of skin loss, classified as acellular or cellular, epidermal, dermal or dermo-epidermal (full-thickness) skin substitutes. Complex full thickness in vitro skin models can, thus, mimic native skin by incorporation of other cell types including melanocytes, endothelial cells, and peripheral neurons. Various different ECM components can also be incorporated in the dermal equivalent. The organotypic skin culture can also be used to study various skin diseases [10]. Platforms for in vitro skin cultures have also been implemented within microfluid chips, ”skin-on-chip model” [11].

**Epithelial-mesenchymal transition**

Epithelial-mesenchymal transition (EMT) is the process that facilitates the derivation of a multitude of functional specialised cells, tissues and organs in the developing embryo. In EMT, the epithelial cells acquire fea-
tures of mesenchymal cells, loose polarity and cell-cell contacts and undergo dramatic cytoskeleton remodeling [12]. EMT is a process that is important in wound healing and tissue remodeling. Transforming growth factor-β (TGF-β) has been identified as a trigger for EMT, inducing differentiation of epithelial cells into myofibroblasts, believed to be important for wound healing [13].

**Major cells in the skin and their interactions**

Keratinocytes, fibroblasts and adipocytes are the main cells in respective layers of the skin. Immunological cells are distributed in all layers. The epidermis contains, except keratinocytes, Langerhans cells (antigen presenting cells), melanocytes (producing melanin pigment), and Merkel cells (sense light touch). The dermis and subcutis contain all blood vessels, lymphatic vessels, nerve fibers, secretory glands and their cellular compartments.

**Fibroblasts**

Fibroblasts are fully differentiated cells, originated from the mesenchyme, most abundant in loose connective tissue and responsible for production of the dermal connective tissue and their precursors. In skin, fibroblasts maintain and support the skin through secretion and degradation of the ECM. They play an important role in almost every skin process during development, tissue homeostasis of the mature skin and in wound healing to restore the barrier function of the skin.

Conventionally, fibroblasts are defined by their spindle-shaped morphology, adhesive growth on culture plastics, expression of mesenchymal markers that include vimentin and collagen I [10]. The lack of reliable and specific molecular fibroblast marker is a limiting factor in studying fibroblast *in vitro*, none are both exclusive to fibroblasts and presented in all fibroblasts, and the best specificity for detecting fibroblast seems to be fibroblast-specific protein-1 (FSP-1) [14]. Fibroblasts are poorly characterized mainly due to the diversity which exists as a product of distinct anatomic locations and their associated microenvironment. Even fibroblasts separated from a single tissue as in dermis show three subpopulations: Superficial (papillary) fibroblasts, reticular fibroblasts and fibroblasts associated with hair follicles. These are morphologically and physiologically distinct, and with an ECM different in terms of their composition and organization [15]. Fibroblasts have a plasticity and a large variability of phenotypes, recruited from resident cell populations, circulating precur-
sors, pericytes and transformed epithelial cells, and do not differentiate along a terminal lineage [12]. Hence, fibroblasts represent a heterogeneous population of cells, with the myofibroblasts as the only sub-phenotype.

Myofibroblasts have an ultrastructural morphology with prominent microfilament bundles in their cytoplasm that distinguish them from “normal” quiescent tissue fibroblasts. Myofibroblasts were first identified in the tissue repair process, where they were capable of changing to a contractile phenotype involving both increased ECM production and contraction [16]. Myofibroblasts have features of both fibroblasts and smooth muscle cells, expressing α-smooth muscle actin (α-SMA) used as a universal marker for the myofibroblast phenotype. Myofibroblasts have been observed in practically all fibrotic conditions involving retraction and reorganization of connective tissue. Myofibroblast differentiation is a complex process, regulated by TGF-β and ED-A (a splice variant of cellular fibronectin), as well as presence of mechanical tension. These factors are thought to be crucial for wound contraction [17]. Many tissues and pathologies with sustained presence of myofibroblasts are also presenting with fibrosis, both in internal organs and in the skin (hypertrophic scars). Also cells with phenotypic features of myofibroblasts have been found in and around a number of epithelial tumors, where they have been named cancer-associated fibroblasts or stromal myofibroblasts [18].

Fibroblasts can also participate in immunological responses in direct response to pro-inflammatory signals in areas such as regulation of normal barrier function of the epithelium, remodeling of infected tissue and regulation of the behavior of infiltrating leukocytes to sites of inflammation. Toll-like receptors (TLR) are essential to the innate immune system, recognizing pathogen-associated molecules, such as bacterial lipopolysaccharides (LPS), were reported to be expressed in fibroblasts [19]. Fibroblasts can respond directly to components of the bacterial flora such as LPS and induce expression or pro-inflammatory such as interleukin (IL)-1α, IL-1β, IL-6, IL-8 and tumor necrosis factor-α (TNF-α) [20].

Fibroblasts undertakes dynamic and reciprocal interactions with other resident cell types e.g. epithelial, endothelial and immunological cells, through direct cell-cell communications, cell-matrix interactions or secretion of growth factors and cytokines [12].

**Keratinocytes**

Keratinocytes are the predominant cells found in the epidermis, derived from the ectoderm. Epithelial tissue is classified, on the basis of their mor-
phology and differentiation specific expression pattern, into three main classes; keratinizing stratified squamous epithelia, stratified non-keratinizing epithelia and simple epithelia. The epidermis of the skin is built up of stratified keratinizing epithelium, in which the keratinocytes undergo a terminally differentiated program. This results in formation of a mechanically resistant and toughened surface composed of cornified cells (squames or corneocytes) that are filled with keratin filament. In the corneocytes, the cell membrane is replaced by a proteinaceous cornified envelope that is covalently crosslinked to the keratin filaments, leading to the formation of a dead superficial cell layer, that are eventually sloughed off [21]. Keratinocytes undergo a program of terminal differentiation, expressing a set of structural proteins, keratins and other interactive proteins, which assemble into filaments and function to maintain cell and tissue integrity. The basal cells are attached to the basement membrane, are proliferative and constitute a compartment characterized by specific antigens and in particular the basal type of keratins, K5 and K14. Keratinocytes that have detached from the basement membrane start to express the earliest markers of terminal differentiation, keratins K1 and K10. Later, upper spinous and granular cells also synthesize the precursors of cross-linked envelopes, involucrin, loricrin and the enzyme responsible for the cross-linking process, the membrane bound transglutaminase. Finally, cross-linking occurs forming the resistant cornified envelope, cellular organelles are discarded and the post-translationally modified keratin 1 and 10 associate with filaggrin forming the stratum corneum [8]. Regulation of keratinocyte stem cell proliferation is an important topic, since the rate of proliferation determines the rate at which differentiated cells enter the upper epidermal layers. Specific agents have been identified, such as Vitamin A and D, calcium and growth factors, which regulate keratinocyte differentiation [22]. Many skin disorders have been shown to result from mutations in keratin genes, demonstrating the importance of these proteins in maintaining the mechanical integrity of epithelial tissue.

Keratinocytes in the epithelia represents the body's first-line defense barrier and significantly contribute to innate immunity. Epidermal keratinocytes can sense pathogens, they express several TLR’s and release numerous anti-microbial peptides when affected, and mediate immune responses by releasing several growth factors and cytokines, including IL-1, IL-6, IL-10, IL-18 and TNF [23].
Interactions between keratinocytes and fibroblasts

During development, tightly controlled mutual interactions between epidermis and the mesenchyme control the skin architecture. Interactions persist in adult life regulating skin homeostasis, being altered during wound healing and in extensively studied in tumor biology. All through the tissue repair process, interactions between different cell types take place allowing for a spatial and temporal control. Cellular interactions become dominated by the interplay of keratinocytes with fibroblasts during mid- and late phase of wound healing, characterized by the gradually shift from an inflammatory to a synthesis-driven granulation tissue. The usage of different in vitro culturing systems have identified networks of growth factors, some of which have been verified in normal skin or in different transgenic and knockout mice [24]. Most growth factors are detected in both the keratinocyte and the mesenchymal cell compartments and effects on cell growth and differentiation were observed in both cell compartments, likely operating in an autocrine and paracrine fashion.

Keratinocyte growth factors/fibroblast growth factor 7 (KGF/FGF7) is rapidly induced in fibroblasts after wounding and exerts growth effects on keratinocytes. Other factors, like platelet-derived growth factor (PDGF), are predominantly expressed in epidermal cells and exerts their action on mesenchymal cells. Still others may have effects on both cell types, by autocrine and paracrine mechanisms. In co-culture models, it became clear that keratinocytes depend on and instruct fibroblasts to synthesis and secrete growth factors and cytokines, such as KGF/FGF7, IL-6 and granulocyte macrophage-colony stimulating factor (GM-CSF). IL-1 derived from keratinocytes was identified as the primary inductor, and addition of IL-1 induced the expression of these factors in fibroblasts [25]. A double paracrine mechanism were demonstrated, where keratinocytes initiate growth factors in fibroblast which themselves stimulate keratinocyte proliferation [26]. Fibroblasts are able to secret IL-6, IL-8, hepatocyte growth factor (HGF) and KGF/FGF7, all which are known to stimulate keratinocyte proliferation and migration [8, 27].

Apart from growth factor regulation, the formation of a new basement membrane zone is another example where the interactions between keratinocytes and fibroblasts are crucial. The basement membrane components are produced by both keratinocytes and fibroblasts. Keratinocytes produce laminin-5 and are primarily producers of collagen VII, to a more extent than fibroblast, whereas nidogen is exclusively derived from fibroblasts. A fully organized basement membrane influences the keratinocyte
phenotype, by matrix-derived signals. Another example of the influence of the ECM is the proteolytic processing that can generate laminin-5 fragment stimulating epidermal growth factor (EGF) signaling [25].

**Wound healing**

Wound healing involves a complex interplay of numerous cell types, modulation of soluble factors, ECM, and blood elements. Wound healing is usually divided into three phases: inflammation, proliferation and remodeling. It consists of a series of consecutive but overlapping events including cell proliferation, migration, ECM deposition (collectively known as fibrogenesis), resolution and remodeling. Each phase is dominated by particular cell types, cytokines and chemokines.

The innate immune system represents the first line of defense against infectious pathogens and aids adaptive responses through antigen presentation, providing a target, specific response and immunological memory. Repair of damaged tissue is a fundamental biological process, which allows the ordered replacement of damage and dead cells after injury. Wound healing becomes pathogenic if it continues unchecked, resulting in accumulation and remodeling of ECM, creating permanent scar tissue. Pathogenic fibrosis typically results from persistent inflammation in the wound, resulting in tissue necrosis, infection leading to persistent myofibroblast and excessive accumulation of ECM components.

**Inflammation**

When skin injury occurs, platelets aggregate and initiate the clotting cascade, triggering the hemostatic process. A blood clot is formed consisting of platelets, neutrophils and monocytes which are embedded in cross-linked embedded fibrin fibers. The fibrin clot covers the wound and acts as provisional matrix for cell attachment and migration during the tissue repair process. The damaged tissue and blood clot release of pro-inflammatory growth factors from, such as PDGF, TGF-β, EGF, and IL-8. Leading to increased vasodilation and vessel permeability, which permits recruiting leukocytes across the provisional ECM. The most abundant inflammatory cell in early stage of wound healing is the neutrophils, which eliminate cell debris, dead cells or pathogens. During this initial leukocyte migration phase, monocytes are recruited to the wound under influence of cytokines and differentiate to tissue macrophages. Macrophages continue the process of wound bed clearance and initiating debridement, by releasing proteases and metalloproteases. In contrast its phagocytic role, macro-
phages have an important regulating role in recruiting and activating of inflammatory cells, by releasing local cytokines. As the inflammatory phase progress, macrophages produce important growth factors, such as KGF/FGF7, TGF-β, vascular endothelial growth factor (VEGF) and PDGF, stimulating growth and migration of keratinocytes and fibroblast. These factors are also monogenic and chemotactic for endothelial cells, enhancing angiogenesis. Subsequently, under the influence of IL-1 macrophages are replaced by T-cell immune infiltration, T-cells have many regulate functions by producing and secreting different factors [28].

**Proliferation**

Construction of a newly formed granulation tissue is fundamental to the next proliferation phase of wound healing. Granulation tissue consists of new vessels that migrate into the wound and the accumulation of fibroblasts and dermal matrix. By influence of growth factors like PDGF, fibroblast growth factor (FGF), fibronectin and TGF-β, fibroblasts migrate into the provisional matrix and synthesizing ECM composed of collagen, glycosaminoglycans, fibronectin and elastin. They are differentiated into α-SMA-expressing myofibroblasts, believed to derive from local mesenchymal cells, epithelial cells undergoing EMT or from peripheral blood fibrocytes. Activated myofibroblasts promote wound contraction, a process aiming the sealing the wound. Endothelial cells migrate into the newly formed matrix forming new blood vessels. Epithelial cells divide and migrate over the basal layers to regenerate the damage tissue, crucial to restore the barrier function of the skin. Growth factors like Insulin growth factor-1 (IGF-1) and EGF effect keratinocyte migration and proliferation. Vital to keratinocyte migration, is the production of several proteases such as collagenases and matrix metalloproteinases (MMP), enabling cell movement. Keratinocytes migrate over the newly formed granulation tissue, completing the reepithelialization process, eventually restoring its stratified morphology. Migration is followed by basement membrane assembly through laminin production [28, 29].

**Remodeling**

The remodeling phase can prolong for months after injury, and is characterized by reduced proliferation and infiltration, active re-organization of the ECM. Myofibroblasts and vascular cells undergo apoptosis clearing the matrix. Collagen fibers become more organized, provisional collagen III is replaced by structural collagen I. Matrix is re-organized by various
MMPs and their inhibitors to restore the damage tissue to its normal appearance.

**Inflammatory cells**

Wound healing is overlapped of well-defined sequence of infiltrating immune cells, of neutrophils, macrophages and lymphocytes, migrating into the wound. Neutrophils are recruited by pro-inflammatory cytokines and chemotactic agents, appearing approximately within 24 h, starts to clean the wound by phagocytosing wound debris and pathogens.

Macrophages migrate into the wound 24-96 hours after injury; regulate fibrogenesis by secreting chemokines that recruit fibroblasts and other inflammatory cells. Macrophages has been proposed as the master regulator of fibrosis, due to its capacity to act both pro and anti-inflammatory, as well as its ability to regulate activation and recruitment of myofibroblasts, and macrophages [30]. Elimination of macrophages is crucial for the transition from the inflammatory to the proliferative phase of wound healing [31].

T-lymphocytes peaks during the late-proliferative/early remodeling phase. T-lymphocytes are major source of cytokines, having a regulatory effect on inflammation and fibrosis. The role of T-lymphocytes is not completely understood and under intensive investigation. However different subsets appears to have different roles, where CD4+ T helper (Th) cells have been found to have positive promoting effects on wound healing, and CD8+ cytotoxic T cells an inhibitory effect [32]. T helper cells have an important role in fibrosis progression and can develop cytokine responses, of either type 1 (Th1) or type 2 (Th2). The Th1-type cytokines tend to produce pro-inflammatory responses dominantly by Interferon-γ (IFN-γ), while the Th2-type cytokines have anti-inflammatory responses, include cytokines IL-4, IL-5, IL-10 and IL-13. Although, inflammation typically proceeds fibrosis, the amount of fibrosis in not necessary linked to the severity of inflammation, suggesting regulation by different mechanisms [33].
Growth factors and cytokines in wound healing

Wound healing is dependent on the recruitment of several cell types that appear in the wound area in a temporally and spatially defined manner. This involves coordinated efforts from several different cell types, such as keratinocytes, fibroblasts, endothelial cells, platelets and immune cells. This complex process is executed and regulated by a network of numerous growth factors, cytokines and chemokines. These agents act to alter the growth, differentiation and metabolism of a target cell, by binding to the receptor, triggering a cascade of molecular events, such as cellular proliferation, differentiation, migration, and adhesion. Molecules act in a paracrine, autocrine, juxtacrine or endocrine mechanisms, resulting in pleotropic effects in multiple cell types. Growth factors can be small molecules such as hormones or macromolecules such as proteins. They can be secreted as fully functional molecules or as molecules that require further post-translational processing in order to be activated. They can be synthesized and secreted by many types of cells; the type of response is dictated by its chemical identity, concentration and duration of action [34].

The initial wound healing phase is initiated by the clot formation, induced hemostasis and influx of inflammatory cells. Platelet degranulation release growth factors like PDGF, TGF-β and EGF. PDGF and pro-inflammatory cytokines, like IL-1, attract neutrophils to the wound site in order to remove contaminants. TGF-β triggers monocyte differentiation to macrophages, which initiate the development of granulation tissue. Macrophages release factors like FGF, TGF-β and PDGF that stimulate fibroblast infiltration. Fibroblasts differentiate into myofibroblasts, by the influence of TGF-β, and develop contractile properties facilitating wound closure [25]. Angiogenesis is assisted by platelets which release VEGF and basic FGF (bFGF or FGF-2) that initiate proliferation of endothelial cells [34]. New vessel formation is vital for the synthesis and reorganization of the ECM, supplying fibroblasts with oxygen and nutrients. Within hours after injury, epithelial cells migrate under the newly formed granulation tissue thus initiating reepithelialization. This process is activated by several growth factors including IL-1. Pre-stored IL-1 is release by keratinocytes upon injury, functioning in an autocrine fashion by inducing keratinocyte migration and proliferation, as well as triggering the inflammation cascade. In addition, IL-1 activates nearby fibroblasts and increases the secretion of KGF/FGF7, which in turn promotes keratinocyte migration and proliferation by the feedback mechanism, mentioned above. TNF-α expressed in keratinocytes have an autocrine effect by stimulating keratino-
cyte migration. This cytokine also work in a paracrine fashion activating fibroblasts and increase secretion of FGF family members. This suggests that it indirectly can promote reepithelialization [25].

During mid and late phase of wound healing, the microenvironment is gradually changing from an inflammatory to a proliferative and synthesizing granulation tissue. The granulation tissue is replaced by a framework of collagen and elastin fibers, proteoglycans and glycoproteins, mostly produced by fibroblasts. The following tissue remodeling involves vascular regression and granulation tissue re-organization. MMP’s produced by fibroblasts, macrophages and neutrophils promote collagen breakdown.

Understanding of the normal tissue repair and how this is regulated, by pro-fibrotic and anti-fibrotic cytokines and their proteins, is fundamental of understanding dysfunctional repair. Dysregulated cytokines and growth factors are of major importance for pathological wound healing, and much attention is focused on the role of these for understanding the fibrogenic process. Notably, the pro-fibrotic TGF-β and connective tissue growth factor (CTGF) are considered master switches for the induction of the fibrotic program. As mentioned above, TGF-β activates fibroblasts to synthesize and contract the ECM, but also induces expression of the critical down-stream mediator CTGF, which further supports TGF-β’s effect [35]. Partly as a downstream effector of TGF-β, CTGF stimulates proliferation, chemotaxis and production of ECM. CTGF is found in almost every fibrotic condition.

The pro-inflammatory cytokine TNF-α is produced by macrophages during the inflammatory phase. At high levels it can be damaging to wound healing, by suppressing ECM proteins while increasing MMPs, having anti-fibrotic properties [34]. T-cells release anti-fibrotic IFN-γ immediately after wounding, which suppress collagen synthesis.

Clinical observations support the view that reepithelialization and epidermal wound coverage counteract excessive scar formation. During the process of reepithelialization, keratinocytes and fibroblasts are dependent on communication with each other to re-establish a functional epidermis and limit fibrosis. Pro-inflammatory IL-1 has been shown to suppress TGF-β induction of α-SMA, collagen and CTGF in vitro, and apparently have an important role in tissue repair which needs further investigation [36].

Over the last decade, there has been a great progress of understanding the mechanistically aspects of TGF-β intracellular signaling. This has implications for the discovery of new therapeutic strategies. It is difficult to
Manipulate the healing process by administrating exogenous cytokines and growth factors due to the complexity *in vivo*. That may in fact disturb the fine balance of other factors that are supposed to act in concert. Thus, by simply adding abundant concentrations of a factor, or adding it in an improper spatial and temporal manner may lead to side effects.

Nevertheless, gene therapy is currently investigated as a growth factor delivery system. Promising factors include VEGF, bFGF and GM-CSF thus candidates for clinical testing. PDGF-BB has been approved by U.S. Food and drug administration to facilitate wound healing, and for use in the treatment of periodontal defects and diabetic ulcers [37].

**Transforming growth factor-β**

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine that causes a diverse array of cellular responses in a variety of cell types [38]. The cellular responses include changes that are important for development, wound healing, immune responses, and the pathogenesis of cancer. Over 33 TGF-β-related genes have been identified in mammalian genomes, including bone morphogenic proteins (BMPs), activing/inhibin, growth and differentiation factors, nodal, and anti-müllerian hormone [40]. The TGF-β super family consists of 3 types (TGF-β1/2/3), with the TGF-β1 being the most abundant isoform in most tissues, including the skin. Each isoform shows a unique expression pattern, suggesting individual distinct function during development [41]. All bind to the type II TGF-β receptor, a serine/threonine receptor kinase, which in turn recruits and phosphorylates type I TGF-β receptor. TGF-β is a multifunctional growth factor with profound regulatory effects on many developmental and physiological processes, as shown in TGF-β knock-down mice, which only survive about 20 days, before they die of autoimmune-like inflammatory responses [42].

**TGF-β activation**

TGF-β is secreted as a latent precursor molecule (LTGF-β) that contains an amino-terminal hydrophobic signal peptide region, the latency associated peptide (LAP), usually also complexed with latent TGF-β-binding protein (LTBP). Proteinases such as plasmin release TGF-β from the complex, rendering TGF-β accessible for receptor binding [Clark and Coker, 1998]. In response to ligand binding to the type II receptor, a stable heterodimeric complex is formed with the type I receptor, allowing its transphosphorylation and thus activation of the type I receptor kinase. In its activated state, the type I receptor directly binds and phosphorylates spe-
cific members of the Smad proteins. All Smad proteins consists of two globular domains, an amino-terminal Mad homology domain 1 (MH1) and a carboxyl-terminal Mad homology domain 2 (MH2), connected by linker sequence. The N-terminal MH1 domain has a DNA binding activity, while the C-terminal MH2 domain have more protein-binding and transactivation properties. This interaction results in activation of the SMAD pathway through which the receptor regulated R-Smads (Smad1, 2, 3, 5, and 8) are phosphorylated, and common mediator (Co-Smad: Smad4) recruited, to form the R-Smad/Co-Smad complexes. These complexes are translocated to the nucleus where Smad proteins interact with sequence-specific transcription factors and with the co-activators CBP and p33, regulating transcription of various TGF-β-responsive genes. The MH1 domain of the R-Smads can bind directly to DNA, the minimal Smad 3/4 binding element (SBE) containing four basepairs, 5′-AGAC-3′ [35, 43].

The inhibitory Smads (Smad 6 and 7) negatively regulate TGF-β signaling, by competing with R-Smads for receptor or Co-Smad interaction and by targeting the receptors for degradation [44, 45]. The Smad signaling pathway is crucial for simultaneous gene expression of the skin fibrillar collagen I, III and V by TGF-β. Besides playing a role in the regulation of expression of ECM components, Smads have been identified as signaling intermediates for the expression of proteases/inhibitors, like Plasminogen activator inhibitor-1 (PAI-1) and MMP-1 [35]. In addition to signaling through the canonical Smad pathway, TGF-β also activates other signaling pathways, including MAP kinases (ERK, p38, and JNK), Rho-like GTPase signaling pathways, and phosphatidylinositol-3-kinase (PI3K)/AKT pathways [46] (Figure 2).
TGF-β in wound healing and fibrosis

TGF-β has an important role in wound healing through its pleotropic effects on cell proliferation, differentiation, ECM production and immune modulation [47]. TGF-β1 predominates during cutaneous wound healing and is released at an early stage of wound healing, prompting e.g. cell growth and ECM production. Granulation tissue is formed and TGF-β induces fibroblast synthesis of key components of the ECM and myofibroblasts differentiation. TGF-β also facilitates the angiogenesis, securing blood supply of the newly formed tissue. During reepithelialization, TGF-β shifts keratinocyte integrin expression profile to promote keratinocyte migration [48]. TGF-β regulates a wide variety of cellular processes, paradoxical TGF-β promotes epithelial cells to undergo growth arrest [39]. There are contradictory results regarding TGF-βs role for keratinocyte proliferation, demonstrating the complexity of signaling participating in wound healing. Overexpression of TGF-β1, during late stages wound healing enhances proliferation of keratinocytes. TGF-β is involved in the final matrix formation and re-modulation phases, producing collagen (mainly...
type I and III), functioning also as a potent inhibitor of MMP, such as MMP-1, MMP-3 and MMP-9 and promoting tissue inhibitor of metalloproteinases (TIMPs) [36].

The second isoform, TGF-β2, is, like TGF-β1, needed for expression and organization of collagen and other major ECM proteins during wound healing. It is involved in recruitment of fibroblasts to the damages area, leading to collagen synthesis and tissue formation.

TGF-β3, however, has been shown to inhibit fibrosis, and improve organization of collagen in vitro [49]. Wound healing of the oral mucosa, which presents with a low level of scar formation, demonstrated an increase in the ratio of TGF-β3 to TGF-β1 [50]. Moreover, exogenous TGF-β3 injection has been shown to reduce scarring after cleft lip repair. The mechanism behind this was reported to be due to restricted myofibroblast differentiation and reduced deposition of collagen I [51]. Also, increased TGF-β3 levels seem to promote wound closure rather than reepithelialization [52].

An elevated TGF-β expression has been observed in keloid tissue, along with increased fibroblast proliferation and collagen deposition [53]. This is supported by the observation that expression of TGF-β is increased in keloid fibroblasts in vitro, compared to dermal fibroblasts [54]. Systemic sclerosis (SSc) is a tissue disorder characterized by pathological remodeling of connective tissue correlated to activation of the TGF-β signaling pathway [35]. Taken together, TGF-β has been implicated as a key mediator in a number of fibrotic diseases in various organs including the skin.

**Connective tissue growth factor**

Connective tissue growth factor (CTGF) is a matricellular protein that plays an essential role in the formation of blood vessels, bone, and connective tissue, during development and in the adult life. CTGF is considered an important molecular mediator of fibrosis, and other cellular processes ascribed to CTGF include proliferation, differentiation, adhesion and ECM synthesis [55]. The 38 kDa polypeptide was discovered as a PDGF-like molecule produced by umbilical vein endothelial cells [56]. CTGF is also known as CCN2, belongs to a family of immediate-early genes collectively denoted CCN (for Cyr61, ctgf, nov).

CTGF consists of four distinct structural modules that orchestrate many biological effects. Module 1 is homologous to insulin-like growth factor binding protein (IGFBP). Module 2 comprises a von Willebrand factor type C (VWF-C) motif, which binds bone morphogenetic proteins and
TGF-β. A hinge region separates the two motifs in the N-terminus domain from those in the C-terminus domains, which is disposed to proteolytic cleavage by proteases. The third module contains thrombospondin-1 (TSP1) and the fourth located at the c-terminal contains a cystein knot motif (CT) [57, 58]. The CT module has been implicated in cell adhesion through a unique integrin and heparin sulfate proteoglycan dependent mechanism [59]. Where proteoglycans containing cell surface heparin sulfate that are necessary co-receptors for CTGFs action, possible by binding to cell surface proteoglycans and secondary through other ligands and receptors, such as TGF-β-receptor [60].

The synthesis of CTGF is stimulated by specific growth factors, like endothelin 1 (ET-1) and TGF-β, and also by environmental changes such as hypoxia and biomechanical stimuli [61]. The CTGF gene seems to be regulated primarily at the level of transcription. The promoter region contains recognition sequences for HIF, Smad, basal control element-1 (BCE-1), Ets-1 and Sp1 as indicated (figure 3). The functional Smad element present in the CTGF promoter is activated by Smad3/4 and Smad 7 suppress the CTGF gene promoter through this motif. TGF-β induction of CTGF also requires protein kinase C and the Ras/MEK/ERK MAP kinase cascade. The Smad element acts in concert with a tandem repeat of ETS elements on the CTGF promoter to confer TGF-β responsiveness [58].

Figure 3. Regulation of the CTGF promotor. The CTGF promotor contains recognition sequences for HIF, Smad, BCE-1, Ets-1 and Sp1. Hypoxia, TGF-β and endothelin-1(ET-1) induces CTGF as indicated. The figure is modified from [58].
CTGF seems also to be regulated post transcriptionally, where the 3’UTR region appears be of importance for the CTGF regulation [60]. CTGF both indirectly and directly stimulates transcription factors and pathways of p42/p44 MAP kinase, Akt/PKB, JNK, Smad and Nuclear factor kappa B (NFκB) [55].

CTGF is important for the formation of connective tissue and angiogenesis during development, as well as tissue remodeling and repair in wound healing. CTGF-deficient mice exhibit severe skeletal and ECM abnormalities, due to impaired chondrogenesis, and die neonatal from respiratory failure. This demonstrates the importance of coordinated expression of CTGF and ECM during development [62].

In adult skin CTGF is not normally expressed unless induced, but is characteristically overexpressed in excessive scarring and most fibrosis disorders. CTGF expression is increased after injury and is involved in granulation tissue formation, reepithelialization and matrix remodeling. It is produced by fibroblasts and in an autocrine way stimulates proliferation and chemotaxis of these cells, as well as being a strong inducer of ECM proteins. In wound healing reepithelialization, CTGF has been proven to be required by promoting cell migration, throw the Ras/MEK/ERK MAPK signaling pathway [63]. CTGF have also been shown to induce proliferation and migration of endothelial cells, thereby contributing to angiogenesis [64].

CTGF has received much attention as a major amplifier of the profibrogenic action of TGF-β, which is considered a central mediator of the fibrotic response and required for persistent fibrosis. TGF-β is a potent inducer of CTGF, but CTGF is not required in fibroblasts for TGF-β to induce collagen or α-SMA expression nor is this matricellular molecule obligatory needed for cutaneous tissue repair. CTGF is not a downstream regulator of TGF-β but rather act as a cofactor to enhance the fibrotic effects of TGF-β [65]. Thus, CTGF represents a molecular target for therapeutic intervention in fibrotic disease. Indeed, inhibition of CTGF by using various strategies appears to block fibrosis induction in several animal models [66].
**Interleukin-1**

The interleukin-1 family of cytokines comprises 11 proteins (IL-1F1 to IL-1F11), primarily associated as major mediators of innate and chronic inflammation. Besides this IL-1 has an important role in wound healing affecting reepithelialization and ECM turnover. IL-1 is produced by a variety of cells such as macrophages, monocytes, dendritic cells, fibroblasts and epithelial cells. IL-1α and IL-1β (IL-1F1 and F2) have potent pro-inflammatory activities regulated at the level of synthesis and release, membrane receptor activation, as well as intracellular signal transduction [67]. IL-1 has a naturally occurring endogenous IL-1 receptor antagonist (IL-1Ra), that blocks the IL-1 receptor type 1 binding of either IL-1α or β. IL-1Ra is used clinically to reduce disease severity in a broad spectrum of inflammatory diseases [68]. IL-1α is synthesized as an active precursor (ProIL-1α), capable of binding to its receptors (IL-1R or TLR) and trigger signal transduction. The precursor of IL-1β (ProIL-1β), on the other hand, requires cleavage by caspase-1, or extracellular neutrophilic proteases, to be active [69]. IL-1α and IL-1β only share a 24% identical amino acid sequence, but have largely identical biological activity [67]. IL-1α or IL-1β bind to IL-1RI which exists in complex with IL-1 receptor accessory protein (IL-1RACP). This ligand-receptor complex recruits an adaptor molecule called MyD88, which supports recruitment of at least one of the two serine/threonine kinases termed IL-1 receptor associated kinases (IRAK) 1 and 2. The IRAKs interact with TNF receptor associated factor 6 (TRAF6). TRAF6 recruits TGF-β activated kinase (TAK1), which in turn recruits and phosphorylates NFκB-inducing kinase. NFκB-inducing kinase activates the IkB kinase complex (IKK), which can phosphorylate IkB, causing a rapid ubiquitination and proteolytic destruction of the latter protein. NFκB is now free to migrate in to the nucleus, where it initiates expression of a variety of inflammatory genes [69, 70].

In response to receptor activation, a complex network of events takes place resulting in activation of NFκB signaling and the JNK and p38 mitogen-activated protein kinase pathways. This interplay cooperatively induces IL-1 responsive genes, like IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1), cyclooxygenase 2 (COX2), inhibitor of nuclear factor B α (IkBα) and Mitogen-activated protein kinase phosphatase-1 (MKP-1). IL-1 is also regulating itself by positive and negative feedback mechanisms [67]. IL-1 is a multifunctional cytokine mainly regulating local and systemic inflammation. Consequently, IL-1 defects results in severe multi organ inflammation. By using neutralizing IL-1Ra (Anakindra) or with neutrali-
zation antibodies, it is possible to control inflammatory conditions such as during autoimmune diseases [69]. In the skin, IL-1 is important to trigger the immune defense, but also to limit the excessive scaring after injury [71]. As mentioned above, IL-1 is an important factor to regulate epithelial-mesenchymal interactions in the skin, thus regulating tissue homeostasis in epidermis. Keratinocytes actively control the production of growth factors in fibroblasts by releasing IL-1, which induces expression of growth factors such as KGF/FGF-7 and GM-CSF in the stromal cells [26]. Both of these latter factors activate keratinocyte proliferation and are upregulated in wound healing [72]. Keratinocytes have been shown to downregulate fibroblasts synthesis of collagen and TGF-β synthesis in cultured skin substitute [73, 74]. IL-1 has also been shown to regulate connective tissue metabolism by promoting degradation of ECM [75]. IL-1α was identified as a keratinocyte-derived factor that mediated CTGF mRNA and protein suppression in human dermal fibroblasts [76]. An antagonistic regulation by IL-1 and TGF-β of target genes important for wound healing has been described, where exogenously added IL-1 was able to suppress TGF-β-induced α-SMA expression in fibroblast co-culture [77]. In addition, using IL-1Ra deficient mice, it has been suggested that increased levels of NFκB is inhibiting TGF-β signaling by decreased Smad phosphorylation [78]. Thus, there seems to be a “crosstalk” between TGF-β and IL-1 signaling where e.g. TAK1 could act as an intermediate between the pathways. TAK1 was first discovered in the context of TGF-β signaling, but seems to function primarily as an essential component of the IL-1 pathway [79]. Induced expression of inhibitory Smad 7 is another possible mediator in such a crosstalk, which, in this case, would to limit TGF-β expression. Pro-inflammatory cytokines are regularly abundant in non-healing wounds. Hence, increased levels of IL-1, along with other pro-inflammatory cytokines such as interleukin-6 and TNF-α, were found to be present in significantly higher concentrations in wound fluid from non-healing compared to healing leg ulcers [80]. It appears that IL-1 has a complex role in relation to fibrosis; both direct anti-fibrotic effects on TGF-β signaling and indirect pro-fibrotic effects via inflammatory cells acting to e.g. enhanced TGF-β secretion may operate simultaneously or in sequence. In support of this view is the observation that IL-1 was elevated in fibroblasts cultures from the skin of systemic sclerosis, a connective tissue disorder of systemic and dermal fibrosis [81].
Interferon

Interferons (IFNs) are cytokines that play a central role in initiating immune responses, especially antiviral and antitumor effects, named for their ability to interfere with growth of live influenza virus [82]. There are three major types; type I consists chiefly of IFN-α and of IFN-β, type II consisting of IFN-γ, and type III consists of the λ interferons. Each type differs in cell types responsible for their production and is characterized by a specific signal transduction pathway. Type I IFN receptors are two membrane spanning proteins IFNαR1 and IFNαR2 that form a complex with the ligand, like Type III the receptors they are associated with Janus kinase (JAK1) and tyrosine kinases (TYK2). The Type II receptor is a heterodimer of two membrane spanning proteins IFNγR1 and IFNγR2. Upon cognate receptor activation, the receptors dimerize and activate JAK1, JAK2 or TYK2, which in turn phosphorylate the signal transducers and activators of transcription (STAT) proteins. STATs dimerize and translocate to the nucleus and induce the expression of IFN-stimulated genes (ISGs) [83]. Interferons share many biological effects due to remarkable overlap in the many genes they regulate by the different types, especially type I and II where almost 70% of genes are mutually regulated, partly explained by the use of the same components of the JAK-STAT signaling pathway [84]. Together they regulate genes resulting in a shared spectrum of biological effects, including regulation of both the innate and the adaptive immune response. As such, dysregulated interferons are major effectors involved in several autoimmune diseases, like systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and systemic sclerosis [85]. There is no clear-cut relation between IFNs and development of fibrosis, although several studies demonstrate anti-fibrotic effects in various approaches. The complexity arises because of the various effects of IFNs effects on various cell types, such as leukocytes and fibroblasts.

IFN-α/β

In humans the IFN-α/β family consists of 13 molecules commonly produced by leukocytes and one (IFN-β) usually produced by fibroblasts or epithelial cells. Innate immune cells, such as dendritic cells or macrophages, sense pathogens by different pattern-recognition receptors (PRR), like TLR, and respond to these pathogens by producing IFN-α and β. In infected and neighboring cells, type I IFNs induces expression of ISG, whose products initiate an intracellular anti-microbial program that limits the spread of infectious agents [86]. Induction of ISGs leads to inhibition of
viral replication, transcription and cell proliferation. IFNs are not essential for ISG induction, because any stimulus capable of activating a member of the IFN regulatory factor (IRF) family of transcription factors has the potential to induce ISGs. Thus, type I IFN signaling use IRF-9 for this purpose, whereas IRF-3 and IRF-7 is essential for activation of type I IFN gene expression and induction of the antiviral state [87]. The predominant STAT complex that is formed in response to type I IFN is the interferon-stimulated gene factor 3 (ISGF3) complex. It is composed of STAT1, STAT2 and IRF9, and binds to IFN-stimulated response element (ISRE) sequences to activate classical antiviral genes [86].

Studies suggest that IFN-α/β may slow down the fibrotic process; intrallesional injections of IFN-α and IFN-β lead to reduction in keloid mass, hypertrophic scarring and dupuytren’s disease nodules [88-90]. At the mechanistic level this may, in part, be caused by induction of myofibroblast apoptosis [91]. The benefit of this treatment seems to be larger in a preventive approach, suggesting that systemic regulation, e.g. involving leukocytes, is required. Approximately, half of the patients suffering from systemic sclerosis have an increased expression of IFN-regulated genes in their peripheral blood (aberrant IFN signature). This exemplifies the complexity regarding IFNs and development of fibrosis. Interleukin-6 is one of the most prominent cytokines activated by the IFN pathway and has an important role in initiation and promotion of fibrosis, e.g. in SSc [92]. In the case of SSc, the IFN profile may trigger excessive interleukin-6 production, and thus overrule other fibrotic effects IFNs block the effects of TGFβ on fibrosis, suggesting that they might actually oppose this aspect of SSc pathogenesis. However activation of TLR on dendritic cells and macrophages also stimulates IL-1, TNF and IL-6 production, and these or other undefined mediators might drive inflammation and fibrosis in SSc [93].

IFN-γ

IFN-γ is the only member of the Type II interferons, produced mainly by natural killer (NK) cells and T-cells. IFN-γ shares no homology with the type I IFNs. IFN-γ exercises its antiviral activity by modulating both the innate and the adaptive immune response. In the adaptive immunity, it is produced by CD8+ T killer cells in order to control the infection, and by CD4+ T helper cells which promote inflammatory responses through clearance of intracellular pathogens. IFN-γ is one of the main cytokines that distinguishes Th1 from other CD4+ subsets, e.g. Th2 [85].
In vitro, IFN-γ inhibits collagen synthesis and negatively regulates the TGF-β signaling pathway [94]. This may be caused by induction of inhibitory Smad 7 [95], or by transcriptional modification via STAT1 [96]. Inflammatory cells secrete TGF-β and IFN-γ at the site of injury, thus exerting dual effects on e.g. collagen synthesis. These antagonistic effects probably play an important role in tissue homeostasis [36].

CD4+ T cells seem to play a role in the fibrosis progression and the role of Th1 and Th2 cytokines for development of fibrosis is under extensive studies. Several studies using cytokine deficient mice show that fibrogenesis is strongly linked to a Th2 CD4+ cell response. However, Th1 cytokines (notably IFN-γ) mediate an equally potent inflammatory response together with a prominent inhibition of fibrosis. Treatment with IFN-γ or IL-12 lead to decreased collagen deposition with no effect on the establishment of infection, a finding that has promoted attempts to find anti-fibrosis strategies, i.e. switching of the pro-fibrotic Th2-cell response in favor of the less damaging Th1 cell response [33].

Fibrosis and pathological conditions

Tissue fibrosis is characterized by excessive accumulation of extracellular matrix, often observed as a by-product of tissue repair. Thus, scarring is frequently a by-product of wound healing, but the scar-less wound healing observed in early human gestational fetuses suggests that it is not an essential component of the response. This has led to a large body of research attempts to understand the mechanisms behind scaring and in turn prevent it [47]. Acute wound healing goes through a linear series of events, whereas if this is not the case there is risk for excessive scarring. The most obvious example of this in the skin is hypertrophic and keloid scarring. Inappropriate healing in internal organs can similarly present with excessive scarring (liver, lung and kidney) [12]. Fibrosis development is largely viewed as a complication of inflammatory processes, since resolution of the inflammatory response is critical to prevent fibrosis. Fibroblasts are triggered by components of both innate and adaptive immunity, as well as from alterations in the microenvironment. Chronic inflammation does not always induce deposition of connective tissue, the magnitude of fibrosis is associated to the development of the Th cell response [33].
**Hypertrophic scars**

Hypertrophic scars are raised, abnormally pigmented and can cause itching or abnormal sensation. Patients suffering from pathological scarring can have cosmetic and functional limitations, especially hypertrophic scars associated with thermal injury that are often accompanied with contractures. Hypertrophic scars remain within the boundaries of the original injury, unlike keloids, and can regress with time. Hypertrophic scars have been shown to have a preponderance of collagen type III fibers oriented parallel to the epidermal surface. They are often composed of nodules containing myofibroblasts, collagen filaments and other ECM molecules. Fibroblast derived from hypertrophic scars have an altered phenotype, compared to normal fibroblasts, expressing more TGF-β, showing prolonged expression of TGF-β receptor and CTGF, suggesting that TGF-β having a possible role in hypertrophic scar formation [47].

**Keloids**

Keloids are defined as pathologic scars that occur in areas of cutaneous injury, and can be described as benign fibroproliferative tumors, with no malignant potential. The incidence is higher among African-Americans, Asian-Americans, Latino-Americans and other darker pigmented ethnicities. The term “keloid” derives from the Greek word chele, which means crab’s claw, an analogy to the horizontal growth of the tissue into the normal skin. Keloids may develop far after the initial injury, persist for extensive periods of time and usually do not regress. They are characterized by an excessive deposition of ECM components, such as collagen, fibronectin, elastin proteoglycans and show presence of a variety of growth factors. They also have an increased fibroblast density and proliferation rates of these cells. Larger, thicker and more wavy collagen fibers are observed in this condition. An increased ratio of type I to type III collagen is seen, unlike hypertrophic scars where type III dominates. The pathogenesis of keloids involves both genetic and environmental factors, but there is still no single unified hypothesis explaining keloid development [97]. For example, it is unclear whether the fibroblast is the causative cell; rather they are modulated by other cell populations in wound repair, such as keratinocytes and macrophages [98].
Other fibroproliferative disorders

Fibrosis is a common outcome of various fibroproliferative disorders, but typically more pronounced in presence of prolonged inflammatory processes. Fibroproliferative disorders are characterized by excessive connective tissue accumulation and slow but continuous tissue contraction. That lead to progressive deterioration in the normal structure and function of affected organs. Fibrosis is the result of fibroproliferative disorders that can lead to permanent scarring, organ malfunction and ultimately death. As seen in end-stage liver disease, kidney disease, idiopathic pulmonary fibrosis and heart failure. Fibrosis is also a major pathological feature of many chronic autoimmune diseases, including systemic sclerosis (SSc), rheumatoid arthritis, Crohn’s disease, ulcerative colitis, myelofibrosis and systemic lupus erythematosus (SLE). Although fibrogenesis is a major cause of morbidity and mortality in most chronic inflammatory diseases, there are few treatment strategies available [29].
AIMS

The aim of this thesis was to investigate the regulation of fibroblast activity by keratinocytes, TGF-β and IL-1α in different in vitro models

The specific aims of the studies were to investigate:

I. The mechanism of IL-1’s inhibitory effect on fibroblasts expression of CTGF.
II. How keratinocytes regulate fibroblast genes involved in ECM remodelling, in a more in vivo-like condition. By using a keratinocyte-fibroblast organotypic skin culture model, to elucidate possible anti-fibrotic effect of keratinocytes during epidermal generation.
III. How IL-1α affects TGF-β regulated genes and pathways in fibroblasts by using transcriptional microarray experiments and gene set enrichment analysis.
IV. How IL-1α may affect TGF-β regulated protein expression and pathways in fibroblasts, by using Quantitative Mass Spectrometry (QMS) and protein set enrichment analysis.
MATERIALS

Patient cells and cell lines

Human dermal fibroblasts were isolated from explanting pieces of dermis [99] from young individuals undergoing plastic reconstructive surgery at Plastic Surgery and Reconstructive Surgery Clinic, Örebro University Hospital. The tissue was removed using standard surgical procedures during elective abdominal or chest surgery, only spare skin for deposition were used without any additional discomfort for the patients. These normal skin specimens were used for immunohistochemistry or isolation of fibroblasts.

Normal human epidermal keratinocytes were isolated from pieces of skin taken from healthy areas of burn wound patients, in the process of \textit{ex vivo} autologous keratinocyte expansion at Plastic and Maxillofacial Surgery, Uppsala University Hospital. During keratinocyte expansions cells were propagated upon lethally irradiated feeder cells, NIH 3T3 fibroblasts cells, in DMEM/Ham’s F12 medium supplemented with 10% Fetal bovine serum with additives (Zn-free insulin, 3.3’, triiodo-D-thyronine, hydrocortisone, cholera toxin, epidermal growth factor, adenine and gentamycin) [100]. Only excess keratinocytes that were not needed for autologous graft procedures were used in this study.

Primary fibroblasts and keratinocytes, with a finite life span, e.g. capable of only limited number of population doublings [101], were used in early passages for experiments (fib 3–10 and keratinocytes 2–5), to avoid phenotype changes.

Embryonic mouse fibroblasts NIH 3T3 (ATCC® CRL-1658™) were purchased from American Type Culture Collection (Wesel, Germany). This cell line was developed from NIH Swiss embryo cultures, and immortalization performed by transfection of Murine sarcoma viruses to form a continuous cell culture, capable of an unlimited number of population doublings [102].

Ethical considerations

This study was approved by the local Ethical committee at Örebro County Council, Sweden (no 2003/0101), and from Ethical committee at Uppsala university, Sweden (no 02/557), and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.
METHODS

Cell cultures

Cells can be grown under controlled conditions outside their natural environment. All cells were adherent cells grown in monolayers in complete cell culture media in a humidified incubator with 5% CO₂. Fibroblasts were sub cultured in medium supplemented 10% serum and antibiotics, until confluence. Fibroblasts were detached by trypsin, afterwards suspended in medium containing serum to inactivate the trypsin, centrifuged and resuspended in fresh medium and reseeded. Keratinocytes were seeded in monolayers in complete keratinocyte medium supplemented with growth factors, instead of using feeder cells. Keratinocytes were removed from the culture surface with trypsin. Since cells were seeded serum free, trypsin was inactivated by a trypsin neutralizing solution, the cells centrifuged and reseeded.

Fibroblast activity could be slowed down by elimination of all serum residues. This was done by washing cells with PBS and medium replaced with serum free-medium. FBS contains a rich variety of proteins that provides the growth supplements for the normal growth of cells. Cell morphology was monitored with a light microscope during regular culturing and cells were counted with a hemocytometer. All cells were cryopreserved in liquid nitrogen in freeze medium containing 10% dimethyl sulfoxide (DMSO) used to accommodate osmotic and mechanical stresses during cryopreservation. The cells were free of easily detected contaminants (as bacterial, yeast and fungi) and routinely tested for mycoplasma contamination. Cell culture was performed by aseptic techniques following standard microbiological practices in cell culture facilities at Biosafety Level 2 [103], recommended for most cell lines by European collection of cell cultures (ECACC).

Organotypic models

An organotypic culture system allows for the in vitro growth of complex biological tissues in a way that replicates part of their function and normal physiology. Only in advanced three dimension models keratinocytes forms stratified well-ordered epithelium, offering an opportunity to analyse cellular mechanisms of tissue formation [104]. Stark et al have established an in vitro skin equivalent model that formed the basis for our system [9]. We modified this organotypic skin model, essentially by using
a serum-free EpiLife medium system, thereby allowing us to adjusting calcium-ion concentration, and by using a glass ring on-top of a collagen gel to seed the keratinocytes within. Several reports describe development of an optimized basal nutrient medium for normal keratinocytes in a growth-supplemented defined medium, for serum free culture and possibility of a gradient calcium ion effect [105, 106]. A scaffold composed of native collagen and fibroblasts was allowed to polymerize in filter inserts placed in a six-deep-well plate. Native collagen type I derived from the hides of beef cattle, was kept at 5-10 degrees to prevent gelation. Fibroblasts were mixed with salt solution and collagen, before added to 3.0 mm filter inserts placed in a six-deep-well plate. The collagen solution with cells was allowed to polymerize, and glass rings placed on top of the gels to allocate the area for seeding the keratinocytes. The incubation was continued for another hour before the gels were submerged in keratinocyte medium. After 24 hours, keratinocytes were added on top of the gels inside the rings, restricting keratinocyte growth inside the ring. After another 24 hours, when keratinocytes have attached, the glass rings were removed. The culture media was changed, CaCl$_2$ was increased to 1.5 mM and by lowering the medium level to the lower part of the gels, cultures were raised to the air–liquid interphase. This restricted nourishment from below. This started the organotypic co-cultures, referred to as day 0, and the cultures continued for 7 days. Co-cultures were stimulated by adding TGF-β to the medium underneath the inserts. Incubation with this cytokine was continued for 48 hours, before analysis of mRNA expression for extracellular matrix genes. For morphological analysis, the co-cultures were snap frozen in cryomold at different time points, or paraffin embedded for immunohistological staining methods.

Fibroblasts and keratinocytes seeded in co-cultures were separated by detaching and removing the keratinocyte layer. The epidermal layers, (keratinocytes), were removed by washing with EpiLifes medium without calcium but containing 2mM EDTA. The keratinocyte layers were removed as a thin sheet from the collagen gels and RNA extracted.
Transfection

There are various methods relying on chemical or physical treatment, other include biological particles (virus) as carriers. Transfection of cells can be carried out by electroporation that uses an electrical pulse to create temporary pores in the membrane allowing uptake of material, or by the use of liposomes which fuse with the cell membrane and deposit their cargo inside. We used a liposome based transfection method to induce a plasmid DNA for promoter studies and electroporation were used to introduce small interfering RNA (siRNA) in to cells for gene silencing or knockdown.

By lipofectamine-promoter studies

Luciferase based assays are methods used to study the analysis of cloned promoter DNA fragments, both for verifying the ability of a potential promoter fragment to drive the expression of a luciferase reporter gene in various cellular contexts, and for dissecting binding elements in the promoter. A reporter gene with a DNA sequence of interest is cloned into an expression vector (with the firefly luciferase activity fragment), that is transferred into cells. Following transfer, the cells are assayed for the presence of the reporter by direct measuring the enzymatic luciferase activity [107]. The expression of the CTGF gene were studied by using a CTGF promoter or TGF-β-responsive element constructs bound to a luciferase reporter construct, that were transfected into fibroblast used a liposome-based method.

The promotor is a region of DNA recognized by RNA polymerase to initiate transcription of a particular gene. Promoter analysis seeks to understand why a particular gene responds to a particular environmental condition or treatment. A 0.8 kb CTGF promoter construct linked to luciferase reporter gene was transfected into NIH 3T3 mouse fibroblasts to analyse the effect of IL-1α and β on CTGF promoter activity. A TGF-β-inducible promoter construct composed of twelve CAGA boxes (Smad binding repeats) generated using pGL3 basic plasmid [43] was transfected to investigate the effect of IL-1 on TGF-β-induced Smad pathway activity. Promoter activity was analysed in NIH 3T3 fibroblasts, because we found that primary human fibroblasts survived poorly lipofectamine-based transfections. Lipofectamine reagent is a transfection reagent that facilitates plasmid DNA into the cells; plasmid DNA is complexed with lipid reagents to mediate efficient delivery into the cell’s nucleus. This process is essential for subsequent protein expression of the gene of interest. Cells were plated
one day before transfection, and 0.2 mg DNA construct was the transfect-
ed into the cells under serum-free conditions according to the kit instruc-
tions. In transient transfections, the introduced nucleic acid exists in the
cells only for a limited period of time. Thus, the expressed transgene can
generally be detected for 1 to 7 days, but cells are usually harvested 1 to 3
days post-transfection. In our study, cells were incubated for 24 h, before
the experiment was performed and the cells were harvested within 48
hours post-transfection, to ensure high levels of expressed protein. Cells
were harvested and protein lysates analysed for luciferase activity accord-
ing to Luciferase assay kit. Luciferase Reporter Assays allow you to inves-
tigate promoter activity by measuring light output from luciferase enzyme
that is expressed under the control of your promoter of interest. In the
presence of ATP luciferase catalyses the oxidation of luciferin and gener-
ates a photon. Emission of light was analysed with a luminometer which
gives a relative value of transcription of the promotor.

**By electroporation -gene knockdown**

RNA interference is a method in which small interfering (si)RNA mole-
cules inhibit gene expression, usually by causing the destruction of specific
mRNA molecules. In principle any gene can be knocked down by a syn-
thetic siRNA molecule. This “gene silencing” involves degradation of
double stranded RNA into siRNA, of about 21 to 25 nucleotides long, by
an RNase III-like activity. The siRNAs join an RNase complex RISC
(RNA-inducing silencing complex), which acts on the complementary
mRNA and degrades it. Silencing with siRNA is causing a transient effect,
having different effectiveness in different cell types [108].

TAK1 mRNA was knocked-down in fibroblasts by electroporation us-
ing gene-specific siRNA molecules obtained from Qiagen AB. One siRNA
duplex (MAP3K7_8) showing best gene knock-down performance selected
from the FlexiTube format of four options was used and siRNA lacking
sequence homology to any known mammalian gene was used as negative
control. Transfection efficiency was determined using quantitative poly-
merase chain reaction (qPCR); TAK 1 knockdown level was 80–85%
relative to negative control siRNA in the 24–48 h range post-transfection
by real-time PCR using MAP3K7 TaqMan Gene Expression Assay (Ap-
plied Biosystems).
RNA extraction, quantification and quality control

Total RNA was isolated from cells grown in monolayer using column-based commercial kits (Qiagen), or from cells embedded in collagen gels by a ready-to-use Trizol reagent (Invitrogen). Type of commercial kits were chosen depending on starting material, amount of cells and desired total RNA yield and was used in paper I, III and IV.

In paper II, RNA from collagen gels was isolated with Trizol Reagent designed to isolate total RNA, as well as DNA and protein. After homogenizing the samples with trizol, chloroform is added, and the homogenate is allowed to separate into a clear upper aqueous layer (containing RNA), an interphase, and a red lower organic layer (containing the DNA and protein, with phenol red as color indicator to facilitate isolation of phases). RNA was precipitated in isopropanol, washed in ethanol, RNA pellets were air-dried and re-suspended in RNase-free water [109].

Quantification and quality of RNA was measured using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technology Inc., Wilmington, DE, USA). The ratio of absorbance at 230, 260 and 280 nm was used to assess the purity of DNA and RNA. An optical density \( A_{260/280} \) ratio of \( \sim 1.8 \) is generally accepted as “pure” for DNA and a ratio of \( \sim 2.0 \) is generally accepted as “pure” for RNA. A lower ratio indicates the presence of protein contaminants that absorb near 280 nm. The \( A_{260/230} \) ratio was used as a secondary measure of nucleic acid purity, which is considered “pure” for nucleic acid are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate presence of organic contaminants like phenol and trizol, which absorb at 230 nm.

Quality of RNA was determined using Agilent Bioanalyzer 2100 (Agilent Technologies), where samples were subjected to miniature gel-electrophoresis on a microfluid RNA chip, RNA molecules were separated and detected by laser induced fluorescence detection [110]. An electropherogram and gel-like image is created showing two distinct ribosomal peaks corresponding to either 18S and 28S for eukaryotic RNA. Besides the ratio, Agilent has introduced a software algorithm that takes the entire electrophoretic data into account to estimate the integrity of total RNA samples. This is converted to “RNA Integrity Number” (RIN) which allows the classification of total RNA, based on a numbering system from 1 to 10, (1 being the most degraded and 10 being the most intact) and a RIN >8 are considered accepted quality.
Quantitative PCR

The qPCR technique was used to study changes in gene expression by relative quantification. With this method, targeted DNA is amplified and detected as the reaction progresses in real time. Fluorescent dyes label the PCR products during thermal cycling and the accumulation of fluorescent signal is measured to quantify the product. To use this technique for RNA quantification, RNA needs first to be reverse transcribed into cDNA, to serve as the template for the PCR reaction. For this purpose, we used the “High Capacity cDNA Reverse Transcription Kit” from Applied Biosystems. Next, we utilized “Gene Expression Assays” (Applied Biosystems) to quantify mRNA expression by PCR. This assay consist of a pair of unlabeled PCR primers and a probe with a FAM™ dye label at the 5’ end and minor groove binder (MGB) and nonfluorescent quencher (NFQ) at the 3’ end. Assays were selected by best coverage recommended by Applied Biosystems. Samples were prepared following universal assay conditions (volume and final concentration) using Taqman PCR master mix, containing Gene Expression Mastermix, Gene Expression Assay, water and cDNA. 96-wells reaction plates were prepared and analysed by 7500 Fast Real-Time PCR System (Applied Biosystems). For each well (sample) a singleplex reaction, where a single primer pair is present, amplifies the targeting sequence or endogenous control (figure 4). Results were analysed by relative quantification using RQ manager software; baseline values for each well where automatically generated as well as the threshold values for the selected detector, using the second derivative maximum method [111]. All quantification cycle values (Cq) reported as greater than 35 or as not detected were considered as a negative call. The none template controls (water) were subjected to the amplification step for each detector and consistently found to be negative. PCR amplification efficiency of each Gene Expression Assays was determined by the slope of a calibration curve constructed from sample dilutions and close to 100% for all genes. This was assumed to be constant for all the genes and the linear dynamic range for the genes covering at least 3 orders of magnitude Log10. Accurate normalization is an absolute requirement for correct measurement of gene expression, involving standardization to one or more constitutively expressed reference genes.
Transcripts of genes of interest were normalized to reference genes, to correct for variations in RNA levels and efficiency of cDNA synthesis. For Paper I and II glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a stably expressed reference gene. For Paper III and IV reference genes were evaluated by the “NormFinder” algorithm [112] to evaluated stably expressed genes among a set of candidate normalization genes, where the best combinations of genes are evaluated based on geometric mean. Out of three reference genes evaluated, GAPDH and TATA binding box protein (TATA) were shown to be the best combination of genes.
Relative level of gene expression (RL) was then calculated using the formula $RL = 2^{(Cq_{ref} - Cq_{test})}$, where $Cq_{ref}$ is the $Cq$ value of an arbitrary reference sample from which all other samples ($Cq_{test}$) are subtracted. By this approach the RL of the reference sample always becomes 1.0 and test sample values directly expressed as fold changes. The assayed gene/GAPDH ratio was calculated for each sample. For paper I and II, fold change in mRNA expression was calculated using this approach, also denoted “comparative $Cq$ ($2^{-\Delta\Delta Cq}$) method”. Paper III and IV mRNA results are presented as relative amount of assayed gene to the reference genes by using ratios of linearized $Cq$ values ($2(Cq_{ref})/2(Cq_{test})$).

**Transcriptional microarray**

Transcriptional microarray measures the expression levels of large numbers of genes simultaneously. Small microscopic spots each containing specific DNA sequences, probes or oligonucleotides sequence, are attached to a surface. To these probes or oligonucleotide sequences, a short section of a gene transcript (cRNA or cDNA) from a sample can hybridize, forming a complex that can be detected and quantified. In paper III we used microarray to investigate the gene expression of fibroblasts with Agilent technologies Microarray system (Santa Clara, CA, USA). The one color procedures were used to generate labelled complimentary RNA (figure 5).

The cRNA samples were hybridized on to Agilent SurePrint G3 Human Gene Expression 8x60K v2 Microarray, analysing 8 samples/chip covering 60 000 spotted probes. To generate cRNA, samples of 50 ng of total RNA were transcribed to cDNA by affinity script reverse transcriptase and Oligo dT-promotor primer, recognizing and annealing to Poly A-tail of mRNA. cRNA are simultaneously generated from the cDNA and amplified at least a 100-fold. As a fluorescent label, a cyanine 3-labeled CTP is incorporated by the use of T7 RNA polymerase recognizing the T7 pro-motor of 2-strand of cDNA. Anti-sense Cyanine-3 labeled cRNA were then purified using RNeasy Mini spin columns (Qiagen), cRNA quantity and concentration were measured. A total of 600 ng of cyanine 3-labeled cRNA was hybridized to the Human Gene Expression Microarray in an Agilent hybridization oven for 16 h at 65 degrees. The slides were washed to remove hybridized sample and scanned with the Agilent Microarray laser Scanner generating a raw image file, where hybridized complexes emit fluorescence. Agilent’s Feature Extraction software (version 10.7.3.1) was used to automatically read and process raw microarray image files. The software finds and places microarray grids for spot recognition and
determines feature intensities and ratios. A quality control report is created for each extraction to evaluating microarray performance. All cRNA preparation, hybridization, washing, and detection procedures were performed as recommended by the supplier, meeting the recommended quality standard. Raw fluorescence intensity values were background corrected and normalized. The latter was achieved by comparing an overall signal value of the microarray of the 75 percentile of all non-control probes, within the Bioconductor Software Collection for R, as previously described [113]. The microarray gene expression data have been deposited in the NCBI Gene Expression Omnibus, an international public repository that distributes genomics data, allowing users to review and download studies and gene expression profiles of interest.
Figure 5. Schematic of the Microarray procedure. (A), RNA is reverse transcribed into cDNA, (B) transcribed into cRNA, amplified and labelled with Cy3, (C) cRNA were purified and (D) cRNA were hybridized to Gene Expression slide. Modified from Agilent Technologies Protocol.
Ingenuity Pathway Analysis

*In silico* analysis of transcriptomic and proteomic data were performed using Ingenuity pathway analysis (IPA). IPA is a web-based software application that enables understanding of complex “omics” data at multiple levels by integrating data from a variety of sources (e.g. disease-derived cells or tissue, or from cellular experiments). It provides insight into e.g. signaling pathways and disease processes in a defined system (cellular or tissue context). The software is backed by the Ingenuity Knowledge Base, a repository of biological interactions and functional annotations forming the core technology behind all Ingenuity applications. In comparison analyses trends can be visualized and similarities across analyses using heat maps for canonical pathways, downstream effects, upstream regulators and diseases and functions. The datasets of molecules that exceeded the chosen cut-offs were analysed in IPA by the “Core Analysis”, to reveal significant pathways and upstream regulators. Both canonical pathways and the upstream analysis gives two different statistical measures: the overlap p-value and the z-score. The overlap p-value (calculated by Fisher’s exact test) measures whether there is significant overlap between the dataset genes and genes that are known to be affected by the pathway or upstream regulator. The z-score is calculated to indicate the direction of the effects activation state (activation or inhibition) of the predicted upstream regulator, such that a score of >2 or < -2 is considered significant.

The Molecule Activity Predictor (MAP) tool enables you to predict the upstream and/or downstream effects of activation or inhibition of molecules in a network or pathway, given a starting set of neighbouring molecules with “known” activity or expression. MAP enables you to visualize the overall effect on a pathway or network and therefore to generate an accurate hypothesis.

Western blotting

Western- or immunoblotting is an analytic method used to detect specific proteins in a sample tissue homogenate or extract, by the usage of antibodies. In paper I, protein expression was analysed by Western blotting to detect CTGF, Smad and Smad-P in fibroblast cell lysate. Fibroblasts were lysed in detergent buffer and protein concentration determined using the colorimetric DC™ protein assay. Proteins were denatured by boiling in Laemmlli sample buffer, giving them an overall negative charge. Samples and molecular size reference proteins were loaded and separated by size in
an SDS-polyacrylamide electrophoresis gel (SDS-PAGE) in mini-protean cell system (Bio-Rad). Proteins were transferred to a Hybond-C nitrocellulose membrane in the mini trans Blot tank system. Detection by antibodies were used to identify specific proteins blotted on the membrane. The membranes were blocked in blocking buffer (bovine serum albumin or skim milk), incubated with primary antibody (against CTGF, Smad and Smad-P), washed, incubated with a secondary antibody coupled to horseradish peroxidase enzyme, and washed again. Chemiluminescent detection by an enzyme-substrate reaction, was made by using Bio-Rad's Immunostar HRP Chemiluminescent Kit. Chemiluminescence imaging was performed with Carge-Coupled device (CCD) camera using ChemiDok instrument (Bio-Rad). To estimate the amount of protein loaded on the gel and transferred to the membrane, antibodies from the membrane were detached and the membrane immunoblotted once again with the use of a primary antibody for β-tubulin.

**Immunohistochemistry**

Immunohistochemistry is a common procedure in laboratory medicine to identify specific tissue proteins, by the interaction of target antigens with specific antibodies tagged with a visible label. Immunohistochemistry enables visualization of localization of specific cellular components within cells and in tissue contexts. In paper II, this method was used to identify involucrin, loricrin, keratin 10 and keratin 14 in the organotypic model and native skin to consider markers of keratinocyte differentiation. Samples were snap-frozen in cryomold (for detection of involucrin or loricrin) or paraffin embedded (for detection of keratin 10 and 14) followed by sectioning. Slides with paraffin sections were deparaffinized and rehydrated, by transferring slides through xylene and graded alcohol series to distilled water. Endogenous peroxidases were blocked and an antigen retrieval step was performed, due to the formation of methylene bridges during fixation, which cross-link proteins and therefore mask antigenic sites. Slides were analysed by first blocking nonspecific binding and then immunohistochemical stained with specific primary antibodies. As a negative control, non-immune mouse IgG1 antibodies were used instead of primary antibodies. Slides were washed and a biotinylated secondary antibody, against the primary antibody host animal added and detected using Vectastain ABC kit, forming an active Avidin/Biotinylated enzyme complex (ABC). The complex was visualized by adding Diaminobenzidine (DAB), a HRP substrate that produces a dark brown reaction product.
Finally, tissue slides were counterstained with hematoxylin and mounted. For detection of keratin 10 and 14, specimens were cryosectioned, fixed in formalin and automatically processed using an autostainer link instrument at the Dept of Pathology, USÖ. The autostainer is a “dual link system” which detect both primary mouse and rabbit antibodies automatically, and visualize the reaction by DAB.

**Quantitative proteomics**

Quantitative proteomics is an analytical technique to determining the amount of proteins in a sample and typically rely on mass spectrometry (MS) to detect, identify and quantitate molecules based on their mass and charge (m/z). Most quantitative proteomic analyses entail the isotopic labeling of proteins or peptides in the experimental groups, which can then be differentiated by MS. Thermo Scientific™ TMT™ labeling technology enable identification and quantitation of proteins in different samples using tandem MS.

In isobaric labeling-based quantification, each sample is derivatized with a different isotopic variant of an isobaric mass tag, from a set, and then the samples are pooled and analysed simultaneously in MS. Since the tags are isobaric, peptides labeled with isotopic variants of the tag appear as a single composite peak at the same m/z value in the first MS1 scan, with identical liquid chromatography (LC) retention time. Ionized peptides are sampled to produce a precursor ion spectrum that represents all ionized peptides in the sample. Individual ions are then selected to undergo collision-induced fragmentation (CID) and a second round of MS (MS2), which yields a fragment ion spectrum for each precursor ion. The fragmentation of the modified precursor ion during MS/MS event generates two types of product ions: (a) reporter ion peaks and (b) peptide fragment ion peaks. The quantification is accomplished by directly correlating the relative intensity of reporter ions to that of the peptide selected for MS/MS fragmentation. The fragment ion peaks observed at higher m/z are specific for peptide amino acid sequence and are used for peptide identifications, which are eventually assigned to the proteins that they represent [114]. These fragment spectra are compared to peptide databases and assigned specific peptide sequences and then computationally organized into the predicted protein sequence. A database search was performed with the Mascot search engine (Matrix Science) using the Homo sapiens SwissProt Database (Swiss Institute of Bioinformatics, Switzerland).
**Statistical analysis**

In paper I, results are presented as means ± standard deviation. Student’s t-test (paired) was used for statistical analysis. P-values equal or <0.05 were considered statistically significant.

In paper II, the distribution of the data were analysed by with Shapiro–Wilk’s W test as well as the Skew and Kurtosis using the SPSS software (IBM Company, Chicago, IL). For data sets with normal distributions, the two-way paired Student’s t-test was used to detect significant changes, with Bonferroni’s corrections whenever multiple comparisons were performed (p<0.05). For data with an abnormal distribution a non-parametric method, Wilcoxon’s matched-pairs signed-rank test was used together with Bonferroni’s corrections, to detect significant changes (p<0.05).

In paper III, the results were assumed to be normal distributed and data were analysed for statistical significance using one-way analysis of variance (ANOVA) and post hoc test with Tukey’s honest whenever multiple comparisons were done. For gene set enrichment analysis (GSEA) the background-corrected and normalized data from the microarray were corrected for multiple testing. The false discovery rate (FDR) estimation [115], yielded q-values to filter single gene candidates for the different comparisons and were considered significant for q-value <0.05. A two-stage hierarchical filtration procedure was also performed on the microarray results to determine gene expression signals at the single gene level [116].

In paper IV, qPCR results were presented as mean ± standard deviation indicated by error bars. Data were analysed for statistical significance using one-way ANOVA and post hoc test with Tukey’s honest in case of multiple comparisons. Quantitative Mass Spectrometry data were analysed as three independent groups (C, TC and IT), forming three biological replicates. Group measurements were compared and p-values were calculated by Student’s t-test.
RESULTS AND DISCUSSION

CTGF regulatory signaling (paper I)

IL-1 inhibited TGF-β-stimulated CTGF mRNA and protein expression in fibroblasts, with IL-1α and β having similar effects. At the mRNA level, suppression of basal and TGF-β induced CTGF mRNA expression were around 30-50% after adding IL-1α. Results were confirmed with western blotting; TGF-β triggered the 38 kDa CTGF protein, and IL-1α inhibited this effect. Thus, the results demonstrated that IL-1 is a potent inhibitor of CTGF, and in consistence with earlier studies in our group [76]. This goes with the hypothesis that keratinocytes have a suppressive role on fibroblasts when it comes to e.g. synthesis of collagen, and supports that the reepithelialization process have a role for limiting excess scar formation.

Role of Smad 3

To find out if this inhibition involves the activation state of Smad 3, we examined how IL-1 affected TGF-β-mediated phosphorylation of Smad 3. Addition of TGF-β for 3 h increased phosphorylation of Smad 3, compared to total Smad3 and the protein loading control β-tubulin, shown by western blotting. Pre-incubation with IL-1α and β suppressed phosphorylation of Smad3, compared to controls. One well-described inhibitor of Smad 3 phosphorylation is the inhibitory Smad 7. Smad 7 is triggered by TGF-β itself and thus part of a negative feedback loop in the TGF-β signaling cascade. Decreased Smad 3 phosphorylation attenuates its complex formation with Smad4, which is required for down-stream signaling [117]. Smad 7 could also interfere with binding of the Smad 3/4 complex to DNA [118], promote degradation of the TGF-β receptor I (TGF-βRI), or mediate deactivation of the TGF-βRI by dephosphorylating enzymes [119, 120].

Interestingly, apart from being induced by TGF-β, we found that Smad 7 were also upregulated by incubation with IL-1α and β, thus providing a mechanism by which the latter cytokines attenuates TGF-β signaling. However, no additive effect was observed with respect to TGF-β and IL-1 on Smad 7 mRNA expression, rather were they anti-synergistic, possible mirroring a self-limitation by the effect of Smad 7 on TGF-β signaling.
Activity of the CTGF promoter

Stimulation of CTGF gene expression by TGF-β involves activation of the CTGF promoter, which harbours several recognition sites, e.g. Smad binding elements composed of multiple CACA-boxes [121]. The suppressive effect by IL-1 on TGF-β induced CTGF were examined by the use of two promoter constructs, one that covered most of the promoter and one that consisted of repeated Smad responsive elements. We found that TGF-β induced the activity of the heterologous CTGF promoter by about three fold, and this induction was reduced to near basal level by IL-1α and β. The other construct served to investigate more specifically the Smad pathway activity. Hence this TGF-β inducible promoter construct was composed of repeated CAGA sequences. IL-1α and β inhibited significantly TGF-β induced activity of the latter construct. These promoter activity experiments were performed in NIH 3T3 fibroblasts, because primary human fibroblasts survived poorly lipofectamine-based transfection. Taken together, the results indicate that IL-1 inhibits TGF-β-induced CTGF expression through inhibition of the Smad cascade.

The role of TAK1

The MAPKKK TGF-β-activated kinase (TAK1) is obligatory in the IL-1 signaling pathway, and is also operative in some down-stream events in the TGF-β pathway [122]. TAK1 has been demonstrated to mediate direct inhibitory effect on TGF-β induced Smad 3 activity [123]. Thus, TAK1 is an interesting intermediate that could link the IL-1 receptor activation and inhibition of TGF-β signaling.

To test this hypothesis, TAK1 mRNA in fibroblasts was knocked down by siRNA technique. Transfected cells were incubated with or without TGF-β and/or pre-treated with IL-1α. CTGF mRNA expression was analysed by qPCR. CTGF mRNA in TAK1 or control siRNA transfected fibroblasts responded equally well to TGF-β, indicating that TAK1 is not necessary for the effect of TGF-β on CTGF expression. In contrast, the effect of IL-1α on CTGF expression was significantly inhibited after TAK1 knockdown in fibroblasts compared to fibroblasts treated with negative control siRNA. Thus, TAK1 appears to contribute to the effect of IL-1 on CTGF expression.

Overall, we identified Smad 7 and TAK1 as two factors that could mediate the inhibitory effect of IL-1 on TGF-β stimulated CTGF expression. This is mirrored by the anticipated effects at the level of promoter activities.
**Features of the organotypic model (paper II)**

The organotypic skin cultures formed a skin equivalent over a 7 day period, as shown by histology sections. Over the 7 days, the keratinocyte compartment adopted an epidermis-like morphology while the collagen matrix became structured and fibroblasts aligned along collagen fibers. Compared to conventional two dimensional cell cultures the usage of organotypic keratinocyte-fibroblast co-culture models allows the keratinocytes to adopt a more *in vivo* like structure expressing markers of differentiation. The basal layer expressed keratin 14 as expected throughout the culture period (native skin biopsies was used as control). At day 7, the co-cultures contained a stratified keratinocyte layer expressing the late epidermal differentiation markers keratin 10, involucrin, and loricrin. This indicates that keratinocytes in the co-culture model are able to differentiate and express relevant markers for skin architecture. Some discrepancies between mature culture model and native skin was seen, such as a broader distribution of keratin 14 and involucrin in cultures compared with native skin. Such features of organotypic cultures have been reported earlier, with some discrepancies between organotypic cultures and native skin [9]. Similarly, a native composition of the ECM in the co-culture dermal compartment is lacking, although it is likely that the fibroblasts synthesize some of their own microenvironment within the collagen matrix. A more obvious limitation of the organotypic model is the lack of other important cell types present during wound healing, notably immune cells and endothelial cells. Also, there is a discrepancy between the model and the *in vivo* wound healing situation in that no wound is generated, i.e., keratinocytes are not migrating out from a pre-existing epidermis. Hence, the organotypic culture model can only be regarded as an approximation to the *in vivo* situation and further *in vivo* experiments are generally warranted.

**Fibroblast response in the co-culture model**

The organotypic model was used to study expression of fibroblast genes involved in ECM modulation regulated by keratinocytes and TGF-β. A steady state of ECM is maintained as a result of a proper balance between production and degradation of the structural proteins that constitute the ECM. Major structural proteins such as collagen I, III and fibronectin, as well as important proteases such as MMP and plasminogen activator were studied at the level of gene expression. Inhibitors of the proteinases, e.g. TIMPs and PAI-1 are also important for the regulation of ECM, and were
included in the studies. α-SMA was used as a marker for myofibroblast differentiation. A total of 13 genes were analysed.

A kinetic study was performed with three independent experiments in order to elucidate when most fibroblast gene expression alterations occurred. Most genes were keratinocyte-regulated at day 2, with nine of the 13 genes showing significant change, compared to day 4 at which 6 genes showed significant regulation. This, reflected an altered keratinocyte activity and/or fibroblast susceptibility with time. A second set of nine experiments were performed analysing fibroblast gene expression in day 2 cultures. When combining all 2-day culture experiments (n=12), eight genes were significantly regulated by TGF-β; CTGF, collagen I, fibronectin, PAI-1, MMP-3, TIMP-1, and TIMP-3 were upregulated, while TIMP-2 were downregulated. Notably, 11 out of 13 genes were significantly regulated by keratinocytes, either in the presence or absence of TGF-β; urokinase-type plasminogen activator (uPA), MMP-1, and MMP-3 were upregulated with keratinocytes, while CTGF, collagen I, collagen III, fibronectin, PAI-1, TIMP-2, TIMP-3, and α-SMA were downregulated. uPA, PAI-1, and TIMP-3 required the presence of TGF-β in order to observe the effects of keratinocytes. Importantly, in the presence of TGF-β, the effects of keratinocytes were consistent with the effects found in absence of TGF-β.

Over all, the results support the view that keratinocytes affect fibroblasts to act catabolically on the extracellular matrix in the reepithelialization process.

**Fibroblast viability in the organotypic model**

An interesting question is how fibroblasts survive and possibly expand in the organotypic model. In order to get a gross picture of this, we inspected RNA yield from the different conditions, mirroring cell number. The results demonstrated a significant increase in fibroblast RNA yield from fibroblasts cultured in presence of keratinocytes (44%) or TGF-β (42%), compared to controls (day 2). To exclude that the former results could be explained by RNA coming from the keratinocytes during preparation of RNA, we analysed the level of keratin 16 mRNA, which is abundant in cultured keratinocytes, but absent in fibroblasts. Keratin 16 mRNA was very low in the preparations, and generally less than 0.5% of the RNA was calculated to come from keratinocytes. Taken together, the results from the RNA yield analysis suggested that keratinocytes and TGF-β provide growth promoting and/or anti-apoptotic cues to fibroblasts.
In conclusion, this study shows that keratinocytes can regulate the expression of fibroblast genes important for the turn-over of ECM in an organotypic co-culture model. Most genes were also regulated by TGF-β, and these were counter-regulated by keratinocytes. Overall, the expression pattern suggests that keratinocytes regulate fibroblasts to act catabolically (anti-fibrotic) on the ECM. This adds to the understanding of how reepithelialization and epithelial transplantation act to reduce scar formation.

**IL-1α counteracts TGF-β regulated genes, proteins and pathways (paper III and IV)**

In order to investigate further the cross-talk between IL-1α and TGF-β, the last two papers dissected the effects of IL-1α on TGF-β-regulated genes and proteins in fibroblasts using “-omics” approaches. These in vitro studies were performed in regular two dimensional cultures, and results from transcriptomics (paper III) and proteomics (paper IV) were analysed with state-of-the-art bioinformatics.

**IL-1α suppresses TGF-β stimulated CTGF expression**

In order to confirm appropriate incubation times and concentrations of TGF-β and IL-1α, we first analysed the effects of these cytokines on CTGF mRNA expression, followed by analysis of global gene expression. CTGF expression increased around 12-fold after addition of TGF-β in fibroblasts compared to control, and was evident in 6-48 h interval. A 16 h TGF-β incubation time was chosen to study the effects of IL-1α. A suppression of TGF-β-induced CTGF mRNA expression of about 60-70% was seen at the two highest IL-1α concentrations, in presence of two concentrations of TGF-β (paper III). This confirms our previous results (paper I and II). This result was corroborated by QMS analysis at the protein level (paper IV).

**IL-1α preferentially counteracts TGF-β regulated genes and proteins**

By using a two-step hierarchical filtering 252 genes were generated that were significantly regulated by IL-1α. It was found that IL-1α preferentially counteracted the effects of TGF-β on gene expression in fibroblasts. This antagonistic effect of IL-1α was seen both with respect to the number of genes affected, as well as the extent of change in gene expressions. Findings at the single gene level that could be anticipated by the addition of TGF-β were e.g. the strong stimulation of α-SMA and CTGF (paper III). IL-1α counteracted this TGF-β-stimulated CTGF and α-SMA mRNA expression in fibroblasts, in accordance to our and others previous results.
KGF/FGF7 is rapidly induced in fibroblasts in experimental systems imitating the wounding situation, and this is suggested to be a response to IL-1 released from the injured epithelium, (“double paracrine loop”). In paper III, expression of KGF/FGF7 mRNA was reduced to half by TGF-β, and this was counteracted by IL-1α, the latter in accordance with the recognized feedback mechanism [26]. Given that keratinocytes secrete abundant IL-1α, this also corroborates the observation that TGF-β secreted by fibroblasts counteracts the mitotic effects of KGF/FGF7 on keratinocytes [27]. Smad 7 mRNA expression was increased around 5-fold by addition of TGF-β in our study. This feedback mechanism on TGF-β signaling is well described [45]. In paper I, we showed that IL-1 and TGF-β both stimulated Smad 7 expression when added separately to the cultures, while together there was no additive effect. Similarly, no significant effect of IL-1α was seen in presence of TGF-β in paper III, a phenomenon probably attributed to the negative feedback within the TGF-β signaling, as supported by the findings in paper I and discussed therein. Stimulation of α-SMA, KGF/FGF7 or Smad 7 proteins by TGF-β could not be detected by the QMS approach in paper IV, possibly due to detection limitations.

**The interferon pathway is activated by IL-1α and inhibited by TGF-β**

The microarray analysis identified 15714 genes that were expressed in all sample groups (C, T, IT). FDR estimates using the same statistical cut-off, yielded 1685 genes eligible in the comparison of T vs C and 2647 genes in the comparison of IT vs C. Gene set enrichment analysis using the Ingenuity Pathway Analysis tool showed that the six top TGF-β regulated pathways that were affected by IL-1α were counteracted by the latter cytokine. The most confident of these pathways was “Interferon signaling” inhibited by TGF-β and activated by IL-1α. There were several interferon related genes that were suppressed by TGF-β and induced by IL-1α in our study (IFIT1, IFIH1, IFI44 and IFIT3). These appear in the identified pathways and as targets for upstream regulators (paper III). IRF7 was the most prominent upstream regulator increased by IL-1α and this gene has been shown to be essential for the induction of IFN-α/β genes [87]. IL-1α-mediated induction of the interferon signaling pathway was validated at the protein level in paper IV. Interferon signaling was the top canonical pathway activated by IL-1α in part overlapping with the second pathway “JAK/Stat signaling”. In paper IV, regulation by IL-1α of interferon signaling molecules IFIT1 and IFIT3 was confirmed on mRNA level. Regulation
of IFIH1 mRNA expression by the two cytokines was also analysed and confirmed the microarray results.

There are many studies on the dysregulation in type I interferons and their inducible genes; e.g. in the pathology of autoimmune fibrotic disease systemic sclerosis. Hence, the anti-proliferative activity of interferons adds to their potential role in controlling various fibrotic conditions, underlining the complexity of the effect on fibrosis by these different cytokines. IFN-γ is a well-described anti-fibrotic cytokine that inhibits collagen synthesis and negatively regulate the TGF-β signaling pathway [94]. Inflammatory cells at site of the injury secrete TGF-β and IFN-γ, exerting opposite effects on e.g. collagen synthesis; their antagonistic interactions probably play an important role in tissue homeostasis [36]. How TGF-β and IL-1α affect IFN-signaling is unclear. Apart from interventions in the downstream signaling from the plasma membrane IFN receptors to the nucleus, other possibilities are e.g. MyD88-dependent mRNA stabilization of interferon-response genes as reported previously [127]. Irrespective of the mechanisms of intervention, extending TGF-β and IL-1α effects to the panorama of IFN responses broadens the implications of the former cytokines. However, it is difficult to predict more precise how this will affect the fibroblasts, since both the IFN type I (IFN α/β) and type II (IFN-γ) pathways were affected by IL-1α. Generally, both pathways (type I and II) may lead to reduced fibrosis, either directly by e.g. effects on TGF-β signaling, apoptosis, or indirectly by regulating Th1/Th2 responses in leukocytes. Thus, IFN triggers complex immunomodulatory responses, and further studies are required shed light on why IFN type I is beneficial in some fibrotic conditions, but not others.

Other pathways regulated by TGF-β and counteracted by IL-α

Other pathways inhibited by TGF-β and activated by IL-α in the transcriptome study (paper III) were “IL-1 signaling”, “death receptor signaling” as well as “retinoic acid mediated apoptosis signaling”. The effects on “IL-1 signaling” confirm the antagonistic actions of the cytokines and support the observations that IL-1α is capable of inducing itself as part of the mechanism of auto-inflammation [68]. The observation that death receptor signaling and retinoic acid mediated apoptosis signaling were suppressed by TGF-β and induced by IL-1α suggests that TGF-β protects cells from some apoptotic cues that are enhanced by IL-1α. Two pathways were involving changes in the actin cytoskeleton and associated molecules regulated in the opposite way, i.e. activated by TGF-β and inhibited
by IL-1α. This counteracting effect of IL-1 adds an extension to the well-known pronounced effects of TGF-β on the actin filaments [128].

Results from upstream analysis in our comparison assay indicated that collectively molecules are acting inhibitory on the cell cycle by TGF-β and activated by IL-1α. Following the same analysis, an increased sensitivity of certain apoptotic cues was suggested following IL-1α signaling. For example, PARP9 was identified as an upstream regulator, inhibited by TGF-β and activated by IL-1α. PARP9 is involved in detection and repair of DNA strand breaks, thus a response to apoptotic events. Moreover, TRIM24 has been reported to be anti-apoptotic, and identified in our study as an upstream regulator activated by TGF-β and inhibited by IL-1α. Thus, the data collectively support the view that IL-1 is triggering pro-apoptotic events.

At the protein level, among upstream regulators controlled by TGF-β that was also affected by IL-1α, seven out of eight were found to be counteracted by IL-1α. These included IL-1β, TGF-β1, TGF-β3, and Smad 4, a downstream molecule from TGF-β signaling. Hence, the upstream regulator data support antagonistic effects of both cytokines on each other. MAPK1 was another upstream regulator positively controlled by TGF-β, and negatively controlled by IL-1α. This kinase was also identified as an upstream regulator in an opposite relation to the cytokines in the transcriptome study, paper III. MAPK1 belongs to one branch of the MAP kinase (MAPK) pathways of the non-canonical pathway induced by TGF-β [46]. The significance of this is unclear but may reflect different effects on susceptibility to apoptosis by the two cytokines [129, 130].

MYCN as upstream regulator was negatively controlled by TGF-β and positively controlled by IL-1α. MYCN is a nuclear oncogene and have been reported to accelerate apoptosis after withdrawal of interleukins [131]. This is in line with the view that TGF-β and IL-1α triggers opposite cues that leads to susceptibility to apoptosis.

**Additional proteins regulated by TGF-β and counteracted by IL-1α**

Using quantitative proteomics, 2572 proteins were identified in Paper IV as expressed in all sample groups. After the chosen cut-offs, 47 proteins was identified as TGF-β regulated, and after IL-1α addition, 125 significantly regulated proteins was identified compared to in presence of TGF-β alone. Comparing these two groups of proteins, we found that nine proteins fulfilled the criteria of being regulated by the above cut off values for both comparisons, whereas eight of this was regulated in opposite direc-
tion by the two cytokines. Proteins that were upregulated by TGF-β and antagonised by IL-1α were CTGF, COL5A1 and SEMA7A. Regulation of CTGF was shown at the protein level in paper I. Semaphorin 7A (SEMA7A) is a membrane-anchoring protein and collagen type V (COL5A1) regulates fibrillogenesis, both previously showed to be induced by TGF-β. IL-1α suppression of these proteins is not to our knowledge known and was also confirmed at mRNA level. Proteins downregulated by TGF-β and induced by IL-1α were NRP1, DCN, LUM, HLA-E and PPAP2B. Neurophilin 1 (NRP1) is a co-receptor for tyrosine kinases including TGF-β receptors, and reduced level by TGF-β indicates a negative feed-back response to this cytokine, which was abrogated by IL-1α. Decorin (DCN) and lumican (LUM) are proteoglycans that localizes with fibrillary collagen in the connective tissue, and demonstrated to have a role in regulating collagen fibril structure [132]. Decorin has anti-fibrotic properties due to its ability to sequester TGF-β in the ECM, which is probably the reason why decorin has a negative effect on CTGF regulation [133]. Lumican are localized to the basement membrane of basal epithelial cells, and its expression in wound epithelium suggests involvement in reepithelialization [134]. This in line with the role of IL-1 in the development of the epithelium. Regulation of decorin and lumican protein levels by the cytokines was validated at the mRNA level. Downregulation of HLA-E by TGF-β may have implications for susceptibility to apoptosis, but the significance of the phosphatidic acid phosphatase PPAP2B is unclear.

In conclusion, these studies provide evidence that IL-1α act antagonistically to much larger extent that acting synergistically, on TGF-β regulated gene and protein expression in fibroblasts. Pathway analysis showed that the top antagonistic effect of IL-1α was the interferon signaling, followed by effects on cell cycle regulators, apoptosis pathways and the actin cytoskeleton. Analysis of fibrosis-related genes suggests that IL-1α acts moderately anti-fibrotic on TGF-β effects. Expression of some novel proteins (SEMA7A, COL5A1, NRP1, CDN and LUM) being affected by TGF-β and antagonised by IL-1α was identified. The study adds to the current understanding on interactions between two cytokines intimately involved in e.g. wound healing processes and fibrosis.
CONCLUSION

The main findings and conclusions are summarised as follows:

I. IL-1 was demonstrated to be a potent inhibitor of CTGF mRNA and protein expression. Both IL-1α and β suppress the TGF-β-induced promotor construct, containing Smad binding element, as well as the phosphorylation of Smad 3. Knock-down experiments demonstrate that TAK1 is mediating a significant part of the IL-1 inhibition of TGF-β induced CTGF expression.

II. Keratinocytes regulate the expression of fibroblast genes important for the turnover of the ECM in an organotypic culture model. Most of the genes were regulated by TGF-β, and counter-regulated by keratinocytes. Overall, the expression pattern suggests that keratinocytes regulate fibroblasts to act catabolically (anti-fibrotic) on the ECM.

III. IL-1α was found to preferentially counteract the effects of TGF-β on gene expression in fibroblasts. The most confident of these pathways was the Interferon signaling, which was inhibited by TGF-β and activated by IL-1α.

IV. The previous transcriptome study was confirm, that IL-1α preferentially counteracts TGF-β effects. Six new proteins involved in matrix synthesis/regulation were identified, being regulated by TGF-β and were counteracted by IL-1α. Pathway analysis confirms counter-regulation of interferon signaling by the two cytokines.
FUTURE PERSPECTIVE

The findings in the present studies on keratinocyte-fibroblast interactions have opened up for further investigations such as:

Role of TAK1 for cross-talk between IL-1 signaling and TGF-β signaling. Studies should include the use of TAK1 knock out cells, and detailed analysis of Smad activation (phosphorylation). Is phosphorylation of non-canonical sites important for the effects of TAK1. Apart from receptor Smads, does TAK1 effect Co-Smads and inhibitory Smads as well?

Detailed studies of how is IL-1 affecting TGF-β signal transduction to bring about the various effects delineated in this thesis (e.g. interferon pathways, myofibroblast differentiation, extracellular matrix molecules, apoptosis pathways, actin remodelling, cell-cycle effects). By using state-of-the art methods such as siRNA, knock-out cells, pharmacological inhibition and overexpression, the role of key components of the TGF-β-signaling pathways can be studied. Relevant pathways include the Smad pathway, as well as MAP kinases (ERK, p38, and JNK), Rho-like GTPase signaling pathways, and phosphatidylinositol-3-kinase (PI3K)/AKT pathways.

To what extent can keratinocytes replace the effects of IL-1α, e.g. with respect to the interferon pathway in fibroblasts? Although IL-1α seems to be a main factor secreted by keratinocytes to affect fibroblast activities, it is likely that other factors also contribute. This could be investigated by e.g. qPCR and microarray experiments that compares effects of keratinocyte-secreted factors with IL-1α on fibroblast responses at the level of genes and pathways.

Regulation of novel single proteins such as the eighth identified extracellular matrix proteins or components of the interferon pathway should be confirmed with other methods such as Western blotting and ELISA. Effects of different concentrations of the cytokines as well as the kinetics of responses should be explored.

How are dysfunctional fibroblasts from e.g. fibrotic conditions and chronic wounds responding to IL-1 and TGF-β? It is possible that such cells are dysregulated with respect to the genes and pathways delineated in this thesis. Given that correct responses are crucial for appropriate functions, such studies may shed more light on pathological tissue repair.
The use of the type of organotypic model that was employed in this thesis is valuable for translating data obtained from two dimensional cultures to more *in vivo* like conditions. Thus, in order to validate our data obtained from two dimensional cultures, it would be informative to perform similar experiments in three dimensional cultures, in presence or absence of keratinocytes, IL-1α or TGF-β.

Responses at the level of *bona fide* cellular functions is lacking in this thesis. Thus, e.g. the proposed effects of IL-1 and TGF-β on fibrosis (collagen production in three dimensional models), susceptibility to certain types of apoptosis, cell-cycle activities and migration (due to actin remodelling) should be further explored by the respective relevant method.
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