TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

*SARJA - SER. D OSA - TOM. 1010*

MEDICA - ODONTOLOGICA

# THE REGULATION AND FUNCTION OF COLLAGENASE-3 (MMP-13) IN CUTANEOUS WOUND HEALING AND SQUAMOUS CELL CARCINOMA

by

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TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2012

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ISBN 978-951-29-4970-0 (PRINT)
ISBN 978-951-29-4971-7 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland, 2012

*”Elämän ymmärtää lopusta alkuun*
*mutta se on elettävä alusta loppuun”*

Søren Kierkegaard

*To my family*

**Mervi Toriseva**
**"The regulation and function of collagenase-3 (MMP-13) in cutaneous wound healing and squamous cell carcinoma"**
The Department of Dermatology and MediCity Research Laboratory, University of Turku, Turku, Finland, and Turku Graduate School of Biomedical Sciences, Turku, Finland; Annales Universitatis Turkuensis, Painosalama, Turku, Finland 2012

# ABSTRACT

Matrix metalloproteinase-13 (MMP-13) is a potent proteolytic enzyme, whose expression has been previously associated with fetal bone development and postnatal bone remodeling and with adult gingival wound healing. MMP-13 is also known to be involved in the growth and invasion of various cancers including squamous cell carcinoma (SCC) of the skin. The aim of this study was to further elucidate the function and regulation of MMP-13 in wound repair and cancer.

In this study, it was shown that fetal skin fibroblasts express MMP-13 in response to transforming growth factor-β in a p38 MAP kinase dependent manner. In addition, MMP-13 was found to be expressed *in vivo* by wound fibroblasts in human fetal skin grafted on SCID mice. Adenovirally delivered expression of MMP-13 enhanced collagen matrix contraction by fibroblasts *in vitro* in association with altered cytoskeletal structure, enhanced proliferation and survival. These results indicate that MMP-13 is involved in cell-mediated collagen matrix remodeling and suggest a role for MMP-13 in superior matrix remodeling and scarless healing of fetal skin wounds. Using an MMP-13 deficient mouse strain, it was shown that MMP-13 is essential for the normal development of experimental granulation tissue in mice. MMP-13 was implicated in the regulation of myofibroblast function and angiogenesis and the expression of genes involved in cellular proliferation and movement, immune response, angiogenesis and proteolysis. Finally, epidermal mitogen, keratinocyte growth factor (KGF) was shown to suppress the malignant properties of skin SCC cells by downregulating the expression of several target genes with potential cancer promoting properties, including MMP-13, and by reducing SCC cell invasion.

These results provide evidence that MMP-13 potently regulates cell viability, myofibroblast function and angiogenesis associated with wound healing and cancer. In addition, fibroblasts expressing MMP-13 show high collagen reorganization capacity. Moreover, the results suggest that KGF mediates the anti-cancer effects on skin SCC.

**Keywords:** MMP, skin, wound, collagen remodeling, KGF, SCC, skin cancer

**Mervi Toriseva**
**”Kollagenaasi-3:n (MMP-13) säätely ja toiminta ihon haavan paranemisessa ja okasolusyövässä”**
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Annales Universitatis Turkuensis, Painosalama, Turku 2012

# TIIVISTELMÄ

Matriksin metalloproteinaasi-13 (MMP-13) on tehokas proteolyyttinen entsyymi. Aiemmin sen fysiologinen ilmeneminen liitettiin ainoastaan sikiövaiheen luunkehitykseen ja luukudoksen muokkaukseen syntymän jälkeen sekä haavan paranemiseen ikenissä. MMP-13 on mukana myös useiden syöpien, kuten ihon okasolusyövän patologiassa.

Tässä tutkimuksessa osoitettiin, että sikiön ihon fibroblastit ilmentävät MMP-13:a transformoiva kasvutekijä-β:n stimuloimana tavalla, joka on riippuvainen p38 MAP-kinaasin toiminnasta ja että MMP-13 ilmentyy myös *in vivo* SCID-hiireen istutetun sikiön ihon haavojen fibroblasteissa. Kuten ikenen haavoissa aikuisella, sidekudoksen muokkaus on näissä haavoissa erityisen tehokasta ja ne paranevat ilman arpea. Osoittaen että MMP-13 on mukana soluvälitteisessä kollageenin muokkuksessa, adenovirusvälitteisesti tuotettu MMP-13 lisäsi huomattavasti fibroblastien kykyä muokata kollageenigeeliä. Kyseisten fibroblastien tukirangassa havaittiin muutoksia ja niiden elinkyky oli kollageenissa lisääntynyt. Käyttäen kokeellista haavan granulaatiokudosmallia osoitettiin *in vivo*, että MMP-13 on välttämätön hiiren normaalille granulaatiokudoksen kehitykselle. MMP-13 liitettiin muun muassa myofibroblastien toimintaan ja verisuonten muodostumiseen sekä tulehdukseen, verisuonten muodostumiseen, protolyysiin ja solujen jakautumiseen ja liikkumiseen liittyvien geenien ilmentymisen säätelyyn.

Tässä tutkimuksessa osoitettiin vielä, että keratinosyyttikasvutekijä (KGF) ei lisää ihon syöpäsolujen jakautumista vaan mahdollisesti heikentää niiden pahanlaatuisuutta esimerkiksi alentamalla useiden mahdollisesti syöpää edistävien kohdegeenien ilmentymistä. Näiden geenien joukossa on MMP-13. Lisäksi KGF vähensi okasolusyövästä eristettyjen solujen invaasiota.

Tulokset osoittavat, että MMP-13:n tehtävä solujen liikkumisen ja jakautumisen, myofibroblastien toiminnan ja angiogeneesin säätelyssä sekä soluvälitteisessä kollageenin järjestelyssä on huomattava, mikä on merkittävää sekä haavan paranemisen että syövän kehittymisen kannalta. Lisäksi tulokset viittaavat siihen, että KGF:llä on ihon levyepiteelisyövän eli okasolusyövän leviämistä estäviä vaikutuksia.

**Avaintermit:** MMP, iho, haava, kollageenin muokkaus, KGF, SCC, ihosyöpä

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# ABBREVIATIONS

| | |
|---|---|
| 3D | three dimension |
| ADAM | a disintegrin and metalloproteinase |
| ADAMTS | a disintegrin and metalloproteinase with thrombospondin motifs |
| BM | basement membrane |
| CXCL | chemokine (C-X-C motif) ligand |
| DMEM | Dulbecco's modified Eagle's medium |
| ECM | extracellular matrix |
| ERK | extracellular signal-regulated kinase |
| f-actin | filamentous actin |
| FCS | fetal calf serum |
| FGFR | fibroblast growth factor receptor |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| HSF | human skin fibroblast |
| IGFBP | insulin-like growth factor binding protein |
| IHC | immunohistochemistry |
| JNK | c-Jun N-terminal kinase |
| KGF | keratinocyte growth factor |
| KGFR | keratinocyte growth factor receptor |
| LPA | lysophosphatidic acid |
| MAPK | mitogen activated protein kinase |
| MEK | MAPK/ERK kinase |
| MKK | MAPK kinase |
| MMP | matrix metalloproteinase |
| MOI | multiplicity of infection |
| MSF | mouse skin fibroblasts |
| MT-MMP | membrane type MMP |
| RAd | recombinant adenovirus |
| ROS | reactive oxygen species |
| SCC | squamous cell carcinoma |
| SCID | severe combined immunodeficiency |
| SMA | smooth muscle actin |
| TGF | transforming growth factor |
| TIMP | tissue inhibitor of metalloproteinases |
| VCS | viscose cellulose sponge |

# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I - IV:

I Ravanti, L., Toriseva, M., Penttinen, R., Crombleholme, T., Foschi, M., Han, J. ja Kähäri, V.-M. (2001). Expression of human collagenase-3 (MMP-13) by fetal skin fibroblasts is induced by transforming growth factor-β via p38 mitogen-activated protein kinase. FASEB J. 15, 1098-1100.

II Toriseva, M., Ala-aho, R., Baker, A.H., Marjomäki, V., Heino, J., and Kähäri, V.-M. (2007). Collagenase-3 (MMP-13) enhances remodeling of three-dimensional collagen and promotes survival of human skin fibroblasts. J. Invest. Dermatol. 127, 49-59.

III Toriseva, M., Laato, M., Carpén, O., Ruohonen S.T., Savontaus, E., Inada, M., Krane, S., and Kähäri, V.-M. MMP-13 deficiency delays growth and modulates gene expression signatures involved in inflammation, vascularization, and cell viability in mouse wound granulation tissue. (Manuscript)

IV Toriseva, M., Ala-aho, R., Peltonen, S., Peltonen, J., Grénman, R., and Kähäri, V.-M. (2012) Keratinocyte growth factor induces gene expression signature associated with suppression of malignant phenotype of cutaneous squamous carcinoma cells. PLoS ONE 7, e33041.

# 1 INTRODUCTION

Wound healing is a dynamic and fundamental process, which enables the reconstruction of a protective barrier of a multicellular organism and is thus indispensable for living on earth. Wound repair is strictly regulated by a multitude of growth factors and cytokines secreted by the various cell types involved in healing as well as by insoluble wound extracellular matrix (ECM) components. However, under certain circumstances, this regulation may fail resulting in chronic ulceration. On the other hand, healing may be exaggerated and lead to the formation of extensive scarring which in some locations may, for instance, restrain movement. Normally in adult skin, a wound matures into a flat, collagenous and relatively acellular scar which, however, never reaches the tensile strength of intact skin. Interestingly, in contrast to normal skin wounds, wounds in human oral mucosa and in the skin of a developing fetus heal with minimal scarring suggesting exceptional remodeling of the collagenous dermis in these wounds.

In normal skin, matrix metalloproteinases (MMPs) can be found only in certain cutaneous structures, but after tissue damage the expression of many MMPs is dramatically induced. The proteolytic activity of MMPs is associated with enhanced motility of cells via their ability to modify ECM molecules as well as through the release and activation of growth factors and other latent MMPs.

MMP-13 is a collagenase, which is not produced in normally healing wounds in adult skin, but it is expressed in chronic wound fibroblasts deep embedded in the collagenous matrix (Vaalamo et al., 1997). Moreover, MMP-13 has been shown to be expressed by fibroblasts in human gingival wounds (Ravanti et al., 1999b) suggesting that MMP-13 may participate in the potent remodeling of the wound ECM resulting in minimal scarring.

Keratinocyte growth factor (KGF) is an epithelial mitogen produced by wound fibroblasts. Its role in epithelial carcinogenesis has been extensively studied, but the effects of KGF on the pathobiology of cutaneous squamous cell carcinoma (SCC), characterized by several features of wound healing, is poorly understood.

The aspiration of thesis work was to find factors regulating scar formation and to uncover the cellular and molecular mechanisms behind the events involved in wound repair focusing on MMP-13 function. The role of KGF in skin SCC was also studied and linked to the regulation MMP expression in cutaneous SCC cells. The results increase our knowledge about the proteolysis involved in wound healing in humans and mice, which is useful for the development of wound management, and adds to our understanding of the nature of cutaneous carcinomas. The results with KGF also provide valuable data to be used when considering KGF as a means of protecting normal tissues during cancer treatments.

# 2 REVIEW OF THE LITERATURE

## 2.1 THE STRUCTURE OF SKIN

Human skin consists of two major compartments: the epidermis, which provides the outermost barrier of the body, and the underlying dermis, which is rich in connective tissue. These two compartments are anatomically separated by a basement membrane zone, which is a thin layer consisting of extracellular matrix (ECM) molecules (Fig.1). The most common skin related or cutaneous appendages in humans include hair follicles, sebaceous glands, sweat glands and arrector pilli, smooth muscles that move the hair (Fig. 1). These structures serve to support skin functions and protect the body against for instance cold, heat and dehydration. The hypodermis consists of subcutaneous adipose tissue surrounded by connective tissue, larger blood vessels and nerves (Wolff, 2009).

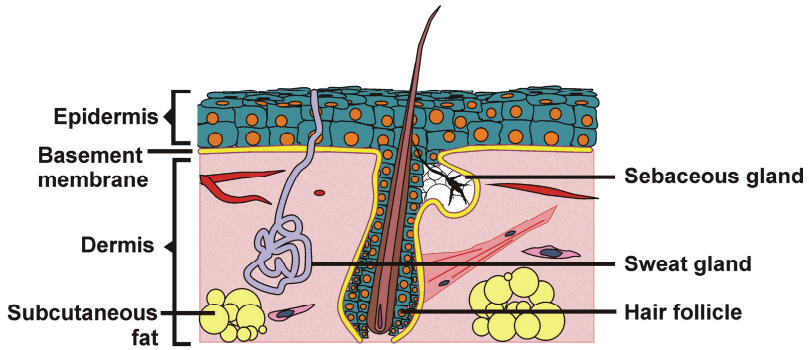


**Figure 1**. The structure of human skin. Modified from an image from the Mayo Foundation for Medical Education and Research.

### 2.1.1 Epidermis

The epidermis forms the outermost compartment of the body and its multi-layered, semi-impermeable structure provides protection against for instance drying, hydration, UV-radiation and a multitude of foreign materials such as bacteria, viruses or different kinds of chemicals. The epidermis consists of epithelial cells (keratinocytes) of progressive differentiation states. Keratinocytes are connected to each other typically by desmosomes and tight junctions. Hemidesmosomes link keratinocytes to the underlying basement membrane. In addition, melanocytes and dendritic cells (Langerhans cells) are found in the epidermis. They provide the skin protection against UV-radiation and microbial antigens, respectively. Based on the morphologic features of keratinocytes, the epidermis is divided into five layers: *stratum basale* (basal layer), *stratum spinosum (*squamous layer*)*, *stratum granulosum (*granular layer*)*, *stratum lucidum* (transitional layer) and *stratum corneum* (cornified layer) listed from the bottom to surface of the epidermis. The basal cells of the basal layer include epidermal

stem cells and their daughter cells, which during differentiation move towards the surface and loose their potential for cell division (Eckert et al., 1997). Keratinocytes change their gene expression profile during this differentiation process (Taylor et al., 2009) and finally at their terminal differentiation state in the cornified layer, the keratinocytes (corneocytes) have undergone apoptosis and lost their cellular organelles including their nucleus (Eckert et al., 1997). Corneocytes are tightly connected to each other and establish a special protective barrier structure with an extensive network of cellular keratins linked to a cornified envelop, which replaces the cell membrane in these cells (Candi et al., 2005).

### 2.1.2 Dermis

While the epidermis is almost exclusively composed of cells, the underlying dermis consists mainly of ECM proteins and proteoglycans, which are produced by resident dermal fibroblasts and give mechanical resistance and elasticity to the skin. The dermis underneath the basement membrane is called the papillary dermis and is relatively abundant in cells. In addition to fibroblasts, dermal cells include neurons and Schwann cells that compose sensory nerves, macrophages and mast cells as well as granulocytes, monocytes and lymphocytes derived from the bloodstream. Most importantly, the blood vessels of the papillary dermis supply oxygen and nutrients to the epidermis, which completely lacks vasculature. The lower layer of the dermis is called the reticular dermis and it contains fewer cells compared to the papillary dermis. The reticular dermis consists of a dense and irregular ECM different from the upper layer (Mauch and Scharffetter-Kockhanek, 2009; Wolff, 2009). The dermis not only provides a structural sponge for blood vessels and dermal cell attachment but also supplies continuous stimulus for the regulation of cell functions such as cell survival and ECM protein gene expression via cellular receptors *e.g.* integrins (Ivaska et al., 1999;2002). Moreover, there is continuous cross-talk between the dermal and epidermal cells via paracrine growth factors and cytokines especially during processes such as wound healing (Werner et al., 2007).

## 2.2 CUTANEOUS EXTRACELLULAR MATRIX

The cutaneous extracellular matrix (ECM) encompasses basically two compartments: the basement membrane, which links the epidermis and the dermis together, and the dermal ECM. In addition, a considerable amount of the large polysaccharide hyaluronan (HA) is located in the narrow spaces between the basal and spinous cells in the epidermis (Tammi and Tammi, 2009). The major source of dermal ECM components are the resident fibroblasts. The main structural component of the dermal ECM is type I collagen, which makes up about 90% of its mass. Like other collagens, type I collagen is made up of three α-chains, the composition and sequence of which differ depending on the collagen type. Type I collagen belongs to fibril-forming or fibrillar collagens characterized by the assembly of the triple-helical collagen molecules into larger fibrils after secretion. The fibrils are subsequently strengthened

by covalent cross-linking. In addition to type I collagen, other collagen types found in skin include types VI and XII, which associate with type I collagen fibrils, as well as type III collagen, which is more abundant in fetal skin compared to post-natal skin (Mauch and Scharffetter-Kockhanek, 2009).

Besides collagens, there are numerous non-collagenous proteins, including glycoproteins in the dermal ECM. Glycoproteins are proteins which typically have a multi-domain structure and several branched oligosaccharide chains linked to certain amino acid residues in the core protein. Fibronectin is one of the most characterized glycoproteins of the dermis and the basement membrane. Together with collagen, fibronectin provides structural support for the dermis. The adhesion of fibronectin to cellular receptors also induces intracellular signals regulating *e.g.* the cell cycle or migration (Mauch and Scharffetter-Kockhanek, 2009).

Two other groups of dermal ECM components, elastic fibers and proteoglycans, provide skin elasticity and a highly hydrated sponge with versatile functions, respectively. The core molecule of elastic fibers is the amorphous and hydrophobic elastin, which is covered by microfibrils composed of large glycoproteins such as fibrillins. Elastic fibers can stretch and snap back giving skin its characteristic elasticity. Proteoglycans are formed from one or more glycosaminoglycans (GAGs) covalently bound to the core protein. GAGs are classified into groups based on their sugar composition, the presence of sulfates and the type of linkage. Generally, GAGs are negatively charged and occupy a lot of space relative to their mass. Proteoglycans can form large polymeric and hydrated complexes, the ground substance, which associates with fibrous proteins such as collagen and elastin as well as with the network-like structure of the basement membrane. Proteoglycans provide a reservoir for a variety of mediators such as growth factors and can inhibit or potentiate their activity. They can also serve as cellular co-receptors augmenting the function of other receptors. The main proteoglycan in the dermal ECM is decorin, which regulates collagen fibril assembly. Other examples of skin proteoglycans include the transmembrane protein syndecan, which is expressed by fibroblasts and functions as ECM receptor and perlecan, which associates with the basement membrane structures of the skin and blood vessels (Mauch and Scharffetter-Kockhanek, 2009).

The basement membrane (BM) is a highly specialized ECM produced by the epidermal keratinocytes and dermal fibroblasts. The BM is a stabilizing as well as a stimulating interface for cell and dermal ECM attachment. In skin blood vessels, a similar basement membrane underlies the endothelium. The BM possesses a ubiquitous structure composing basically from four molecular elements. 1) The laminin network, which is needed for initial BM formation (*lamina lucida*). Below the *lamina lucida* 2) type IV collagen molecules are covalently cross-linked into a network (*lamina densa*). 3) Nidogen functions in BM assembly and cross-linking, and a heparan sulphate proteoglycan 4) perlecan regulates the function of keratinocytes. Epidermal basal keratinocytes are anchored to the BM by hemidesmosomes, where cellular $\alpha 6\beta 4$ integrins adhere to the BM laminin. Together with the transmembranous type XVII collagen, hemidesmosomes form anchoring filaments linking the cellular cytoskeleton to the BM. Type VII collagen constitutes anchoring fibrils, which link BM components to the underlying dermal fibrillar collagens (Breitkreutz et al., 2009).

## 2.3 MATRIX METALLOPROTEINASES (MMPS)

### 2.3.1 Introduction of the MMP family

MMPs are structurally related enzymes, which together cleave practically all types of ECM molecules and a multitude of other substrates including other proteases, growth factors and cytokines, which MMPs often activate (Sternlicht and Werb, 2001). Twenty-three MMPs have been identified in humans. They are classified into six subgroups based on the substrate specificity and structural similarities (Table 1, see also MEROPS database, subfamily M10A, http://merops.sanger.ac.uk/). Today, numerous physiological and *in vitro* substrates are known for most MMPs. Especially proteomics techniques have greatly expanded the lists of MMP substrates (Morrison et al., 2009). Many MMP substrates have been collected into public databases such as CutDB (http://cutdb.burnham.org/) which are valuable data sources for scientists. Databases such as the Degradome (http://degradome.uniovi.es/) provide information on *e.g.* MMP-related diseases.

**Table 1. Human MMPs.**

| *Subgroup* | *MMP* | *Trivial name* |
|---|---|---|
| **Collagenases** | MMP-1 | Collagenase-1 (Human interstitial collagenase) |
| | MMP-8 | Collagenase-2 (Neutrophil collagenase) |
| | MMP-13 | Collagenase-3 |
| **Gelatinases** | MMP-2 | Gelatinase A (72 kDa) |
| | MMP-9 | Gelatinase B (92 kDa) |
| **Stromelysins** | MMP-3 | Stromelysin-1 |
| | MMP-10 | Stromelysin-2 |
| | MMP-11 | Stromelysin-3 |
| **Matrilysins** | MMP-7 | Matrilysin-1 |
| | MMP-26 | Matrilysin-2 |
| **MT-MMPs** | MMP-14 | MT1-MMP |
| | MMP-15 | MT2-MMP |
| | MMP-16 | MT3-MMP |
| | MMP-24 | MT5-MMP |
| | MMP-17 | MT4-MMP |
| | MMP-25 | MT6-MMP |
| **Other MMPs** | MMP-12 | Metalloelastase |
| | MMP-19 | - |
| | MMP-20 | Enamelysin |
| | MMP-21 | - |
| | MMP-23 | CA-MMP |
| | MMP-27 | - |
| | MMP-28 | Epilysin |

**Table 2. Selected human MMPs and substrates.**

| *MMP* | *ECM substrates* | *Other substrates* |
|---|---|---|
| MMP-1 | aggrecan, collagen types I/II/III/VII/VIII/X/XI, entactin, gelatin, FN, laminin, link protein, myelin basic protein, tenascin, versican, VN | C1q, α2M, SAA, IGFBPs, IL-1β, ovostatin, proTNF-α, α1-ACT, α1-AT, PAR1, proMMP-1, -2 and-9, MMP-1 |
| MMP-13 | aggrecan, collagen types I/II/III/IV/VI/IX/X/XIV, collagen telopeptides, gelatin, FN, large tenascin-C, SPARC | C1q, α2M, factor XII, α1-ACT, fibrinogen, MCP-3, proTGF-β, CXCL12, pro-MMP-9 and -13, MMP-13 |
| MMP-2 | aggrecan, collagen types I/II/III/IV/V/VII/X/XI, galectin-3, gelatin, decorin, entactin, elastin, FN, laminin, link protein, myelin basic protein, SPARC, tenascin, versican, VN | α2M, SAA, myosin light chain, troponin I, PARP, adrenomedullin, MCP-3, big endothelin-1, FGFR1, latent TGF-β, LTBP-1 (Dallas et al., 2002), IGFBPs, IL-1β, proTNF-α, substance P, plasminogen, α1-ACT, α1-AT, proMMP-1, -2, -9, - and -13, MMP-2 |
| MMP-9 | aggrecan, collagen types IV/V/XI/XIV, galectin-3, gelatin, decorin, elastin, laminin, link protein, myelin basic protein, SPARC, versican, VN | α2M, IL-2Rα, latent TGF-β, LTBP-1 (Dallas et al., 2002), IL-1β, proTNF-α, ovostatin, angiotensin I and II, substance P, carboxymethylated transferrin, release of VEGF, plasminogen, α1-AT, MMP-9 |
| MMP-3 | aggrecan, collagen types III/IV/V/VII/IX/X/XI, collagen telopeptides, gelatin, decorin, entactin, elastin, FN, fibrin, fibulin, laminin, link protein, myelin basic protein, perlecan, SPARC, tenascin, versican, VN | α2M, osteopontin, E-cadherin, SAA, IGFBP-3, IL-1β, proTNF-α, pro-HB-EGF, ovostatin, substance P, T-kininogen, carboxymethylated transferrin, antithrombin-III, α2-AP, plasminogen, PAI-1, fibrinogen, u-PA, α1-ACT, α1-AT, proMMP-1, -3, -7, -8, -9 and -13, MMP-3 |

Based on Nagase et al. (2006) and Visse and Nagase (2003). α1-ACT, α1-antichymotrypsin; α2M, α2-macroglobulin; α1-AT, α1 antitrypsin; α2-AP, α2-antiplasmin; FGFR, fibroblast growth factor receptor; FN, fibronectin; IGFBP, insulin growth factor binding protein; LTBP, latent TGF-β binding protein; MCP, monocyte chemoattractant protein; PAI, plasminogen activator inhibitor; PAR, protease-activated receptor; pro-HB-EGF, pro-heparin-binding EGF-like growth factor; SAA, serum amyloid A; SPARC, secreted protein, acidic and rich in cysteine (osteonectin); u-PA, urokinase plasminogen activator; VN, vitronectin

The MMPs that are particularly relevant for this thesis with a selection of identified substrates are listed in Table 2.

Figure 2 represents the overall structure of MMPs. All MMPs have a pro-domain and a catalytic domain. They are synthesized with a signal peptide, which is eliminated during translation. Generally MMPs are secreted from cells as latent proenzymes or zymogens. The latency is preserved by the interaction of a conserved cysteine residue in the pro-domain with the catalytic Zn2+ ion bound to a highly conserved sequence in the catalytic domain (cysteine switch). During the proteolytic activation of proMMPs, the "bait" region of the pro-domain is first cleaved generating an MMP intermediate, which is then processed by removal of the remnants of the pro-domain by the MMP intermediate itself or by other active proteinases. The process generates a fully active MMP (Kessenbrock et al., 2010).

Most MMPs are activated after secretion, but membrane type MMPs (MT-MMPs), MMP-11, -21, -23 and -28 contain a cleavage site for furin-like proteases between the pro- and catalytic domains and are likely to be activated intracellularily. MMPs can be also activated *in vitro* by chemicals that disturb their molecular structure, such as

mercurial compounds (*e.g.* APMA) and thiol-group (-SH) containing reagents or by reactive oxygen species (ROS) reacting with the cystein of the cystein switch (Kessenbrock et al., 2010).

MMPs, except matrilysins and MMP-23, possess a C-terminal hemopexin domain, which mediates molecular interactions and is involved in substrate recognition. The catalytic domains of the gelatinases MMP-2 and -9 also contain three repeats of a fibronectin type II sequence, which mediate the recognition of large ECM proteins such as type IV collagen, gelatin and elastin. MMPs are mainly secreted as soluble proteins but MT-MMPs possess a type 1 transmembrane domain (MMP-14, -15, -16 and -24) or glycosylphosphatidylinositol (GPI) anchor (MMP-17 and -25) rendering the molecule to remain attached to plasma membrane. MMP-23 associates with the cell membrane via an N-terminal signal anchor and is secreted after the activating cleavage (Nagase et al., 2006).

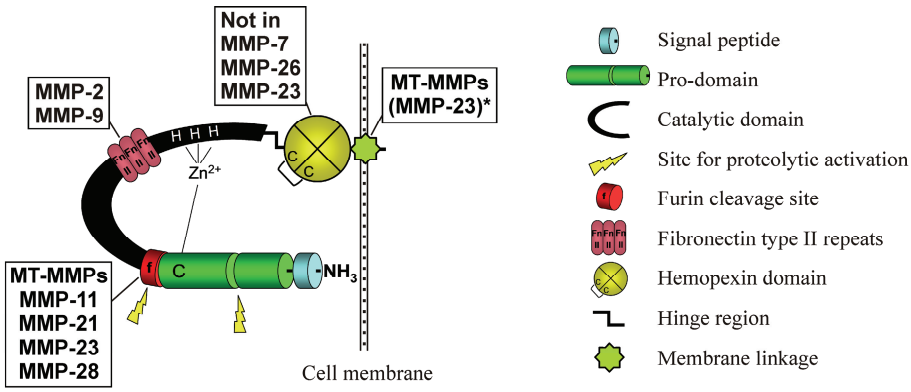


**Figure 2.** A schematic presentation of the general structure of a matrix metalloproteinase. *MMP-23 contains an N-terminal transmembrane domain

### 2.3.2 The function of MMPs

The physiological and pathological roles of MMPs depend on their capacity to specifically cleave a variety of proteins, including ECM macromolecules, pro-cytokines, pro-growth factors and their inactivating binding proteins, receptors as well as other pro-proteinases and proteinase inhibitors (Kessenbrock et al., 2010). Thus, in addition to their ability to cleave ECM barriers, MMPs are now recognized as modulators of cellular signaling in health and disease. More recently, even functions that are not associated with proteolysis at all have also been described for some MMPs exemplified by MMP-11, which can act as a transcription factor-like molecule in cells regulating the expression of CTGF/CCN2 (Eguchi et al., 2008).

The action of MMPs is often associated with pathological conditions with focal tissue destruction such as osteo- and rheumatoid arthritis (Burrage et al., 2006), heart and vascular diseases (Newby, 2007), lung diseases (Gueders et al., 2006),

periodontitis (Sorsa et al., 2006) and chronic cutaneous and intestinal ulcerations (Salmela et al., 2004; Pirilä et al., 2007; Vaalamo et al., 1997). A multitude of studies have addressed the expression and activities of MMPs in the development and progression of different malignancies (Kessenbrock et al., 2010). For example MMP-13 and MT1-MMP are implicated in tumor invasion due to their ability to degrade the ECM (Ala-aho et al, 2004;Sabeh et al., 2004), and the over-expression of MMP-1 is associated with poor a prognosis in a variety of cancers (Ala-aho and Kähäri, 2005). MMP-9 is shown to be markedly important for tumor angiogenesis by increasing the bioavailability of vascular endothelial growth factor (VEGF) (Du et al., 2008), but also *e.g.* MMP-2 and MMP-13 have been shown to regulate of tumor vascularization (Itoh et al., 1998;Lederle et al, 2010). Moreover, inflammation is highly important for cancer suppression, and several MMPs are recognized for their ability to regulate inflammation by modulating the activity and availability of cytokines and chemokines (Kessenbrock et al., 2010). To exemplify the pattern of over-expressed MMPs in cancer tissues, a comprehensive expression analysis of squamous cell carcinomas of the head and neck of a large number of patients found the expression of most MMPs to be elevated compared to normal mucosal tissues, including MMP-1, -3, -9, -7, -10, -13 and -14 (Stokes et al., 2010). However, although the expression levels of MMPs are usually low in normal tissues, their expression and function is elevated also in various dynamic physiological events such as in different events during embryonic tissue development and wound healing (Page-McCaw et al., 2007; Toriseva and Kähäri, 2009). Proteolysis, in terms of wound healing, will be discussed in detail below.

### 2.3.3 The regulation of MMP activity

*In vivo*, the activity of MMPs is regulated basically at three levels: 1) by proteolytic or chemical (ROS) activation of the zymogen as described above, 2) by inhibitors that bind to active MMPs and 3) through the transcriptional control of MMP genes. Secreted MMP zymogens are typically activated by serine proteases *e.g.* plasmin, and for example MMP-1, -2, -3, -7 and -10 are capable of activating several proMMPs (see Table 2) (Kessenbrock et al., 2010). In addition, most MMPs display positive auto-regulation by degrading serine proteinase inhibitors (serpins) such as α1-antitrypsin (α1-AT) thus increasing the availability of functional plasmin. On the other hand, many MMPs can degrade plaminogen, which in turn reduces reservoir of proteins that can activate proMMPs (see Table 2).

Mammals are known to have four endogenous tissue inhibitors of MMPs (TIMPs), namely TIMP-1, -2, -3 and -4. All TIMPs bind all MMPs and inhibit them with a 1:1 stoichiometry, although TIMP-1 only poorly inhibits MMP-19 and certain MT-MMPs (Murphy and Nagase, 2008). TIMPs are secreted proteins, which are primarily found in soluble form in the extracellular space. However, the bioavailability of TIMP-3 is regulated by a strong association with the ECM (Yu et al., 2000). It is of note that TIMPs can bind to proMMP-2 and proMMP-9 with their non-inhibitory C-terminal domain (Murphy and Nagase, 2008). When TIMP-2 is bound to active MT1-MMP the complex functions as a receptor for proMMP-2, which then in turn is subjected to activation by an adjacent active MT1-MMP (Butler et al., 1998). This property seems

to be specific for the MT1-MMP-TIMP-2-proMMP-2 complex and extends the role of TIMP-2 from an inhibitor of activation (at high concentrations) to an activator (at low concentrations) of MMP-2. In addition to TIMPs, MMPs are inhibited by binding to plasma α2-macroglobulin, which is also degraded by most MMPs providing a mechanism for positive auto-regulation. Moreover, other inhibitors of MMP activity have been reported, for instance secreted β-amyloid precursor protein (MMP-2) and RECK, a GPI-anchored glycoprotein that suppresses angiogenesis (MMP-2, MMP-9 and MMP-14) (Murphy and Nagase, 2008).

The regulation of MMP gene expression has recently been comprehensively reviewed by Yan and Boyd (Yan and Boyd, 2007). Shortly, at the transcriptional level, the expression of MMPs is initially regulated by extracellular signals that are generated 1) via ligand binding to a growth factor or a cytokine receptor or 2) to integrin receptors, or 3) by alterations in cadherin-mediated cell-cell junctions. These events triggers cellular signals *e.g.* the activation of the mitogen-activated protein kinase (MAPK) pathway, the Smad-pathway or the NF-κB-pathway by a growth factor or a cytokine. Or they lead to the formation of a focal adhesion complex resulting in a variety of signals transduced by integrin activation, or the activation of β-catenin by cadherins. Ultimately these lead to the activation/inactivation of an appropriate gene promoter region and a change in MMP expression. Many MMP promoters share similar properties and are simultaneously expressed (Yan and Boyd, 2007).

For example in a skin wound, transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and keratinocyte growth factor (KGF) regulate MMP expression. MMP-1, which is induced by collagen contact in migrating wound keratinocytes (Pilcher et al., 1997a; Vaalamo et al., 1997), is down-regulated *in vitro* by bFGF and KGF in keratinocytes (Pilcher et al., 1997b). Moreover the expression of MMP-1 in human fibroblasts is enhanced *e.g.* by PDGF (Bauer et al., 1985) and by α2β1 integrin mediated ligation with type I collagen in a 3D culture (Langholz et al., 1995), whereas TGF-β down-regulates MMP-1 expression (Yuan and Varga, 2001). In skin fibroblasts, extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 MAP kinases regulate MMP-1 expression in distinct ways. Activation of the ERK1/2 pathway rapidly up-regulates transcription of MMP-1, whereas activation of p38α restrains signaling via the ERK1/2 pathway and enhances the expression of MMP-1 by increasing mRNA stability (Reunanen et al., 2002; Westermarck et al., 2001). As one stimulus can induce multiple signaling cascades with different effects, and because in tissues cells respond to variety of stimuli simultaneously, it is the amplitude and duration of the signal, taking into account the simultaneous inhibitory signals, that determine the end result of the regulation.

Finally, the expression of certain MMP genes is regulated by epigenetic modification of chromatin, such as methylation or the packing of DNA around the histones, as well as post-transcriptionally by stabilizing or destabilizing the MMP mRNA (Yan and Boyd, 2007) as described above for MMP-1.

## 2.4 CUTANEOUS WOUND REPAIR

Prompt and efficient wound repair is one of the fundamental processes evolved in organisms to protect the body from blood loss, drying and the invasion of pathogens. In mammals, cutaneous wound healing is a complex and highly coordinated event involving the dynamic interplay of cells of distinct tissue patterns of the skin. Classically, wound healing is divided into three major phases: 1) hemostasis and inflammation, 2) re-epithelialization and granulation tissue formation, and 3) tissue remodeling (Clark, 1995). These phases are histologically and functionally separate but temporarily overlapping, and the overall healing rate always depends on the size of the wound (Fig. 3). Stress and damage directed to a tissue by injury lead to the activation of signal pathways such as the p38 and ERK MAPK pathways (Kobayashi et al., 2003). Moreover, growth factors, cytokines and a variety of other factors derived from the plasma and cells as described below induce cellular signals resulting in a response in gene expression and, for instance, cell motility. Transcriptional profiling of *in vivo* wounds in mice and humans has been carried out in a number of studies, which show hundreds or even thousands of differentially regulated genes in wounds at different steps of healing. These include genes involved in the inflammatory response, pathogen recognition, endopeptidase activity, ECM composition and a variety of regulatory events in cells (Chen et al., 2010; Cole et al., 2001; Deonarine et al., 2007; Roy et al., 2008).

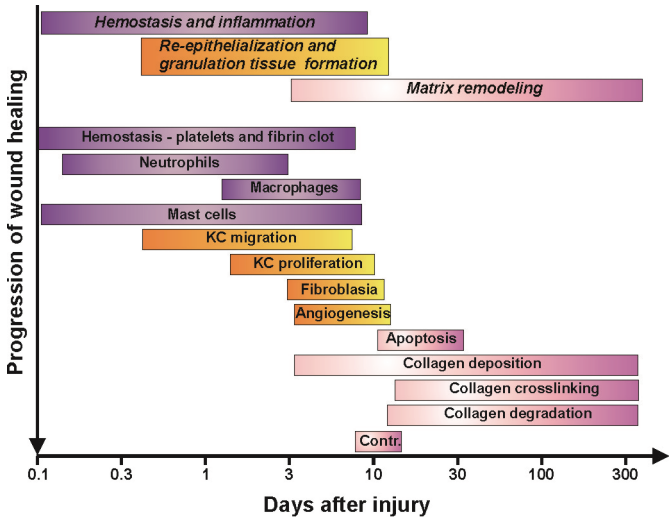


**Figure 3.** The phases and functional events of cutaneous wound healing. KC, keratinocyte; Contr., contraction. Modified from Toriseva and Kähäri (2009).

### 2.4.1 Hemostasis and inflammation

Immediately after a skin injury, which extends to the vascularized dermal layer, blood extravasates to the open wound from disrupted blood vessels and a variety of vasoactive factors are released into the tissue. The inflammatory phase is initiated by vasoconstriction, and adhesion, aggregation, and degranulation of platelets leading to hemostasis. The activation of a coagulation cascade then leads to the cleavage of fibrinogen by thrombin and the formation of a fibrin network, which provided a sponge for platelet adhesion and serves as a physical plug to stop bleeding. The blood clot includes fragments of coagulation factors, complement components, and growth factors derived from ruptured cells and activated platelets, which induce vasodilation and increase vessel permeability as well as proffer chemotactic stimuli for inflammatory cells, keratinocytes, fibroblasts and endothelial cells. As the wound healing progresses, a provisional matrix composed of fibrin and featured by plasma fibronectin and vitronectin is invaded by migrating keratinocytes, degraded by proteinases, mainly by plasmin, and finally disengaged from the wound site as a scab (Shaw and Martin, 2009).

The innate immune system is activated at the wound site in only few hours after injury. The first cells to migrate to the wound are blood neutrophils (Eming et al., 2009) apparently, attracted in part by the resident mast cells (Egozi et al., 2003; Weller et al., 2006). The main function of the neutrophils is the phagocytosis of infectious agents and the devitalized tissue in the wound. Upon degranulation of neutrophils a number of factors (*e.g.* ROS and proteases) that are needed for obliterating damaged tissue and invaded pathogens are released into the wound bed. They also secrete various compounds that amplify inflammation (Eming et al., 2009).

Within two days of an injury blood monocytes immigrate to the wound site and become activated macrophages, which function as antigen-presenting cells and phagocytes, and contribute to the regulation of wound healing by secreting numerous growth factors, such as TGF-β, TGF-α, bFGF, PDGF and VEGF (Eming et al., 2009). Macrophages are indispensable for normal wound healing (Leibovich and Ross, 1975). Their competence is elucidated by recent results showing that macrophages of a distinct activation state predominate temporally in cutaneous wounds. Both macrophage subtypes, M1 (classically activated) and M2 (alternatively activated) are present during early wound healing, whereas at later stages M2 macrophages predominate. Accordingly, M1 macrophages exert proinflammatory activities while M2 macrohages are pro-angiogenic and involved in tissue remodeling and the resolution of inflammation (Deonarine et al., 2007).

As wound healing progresses the resolution of inflammation becomes extremely important in terms of normal wound closure demonstrated *e.g.* by prolonged inflammation associated with chronic ulcers. The resolution of inflammation during wound repair is not fully understood but the reduction of pro-inflammatory molecules and an increase in anti-inflammatory factors in the wound environment as well as the apoptosis and phagocytosis of leukocytes are involved in the process (Eming et al., 2009).

### 2.4.2 Re-epithelialization

Concurrent with the inflammatory phase of wound healing, re-epithelialization and granulation tissue formation are initiated. Within a few hours after injury, epidermal keratinocytes at the wound edge and in the remnants of the skin appendages, *e.g.* hair follicles, detach from the underlying basement membrane and from adjacent cells, and start to migrate into the wound, typically underneath the scab (Shaw and Martin, 2009). In two days, wound keratinocytes distant from the wound edge develop into a hyperproliferative phenotype providing cells to fill the gap in the epithelium and restore skin integrity. Gene expression studies of keratinocytes in scratch wound assay *in vitro* have given a picture, although simplified, of the genes induced during keratinocyte migration (Cheng et al., 2008) and during "re-epithelialization" (Fitsialos et al., 2007). In combination with functional experiments, the ERK and p38 MAPK pathways were shown to mediate positive signal for "wound" closure and PI3K to mediate the opposite signal (Fitsialos et al., 2007). Obviously, scratch assays lack the stimuli derived from the wound ECM and the soluble surroundings. However, they provide interesting data to be taken into account when trying to understand the re-epithelialization process.

It appears that epithelial stem cells in the hair follicle bulge regions adjacent to the wound site supply the proliferative potential needed during re-epithelialization, whereas stem cells in the interfollicular skin epidermis are responsible for the maintenance of the intact epidermis (Cotsarelis, 2006). Once the wound gap is closed, the basement membrane is reestablished and the cellular contacts are re-formed, keratinocytes differentiate to constitute the multi-layered epidermis of the skin.

### 2.4.3 Granulation tissue formation and vascularization

Wound granulation tissue contains numerous new blood vessels, fibroblasts and leukocytes embedded in the wound ECM. The formation of the granulation tissue begins within a few days after injury by fibroplasia - the proliferation of fibroblasts and their invasion into the wound provisional matrix. Fibroplasia is promoted by growth factors secreted by platelets and inflammatory cells, and ligation with ECM molecules. PDGF-BB is the most effective stimulator of fibroblast migration in a two-dimensional culture (Li et al., 2004). Inhibition of the PDGF receptor-ß *in vivo* delays early wound healing in mice by reducing the proliferation of fibroblasts and blood vessel pericytes and the recruitment of myofibroblasts (Rajkumar et al., 2006). The mitogenic effect of PDGF-BB requires cellular contact with type I collagen and involves metalloproteinase activity (Li et al., 2004). PDGF-BB was shown to stimulate fibroblast migration also in a clever 3D cell culture model, the nested collagen matrice, in a manner involving the activity of Rho kinase, myosin II and metalloproteinases (Grinnell et al., 2006).

It is thought that the majority of the wound fibroblasts originate from the adjacent undamaged dermis next to the injury (Hinz, 2007). A portion of the fibroblasts is, however, derived from the bone marrow-derived mesenchymal progenitor cells

(Opalenik and Davidson, 2005), and it is also likely that multipotent dermal resident skin-derived precursor cells (Toma et al., 2005) differentiate into wound fibroblasts.

Wound fibroblasts deposit most of the granulation tissue ECM, which differs from intact dermal ECM in composition. Initially, the wound ECM consists mainly of fibronectin and hyaluronan, both of which stimulate cell migration. At a later stage, proteoglycans and type III and I collagens are deposited and become the major components of the wound ECM (Clark, 1995). In addition, wound granulation tissue is characterized by the presence of matricellular proteins that act temporally and spatially to proffer signals that trigger certain cellular activities within the wound (Midwood et al., 2004). Matricellular proteins found in cutaneous wounds include galectins, osteopontin, SPARC (osteonectin), syndecans, tenascins, thrombospondins and vitronectin, which interact with cell surface integrins and participate in the regulating cell functions such as migration and matrix remodelling and contraction (Midwood et al., 2004). At least syndecans, osteopontin and thromposbondins are associated with positive and negative regulation of angiogenesis (Alexopoulou et al., 2007; Bornstein et al., 2004; Dai et al., 2009).

Tissue destruction and hypoxia induces sprouting angiogenesis, that is migration and proliferation of the endothelial cells of the existing vessels. Subsequently, angiogenesis is stimulated by various soluble factors, such as bFGF, VEGF and TGF-β, which are secreted by wound macrophages, keratinocytes and endothelial cells (Singer and Clark, 1999). Simultaneously, the early granulation tissue, rich in blood clot fibrin, fibronectin and hyaluronan, stimulates the sprouting of new blood vessels into the wounded tissue (Hynes, 2007; Potter et al., 2006; Slevin et al., 2007). Recently, using implanted experimental fibrin-collagen "granulation tissue" on chick chorioallantoic membrane and the healing mouse cornea, it was shown that the mechanical tension generated by fibroblast/myofibroblast-mediated tissue remodelling is able to expand and translocate pre-existing vasculature, and in this way stimulate rapid neovascularisation (Kilarski et al., 2009). It is well established, that wound angiogenesis is dependent on the proteolytic activity of plasmin and matrix metalloproteinases (MMPs) (Pepper, 2001). Granulation tissue formation has been studied in a well defined experimental model of a subcutaneously implanted, rectangular or cylindrical cellulose sponge. The sponge is rapidly invaded by granulation tissue-like components such as inflammatory cells, fibroblasts and blood vessels as well as ECM components (Holund et al., 1979; Tommila et al., 2008).

### 2.4.4 Dermal remodeling and wound maturation

The remodeling of granulation tissue is essential for the restoration of a functional dermis. In wound granulation tissue fibroblasts deposit collagen, which will become the most abundant molecule in the matured dermis. The remodeling of the collagenous newly formed dermis is mediated by a traction force exerted by fibroblasts. It leads to the proper assembly and orientation of collagen fibrils in a way that provides sufficient tensile strength to skin. The remodeling is regulated by factors such as fibroblast-myofibroblast transformation, TGF-β and PDGF, serum lipid growth factor

lysophosphatidic acid (LPA) and by the mechanical tension of the matrix as demonstrated by a variety of *in vitro* experiments within 3D cell culture models (Grinnell and Ho, 2002; Lee et al., 2003). These models possess molecular and structural properties resembling native fibrous connective tissue *in vivo*.

As part of tissue remodeling, in the second week of healing, likely stimulated by TGF-β, mechanical tension generated by the open wound, and possibly also by low oxygen levels, fibroblasts obtain a myofibroblast phenotype characterized by the expression of α-smooth muscle actin (αSMA) (Hinz, 2007; Modarressi et al., 2010). These cells promote wound closure by tissue contraction and by pulling the wound edges closer to each other.

In the last decade, there has been marked development in understanding fibroblast-mediated remodeling of the collagenous matrix in more detail (Grinnell and Petroll, 2010). In addition to myofibroblasts, wound fibroblasts are able to exert collagen fiber organization by migratory movement rather than cell contraction or shortening in a 3D matrix (Miron-Mendoza et al., 2008). Matrix remodeling by wound fibroblasts is based on cell protrusion, adhesion to collagen and retraction, and it involves a functional actin cytoskeleton. The signaling events inside the cell depend on the stiffness of the tissue and the presence of stimulating growth factors (LPA, PDGF) (Grinnell and Petroll, 2010). An *in vitro* 3D cell culture model of a low-tension collagen matrix induces a quiescent fibroblast phenotype with little proliferation and a typical cell morphology with long protrusions (Grinnell et al., 2003; Rosenfeldt and Grinnell, 2000). In similar but restrained cultures fibroblasts become activated with stimulated ECM biosynthesis (Eckes et al., 2006). Growth factors such as LPA or PDGF induce the cellular translocation of collagen fibers eventually developing tension in the matrix. This subsequently results in the formation of lamellar structures, further activation of cells and the formation of actinic stress fibers enabling more efficient matrix movement by cell contraction (Grinnell and Petroll, 2010). Extrapolated into the *in vivo* scenario, it is thought that the mechanically unloaded culture model resembles the situation in intact dermis. Dermal injury and the formation of granulation tissue exerts tension into the matrix resulting in fibroblast activation, which is further stimulated by the appropriate growth factors. While myofibroblasts appear relatively late in the wound tissue, it is likely that the initial remodeling of granulation tissue results from the function of wound fibroblasts which then, as a result of increased matrix tension and TGF-β, differentiate into myofibroblasts. Following contraction by myofibroblasts and wound closure, the maturing wound dermis is released from mechanical tension.

Using different function blocking constructs and transfected cell lines, the collagen receptor integrins α1β1, α2β1, α11β1 as well as αVβ3, which binds to Arg-Gly-Asp residue of denatured collagen molecules, have been implicated in the mediation of collagen remodeling. This can be measured as collagen matrix contraction (Cooke et al., 2000; Racine-Samson et al., 1997; Schiro et al., 1991; Tiger et al., 2001). Ligation of *e.g* α2β1 integrin to collagen at a rigid substratum results in the formation of relatively large focal adhesion sites, where numerous signaling molecules and cytoskeletal components are associated with the ECM via integrins (Petit and Thiery, 2000; Wang et al., 2003), though these structures may differ during ligation with a 3D substratum (Cukierman et al., 2001). It has been shown that tension relaxation results

in disruption of ERK signaling (Rosenfeldt and Grinnell, 2000) and subsequent apoptosis (Niland et al., 2001; Tian et al., 2002). Indeed, α2β1 integrin appears to function as a mechanoreceptor, which senses stress-release in the ECM and induces a signaling cascade leading to the dephosphorylation of focal adhesion kinase (FAK) followed by the dephosphorylation of phosphatidylinositol 3-kinase (PI3K) and Akt (Ivaska et al., 2002; Niland et al., 2001; Xia et al., 2004). An alternative convention for the negative regulation of Akt is proposed via the demonstrated activation of protein serine/threonine phosphatase 2A (PP2A) by α2β1 in fibroblasts cultured in a mechanically unloaded collagen matrix (Ivaska et al., 2002).

The regenerative phase of cutaneous wound healing is followed by the maturation of the wound, leading to the formation of a collagenous scar. While the persistent and excess presence of myofibroblasts in tissues is distinctive of pathologic contractures of hypotrophic scars and other fibrotic diseases (Hinz, 2007), myofibroblasts are eliminated in physiological healing conditions via apoptosis (Desmouliere et al., 1995). Concurrently, the majority of the blood vessels are disintegrated via apoptosis (Desmouliere et al., 1995). At least for fibroblasts, this is likely to take place in a manner involving relaxation-recognition by α2β1 and the subsequent inactivation of one of the major survival signal pathway, the PI3K/Akt pathway, as described above, and the disruption of ERK signaling, as well as by special cell-cell contacts and different growth factors.

The resident fibroblasts in the maturing wound continue the remodeling of the collagenous dermis for several months by depositing and degrading collagen molecules and arranging collagen fibers into an orientation that supports the functionality of the skin. However, only about 70% of the original tensile strength of intact skin is accomplished (Clark, 1995).

### 2.4.5 Growth factors and cytokines regulating wound healing

The highly coordinated function of different cell types during wound repair is orchestrated by signals generated by cell-ECM contacts, intercellular contacts and by changes in cellular mechanical stress. Moreover, there are a number of different growth factors and cytokines that regulate cell behavior. They are available as soluble molecules or they can be associated with the cell surface or the ECM and released proteolytically. As shown by wound healing studies with genetically modified animals and neutralizing antibodies against specific growth factors, it is obvious that many of growth factors and cytokines, *e.g.* HGF/SF, are necessary for proper wound healing (Chmielowiec et al., 2007; Werner et al., 2007). The major growth factors and cytokines involved in cutaneous wound repair and the best characterized effects are listed in Table 3. Generally, PDGF and TGF-β can be considered the two main growth factors regulating the formation of granulation tissue while HGF, TGF-α and HB-EGF are the most important growth factors for re-epithelialization. The role of KGF in wound healing and especially in cutaneous SCC will be discussed later in the text in more detail.

In addition to the growth factors and cytokines listed in Table 3, a number of additional soluble factors are implicated in wound healing events in humans and mice. These include *e.g.* activins, bone morphogenetic factors (BMPs), leptin and a variety of cytokines, which mainly act by attracting leukocytes to the site of injury as well as regulating the formation of granulation tissue and re-epithelialization, such as macrophage chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1-α (MIP-1α), growth-related oncogene-α (GRO-α), IL-8, IFN-γ, IL-6 as well as anti-inflammatory IL-10 (Werner and Grose, 2003).

**Table 3. The major growth factors and cytokines affecting wound repair.**

| *Growth factor/ Cytokine* | *Major source* | *Regulated wound healing events* | *References* |
|---|---|---|---|
| PDGF | platelets, macrophages, KC | fibroplasia, leukocyte recruitment, ECM production, contraction, angiogenesis | (Uutela et al., 2004; Werner and Grose, 2003) |
| TGF-β | platelets, macrophages, KC, fibroblasts | inhibition of KC proliferation, fibroplasia, ECM production, contraction, leukocyte recruitment | (Werner et al., 2007) |
| FGF-1 | KC | pleiotropic mitogen, angiogenesis | (Werner and Grose, 2003) |
| FGF-2 | KC, fibroblasts, macrophages, endothelial cells | pleiotropic mitogen, angiogenesis, ECM production | (Werner and Grose, 2003) |
| FGF-7 (KGF) | fibroblasts | KC proliferation and differentiation | (Andreadis et al., 2001; Werner, 1998) |
| EGF | platelets, leukocytes | epithelialization, fibroplasia, ECM production and degradation | (Brown et al., 1989; Laato et al., 1987; Mimura et al., 2006; Werner and Grose, 2003) |
| TGF-α | macrophages, granulocytes, KC | epithelialization | (Li et al., 2006; Werner and Grose, 2003) |
| HB-EGF | KC | KC migration | (Shirakata et al., 2005) |
| HGF/SF | fibroblasts, KC | epithelialization, leukocyte recruitment, angiogenesis | (Bevan et al., 2004; Chmielowiec et al., 2007) |
| VEGF | platelets, leukocytes, SM-cells, KC, fibroblasts | angiogenesis, leukocyte recruitment, expression of growth factors | (Bao et al., 2009) |
| CTGF | platelets, fibroblasts | fibroplasia, ECM production, angiogenesis | (Werner and Grose, 2003) |
| IGF-I | fibroblasts, KC, plasma | epithelialization, ECM production | (Edmondson et al., 2003) |
| TNF-α | leukocytes, KC | expression of growth factors, leukocyte recruitment, ECM degradation | (Werner et al., 2007) |
| IL-1 | leukocytes, KC | expression of growth factors, leukocyte recruitment, ECM degradation, regulation of contraction | (Werner et al., 2007) |

Modified from Toriseva and Kähäri (2009); CTGF, connective tissue growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; HB-EGF, heparin-binding EGF-like growth factor; HGF/SF, hepatocyte growth factor/scatter factor; IGF, insulin-like growth factor; IL, interleukin; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; ECM, extracellular matrix; KC, keratinocyte; SM-cell, smooth muscle cell; KC, keratinocyte; SM-cell, smooth muscle cell

### 2.4.6 Proteolysis in wound healing

Plasmin and metalloproteinases comprise the proteolytic team essential for wound healing. The roles of plasmin and MMPs are well established, but increasing data are emerging also on the function of ADAMs (a disintegrin and metalloproteinases) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) proteases in wound healing (Toriseva and Kähäri, 2009).

Plasmin is a serine protease responsible for fibrin homeostasis, and it is capable of cleaving *e.g.* laminin and fibronectin and it can activate several latent MMPs including MMP-1, -8, -13, -9, -3, -7 and MT1-MMP at least *in vitro* (Toriseva and Kähäri, 2009). For more molecular information on plasmin and its substrates, see the databases MEROPS (subfamily S1A, http://merops.sanger.ac.uk/) and CutDB (http://cutdb.burnham.org/). Plasmin is abundantly present in the blood stream. It is produced from inactive plasminogen (plg) by urokinase plg activator (uPA). Thereafter, the activity of plasmin can be inhibited by serine protease inhibitors (serpins) (Cesarman-Maus and Hajjar, 2005). In human wounds uPA is expressed in migrating keratinocytes, macrophages and fibroblasts (Schäfer et al., 1994; Vaalamo et al., 1996) suggesting the presence of active plasmin. The importance of plasmin activity in wound healing was demonstrated using plg-deficient mice, which displayed very severely impaired wound closure due to disabled keratinocyte migration through the provisional matrix. Treatment of these wounds with a wide spectrum metalloproteinase inhibitor galardin virtually prevented wound closure (Lund et al., 1999). Since galardin can inhibit several MMPs, but also for instance certain ADAMs, the result demonstrates the overlapping activities of plasmin and metalloproteinases in wound healing. The wound healing phenotypes for protease-deficient mice are collected into Table 4.

ADAMs are a large family of transmembrane proteins, most of which display proteolytic, adhesive and putative signaling activities (Edwards et al., 2008) (See also MEROPS subfamily M12B, http://merops.sanger.ac.uk/). One of the most typical functions of ADAMs is cytokine and growth factor shedding, that is the release of membrane bound protein into the extracellular space, which often results in protein activation. The expression of ADAMs in skin wounds is still poorly understood. At least, ADAM-9, -10 and -17 appear to function in keratinocyte biology (Franzke et al., 2002; Maretzky et al., 2005; Zigrino et al., 2007). ADAM-9 is expressed in normal epidermis and a variety of dermal cells in mice and its expression in the epidermis is enhanced after injury (Mauch et al., 2010). Interestingly, ADAM-9-deficient mice were reported to display enhanced wound closure due to a faster migration rate of keratinocytes (Mauch et al., 2010).

ADAMTS proteases are widely expressed in tissues. They are structurally related to ADAMs but in contrast to ADAMs, ADAMTS are secreted proteins and prefer ECM substrates. Collectively, they are involved in ECM organization, blood clotting, angiogenesis, cell migration and inflammation (Porter et al., 2005). So far, only the expression of ADAMTS-1 and -5 has been described in skin wound healing. ADAMTS-1 is constitutively expressed in mouse epidermis and it is enhanced upon wound closure. It has been suggested to regulate epidermal differentiation (Krampert et

al., 2005). It was also detected in wound macrophages and fibroblasts possibly regulating cell migration (Krampert et al., 2005). Moreover, ADAMTS-1-deficient mice display defective wound closure accompanied by increased angiogenesis (Lee et al., 2006b). Thus ADAMTS-1 is likely to inhibit angiogenesis in wound repair. Recently, ADAMTS-5 was found to have a significant role in mouse wound healing. ADAMTS-5 deficient mice were reported to develop fibroblast aggregates surrounded by aggregan, versican and HA in the granulation tissue resulting in impaired deposition of collagen and wound contraction. This was suggested to be due to an unresponsiveness of fibroblasts to TGF-β (Velasco et al., 2011).

As MMPs and particularly MMP-13 are the main focus of this thesis, the following section is devoted to reviewing the expression and function of MMPs in cutaneous wounds.

### 2.4.7 MMP-mediated proteolysis in wound healing

MMPs participate in all the phases of the wound healing process in the skin. They function in the physical clearance of the ECM out of the way of migrating cells at same time releasing activating (or inactivating) bioactive products. Importantly, the proteolytic activity of MMPs results in alterations in cell-ECM contacts, which regulate cell behavior. Changes in the strictly regulated proteolysis are associated with scarring and defects in wound closure.

The expression levels of MMPs in normal uninjured skin are very low. MMP-7 and MMP-19 are constitutively expressed in sweat and sebaceous glands (Saarialho-Kere et al., 1995; Sadowski et al., 2003b) and MMP-19 is also detected in basal keratinocytes and in hair follicles, as well as in blood vessels (Sadowski et al., 2003b). Moreover, low levels of MMP-2 and MT1-MMP expression have been detected in intact dermis in animal models (Madlener et al., 1998; Ågren, 1994). However, the chemical and physical changes in the microenvironment of an injured tissue induce the up-regulation of multiple MMPs. MMPs that have been identified in human acute cutaneous wounds include the collagenases MMP-1 and MMP-8 (Inoue et al., 1995; Nwomeh et al., 1999), the gelatinases MMP-2 and MMP-9 (Mirastschijski et al., 2002a), the stromelysins MMP-3 and MMP-10 (Vaalamo et al., 1996), the metalloelastase MMP-12 (reported in mice) (Madlener et al., 1998), MT1-MMP (Mirastschijski et al., 2002a), MMP-19 (Hieta et al., 2003), MMP-26 (Ahokas et al., 2005) and MMP-28 (Lohi et al., 2001). The expression of TIMP-1, -2 and -3 have also reported in acute skin wounds (Vaalamo et al., 1999b). The expression and cellular source of MMPs in an acute cutaneous wound are illustrated in Figure 4.

In cutaneous wounds associated with the interference of the basement membrane, keratinocytes at the wound edge pertain to the dermal ECM and as a consequence start to produce MMP-1. The expression peaks 24 h after injury (Inoue et al., 1995; Saarialho-Kere et al., 1993). It has been shown that native type I collagen induces MMP-1 expression in migrating keratinocytes *in vitro,* whereas basement membrane proteins, fibronectin or type III collagen do not (Sudbeck et al., 1997). *In vivo*, this is thought to be important especially for the immediate initiation of re-epithelialization

when keratinocytes have lost their attachment to the basement membrane, come into contact with type I collagen, and when the provisional matrix has not yet been formed. Indeed, the activity of MMP-1 appears to be indispensable for keratinocyte migration on native type I collagen and the expression of MMP-1 is positively regulated by ligation of α2β1 integrin with type I collagen (Pilcher et al., 1997a). MMP-1 cleaves type I collagen, generating fragments that at body temperature denature to gelatin, which is a less adhesive ligand for α2β1 than is native collagen and therefore supports migration better. Notably, while collagen ligation with α2β1 and the subsequent expression of MMP-1 and the formation of the α2β1-MMP-1 complex are suggested to promote the migration of keratinocytes on type I collagen during re-epithelialization in humans, α2-deficient mice still show normal re-epithelialization. In mouse skin wounds, MMP-1 is virtually substituted by murine MMP-13 which displays comparable expression but partially distinct substrate specificities. In the skin of α2-deficient mice, the expression of MMP-13 is actually enhanced after wounding (Grenache et al., 2007). This may reflect differences in the structure of the skin and/or the composition of the dermis between humans and mice and the related adaptation and function of the integrin, or it may be that other collagen receptor integrins compensate for the function of α2β1. Moreover, although MMP-13 is expressed analogously to human MMP-1 in murine cutaneous wounds (Madlener et al., 1998; Saarialho-Kere et al., 1993) and MMP-13 is able to cleave dermal collagens and promote keratinocyte migration (Hattori et al., 2009), its regulation by cell-ECM contacts may differ from human MMP-1 in keratinocytes.

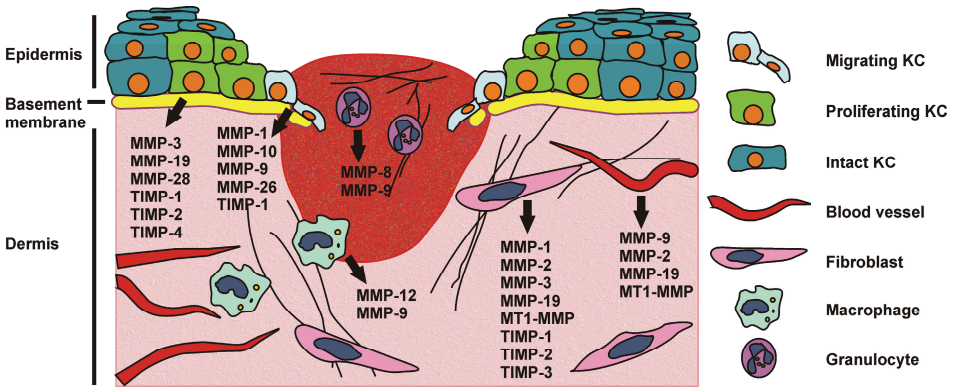


**Figure 4.** The expression and cellular source of matrix metalloproteinases (MMP) and tissue inhibitors of MMPs (TIMP) in a normal cutaneous wound. Note that in murine skin MMP-1 is virtually substituted by MMP-13. KC, keratinocyte.

In human skin wounds, the expression of MMP-1 is subsided by the completion of re-epithelialization (Inoue et al., 1995). The importance of the strict regulation of MMP-1 activity during wound repair was demonstrated by overexpressing human MMP-1 in mouse epidermis. These mice displayed markedly delayed closure of a full-thickness wound and a hyperproliferative epidermis (Di Colandrea et al., 1998) (Table 4). On the other hand, in mice with a collagenase-resistant mutation in type I collagen

the closure of a incisional skin wound was severely impaired due to defective wound contraction and delayed re-epithelialization (Beare et al., 2003). Thus, controlled epidermal as well as dermal collagenolysis is essential for proper wound healing.

**Table 4. Proteinase gene targeting – skin wound phenotypes**

| *GENE* | *Modification* | *Wound phenotype* | *Ref.* |
|---|---|---|---|
| hMMP-1 | overexpression in KCs | Delayed re-epithelialization | (Di Colandrea et al., 1998) |
| MMP-8 | knockout | Delayed re-epithelialization, delayed onset and persistent inflammation | (Gutierrez-Fernandez et al., 2007) |
| MMP-13 | knockout | 1) Unaltered (small excisional)<br>2) Delayed re-epithelialization, reduced vascularization, reduced wound contraction (large exicisional) | 1) (Hartenstein et al., 2006)<br>2) (Hattori et al., 2009) |
| MMP-2 | knockout | Unaltered | (Frøssing et al., 2010) |
| MMP-9 | knockout | 1) Delayed re-epithelialization<br>2) Delayed re-epithelialization, reduced clearance of fibrin clots | 1) (Hattori et al., 2009)<br>2) (Kyriakides et al., 2009) |
| MMP-9/13 | double knockout | Delayed re-epithelialization, reduced vascularisation, reduced wound contraction | (Hattori et al., 2009) |
| MMP-3 | knockout | Impaired wound contraction | (Bullard et al., 1999) |
| MMP-10 | overexpression in KCs | Unaltered closure, scattered epithelial sheet | (Krampert et al., 2004) |
| MT1-MMP | knockout | Unaltered, impaired epithelialization ex vivo | (Mirastschijski et al., 2004b) |
| ADAM-9 | knockout | Enhanced re-epithelialization | (Mauch et al., 2010) |
| ADAMTS-1 | knockout | Delayed re-epithlialization, increased angiogenesis | (Krampert et al., 2005) |
| ADAMTS-5 | knockout | Impaired closure, accumulation of aggregan, defected response to TGF-β by fibroblasts | (Velasco et al., 2011) |
| PLG | knockout | Severely impaired closure | (Rømer et al., 1996) |
| PLG/MMP-13 | double knockout | Increased effect compared to plg-deficiency | (Juncker-Jensen and Lund, 2011) |

Modified from Toriseva and Kähäri (2009). ADAM, a disintegrin and metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs KC, keratinocytes; MMP, matrix metalloproteinase; PLG, plasminogen.

MMP-1 is also expressed by fibroblasts in granulation tissue (Inoue et al., 1995; Vaalamo et al., 1997), where it is thought to participate in the remodeling of the collagenous ECM (Pins et al., 2000). Accordingly, mouse MMP-13 has been shown to play a role in dermal wound healing (Hattori et al., 2009). It was recently shown that deletion of MMP-13 in plg-deficient mice additionally impaired wound healing although not nearly as much as did the addition of a broad spectrum metalloproteinase inhibitor (Juncker-Jensen and Lund, 2011; Lund et al., 1999) (Table 4). This suggests a functional overlap of plasmin and MMP-13 yet emphasizing the function of other metalloproteinases in wound healing.

Moreover, MMP-2 and MT1-MMP, which both are also capable of cleaving fibrillar collagens, are present in human and murine skin wounds (Madlener et al., 1998; Mirastschijski et al., 2002b). In fact, MT1-MMP appears to be one of the major collagenases in fibroblasts (Lee et al., 2006a; Sabeh et al., 2009) that mediate cell migration (Sabeh et al., 2004). Surprisingly, although MT1-MMP null mice display severe abnormalities in bone development and defective angiogenesis in cartilage and cornea (Zhou et al., 2000), cutaneous wound healing is virtually normal in 3-day-old animals (Mirastschijski et al., 2004b). It is of note that the skin of newborn mice is markedly thinner than that of adults, which is likely to reflect the collagen content of the dermis and thus it is possible that collagenases become more important in adult mouse wound healing compared to young animals.

MMP-8 (neutrophil collagenase, collagenase-2) is mainly expressed by neutrophils, where it is stored in cellular granules and secreted upon neutrophil activation (Hasty et al., 1986). In human excisional wounds of the skin, MMP-8 was found to be the most abundant collagenase (Nwomeh et al., 1999; Pirilä et al., 2007). It was suggested to compensate for the function of MMP-13 in small cutaneous wounds in MMP-13 deficient mice (Hartenstein et al., 2006) and recent studies with MMP-8-deficient mice have revealed a significant delay in wound healing due to impaired re-epithelialization, a lag in neutrophil infiltration and persistent inflammation (Gutierrez-Fernandez et al., 2007). These studies suggest that neutrophil-derived MMP-8 acts in keratinocyte migration together with an epidermal collagenase, and in the migration and resolution of wound neutrophils.

Stromelysins, MMP-3 and MMP-10 are expressed by keratinocytes during wound repair in human and mouse wounds. MMP-3 is expressed by the basal proliferating keratinocytes behind the migrating cells, while MMP-10 is produced exclusively at the tip of the migrating keratinocyte sheet (Madlener et al., 1998; Rechardt et al., 2000; Vaalamo et al., 1996). In addition, MMP-3 is produced by wound fibroblasts (Madlener et al., 1998; Vaalamo et al., 1996). Providing evidence for the competence of MMP-3 in the assembly and organization of f-actin-containing fibroblasts to facilitate wound contraction, MMP-3-deficient mice display delayed wound closure associated with impaired wound contraction (Bullard et al., 1999). The function of MMP-3 in the epithelium remains unclear, since these animals show normal re-epithelialization (Bullard et al., 1999). However, MMP-3 can increase the activity and bioavailability of cytokines and growth factors such as HB-EGF and TGF-β, and activate several other proMMPs (Imai et al., 1997; Visse and Nagase, 2003). It also cleaves basement membrane proteins and a variety of other ECM proteins and has been shown to modulate intercellular contacts resulting in enhanced invasion (Noe et al., 2001; Visse and Nagase, 2003). Thus, MMP-3 may act to loosen the epithelial structure of the epidermis as a prerequisite for keratinocyte proliferation and migration.

Epidermal MMP-10 is induced about three days post-wounding in humans, and at least *in vitro* it seems to be regulated by cytokines such as EGF, TGF-β1 and TNF-α (Rechardt et al., 2000). MMP-10 is thought to regulate keratinocyte migration during re-epithelialization, since the over-expression of active MMP-10 in the basal keratinocytes of transgenic mice severely scatters the migrating epithelial sheet (Krampert et al., 2004).

MMP-2 and MMP-9 (gelatinase A and B) exhibit a distinct expression pattern during skin wound healing. MMP-9 is detected at the migrating epithelial front whereas MMP-2 is exclusively expressed in the dermis by fibroblasts and endothelial cells (Madlener et al., 1998; Mirastschijski et al., 2002a). MMP-9 is also present in inflammatory cells including T cells, neutrophils and macrophages (Inkinen et al., 2000; Leppert et al., 1995; Okada et al., 1997). The relatively stable and long-lasting expression of MMP-2 in wound granulation tissue suggests participation in prolonged ECM remodeling (Frøssing et al., 2010; Ågren, 1994). Indeed, in addition to the direct processing of dermal ECM proteins, several studies have implicated MMP-2 as an enhancer of the activity of TGF-β, a potent growth factor regulating matrix deposition and remodeling, via different mechanisms (Dallas et al., 2002; Imai et al., 1997; Yu and Stamenkovic, 2000). However, MMP-2-knockout mice show slightly but not significantly delayed wound closure and otherwise normal architecture of a healing skin wound (Frøssing et al., 2010) suggesting possible enzymatic redundancy due to other proteinases. Cutaneous wound healing studies with MMP-9-deficient mice have provided somewhat contradictory results. First, MMP-9 was reported to inhibit epidermal wound healing and MMP-9-deficient mice showed slightly accelerated wound closure, which was suggested to be due to enhanced cell proliferation (Mohan et al., 2002). This could be explained *e.g.* by the ability of MMP-9 to activate TGF-β (Dallas et al., 2002; Yu and Stamenkovic, 2000). However, what is confusing is that wound healing in the wild type mice was atypically slow, maybe because of the experimental settings, which differed from the common practice. In two more recent studies with more typical implementations, MMP-9 deficiency was reported to significantly delay re-epithelialization and this was suggested to result from impaired migration of keratinocytes (Hattori et al., 2009; Kyriakides et al., 2009).

Both MMP-2 and MMP-9 are expressed in the blood vessel endothelium as are MT1-MMP and MMP-19 (Aplin et al., 2009; Hieta et al., 2003; Mirastschijski et al., 2002a; Oikarinen et al., 1993). MMP-2 and MMP-9 have been shown to mediate both physiologic and tumorigenic angiogenesis (Bergers et al., 2000; Itoh et al., 1998; Kato et al., 2001; Vu et al., 1998). Essential for the sprouting of blood vessels, gelatinases digest various components of the vascular basement membrane and activate or release angiogenic cytokines and growth factors, such as TNF-α (Gearing et al., 1994) and VEGF (Bergers et al., 2000; Dean et al., 2007). Importantly, gelatinases, as well as several other MMPs, also regulate angiogenesis by generating anti-angiogenic peptides by the proteolytic cleavage of other proteins such as type IV and XVIII collagens (tumstatin and endostatin, respectively) and plasminogen (angiostatin) (Cornelius et al., 1998; Hamano et al., 2003; Heljasvaara et al., 2005; O'Reilly et al., 1999). In addition, MMP-2 can regulate mitogenic and angiogenic signals induced by FGFR-1 by shedding and still preserving the receptor capable of binding FGF (Levi et al., 1996). Thus, MMP-2 may regulate FGF-mediated angiogenesis by affecting the amount of functional cell surface receptors and the availability of active FGF. MT1-MMP appears to be pivotal for angiogenesis (Chun et al., 2004). During wound healing its fibrinolytic and collagenolytic activities are needed for vessel invasion through fibrin and collagenous barriers (Chun et al., 2004; Genis et al., 2007; Hiraoka et al., 1998) and TIMP-2-mediated activation of proMMP-2 by MT1-MMP potentiates MMP activity in tissues (Strongin et al., 1995). MMP-19 has been detected in proliferating

epithelia, microvascular endothelial cells, fibroblasts and in macrophages during cutaneous wound healing in humans. It has been suggested to regulate keratinocyte migration, proliferation and differentiation (Beck et al., 2007; Sadowski et al., 2005; Sadowski et al., 2003a) and it may inhibit angiogenesis by destabilizing the ECM necessary for capillary morphogenesis (Jost et al., 2006).

MMP-12 (metalloelastase) is produced by wound macrophages in acute murine excisional wounds (Madlener et al., 1998). In humans, abundant MMP-12 expression has been detected in various cutaneous diseases (Salmela et al., 2001; Suomela et al., 2001; Vaalamo et al., 1999a), although the authors could not find MMP-12 in a majority of the examined acute or chronic skin wounds despite the presence of macrophages (Vaalamo et al., 1999a). MMP-12 possesses bactericidal activity (Houghton et al., 2009), and its ability to generate angiostatin suggests a role in regulation of angiogenesis (Cornelius et al., 1998). MMP-26 and MMP-28 (matrilysin-2/endometase and epilysin, respectively) are detected in the epidermis during the re-epithelialization of a cutaneous wound. MMP-26 was detected in the epithelial tip bordering the wound gap (Ahokas et al., 2005) and it has been suggested to coordinate the cell–cell adhesion involved in keratinocyte migration (Pirilä et al., 2007). MMP-28 was detected in the suprabasal keratinocytes distal to the epithelial tip in a region occupying a virtually intact basement membrane (Saarialho-Kere et al., 2002). In lung adenocarcinoma cells, the expression of MMP-28 resulted in an increase in TGF-β activity accompanied by the loss of E-cadherin and increased cell motility (Illman et al., 2006). Thus, the localized expression of MMP-28 during wound repair is likely to regulate the proliferation and migration of keratinocytes.

The expression of TIMP-1, -2 and -3 has been detected in the wound epithelium and dermal fibroblast- and macrophage-like cells in humans (Vaalamo et al., 1999b; Vaalamo et al., 1996). Occasional blood vessels of acute wounds were also found to be positive for TIMP-1 and TIMP-3. No TIMP-4 expression was detected in acute human wounds (Vaalamo et al., 1999b).

Finally, although the single MMP gene knock-out studies have not revealed baneful wound healing phenotypes, numerous studies using wide spectrum small-molecule chemical inhibitors of MMPs have proven the importance of the collective action of MMPs in wound healing *in vitro* and *in vivo*. For instance, treating fibroblasts cultured in a 3D collagen matrix with marimastat (BB-2516) or galardin (GM6001, ilomastat) impaired fibroblast-mediated collagen contraction (Martin-Martin et al., 2011; Scott et al., 1998), and batimastat (BB-94) totally blocked the TGF-β-promoted migration of keratinocytes *in vitro* (Mäkelä et al., 1999). Furthermore, systemic treatment of murine skin wounds with galardin attenuated wound contraction and interfered with the migration of keratinocytes (Lund et al., 1999; Mirastschijski et al., 2004a). And topical treatment of rat excisional wound with batimastat significantly delayed wound closure, the appearance of myofibroblasts and wound contraction (Mirastschijski et al., 2010). However, it should be noted that the specificity of these inhibitors is not restricted to MMPs but *e.g.* galardin can also inhibit certain ADAMs (Moss and Rasmussen, 2007).

## 2.5 ABERRANT WOUND HEALING IN SKIN

### 2.5.1 Chronic ulcers

The majority of chronic wounds appear in the lower extremities and involve ischemia associated with vascular disorders, metabolic diseases such as diabetes, or pressure and shearing (Eming and Scharffetter-Kochanek, 2009). Chronic wounds are characterized by delayed or insufficient closure, where the wound remains open for more than one month. It is estimated, that in Western Europe about 1.3–3.6 % of the population have a chronic leg ulcer at some point in life with an increasing susceptibility with age (Vaalasti et al, 2007). Patients with chronic wounds often experience pain, which decreases their quality of life, and the most severe ulcerations may lead to amputation and increased mortality (Menke et al., 2007). A severe but fortunately rare complication of a chronic wound is malignant transformation (Menke et al., 2007; Trent and Kirsner, 2003). The health care expenditures of treating chronic ulcer are significant (Menke et al., 2007; Seppänen and Hjerppe, 2008). While conventional local wound healing therapy such as debridement and different kinds of dressings are useful in chronic wound care, understanding the cellular and molecular mechanisms of ulceration is clearly needed for helping the development of novel strategies to further improve healing.

Chronic wounds are characterized by pathologic inflammation, fibroblast senescence and uncontrolled proteolysis (Menke et al., 2007). This may disturb cell-ECM contacts or lead to the degradation of growth factors and their receptors and thus promote the chronic nature of an ulcer. A number of studies have shown that the expression levels of MMPs, *e.g.* MMP-2, -9, -1 and -8, are elevated in chronic ulcers, while the expression of TIMPs is decreased compared to acute wounds (Bullen et al., 1995; Lobmann et al., 2002; Nwomeh et al., 1999). The increased amount of MMPs in chronic wounds can be partially explained by the dramatic infiltration of inflammatory cells secreting MMPs such as MMP-8 and MMP-9 (Mirastschijski et al., 2002a; Nwomeh et al., 1999; Pirilä et al., 2007) and a variety of growth factors, which in turn are likely to regulate the MMP expression of the cells. Moreover, compounds derived from *Staphylococcus aureus*, a common infectious microbe in chronic leg ulcers, may up-regulate the expression of multiple MMPs, namely MMP-1, -2, -3, -7, -10, -11, and -13, as well as TIMP-1 and TIMP-2 by normal dermal fibroblasts (Kanangat et al., 2006). A distinct feature of chronic ulcers is the expression of MMP-13 by wound fibroblasts embedded in the collagenous stroma (Vaalamo et al., 1997).

The reduction of inflammation and any fulminant bacterial infection is a major objective in the care of chronic wounds. In addition, novel approaches for MMP inhibition are being considered in chronic wound care. Limited results using protease absorbing matrices, especially combined with autologous growth factors, on chronic venous and diabetic ulcers appear somewhat promising (Kakagia et al., 2007; Vin et al., 2002). Furthermore, doxycyline, a tetracycline antibiotic with MMP and TACE inhibiting properties, has been suggested as a potential compound for neutralizing the detrimental protease activity in chronic wounds (Stechmiller et al., 2010). However, it

should be kept in mind that a certain amount of MMP activity is still needed for normal wound healing (Mirastschijski et al., 2004a; Mirastschijski et al., 2010; Ågren et al., 2001).

### 2.5.2 Fibrotic wounds

Fibrotic wounds, characterized by an excessive formation of collagenous scar tissue, can be seen as healing too effectively. The risk factors for a wound to develop into a fibrotic one include large size, delayed closure, infection and tissue tension, and as a result, the skin loses its elasticity and in the extremities, this may lead to contractures and functional disability. Hypertrophic scars and keloids are two types of local fibrosis. In hypertrophic scars the skin has a thickened appearance, but is confined to the boundaries of the initial injury. Keloids in turn are fibrous, reddish and firm nodules that grow beyond the borders of the original wound,. Hypertrophic scars are smaller than keloids but may develop severe contractures that can affect tissue functionality. Keloids are rarer than hypertrophic scars and tend to be genetically inherited (Bran et al., 2009).

Hypertrophic scars and keloids also shear common properties: they are only seen in humans, and their histopathological markers include the accumulation of collagen and fibroblasts. Interestingly though, fibroblasts proliferate actively in keloids, while myofibroblasts colonize hypertrophic scars (Bran et al., 2009; Ehrlich et al., 1994). The molecular mechanisms behind the shift from normal healing to fibrotic scarring remain largely unknown. The pro-fibrotic growth factor TGF-β is commonly thought to enhance scarring conditions. This notion is supported by reports showing the up-regulation of TGF-β in hypertrophic scars and keloid fibroblasts (Ghahary et al., 1993; Lee et al., 1999; Peltonen et al., 1991; Wang et al., 2000) and elevated levels of TGF-β receptors in keloid fibroblasts (Chin et al., 2001). This is likely to contribute to the enhanced collagen synthesis detected in keloid and hypertrophic scar fibroblasts (Fujiwara et al., 2005; Ghahary et al., 1993) and may stimulate the differentiation of fibroblasts to myofibroblast in hypertrophic scars (Ehrlich et al., 1994). Moreover, hypertrophic scar fibroblasts show reduced levels of MMP-1 mRNA (Ghahary et al., 1996) while the up-regulation of MMP-1, MMP-2 and TIMP-1 proteins has been reported in keloid fibroblasts (Fujiwara et al., 2005) and of MMP-13 in keloid tissues (Kuo et al., 2005). Tissue extracts from both lesions show markedly increased levels of MMP-2 activity compared to normal skin samples, and low levels of MMP-9 (Neely et al., 1999). The elevation of collagenase expression in keloids may reflect an attempt to remove the excess collagen in the tissue. In hypertrophic scar fibroblasts, the down-regulation of MMP-1 is suggested to be due to IGF-1 (Ghahary et al., 1996). It is also possible that fibrotic scar collagen receptor integrins are dysfunctional during collagen ligation, resulting in abnormal regulation of collagenases and collagen synthesis (Heino, 2000). In keloid fibroblasts this may counteract the negative-regulation of MMP-1 expression by TGF-β.

### 2.5.3 Wound healing in fetal skin

Whereas scarring is normal for cutaneous wound repair in adults, in fetal skin, up to about 24 weeks of gestation, wounds heal without or with only minimal scar formation and the repair process in general is dynamic and rapid (Buchanan et al., 2009). This fact has encouraged scientists to explore the mechanism of fetal wound healing in terms of trying to find new therapeutic strategies for improving impaired wound healing. At early gestation, fetal wounds possess different composition of ECM and cells, inflammation response, soluble modulators and gene expression pattern compared to postnatal or adult wounds (Table 5). The sterile environment of the fetus minimizes the risk of pathogen invasion. Moreover, fetal platelets are less active compared to adult platelets (Olutoye et al., 1997; Olutoye et al., 1996), which may partially result in the remarkably mild inflammation reaction characteristic of fetal skin wound repair (Buchanan et al., 2009). As inflammation is one of the factors increasing scarring and its reduction hinders scar formation in adults, the virtually absent inflammation in fetal skin wounds is implicated to scarless healing.

**Table 5. Specific properties of fetal skin wound healing.**

| | ***Fetal wound in comparison to adult*** | ***Ref.*** |
|---|---|---|
| ECM content | High levels of hyaluronan<br>High ratio of type III to type I collagen, low level of type I collagen crosslinking<br>- increased cell motility | (Estes et al., 1993; Lovvorn et al., 1999; Merkel et al., 1988) |
| ECM modulators | High ratio of MMP level to TIMP level<br>- increased ECM remodeling and turnover | (Dang et al., 2003) |
| Cells | Few inflammatory cells - less leukocyte-derived reactive compounds, less inflammatory cytokines<br>High dermal cellularity - potential for fast recovery<br>Characteristic fibroblast phenotype - faster migration rate, more dynamic production of ECM, efficient ECM remodeling<br>Few myofibroblasts - no wound contraction | (Buchanan et al., 2009; Frantz et al., 1992; Sandulache et al., 2007) |
| Soluble factors | Decreased expression of pro-inflammatory cytokines IL-6 and IL-8 - diminished inflammation<br>Low levels of TGF-β1 and -β2, increased TGF-β3<br>- balance on non-fibrotic TGFs | (Chen et al., 2005; Liechty et al., 2000; Liechty et al., 1998; Nath et al., 1994) |
| Gene expression | Rapid up-regulation of growth-related genes | (Colwell et al., 2008) |

A histological landmark for a scar is the dermal accumulation of thick and tense collagen bundles, thus a potent element in scarless repair should be efficient ECM remodeling. Many collagen gel contraction studies have shown that collagen matrix remodeling is increased by fetal dermal fibroblasts *in vitro* in comparison to their adult counterparts (Irwin et al., 1998; Sandulache et al., 2007). Moreover, a high expression ratio of TGF-β3 to its fibrogenic isoforms TGF-β1 and -β2 in fetal skin in early gestation is associated with scarless repair (Chen et al., 2005; Nath et al., 1994). Accordingly, adding TGF-β3 exogenously, and extinguishing both TGF-β1 and -β2 with function blocking antibodies, reduced scarring in a rat model (Shah et al., 1995).

There are indications that the expression of several MMPs is increased in skin over time during fetal development (Chen et al., 2007; Dang et al., 2003; Peled et al., 2002), However, it is interesting that in early gestation in fetal rat skin, an injury induces higher expression of the interstitial collagenase, MMP-2, MMP-9 and MMP-14 and lower expression of TIMPs in comparison with late gestation rat. This results in markedly higher MMP:TIMP ratio in scarless wounds than in scarring wounds (Dang et al., 2003). Thus, the distinct proteolytic profile associated with scarless wound repair in fetal skin and together with a specific ECM composition may contribute to the superb restoration of dermis observed in fetal skin. To conclude, the exact factors leading to scarless healing in fetal skin are still rather obscure and it is likely that several different aspects are involved in the process, including cellular dynamics and the metabolism of the wound environment, that collectively participate in the tissue remodeling resulting in scarless wound repair.

## 2.6 SQUAMOUS CELL CARCINOMA OF THE SKIN

Cutaneous squamous cell carcinoma (SCC) is defined by the malignant growth of epidermal keratinocytes and it comprises several cancer types including SCCs *in situ* (Bowen's disease) and invasive SCC, which has metastatic potential (Madan et al., 2010; Weinberg et al., 2007). Skin SCCs are more common among older adults and in territorial locations with high UV-radiation. In Finland, about 1500 new cases were diagnosed in 2010 ranking non-melanoma skin cancer excluding basal cell carcinomas as the fifth and fourth common cancer in women and men, respectively (Finnish Cancer Registry, www.syoparekisteri.fi). SSC was the cause of about 43 deaths annually between 2005 and 2009 in Finland (Engholm et al., 2011). The majority of invasive SCCs evolve from precursor lesions such as actinic keratoses and SCC *in situ*. The risk factors implicated in skin SCC include genetic factors, UV-radiation, human papilloma virus infection, tobacco and alcohol, immunosuppression and the presence of a chronic injury or inflammation of the skin (Madan et al., 2010).

For the majority of primary cutaneous SCCs, the standard treatment is surgical excision. Other possibilities for treating SCCs include non-surgical treatments such as cryotherapy, which can be used for small low-risk tumors, and radiotherapy for patients with high operative risk or as an additional method with surgery (Neville et al., 2007). Chemotherapy is used for treating metastasized SCC (Weinberg et al., 2007).

As exemplified by Marjolin's ulcer, which is a skin SCC evolved in a chronic ulceration (Trent and Kirsner, 2003), malignant tumors tend to develop at sites of chronic injury and inflammation (McGrath et al., 1992; Trent and Kirsner, 2003). On the other hand, tissue injury and skin ulceration are notable players in the pathogenesis of cutaneous SCC (Madan et al., 2010). Since processes, such as active cell migration and proliferation, inflammation, angiogenesis and proteolysis are all common factors for both wound healing and cancer, tumors have been referred as wounds that do not heal (Dvorak, 1986). That is, in cancer these events are not under physiological control, which leads to un-controlled growth, invasion and metastasis. For instance, in the reepithelializing wound epidermis the migrating keratinocytes undergo changes in

the expression pattern of their receptor integrins. They loose hemidesmosomal contacts with the underlying basement membrane zone, many intercellular contacts and their apical–basal polarity (Garrod et al., 2005; Larjava et al., 1993). These alterations resemble the epidermal-mesenchymal transition (EMT) detected often in epidermal tumor cells (Kalluri and Weinberg, 2009). However, in tumors many of these changes result from a persistent stimulus from growth factors such as TGF-β, and are associated with genomic mutations resulting in *e.g.* the activation of the Ras-oncogene (Janda et al., 2002). Moreover, as wound myofibroblasts contract granulation tissue to get wound edges closer to each other (Hinz, 2007), abundant myofibroblasts are typically detected also around epithelial tumors (van Kempen et al., 2004). These cells and the surrounding ECM, provide a tumor microenvironment, which is involved in stimulating cancer growth and invasion (Orimo et al., 2005; Zigrino et al., 2009).

### 2.6.1 Matrix metalloproteinases in cutaneous SCC

The importance of plasmin and MMP activity has been established for wound healing (Lund et al., 1999). Similarly, proteolytic activity is fundamentally connected to different aspects of cancer invasion and metastasis (Kessenbrock et al., 2010). The expression of several MMPs, in particular MMP-7, MMP-13 and MT1-MMP is associated with the malignant transformation of epidermal cells (Airola et al., 1997; Impola et al., 2005; Kerkelä et al., 2001; Vosseler et al., 2009). Moreover, the expression of MMP-1 and MMP-3 mRNA in skin SCC tumor cells and stromal fibroblasts at the invasive area and of MMP-2 mRNA in stromal fibroblasts has been reported, and appears to correlate with a poor differentiation stage of the cancer (Airola et al., 1997; Kerkelä et al., 2001; Tsukifuji et al., 1999). In accordance, using an epidermal xenograft cancer model established by Ha-*ras*-transformed HaCaT cells with increasing tumorigenic potential, Vosseler *et al*. (2009) discriminated tumor-derived (human) and stromal (mouse) MMPs in the distinct stages of cancer progression. They detected a high expression of MMP-1, -2 and -14 mRNA and protein in the tumor cells, and a high expression MMP-2, -3, -9 -13 and -14 mRNA and protein in the stromal cells, where the expression of MMP-9 was mostly restricted to neutrophils in the vicinity of blood vessels (Vosseler et al., 2009). The up-regulation of all MMPs was correlated with increasing tumor malignancy and the invasion state of the malignant cancer. Only the expression of MMP-2 was detected in tumors made up of the corresponding benign cells (Vosseler et al., 2009). In addition, the expression of MMP-10 mRNA has been detected in tumor cells in a portion of cutaneous SCCs (Kerkelä et al., 2001). Interestingly, in contrast with the conclusion that MMPs function only in the cancerous progression of SCCs, ablation of some MMPs is associated also with the dedifferentiation of skin SCCs (Ahokas et al., 2005; Impola et al., 2003). High expression levels of several MMPs in cancers is likely to result from alterations in microenvironmental stimuli *e.g.* in the patterns of active growth factors or changes in cellular signaling pathways. Or they may be due to genetic polymorphisms in the MMP promoter sequences, which could affect the transcriptional activity of MMP genes (Westermarck and Kähäri, 1999; Ye, 2000). In addition, there are indications for MMP gene amplifications in cancer (Roman et al., 2008).

The action of MMPs in cancer has been demonstrated using transgenic mouse models (Murphy and Nagase, 2008). For instance, a deficiency in MMP-9 results in reduced keratinocyte proliferation in neoplastic lesions and a decreased incidence of invasive cutaneous tumors in a mouse model of multi-stage tumorigenesis elicited by HPV16 oncogenes (Coussens et al., 2000). Over-expression of the human MMP-1 transgene in mouse skin sensitized the animals to epidermal carcinogenesis (D'Armiento et al., 1995). Moreover, MMP-10 null mice possess reduced carcinogen-induced epithelial tumorigenesis, which was demonstrated to be, at least partially due to the loss of fibroblast-derived MMP-10 activity (Masson et al., 1998). Interestingly, stromal MMP-13 appears to be essential for the maintenance of tumor angiogenesis and invasive growth in a mouse cutaneous SCC model (Lederle et al. 2010).

MMP-13 was initially cloned from a breast cancer cDNA library (Freije et al., 1994). It is a collagenase with relatively wide substrate specificity and a restricted physiologic expression pattern (Ala-aho and Kähäri, 2005). While normal epidermal keratinocytes do not express MMP-13 (Vaalamo et al., 1997), MMP-13 is produced by transformed keratinocytes such as benign HaCaT cells and SCC cells *in vitro* and *in vivo* (Airola et al., 1997; Johansson et al., 1997c) which suggests critical alterations in the signaling pathways that regulate MMP-13 expression in epidermal cells. Providing evidence for the pivotal effects of MMP-13 on cutaneous SCC growth and invasion, the inhibition of MMP-13 in skin SCC tumors by adenovirally introduced MMP-13 antisense ribozyme markedly reduced tumor growth and proliferation in a mouse xenograft cancer model and strongly inhibited SCC cell invasion *in vitro* (Ala-aho et al., 2004). In accordance, inducing MMP-13 expression in fibrosracoma cells enhanced cell invasion through matrigel and a collagen matrix *in vitro* (Ala-aho et al., 2002).

Traditionally, MMPs have been thought to promote cancer growth and invasion by primarily degrading protein structures out of the way of invading cells. However, it is now well understood that the proteolytic processing of a variety of substrates by MMPs results in the release of bioactive molecules from the ECM, and the subsequent activation and inactivation of growth factors, cytokines, chemokines and their receptors as well as other proteases. This will either positively or negatively influence for example angiogenesis and the inflammatory state of tumors, and is at least an equally important function of MMPs during cancer progression as is matrix degradation (Kessenbrock et al., 2010).

## 2.7 KERATINOCYTE GROWTH FACTOR IN TUMORIGENESIS

### 2.7.1 Keratinocyte growth factor/fibroblast growth factor-7

Keratinocyte growth factor (KGF, fibroblast growth factor-7/FGF-7, ~28 kDa) is one of the 22 FGFs identified in humans and was originally found in a human embryonic lung fibroblast culture medium (Ornitz and Itoh, 2001; Rubin et al., 1989). Subsequently KGF was characterized as a paracrine mediator produced by cells of mesenchymal origin, *e.g.* by skin and intestinal fibroblasts and by epidermal γδ T cells

(Finch et al., 1989; Jameson et al., 2002). There are four genes that code for FGF receptors (FGFR1–FGFR4). FGFRs consist of three extracellular immunoglobulin domains, a transmembrane domain and a cytoplasmic tyrosine kinase domain (Eswarakumar et al., 2005). KGF binds exclusively to a specific cell surface receptor, which is a splicing variant of FGF receptor-2, called IIIb (FGFR2-IIIb) or alternatively KGFR (Miki et al., 1992). FGFR2-IIIb is expressed specifically by epithelial cells in adults, and in the most epithelial organs during development (Orr-Urtreger et al., 1993), for example in the skin (Marchese et al., 1995; Werner et al., 1992), bladder (Yi et al., 1995), stomach and intestine (Housley et al., 1994) and in liver hepatocytes (Steiling et al., 2004), where it enables the paracrine function of KGF. Similarly to other FGFs, KGF also binds to the low-affinity co-receptors heparin and heparan sulphate proteoglycans (HSPG) that do not mediate cellular signals, but rather influence ligand binding and receptor activation (LaRochelle et al., 1999; Ye et al., 2001). More recently FGF binding protein, which is produced by epidermal keratinocytes and up-regulated upon wound healing, was shown to interact with KGF and enhance ligand activity at low concentrations (Beer et al., 2005). A schematic picture of FGFR activation is presented in Figure 5.

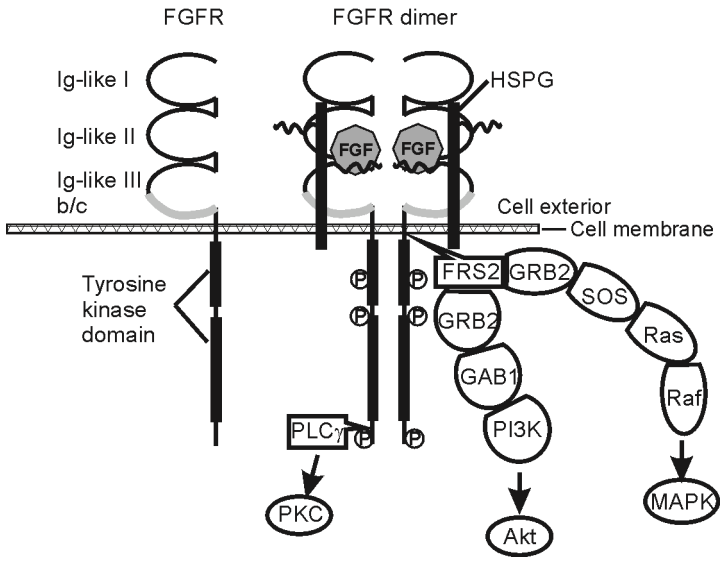


**Figure 5.** Fibroblast growth factor receptor (FGFR) signaling. The extracellular part of FGFR contains three Ig-like domains (I-III). The third Ig-like domain is alternatively spliced (b/c) in FGFR1-3. FGF associates with HSPG and FGFR resulting in the dimerization of two ternary complexes and the subsequent phosphorylation of intracellular tyrosine residues. This leads to the activation of signaling cascades including MAPK pathways. Ig, immunoglobulin; HSPG, heparan sulfate proteoglycan; MAPK, mitogen-activated protein kinase. (Based on Wesche et al., 2011).

The mitogenic effects of KGF have been shown by numerous studies on a variety of epithelial cells (Housley et al., 1994; Rubin et al., 1989; Yi et al., 1994). It also regulates epithelial morphogenesis, differentiation and homeostasis (Andreadis et al.,

2001; Grose et al., 2007; Guo et al., 1993; Orr-Urtreger et al., 1993) and keratinocyte migration (Karvinen et al., 2003; Putnins et al., 1999). KGF expression is often associated with epithelial injury (Brauchle et al., 1996; Marchese et al., 1995; Werner et al., 1992). In a normal cutaneous wound, the expression of KGF is markedly increased early after injury by dermal fibroblasts (Marchese et al., 1995; Werner et al., 1992), which is likely to result from stimulation by keratinocyte and leukocyte-derived growth factors such as transforming growth factor-α (TGF-α) and pro-inflammatory cytokines such as IL-1 and TNF-α. They have been shown to stimulate expression of KGF by fibroblasts *in vitro* demonstrating the cross-talk between the epithelium and the dermal compartment during tissue repair and homeostasis (Brauchle et al., 1994; Chedid et al., 1994; Hubner et al., 1996). In intact epidermis, FGFR2-IIIb is more persistently expressed by differentiating keratinocytes in the spinous layer and also to some extend in the basal layer (LaRochelle et al., 1995).

FGFR2-IIIb signaling has a pivotal role in embryonal development, as the gene deficient mice die at birth and display gross abnormalities in the organogenesis of *e.g.* their kidneys, salivary and adrenal glands, thymus, pancreas, skin, hair follicles and stomach as well agenesis of the lungs, limbs and teeth (Revest et al., 2001). Particularly in the skin, the functional knock-down of FGFR2-IIIb by the overexpression of a dominant negative FGFR2-IIIb mutant under the control of the cytokeratin 14 promoter in mouse stratified epithelium caused epidermal thinning, dermal fibrosis, and abnormalities and a reduction in the number of hair follicles (Werner et al., 1994). These mice also showed delayed wound re-epithelialization associated with reduced keratinocyte proliferation (Werner et al., 1994). However, as FGF-1, FGF-10 and FGF-22 are also ligands for FGFR2-IIIb (Zhang et al., 2006) the specific effects of KGF was not verified in this study. The issue was addressed by generating transgenic mice deficient for KGF (Guo et al., 1996). In accordance with studies showing altered hair follicle development in FGFR2-IIIb-deficient animal models, KGF-deficient mice displayed some abnormalities in hair growth. However, no major phenotype was observed. Unexpectedly, epidermal wound healing was completely comparable with that of wild type mice (Guo et al., 1996). In contrast, the overexpression of KGF under the control of the cytokeratin 14 promoter in mice caused epidermal thickening associated with incomplete differentiation of cornified layer cells. This was suggested to result from hyperproliferation (Guo et al., 1993). Similarly to functionally FGFR2-IIIb -deficient mice, hair follicle development in KGF-overexpressing animals was severely suppressed, and with increasing age, the mice started to show early signs of epithelial transformation in their tongue (Guo et al., 1993). Thus, appropriate KGF levels are needed for normal tissue morphogenesis and homeostasis. The beneficial competence of KGF as a stimulator of cutaneous wound healing has been demonstrated by studies employing different methods for delivering exogenous KGF into the wound tissue (Escamez et al., 2008; Geer et al., 2005; Marti et al., 2004). However, the studies with FGFR2-IIIb and KGF-deficient mice demonstrate that the regulation of epidermal re-generation involves several growth stimulatory factors and suggests high functional redundancy of these factors during wound healing.

During the last decade, KGF has been recognized for its potent protective properties on various epithelial tissues as reviewed in (Finch and Rubin, 2004). Using rodent

models, pretreatment with KGF protected the lung epithelium from injury induced by toxic factors such as hyperoxia, acid instillation or by anti-cancer agents, radiation and bleomycin. KGF also protected the bladder from developing hemorrhagic cysts induced by *e.g.* radiation or cyclophosphamide. Moreover, several studies have shown that KGF administration reduces radiation- and chemotherapy-induced injuries of the gastro-intestinal tract, *e.g.* mucositis in gut and mouth, thus established the ability of KFG to protect normal epithelial tissue from anti-cancer treatments. (Farrell et al., 1998; Spielberger et al., 2004; Henke et al., 2011). KGF was also reported to protect normal keratinocytes from apoptosis induced by ROS-producing and electrophilic toxic compounds and by UV irradiation *in vitro* and *in vivo* (Braun et al., 2006). Because of these beneficial properties of KGF, recombinant KGF (palifermin, Kepivance®) has already approved for clinical use to prevent the incidence and severity of oral mucositis in hematologic cancer patients, who receive chemotherapy and radiation therapy before a bone marrow transplant. However, in the case of treatment of solid tumors and especially carcinomas with chemo- and radiotherapy causing severe mucositis, the use of KGF for protection of normal tissue is restrained. This is because of the uncertainty of the impact of KGF on cancer cells that often express FGFR2-IIIb by definition.

### 2.7.2 The role of KGF and FGFR2-IIIb in cancers

As it acts as a mitogen in epithelial cells, the activities of KGF in malignant transformation and cancer progression have been extensively studied. However, the results have not always been convergent and it is now becoming clear that the expression levels of KGF and KGFR as well as the response of the cancer cells to KGF may differ depending on the cancer type. The topic has been comprehensively reviewed in (Finch and Rubin, 2006).

While the physiological expression of KGF is restricted to the stromal cells of epithelial organs, the expression of KGF as detected at the mRNA and protein levels is reported for some cancerous epithelial cells including estrogen receptor positive breast cancer cells (Tamaru et al., 2004), pancreatic ductal carcinoma cells (Ishiwata et al., 1998; Liu et al., 1998) and prostate glandular epithelial cells (McGarvey and Stearns, 1995). As expected, because of their epithelial origin, many carcinoma cell lines express FGFR2-IIIb at least at the mRNA level (Brake et al., 2008). The overexpression of KGFR and KGF in malignant tissues has been reported for example in pancreatic carcinoma (Ishiwata et al., 1998), prostate cancer (McGarvey and Stearns, 1995) and lung cancer (Yamayoshi et al., 2004). In contrast, the level of KGF mRNA has been found to be lower for example in tumor samples from advanced SCC of the head and neck compared to normal mucosa from the same patient (Knerer et al., 1998). Moreover, to exemplify the complexity of the results, FGFR2-IIIb mRNA was reported to be higher in colon cancer samples compared to normal tissues (Watanabe et al., 2000) but in another study no differences in expression levels were found (Otte et al., 2000). However, in the latter study, FGFR2-IIIb protein levels were found to be increased in well and moderately differentiated colorectal tumors compared to poorly differentiated tumors or normal tissue (Otte et al., 2000). Especially in terms of

transcript levels in cancer tissues, some variation in the results is likely to result from different cellular composition in the tissue specimens. In addition, the lack of specific antibodies against FGFR2-IIIb is still a problem.

In functional assays, KGF has been shown to stimulate the migration and invasion of a transformed but nontumorigenic pancreatic ductal epithelial cell line (Niu et al., 2007) and the proliferation of an estrogen receptor positive breast cancer cell line (MCF7) (Rotolo et al., 2008). Moreover, KGF appears to counteract the anti-proliferative and proapoptotic effects of the chemotherapeutics 5-fluorouracil and tamoxifen on MCF7 cells (Rotolo et al., 2008). In sharp contrast, KGF was reported not to stimulate the proliferation of colorectal cancer cell lines (Otte et al., 2000) nor FGFR2-IIIb positive hepatocellular carcinoma (HCC) cells (Amann et al., 2010). In fact, the expression of FGFR2-IIIb is decreased in HCC tissues and cell lines as compared to comparable normal tissues. And in relation to clinicopathological parameters, FGFR2-IIIb negative HCC showed a significantly higher proliferative index and invasiveness compared to FGFR2-IIIb positive HCC. In accordance, the re-expression of FGFR2-IIIb in HCC cells resulted in the decreased growth of HCC tumors in a mouse xenograft model *in vivo* (Amann et al., 2010). Interestingly, decreased expression of FGFR2-IIIb compared to normal tissue has also been described at least for oral cavity SCC (LaRochelle et al., 1995) although the number of examined specimens was not disclosed. Moreover, supporting the tumor suppressive effects of FGFR2-IIIb, transgenic mice lacking FGFR2-IIIb in the epidermal basal layer develop spontaneous papillomas with an enhanced progression to invasive SCC (Grose et al., 2007). These mice are also highly sensitive to chemical carcinogenesis and they display increased cutaneous inflammation (Grose et al., 2007).

Finally, the effect of exogenously administered KGF on the tumor growth and on the response of cancer cells to a variety of chemotherapeutic substances was analyzed by Brake *et al*. (2008) using mouse xenograft tumor models induced with SCC of the head and neck cells and colon carcinoma cells (Brake et al., 2008). In this study, KGF did not affect the growth of the tumors and did not interfere with the action of chemotherapeutics when administered alone or in manner comparable to the clinical use when treating hematologic cancer patients (Brake et al., 2008).

To conclude, for some epithelial cancers, such as breast and pancreatic cancer, there is evidence that the epithelial expression of KGF may lead to autocrine stimulation of FGFR2-IIIb, which can be involved in cancer growth. KGF also appears to protect some, but not all, tumor cells from anti-cancer treatments. Moreover, the overexpression of KGF and/or its receptor in cancer tissues may augment cancer cell proliferation. However, some cancer cells appear to be unresponsive to the mitogenic stimulus of KGF and the loss of KGFR expression in tumor cells may even promote cancer progression. Thus, in terms of using recombinant KGF to protect normal tissues during cancer treatment, the cancer type is of pivotal importance. Additional studies are needed to examine the role of KGF and its receptor in the malignancies of specific types of cancers taking also into account the possible effects of other FGFR2-IIIb ligands, particularly FGF-10 and FGF-22.

# 3 AIMS OF THE STUDY

1) To study the expression and regulation of MMP-13 in fetal wound repair.

2) To elucidate the effects of MMP-13 on the remodeling of a collagen matrix by dermal fibroblasts.

3) To study the activities of MMP-13 in the formation of granulation tissue in a mouse model.

4) To study the role of KGF in the regulation of cutaneous SCC cell biology associated with MMP expression.

# 4 MATERIALS AND METHODS

## 4.1 ETHICAL CONCIDERATIONS

Human skin SCC tumor samples (n=6) and normal skin samples (n=6), and human primary fibroblast and keratinocyte and SCC cell cultures were all received from Turku University Hospital. Their use was approved by the Joint Ethical Committee of the Turku University Hospital and the University of Turku, Turku, Finland. Participants gave their written informed consent, and the studies were conducted according to the Declaration of Helsinki principles. Animal experiments were performed according to institutional guidelines and with permission from the animal test review board of the University of Turku, Finland (III) and by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia, Philadelphia, PA, United States, in accordance with NIH guidelines (I).

## 4.2 EXPERIMENTAL METHODS

The methods used in the thesis are described in more detail in the original publications (I-IV), as indicated.

**Table 6. Methods used in the original publications.** *(Continued on the next page)*

| *Method* | *Description* | *Used in* |
|---|---|---|
| Cell culture | See Table 7. | I-IV |
| 3D cell culture | Fibroblasts, collagen gel contraction | II,III |
| Production of rMMP-13 | Construction of recombinant baculovirus; production of recombinant protein in Sf9 cells | II |
| Analysis of collagenolytic activity | Fluorometric methods | II |
| cDNA cloning and construction of recombinant adenovirus | Cloning of KGF cDNA into pCA3 shuttle vector and co-transfection into 293 cells | IV |
| Adenoviral infection of cultured cells | Overexpression of transgene | I-II,IV |
| total RNA extraction | RNeasy Mini Kit (Qiagen); CsCl-gradient, isopropanol-precipitation | I,III-IV |
| Northern blot | mRNA expression analysis | I |
| Reverse-transcription PCR (RT-PCR) | mRNA expression analysis | IV |
| Real-time RT-PCR (Taqman) | Quantitative mRNA expression analysis | III,IV |
| Microarray analysis, bioinformatics | Genome wide mRNA expression analysis, Ingenuity pathway analysis | III,IV |
| Western blot | Immunodetection of protein | I-II,IV |
| Fluorescent labelling of cells | Detection of protein | II,III |
| Microscopy | Conventional light and confocal | I-IV |
| Immunohistochemistry | immunodetection of protein in tissue sample sections; DAKO or Vectastain ABC Kit | I,III |

| *Method* | *Description* | *Used in* |
|---|---|---|
| Fetal skin grafting into mouse back skin and wound creation | Analysis of human MMP-13 expression in fetal skin wound *in vivo* | I |
| Subcutaneous induction of granulation tissue in mice | Analysis of the effects of MMP-13 deficiency for granulation tissue formation | III |
| Cell proliferation assay s | Colorimetric quantification of DNA-synthesis (BrdU-labelling) | II,IV |
| | Colorimetric quantification of viable cells (metabolic modification of WST-1) | IV |
| TUNEL-assay | Terminal deoxynucleotidyl transferase - mediated dUTP nick end labeling of apoptotic cells | II |
| Invasion assay | SCC cell invasion through collagen gel | IV |
| Statistical analysis | Mann-Whitney, t-test, Pearson's $\chi^2$ | II-IV |

## 4.3 CELL CULTURES AND REAGENTS

The use of cells and reagents is described in more detail in the indicated original publications (I-IV). The sequences of primers and probes used for quantitative real-time RT-PCR (TaqMan®) (human β-actin, KGFR, KGF, MMP-7, -13, matrillin 2, CXCL10, IGFBP3, DUSP4 and DUSP6; mouse β-actin, MMP-2, -3, -9, ADAMTS-4) used in III and IV, and for conventional RT-PCR (human KGFR and GAPDH) used in IV, can be found in the original publications.

**Table 7. The cells and culturing conditions used in the original publications.**

| *Cells* | *Description* | *Culture medium* | *Used in* |
|---|---|---|---|
| Fibroblasts | From human adult skin, primary cells | DMEM, 10% FCS, 2 mM L-glutamine, antibiotics | II |
| Fibroblasts | From human fetus (17-week-old) skin, primary cells | as other fibroblasts | I |
| Fibroblasts | From mouse skin, primary cells | as other fibroblasts | III |
| HaCaT | Human keratinocytes, immortalized, benign (Fusenig and Boukamp, 1998) | DMEM, 10% FCS, 2 mM L-glutamine, antibiotics | IV |
| A5 | Ha-*ras*-tranfected HaCaT cells, similar to HaCaT cells (Boukamp et al., 1990) | as HaCaT cells, with 200 µg/ml geneticin | IV |
| II4 | Ha-*ras*-tranfected HaCaT cells, locally invasive (Boukamp et al., 1990) | as A5 cells | IV |
| RT3 | Ha-*ras*-tranfected HaCaT cells, highly invasive (Fusenig and Boukamp, 1998) | as A5 cells | IV |
| UT-SCC (7,12A,59A,91A, 105,111,115,118) | Cutaneous squamous cell carcinoma cells (Lansdorf et al., 1999) | as HaCaT cells, with 100 µM non-essential amino acids (GIBCO) | IV |
| Keratinocytes | Human skin, primary cells | KGM-2 with SupplementMix (PromoCell) | IV |
| Keratinocytes | Human skin, primary cells (PromoCell) | as other primary keratinocytes | IV |

**Table 8. Reagents used in the original publications.**

| *Reagent* | *Description* | *Supplier* | *Used in* |
|---|---|---|---|
| anti- α1-integrin | SR-84, function blocking antibody | BD Biosciences Pharmingen | II |
| anti- α2-integrin | AK-7, function blocking antibody | BD Biosciences Pharmingen | II |
| anti- αV-integrin | L230, function blocking antibody | ATCC (Producer cell line) | II |
| FITC-conjugated type I collagen | D-12060, from bovine skin | Molecular Probes, Inc. | II |
| Type I collagen | Cellon, Vitrogen 100, PureCol | Cellon; Allergan, Inc. | II,III |
| TGF-β1 and TGF-β3 | recombinant growth factors | Sigma Chemical Co. | I,III |
| KGF | recombinant growth factor | Sigma Chemical Co. | IV |
| Pyrimidine-4,6-dicarboxylic acid, bis-(4-fluoro-3-methyl-benzylamide) | MMP-13 inhibitor | Calbiochem | II |
| PD98059 | MEK1 inhibitor | Calbiochem | I,IV |
| SB203580 | p38 inhibitor | Calbiochem | I,IV |

**Table 9. Antibodies used in immunohistochemistry (IHC).**

| *Antigen* | *Product No.* | *Supplier* | *Conc./ Antigen Retrieval* | *Used in* |
|---|---|---|---|---|
| MMP-13 | IM44 | Calbiochem | 5 µg/ml /MW | I |
| α-SMA | A2547 | Sigma-Aldrich | 1:2000 | III |
| CD34 | sc-18917 | Santa Cruz Biotechnology | 1:500 (0,4 µg/ml) /MW | III |

**Table 10. Antibodies used in immunoblotting.**

| *Antigen* | *Product No.* | *Supplier* | *Used in* |
|---|---|---|---|
| MMP-13 | IM44 | Calbiochem | I, II |
| MMP-13 | IM64L | Calbiochem | IV |
| MMP-1 | AB806 | Chemicon | II, IV |
| MMP-2 | AB809 | Chemicom | IV |
| TIMP-1 | AB800 | Chemicon | I, II |
| FGFR2 | F0300 | Sigma-Aldrich | IV |
| FGF-7 | sc-1365 | Santa Cruz | IV |
| p38 | #9212 | NEB/Cell Signaling Tech. | II, IV |
| p-p38 | #9211 | NEB/Cell Signaling Tech. | II, IV |
| ERK | #9102 | NEB/Cell Signaling Tech. | II, IV |
| p-ERK | #9101 | NEB/Cell Signaling Tech. | II, IV |
| Akt | sc-1618 | Santa Cruz | II |
| p-Akt | #9271 | Cell Signaling Tech. | II |
| β-actin | A1978 | Sigma-Aldrich | II |
| p-Creb | #9191 | Cell Signaling Tech. | IV |

## 4.4 RECOMBINANT ADENOVIRUSES

**Table 11. Previously described replication-deficient recombinant adenoviruses.**

| ***Construct name*** | ***Description*** (all Δ E1) | ***Reference*** | ***Used in*** |
|---|---|---|---|
| RAd66 | Control virus, no gene coded | (Wilkinson and Akrigg, 1992) | I |
| RAdLacZ | Control virus, codes for E. coli β-galactosidase | (Wilkinson and Akrigg, 1992) | II |
| RAdpCA3 | Control virus, non-coding sequence of the shuttle vector donated during recombination, ΔE3 | (Leivonen et al., 2002) | II,IV |
| RAdN17rac2 | codes for human Rac1, dominant negative | (Sulciner et al., 1996) | I |
| RAdMEK1CA | codes for human MEK1, constitutively active | Foschi, 1997 #613} | I,IV |
| RAdMKK6bE | codes for human MKK6b, constitutively active | (Wang et al., 1998) | I |
| RAdp38AF | codes for human p38α, dominant negative | (Wang et al., 1998) | I |
| RAdMMP-13 | codes for human proMMP-13 | (Ala-aho et al., 2002) | II |
| RAdMTIMP-1 | codes for human TIMP-1 | (Baker et al., 1996) | II |

### 4.4.1 Construction of RAdKGF recombinant adenovirus (IV)

The construction of replication-deficient (E1 and E3 deletions) recombinant adenovirus was performed as described previously (Ala-aho et al., 2002). Human KGF coding sequence was amplified from dermal fibroblast total RNA and cloned into the pCA3 shuttle vector under the cytomegalovirus early immediate (CMV IE) promoter (Microbix Biosystems, Toronto, ON, Canada) (Fig. 6). Human embryonic kidney (HEK) 293 cells were co-transfected with the pCA3 construct and the pBHG10 plasmid, which contains the adenovirus genome, using the CalPhosMaximizer kit (Clontech, Palo Alto, CA, USA). After three weeks, successful co-transfection and recombination of plasmids was apparent as the formation of virus-induced plaques into the HEK-293 cell layer. Virus clone purification was performed by diluting and plaque purification. Positive recombinants were identified by PCR with pCA3-specific primers (Leivonen et al., 2002). One positive virus clone was chosen for the preparation of a CsCl-purified high titer stock for the expression of KGF (RAdKGF). The functionality of RAdKGF was verified by infecting serum-starved dermal fibroblasts in suspension at selected multiplicity of infection (MOI) units followed by detection of transgene expression at the mRNA level by northern blotting (data not shown) and by western immunoblotting for KGF (IV, Fig. 2C).

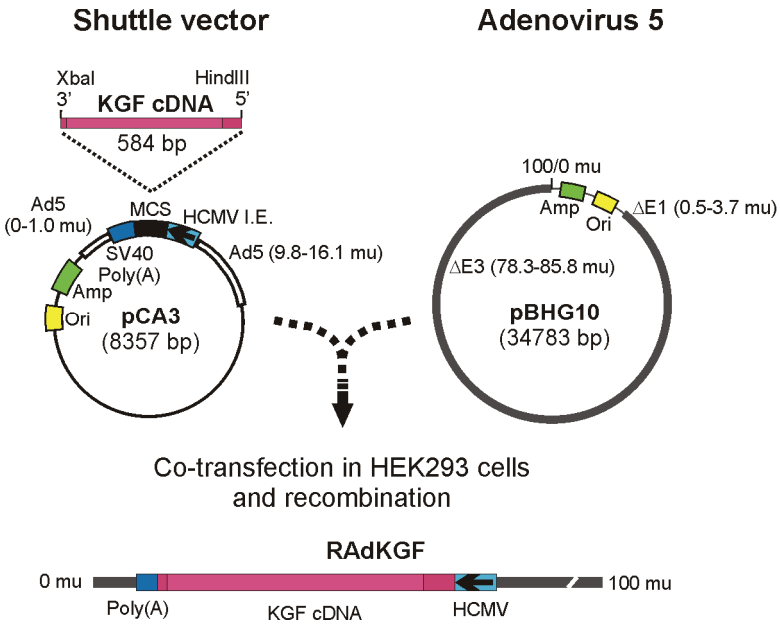


**Figure 6.** Construction of recombinant adenoviruses harboring human KGF sequence. Human KGF cDNA protein coding sequence was cloned into the pCA3 shuttle vector under the CMV IE promoter. HEK 293 cells were co-transfected with the pCA3 construct and the adenoviral backbone pBHG10 plasmid, resulting in the recombination and formation of adenoviruses.

# 5 RESULTS

## 5.1 THE EXPRESSION OF MMP-13 IN FETAL SKIN WOUNDS AND INDUCTION IN FETAL FIBROBLASTS BY TGF-β (I)

### 5.1.1 MMP-13 is expressed by human fetal fibroblasts *in vivo*

MMP-13 (collagenase-3) is an effective modulator of the cellular environment and capable of cleaving a variety of ECM proteins, such as fibrillar collagens, as well as many soluble proteins including TGF-β (D'Angelo et al., 2001; Knäuper et al., 1996). MMP-13 is not present in normally healing human cutaneous wounds (Vaalamo et al., 1997). However, it was previously shown that MMP-13 is expressed by fibroblasts in normal human gingival wounds characterized by minimal scar formation (Häkkinen et al., 2000; Ravanti et al., 1999b). To examine the role of MMP-13 in human fetal wound repair, comparably characterized by minimal scarring (Bullard et al., 2003), the expression of MMP-13 was first monitored in a well-characterized model, where an incisional wound is created on human fetal skin grafted onto a SCID mouse (Lorenz et al., 1992). Human MMP-13 was detected in the dermal fibroblasts of 4-day-old fetal skin wounds (I, Fig. 1A and B) and MMP-13 positive fibroblasts were especially located at the edge of the fetal skin graft, whereas dermal fibroblasts in the adjacent mouse skin were negative for human MMP-13 (I, Fig. 1C). The result shows a marked difference in MMP-13 expression between fetal and adult skin wounds and suggests a fundamentally different mode of regulation for MMP-13 expression in fetal skin fibroblasts compared to their adult counterparts.

### 5.1.2 TGF-β induces the expression of MMP-13 in fetal fibroblasts

In contrast to adult human skin fibroblasts, human gingival fibroblasts in culture express MMP-13 in response to TGF-β1 exposure (Ravanti et al., 1999b). Since fetal skin wounds display remarkable similarities with adult gingival wounds in terms of minimal scar formation and the expression of MMP-13 by dermal fibroblasts (Bullard et al., 2003 and I, Fig. 1), the regulation of MMP-13 expression in human fetal skin fibroblasts by TGF-β was studied. Fibroblasts from human fetal skin at a gestational age of 17 wk and from neonatal skin were treated with TGF-β1 (5 ng/ml) for 48 h, and the production of MMP-13 into conditioned media was monitored by Western immunoblotting. In accordance with earlier observations in adult dermal fibroblasts (Ravanti et al., 1999a), the neonatal fibroblasts did not produce detectable amounts of proMMP-13, while TGF-β1 down-regulated the production of proMMP-1 (I, Fig. 2A). In contrast, TGF-β1-treatment induced the production of proMMP-13 in fetal skin fibroblasts without affecting the level of proMMP-1 production (I, Fig. 2A). Concomitant with down regulation of MMP-1 in adult dermal fibroblasts (Verrecchia et al., 2001) and verifying that these cells are responsive to TGF-β1, TIMP-1

production was slightly enhanced by TGF-β1 in both fetal and neonatal skin fibroblasts (I, Fig. 2A). At the mRNA level, TGF-β1 as well as TGF-β3 enhanced the expression of MMP-13 in fetal skin fibroblasts, whereas no induction of MMP-13 mRNA was detected in neonatal skin fibroblasts (I, Fig. 2B).

### 5.1.3 MAPK p38 mediates the TGF-β-induced expression of MMP-13 in fetal skin fibroblasts

To study the signaling mechanisms responsible for the regulation of MMP-13 expression in fetal skin fibroblasts by TGF-β, the activation of mitogen-activated protein kinase (MAPK) p38 and extracellular signal-regulated kinase 1/2 (ERK1/2) were explored. Using Western immunoblotting, potent phosphorylation of p38 in fetal skin fibroblasts was detected in 30 minutes after TGF-β-treatment, whereas ERK1/2 was not activated (I, Fig. 3A and B). In contrast, in neonatal skin fibroblasts, a rapid activation of ERK1/2 was observed (I, Fig. 3C and D). In addition, compared to fetal fibroblasts, TGF-β1 induced a weaker and less persistent activation of p38 in neonatal fibroblasts, which reached the maximum after 1 h of incubation (I, Fig. 3C and D).

It has been previously shown, that p38 MAPK activity is required for the induction of MMP-13 expression by TGF-β1 in human gingival fibroblasts (Ravanti et al., 1999b). To study the specific activities of MAPKs in mediating the induction of MMP-13 expression by TGF-β1 in fetal skin fibroblasts, the small molecular inhibitors PD98059 (PD, 30 μM) and SB203580 (SB, 10 μM) were first used to preclude the functionality of ERK1/2 and p38 MAPK pathways, respectively. While inhibition of ERK1/2 had no effect on the elevation of proMMP-13 production by TGF-β1, the selective inhibitor of p38 entirely inhibited the induction of proMMP-13 by TGF-β1 (I, Fig. 4A). Treatment of fetal skin fibroblasts with TGF-β1 did not affect proMMP-1 production. The expression of TIMP-1 was slightly enhanced by TGF-β1, but this stimulation was not affected by PD or SB (I, Fig. 4A).

To further examine MAPK signaling pathways mediating the induction of MMP-13 gene expression by TGF-β1, we used recombinant replication-deficient adenoviruses to specifically inhibit and activate endogenous MAPK signaling cascades. We infected fetal skin fibroblasts with adenoviruses coding for dominant negative forms of the small GTPase Rac1, which is involved in the activation of JNK and p38 (Minden et al., 1995) and of p38α. Compared to RAd66-infected fibroblasts, the adenoviral expression of a dominant negative Rac1 (RAdN17rac1) had no marked effect on the induction of proMMP-13 production by TGF-β1 (I, Fig. 4B). However, the adenoviral expression of a dominant negative p38α (RAdp38AF) diminished the induction of proMMP-13 production by TGF-β1, in comparison with RAd66 infected cells (I, Fig. 4B), further emphasizing the role of p38 MAPK in mediating the induction of MMP-13 expression in fetal skin fibroblasts by TGF-β1.

Finally, to examine the direct effect of ERK1/2 and p38 MAPK activation alone in the regulation of MMP-13 expression, adenoviral gene delivery of constitutively active MEK1 (RAdMEK1CA) and MKK6b (RAdMKK6bE) was employed to activate ERK1/2 and p38 MAPK, respectively, in fetal skin fibroblasts. As indicated by

Western immunoblotting, the adenoviral infection of fibroblasts with RAdMEK1CA and RAdMKK6bE induced strong phosphorylation of ERK1/2 and p38, respectively (I, Fig. 5A). However, the artificial activation of ERK1/2 or p38 MAPK pathways by RAdMEK1CA or RAdMKK6bE was not sufficient to induce proMMP-13 production in fetal skin fibroblasts (I, Fig. 5B). Interestingly, MMP-1 production was enhanced by ERK1/2 activation, whereas it was not affected by the activation of p38 (I, Fig. 5B) indicating different patterns of regulation for MMP-13 and MMP-1 in fetal skin fibroblasts. Production of TIMP-1 was up-regulated in fibroblasts by activated ERK1/2, whereas the activation of p38 did not alter TIMP-1 production (I, Fig. 5B).

## 5.2 MMP-13 ENHANCES THE COLLAGEN GEL CONTRACTION CAPACITY OF ADULT SKIN FIBROBLASTS AND PROTECTS THEM FROM APOPTOSIS IN 3D COLLAGEN (II)

### 5.2.1 MMP-13 increases the remodeling of 3D collagen by skin fibroblasts

MMP-13 is not present in normally healing adult human skin wounds (Vaalamo et al., 1997). However, MMP-13 is expressed by fibroblasts during adult gingival and fetal skin wound repair (Ravanti et al., 1999b and I, Fig. 1), both characterized by minimal scar formation. This lead to the hypothesis that MMP-13 functions as an effective remodeller of the collagenous tissue involved in scarless healing. The hypothesis was addressed using RAdMMP-13 recombinant adenovirus to obtain high-level expression of proMMP-13 in primary adult human skin fibroblasts (HSFs) and by studying the 3D collagen matrix contraction capacity of these cells. Normally skin fibroblasts express MMP-13 at low level, and only when cultured inside 3D collagen (Ravanti et al., 1999a). Infection with RAdMMP-13 resulted in a marked dose-dependent production of proMMP-13 protein by HSFs in collagen (II, Fig. 1A). This was accompanied by a marked and dose-dependent increase in collagen gel contraction (by up to 60% at day 4), as compared to cultures transduced with a control virus, RAdpCA3 (II, Fig. 1B). In accordance, the addition of exogenous recombinant proMMP-13 protein to the culture medium increased the collagen contraction exerted by HSFs in a dose-dependent manner (II, Fig. 1C and D). The collagen gel contraction induced by recombinant MMP-13 was not, however, as potent as that achieved by adenovirally produced MMP-13, which was assumed to be due to the distribution of recombinant MMP-13 in the culture (see restricted collagenolysis by adenovirally produced MMP-13 below).

### 5.2.2 Adenovirally produced MMP-13 is activated by fibroblasts and induces pericellular collagen cleavage

Generally, MMPs are secreted as latent zymogens and activated in the pericellular space. Using Europium- (Eu) and a quencher-labeled substrate peptide for MMPs we showed that the adenovirally produced MMP-13 secreted from HSF is activated and exerts collagenolytic activity (II, Fig 2A). In addition, the activated form of MMP-13

in the corresponding sample medium was detected by Western immunoblot analysis (II, Fig. 2B). Moreover, by culturing RAdMMP-13-infected HSF in a fluorescently-labeled 3D type I collagen gel we detected *in situ* that collagen was cleared from around the MMP-13-expressing fibroblasts, indicating pericellular collagenolysis by active MMP-13 in RAdMMP-13-infected fibroblast cultures. In the controls, the fluorescent collagen was not cleaved significantly (II, Fig. 2C). The release of fluorescence in the cell culture media in RAdMMP-13-infected fibroblast cultures increased about 20% (n=6, $P<0.005$, Mann–Whitney U-test) compared to control cell cultures, as measured by a fluorometer. The expression levels of MMP-1 were not altered by MMP-13 as detected by Western immunoblotting (Fig. 7).

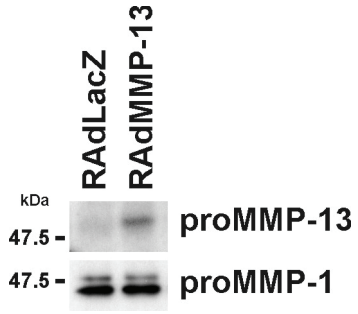


**Figure 7**. Western blot analysis of MMP-13 and MMP-1 secreted by HSFs cultured in a fluorescent-labeled 3D collagen gel.

### 5.2.3 MMP-13-enhanced collagen gel contraction is repressed by inhibiting MMP-13 activity

To assess whether the proteolytic activity of MMP-13 is required for enhanced collagen gel contraction by RAdMMP-13-infected HSFs, we used a specific chemical inhibitor of MMP-13 activity, pyrimidine-4,6-dicarboxylic acid, bis-(4-fluoro-3-methyl-benzylamide) and co-infected cells with RAdTIMP-1, which codes for a tissue inhibitor of metalloproteinases-1, to restrain the activity of MMP-13 during cell culture in a floating 3D collagen gel. The MMP-13 inhibitor (2 μM) reduced MMP-13-enhanced collagen gel contraction effectively by 60% ($P<0.05$), whereas contraction by RAdLacZ-infected cells was not affected (II, Fig. 3A). In accordance with the results obtained with the inhibitor, MMP-13-enhanced collagen gel contraction was inhibited by the adenoviral co-expression of TIMP-1 (II, Fig. 3B). These results indicate that MMP-13 possesses an enzymatic activity needed for the contraction process. The level of endogenous TIMP-1 expression was not altered by the MMP-13 inhibitor or by MMP-13. However, the level of endogenous MMP-1 expression was not altered by the MMP-13 inhibitor but was decreased by the adenoviral expression of MMP-13 and TIMP-1 (II, Fig. 3C).

### 5.2.4 Adenoviral expression of MMP-13 induces altered cell morphology and f-actin clustering in fibroblasts cultured in 3D collagen

Collagen gel contraction by fibroblasts depends on the functional structure of cellular filamentous actin (f-actin) and the attachment to the surrounding matrix and other cells (Cooke et al., 2000; Ehrlich et al., 2000; Grinnell and Ho, 2002). Fibroblasts cultured in a floating 3D collagen gel acquire a dendritic phenotype in contrast to the myofibroblastic phenotype typical for fibroblasts in mechanically loaded collagen (Grinnell et al., 2003). To investigate the morphology of MMP-13-expressing HSFs in detail, cells were cultured in a floating collagen gel for 2 days, fixed, and stained for f-actin with fluorescently labeled phalloidin. In concert with previous reports, control fibroblasts displayed a dendritic morphology with fine and long f-actin containing projections and few stress fiber-like structures (II, Fig. 4A and C). Likewise, few stress fibers were detected in MMP-13-expressing HSFs. However, the f-actin of MMP-13-expressing HSFs was organized into numerous brush- or hair-like structures reaching from the cell body to adjacent cells and collagen. Compared to those seen in control cells, these filaments were clearly shorter and often more complex in structure (II, Fig. 4B). Moreover, specific patch-like structures, which contained f-actin were also observed along the cell projections of RAdMMP-13-infected fibroblasts, suggesting an alteration in the interaction between the cells and the surrounding collagen, whereas in control cells f-actin was evenly distributed at the cortical areas of the cells (II, Fig. 4C and D). Quantification of the relative number of fibroblasts with normal and altered f-actin morphology from three separate experiments demonstrated that the majority of cells in 3D collagen, which expressed MMP-13, displayed altered f-actin morphology, as compared to RAdLacZ-infected control cultures (II, Table 1).

Previous studies have shown that collagen gel contraction by fibroblasts is mediated via the function of the collagen receptor integrin α2β1, as well as the Arg-Gly-Asp-binding integrin αVβ3 (Cooke et al., 2000; Schiro et al., 1991). Incubation with a function blocking α2 integrin antibody reduced basal contraction by control adenovirus-infected fibroblasts by 20%, whereas MMP-13-enhanced collagen gel contraction was inhibited by up to 40%, indicating that the MMP-13-enhanced contraction is dependent on functional cell attachment to collagen via α2β1 integrin (Fig. 8B). The antibody against α1-integrin had no effect on collagen contraction at the concentration used (Fig. 8B). The antibody against αV-integrin had no effect on MMP-13-enhanced contraction, but it partly inhibited basal collagen contraction (Fig. 8C). The overall levels of α1 and α2 integrins were relatively low under both culture conditions and no marked differences were detected in the expression levels of α1 or α2 integrins after 24–72 hours in cells expressing MMP-13 (Fig. 8A; data not shown for 48 and 72 h time points).

### 5.2.5 Adenoviral expression of MMP-13 promotes the survival, proliferation and Akt signaling of adult dermal fibroblasts in 3D collagen

Fibroblasts have been reported to acquire a quiescent phenotype and undergo apoptosis in a mechanically unloaded collagen gel (Fluck et al., 1998). In this context, we

investigated the effect of MMP-13 expression on the survival and proliferation of HSFs in a floating 3D collagen gel. Verifying the previous observations, 25% of uninfected and RAdLacZ-infected control cells displayed an apoptotic phenotype with condensed nuclei and fragmented DNA as detected with Hoechst and TUNEL staining, respectively, after culturing in collagen for 48 hours (II, Fig. 5A). In contrast, the RAdMMP-13-infected HSFs showed normal nuclear morphology and a minimal number of apoptotic TUNEL-positive cells (6%) (II, Fig. 5A).

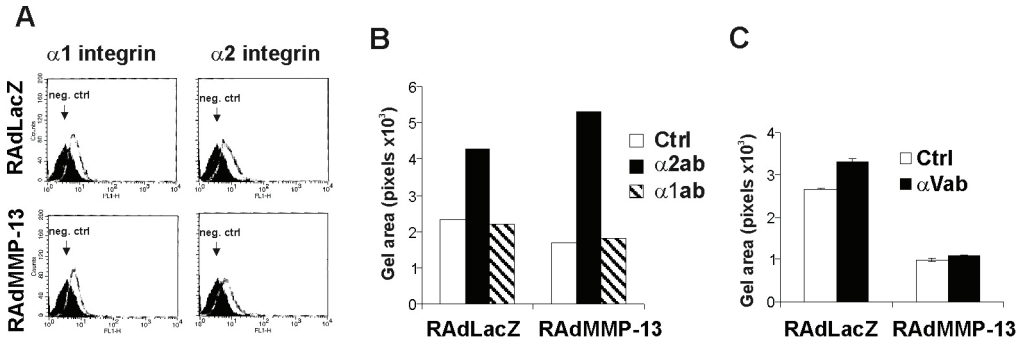


**Figure 8**. MMP-13-enhanced collagen contraction is dependent on the functional attachment of cells to collagen via α2β1 integrin. A) Flow cytometric analysis of α1 and α2 integrins expressed on RAdLacZ and RAdMMP-13-infected fibroblasts cultured in 3D collagen gel for 24 h. The black histogram shows the level of background signal and the transparent histogram shows the level of integrin expression. B and C) HSF were infected with RAdLacZ or RAdMMP-13 and cultured in a floating 3D collagen gel in the presence of function blocking antibodies against α1, α2 or αV (AK-7, SR-84 and L230) integrins. Collagen gel contraction was analyzed after 4 days.

To determine whether the increased survival of MMP-13-expressing fibroblasts in the collagen gel resulted in increased cell proliferation, we quantified DNA synthesis by BrdU labeling. The results showed that MMP-13 expression was accompanied by a 20% increase in DNA synthesis in the HSFs ($P<0.01$) (II, Fig. 5B), indicating enhanced cell proliferation and increased cell number in the gels. When fibroblasts infected with a control adenovirus or with RAdMMP-13 were cultured as a monolayer on plastic cell culture dishes, no alteration in DNA synthesis was observed.

The mechanisms behind the enhanced cell survival and proliferation by MMP-13 were addressed by monitoring cellular survival signals. Thus, the phosphorylation statuses of Akt, ERK1/2 and p38, signal molecules involved in the regulation of cell survival and proliferation (Chang and Karin, 2001; Franke et al., 1997), were studied. Immunoblot analysis of cell lysates of MMP-13-expressing HSFs showed a marked increase in the levels of phosphorylated (serine 473) Akt, indicating that activation of the PI3K pathway was responsible for enhancing cell survival in 3D collagen (II, Fig. 5C). In addition, the expression of MMP-13 resulted in the phosphorylation and activation of ERK1/2, which is associated with increased cell proliferation. In contrast, the phosphorylation of p38 MAPK was unaltered (II, Fig. 5C).

## 5.3 THE LACK OF MMP-13 DISTURBS NORMAL GRANULATION TISSUE GROWTH IN MICE (III)

### 5.3.1 MMP-13-knockout mice show delayed growth of granulation tissue in a standardized experimental wound healing model

MMP-13 was shown to modify the ability of human fibroblasts to remodel a collagen matrix. Also, the viability and proliferation of MMP-13 expressing fibroblasts was increased, and the morphology of the cells suggested enhanced interaction with the matrix and adjacent cells. Fibroblasts are key players in granulation tissue formation during wound healing. While MMP-13 is not expressed in normal skin wounds, it is produced by mouse skin fibroblasts. Thus, an MMP-13 deficient (KO) mouse strain was used to study the function of MMP-13 *in vivo* in cutaneous wound healing, (Inada et al., 2004) , and the role of MMP-13, specifically in granulation tissue formation, was examined. The granulation tissue was induced by subcutaneous implantation of a viscose cellulose sponge (VCS). The model is well established and stimulates the tissue growth highly comparable to wound associated granulation tissue as defined by its histological and chemical properties (Laato et al., 1987; Märtson et al., 1998). VCS induced granulation tissues were harvested for histological and transcriptional analysis at the time points of 7, 14 and 21 days (7d, 14d and 21d, respectively).

Initial granulation tissue development was observed at similar level in wild type (WT) and KO mice at 7d. Granulation tissue growth was defined by the influx of numerous leukocytes including foreign body-type macrophages around VCS structures, fibroblast-like cells and vessel structures as identified based on cell morphology in a basic histological staining (III, Fig.1). At 14d, cellular tissue was detected in all samples. No marked alterations were observed in the tissue growth or cellular composition between WT and KO at 7d or 14d as determined by hematoxylin and eosin staining. During the third week, a marked difference in tissue growth evolved, showing 42% less cellular granulation tissue in KO mice compared to WT at 21d, as assessed by a morphometrical analysis (III, Fig.1). The result indicates that MMP-13 is an important factor regulating cellular events related to granulation tissue growth. It suggests that the initiation of granulation tissue growth is MMP-13-independent, but the activities of MMP-13 become more important in later events such as cell proliferation and penetration into the tissue.

### 5.3.2 Defective granulation tissue growth is accompanied by impaired organization of myofibroblasts and altered composition of vessels

Myofibroblasts exert a contractile force in an early skin wound bringing wound edges closer to each other. To examine whether MMP-13 has any effect on myofibroblast differentiation in VCS-induced granulation tissues, KO and WT granulation tissue sections were processed for α-SMA immunohistochemisty. At 7d, α-SMA was detected in fibroblastic cells close to the VCS surface and the staining pattern was

typically dense and oriented parallel to the surface in WT tissues (III, Fig. 2A) possibly reflecting the contractile function of myofibroblasts. In contrast, the staining pattern of α-SMA appeared generally weaker in KO tissues, although no statistical significance was reached (III, Table 1), and as assessed by quantitative evaluation, α-SMA-positive myofibroblasts showed a markedly thinner collective lining (III, Fig.2A, Table 2) suggesting defects in myofibroblast assembly.

However, suggesting that activity of MMP-13 can be compensated by other factors *in vivo,* at 14d both genotypes showed a strong α-SMA staining pattern, reaching throughout the cellular area, and a strong parallel orientation especially in the areas close to VCS surface. Interestingly, at 21d the difference in the α-SMA staining pattern was again evident showing that while in KOs the α-SMA was still largely and strongly detected throughout the cellular tissue, in WTs the α-SMA expression was clearly diminishing showing positivity in the inner parts of the granulation tissue but markedly less expression in the areas close to VCS surface and assumingly representing the most matured granulation tissue (III, Fig. 2B). Similar shift in the α-SMA expression pattern was also noted in two KO samples with relatively strong tissue ingrowth.

To assess angiogenesis in VCS-induced granulation tissue in WT and MMP-13 KO mice, tissue sections were immunostained for CD34 and analyzed morphometrically. Generally, the density of vessels showed relatively high variation in any sample group. At 7d CD34 positive blood vessels were abundantly present in both genotypes in areas with clear tissue growth with no statistically significant differences in the vessel density (III, Fig. 4). CD34 positive blood microvessels (<10 μm in diameter) were abundantly present in both WT and KO tissues at all time points. However, microvessel density was significantly increased in KO samples at 14d. The density of the medium sized vessels was the same in WT and KO at all the examined time points (III, Fig. 4). The most striking histological difference was observed at 21d when KO granulation tissues were found to almost completely lack large vessels (>40 μm in diameter), which are likely to represent venules or arterioles, which in contrast, were commonly presented in WT tissues (III, Fig. 4). When the total number of large vessels in tissue sections at 21d was counted, an about 7-fold decrease was detected in KOs.

### 5.3.3 Gene expression profiling of VCS-induced granulation tissues

It was noted that MMP-13 KO granulation tissues show delayed growth especially during the third week of VCS implantation. To explore the possible explanations for this observation, WT and MMP-13 KO granulation tissues were subjected to genome wide gene expression profiling. Total RNA samples from time points 7d, 14d and 21d were processed for DNA microarray analysis. The data were analyzed for two aspects: 1) differential gene expression comparing the genotypes KO/WT at a given time point and 2) temporally comparing differentially expressed genes at 14d/7d and at 21d/14d primarily in WT.

The comparison of KO granulation tissues with WT at specific time points resulted in 1303, 3560 and 1984 genes with different expression levels with statistical significance at 7d, 14d and 21d, respectively. Of these 87, 96 and 95 genes,

respectively, exhibited FC>0.75 and these genes are listed in a heat map in Figure 5 (III). Apart from the over-expression of angiogenesis-related *Adamts4* (Kahn et al., 2000) and downregulation of the serine/cysteine peptidase inhibitors *Serpina1b* and *Serpina3n*, no other signs of possible enzymatic redundancy for MMP-13 were recognized. The classification of the differentially expressed genes (P<0.05, FC>0.75) for all time points based on Ingenuity Pathway Analysis (IPA) is presented in III, Figure 5B. Majority of the transcripts were up-regulated in KO granulation tissues at 7d, whereas reduced expression predominated at 14d and 21d.

To get an insight into the biological activities associated with differentially expressed genes, IPA functional analysis was performed. To gain larger groups of differentially regulated genes between the genotypes in a given time point for this analysis, the thresholds P<0.05, FC>0.5 were used. For each time point, the molecular network directly related to MMP-13 was created and it shows the expression ratio as a heatmap of every gene in KO compared to WT (III, Fig. 6). These genes include collagens such as *Col8A1, Col8A2* and *Col14A1*, several MMPs and also hepatocyte growth factor receptor *Met* and a proteinase inhibitor α2-macroglobulin. Moreover, several chemokines and cytokines including *Il6, Il1, Ccl7, Ccl4* and *Ccl13* were related to MMP-13 by IPA (III, Fig. 6). The results of the functional analysis for differentially expressed genes between the genotypes in a given time point are summarized in Tables 3-5 (III).

At 7d, the most highly up-regulated genes in KO compared to WT included interferon activated gene 202B (*Ifi202b*, *p202*, FC~8) and cell adhesion molecule 1 (*Cadm1*; FC~2.5), which are implicated *e.g.* in the inhibition of cell growth (Nowacki et al., 2008; Xin et al., 2003) (III, Fig. 5A). The functional analysis of the differentially regulated genes (P<0.05, FC>0.5) at 7d showed highly significant association with cell proliferation, migration, inflammation and cell death (III, Table 3). Actually, these biofunction categories came up in every time point as the most significantly associated with the differentially expressed genes. In accordance with the up-regulation of *e.g.* angiogenic *Adamts4* (Kahn et al., 2000), *Met* and *Vegfc,* the biofunction vasculogenesis was predicted to be up-regulated in KO at 7d although statistical significance was not quite reached. This is in accordance with increased density of microvessels noted in KO granulation tissues at 14d by immunohistochemistry. In accordance with the statistics of the immunohistochemical observations, evidence specifically for the delayed appearance of myofibroblasts in KO granulation tissues was not found.

At 14d, a strong up-regulation of neuropeptide Y (*Npy*; FC~5) was observed, and the expression of *Cadm1* and *Ifi202b* remained up in KO compared to WT. In contrast, a variety of genes associated with collagen metabolism and fibrillogenesis (*Mmp2, -3, -9, Tnxb, Col14a1* as well as *Mmp13*), cell adhesion and motility and angiogenesis (*e.g. Thbs3, Dsp, Selp, Vtn, Pdgfd, Tnxb*, and *Mmp9*) were downregulated in KO granulation tissues (III, Fig. 5A).

In the functional analysis of differentially regulated gene sets at 14d, one of the most obvious biofunctions that came up was the cell movement of leukocytes (III, Table 4). Apparently, cell movement of granulocytes was predicted to be significantly reduced in KO granulation tissues as compared to WT (III, Table 4, Fig. 7A). In

accordance with the marked downregulation of several MMPs in KO tissue at 14d, the functional analysis predicted the biofunction proteolysis involved in metabolism of protein to be significantly downregulated (III, Table 4, Fig. 7B). Interestingly, despite of *e.g.* the high up-regulation of *Npy* expression, as well as enhanced expression of angiogenic *Met, Cyr61* and *Fgf13* (FGF-2), angiogenesis was systematically predicted to be slightly downregulated, although not significantly, in KO granulation tissues (III, Table 4, Fig. 7C). This may reflect the immunohistological observation on reduced density of the large blood vessels at 21d. The temporal expression pattern of MMPs in WT and KO samples is illustrated as a heatmap in III, Supplementary figure 5.

At 21d, several genes involved in angiogenesis (*e.g. Col8a1, Col8a2*, *Angpt1* and *Figf)* were downregulated in KO granulation tissues compared to WT (III, Fig. 5A). In accordance, the functional analysis of the differentially expressed genes (P-value<0.05, FC>0.5) revealed a strong association with angiogenesis (P<5.27E-11). Parallel to the immunohistological observations, angiogenesis tended to be downregulated at 21d although no statistical significance was reached (III, Table 5). Interestingly, apoptosis was predicted to be downregulated and proliferation of different cell types (except endothelial cells) up-regulated in KO tissues compared to WT with high significance (III, Table 5). In contrast to 14d, certain functions related to inflammation were predicted as up-regulated in KO tissues at 21d. The functional analysis of the differentially regulated genes at 21d time point suggests high fibroblast viability and proliferation in KO granulation tissue, whereas cell apoptosis in WT is increased. This may reflect the altered distribution of myofibroblasts noted by immunohistochemistry. Supplementary figure 2 (III) represents the top 1 molecular networks of differentially expressed genes at each time point created and scored by IPA analysis.

It is of note that caspase 4, one of the "inflammatory caspases" and PDGF-D, a potent inducer of cell proliferation and angiogenesis (Uutela et al., 2004; Wang et al., 2007) were downregulated at mRNA level with high significance at all of the time points.

To take an advantage of the experimental model of granulation tissue growth in WT mouse, the IPA functional analysis was performed also on genes showing significant differential expression (P<0.05, FC>1) between the two consecutive time points. In the gene expression comparison of 14d to 7d, the functional analysis identified the differentially expressed genes to be associated with the biofunctions involved in cellular movement, cell growth and proliferation and cell death in a highly significant manner (III, Supplementary Table 2). However, only the biofunctions related to inflammation actually showed significant systematic regulation (down). The molecular networks with the highest scoring contained several expected molecules with up-regulation (III, Supplementary Fig. 3). The two molecular networks with the highest score included genes *e.g.* for basement membrane molecules *Col4a1* and *Col4a2*, angiogenesis associated *Col8a1* and *Col8a2*, *Mmp9*, *Tgfb*, *Pdgfrb*, myofibroblast associated *Actg2* (γ-SMA) and *Tagln* (Transgelin, Sm22) as well as *Mmp13*, *Mmp3* and *Mmp11*, *Igfbp2*, *-3* and *-4*, *Eln* (elastin), *Fbn2* (fibrillin 2), *Fbln1* (fibulin 1) and *Nid2* (nidogen 2). The up-regulation of macrophage MARCO receptor and hemoglobin alpha chains are likely to reflect the influx of macrophages into the granulation tissue between 7d and 14d (Tommila et al. 2011) (III, Supplementary Fig. 3).

The functional analysis of different gene expression at 21d to 14d identified the biofunctions inflammation, cell movement of monocytes and chemotaxis of neutrophils as significantly downregulated (III, Supplementary Table 3). Also, while the contraction of muscle cells was predicted to be up-regulated at d21, the differentiation of muscle cells appeared significantly downregulated. The most significant molecular networks of molecules differentially expressed at 21d as compared to 14d, are presented in Supplementary Figure 4 (III).

### 5.3.4 Verification of *Mmp2, -3, -9, Adamts4* and *Npy* expression in granulation tissues by quantitative RT-PCR

MMP-2 and MMP-3 are detected in granulation tissue fibroblasts in skin wounds in humans and mice (Madlener et al., 1998; Mirastschijski et al., 2002a; Vaalamo et al., 1996). Both MMPs are implicated in wound contraction and matrix remodeling (Bullard et al., 1999;Madlener et al., 1998; Ågren, 1994). As matrix remodeling typically involves cellular movement, MMP-2 and MMP-3 may participate in fibroblast migration in VCS. MMP-9 which in wound is predominantly expressed by inflammatory cells (Inkinen et al., 2000; Okada et al., 1997), and MMP-2, are implicated in physiological as well as malignancy-associated angiogenesis (Bergers et al., 2000; Itoh et al., 1998; Kato et al., 2001; Vu et al., 1998). Moreover, although the process itself was predicted to be downregulated at 14d, several genes involved in angiogenesis, including *Adamts4* and *Npy* (Kahn et al., 2000; Movafagh et al., 2006), were up-regulated in KO tissues at 7d and 14d, proposing candidate genes involved in increased microvessel density observed in KO tissues at 14d. Thus, quantitative real-time RT-PCR was used to verify the expression of these interesting genes, which may partially be responsible for the histological observations.

In accordance with the microarray data, the expression levels of *Mmp2, Mmp3* and *Mmp9* were significantly increased in WT during the second week of granulation tissue growth and remained high until 21d. No marked enhancement was observed in KO by 14d meaning significantly higher expression levels in WT than in KO at 14d (III, Fig. 8). The expression of *Mmp2* was significantly increased in KO at 21d approaching the level in WT, suggesting a delay in MMP induction, (III, Fig. 8). The expression of *Mmp3* and *Mmp9* was not significantly altered in KO during granulation tissue formation, although a slight increase could be detected.

The expression level of *Npy* started to increase in KO tissues at 7d, and it reached a marked and significant up-regulation at 14d compared to WT, although the expression level of *Npy* generally was relatively low (III, Fig. 8). At 21d, the expression of *Npy* was already decreased in KO but increased in WT suggesting earlier peak in *Npy* expression level in KO. In accordance with the microarray results, *Adamts4* was up-regulated in KO compared to WT at 7d and remained at the same level throughout the study (III, Fig. 8). In WT, the expression of *Adamts4* mRNA was enhanced later, at 14d, to a comparable level suggesting expedited induction in KO.

### 5.3.5 Mouse skin fibroblasts exhibit reduced collagen gel contraction and reduced expression of *Mmp3 in vitro*

Contraction of a mechanically unloaded 3D collagen gel by fibroblasts is considered to reflect primarily their motile activity related to cell adhesion (Grinnell and Petroll, 2010). To examine the motile activity of KO mouse skin fibroblasts (MSF), fibroblasts were seeded in a floating 3D collagenous matrix, and their cellular morphology and the collagen contraction capacity were studied. Here, KO MSF showed about 60% less collagen gel contraction compared to WT in 48 h in the presence of FCS (III, Fig. 3B). Moreover, while WT MSF assumed a stellate morphology with thick cell extensions reaching to the matrix and other cells in response to FCS and TGF-β, KO MSF showed only a weak response to TGF-β (III, Fig. 3A). This suggests reduced sensitivity to TGF-β and possibly other serum factors, and disturbed cellular contractile activity. Similarly, KO MSF showed reduced contraction of a stressed collagen gel after stress relaxation (III, Fig. 3C) which is a better model to study contractile activity of cells as compared to the floating collagen gels (Grinnell and Petroll, 2010).

To clarify the gene expression of *Mmp2, Mmp3* and *Mmp9* is KO MSF *in vitro,* total RNA from MSF established from three individual WT and KO mice were analyzed with qRT-PCR. *Mmp9* transcript was not detected in MSF which is in accordance with data showing inflammatory cells as the source of *Mmp9* in rat granulation tissue (Inkinen et al., 2000; Okada et al., 1997). The expression of *Mmp2* and *Mmp3* mRNAs was clearly detected in MSF of both genotypes. The expression of *Mmp3* was significantly downregulated ($P<0.03$) in KO MSF compared to WT, whereas the expression level of *Mmp2* was not significantly affected by MMP-13 deficiency *in vitro* (III, Fig. 9). The result suggests a marked role for MMP-13 as a regulator of proteolysis. This could be due to soluble mediators that are processed by MMP-13, which subsequently affects their activities.

## 5.4 KERATINOCYTE GROWTH FACTOR ASSOSIATES WITH REDUCED MALIGNANT PHENOTYPE OF CUTANEOUS SQUAMOUS CARCINOMA CELLS (IV)

### 5.4.1 KGF and KGF receptor are expressed in skin SCC tumors *in vivo*

Keratinocyte growth factor (KGF) is an epithelial mitogen, which is up-regulated during wound healing (Rubin et al., 1989; Marchese et al., 1995; Werner et al., 1992). Because of its ability to protect normal epithelial tissues, there is an interest to study whether this growth factor could be used for protecting normal epithelium during carcinoma treatment. KGFs role in the progression of different epithelial cancers appears to largely depend on the cancer type (Finch and Rubin, 2006). Previously, there was virtually no data on the effects of KGF on the biology of cutaneous squamous cell carcinoma (SCC) cells. Here, the expression of KGF and that of its receptor KGFR were first studied in cutaneous SCC tumors at the mRNA level using

quantitative real-time RT-PCR. The expression level of KGF mRNA in skin SCCs did not differ significantly from normal tissues (IV, Fig. 1A, upper panel). Also KGFR mRNA was detected in SCC samples. However, the level of KGFR mRNA expression was reduced in SCCs ($P<0.003$) compared to normal skin samples (IV, Fig. 1A, lower panel).

### 5.4.2 The expression of KGFR in cutaneous SCC cell lines

To examine the effect of KGF on SCC cell biology *in vitro*, we first determined the expression level of KGFR mRNA in skin SCC cell lines. Using semi-quantitative reverse transcription PCR (RT-PCR) for KGFR mRNA expression, a specific 150 bp fragment representing the specific IIIb-type exon of the FGFR2-receptor transcript was amplified in 4/5 skin SCC cell lines, in HaCaT cells and in epidermal keratinocytes from two individuals. As expected, dermal fibroblasts were negative for the KGFR-transcript (IV, Fig. 1B, upper panel). A quantitative analysis of KGFR mRNA revealed markedly increased levels of the transcript in 2/8 cutaneous SCC cell lines compared to normal keratinocytes (IV, Fig. 1B, lower panel). Interestingly, in 2/8 cutaneous SCC cell lines KGFR mRNA was virtually absent as demonstrated also with RT-PCR in UT-SCC-111 cells (IV, Fig. 1B). KGF mRNA was virtually absent in skin SCC cell lines practically ruling out the possibility of autoregulation via KGFR in these cells (IV, Fig. 1C).

### 5.4.3 Cellular response of cutaneous SCC cells to KGF-treatment

*Lack of mitogenic response to KGF in skin SCC cells*

As KGF is a well recognized mitogen for normal keratinocytes, we tested the potential of KGF to enhance cutaneous SCC cell proliferation. We first stimulated UT-SCC-12A and UT-SCC-7 cells (from primary and metastatic tumors, respectively), normal primary keratinocytes and HaCaT cells with rKGF, and analyzed their DNA-synthesis. KGF potently and significantly enhanced DNA-synthesis in keratinocytes and HaCaT cells in a concentration dependent manner, while it had no effect on the DNA-synthesis in the tested SCC cell lines (IV, Fig. 2A). KGF also increased the number of viable cells in keratinocyte and especially in HaCaT cell cultures, but not in SCC cell cultures (IV, Fig. 2B). The negative result for KGF on SCC cell replication was verified by treating SCC cells and HaCaT cells with conditioned medium from human primary dermal fibroblasts infected with a recombinant RAdKGF adenovirus or with a control adenovirus, RAdpCA3. The undiluted conditioned media contained 15.1 ng/ml and 0.1 ng/ml KGF as determined by an ELISA-based assay (IV, Fig. 2C). HaCaT cells showed strong and concentration-dependent stimulation of DNA-synthesis by the medium from RAdKGF-infected firoblasts (IV, Fig. 2D), while in SCC cells, media from both RAdpCA3 and RAdKGF-infected fibroblasts had a similar effect on DNA-synthesis (IV, Fig. 2, E and F). UT-SCC-7 cells showed a significant enhancement of DNA-synthesis by 1% conditioned medium from RAdKGF-infected fibroblasts

compared to control medium. However, higher concentrations of conditioned medium from RAdKGF-infected fibroblasts stimulated DNA-synthesis in SCC cells less than the control medium and appeared to even reduce DNA-synthesis (IV, Fig. 2, E and F).

*KGF induces a specific gene expression signature in skin SCC cells*

It was demonstrated that KGFR is expressed by cutaneous SCC cells *in vivo*, although its level of expression may be lower than in normal skin. To elucidate the activities of KGF in the biology of SCC cells, three KGFR-positive cutaneous SCC cell lines cultured in serum-free medium were treated with rKGF for 24 h. Total RNA from KGF-treated SCC cells, untreated SCC cells and from two lines of untreated normal keratinocytes cultured in defined growth medium samples were analyzed for differential gene expression by Affymetrix microarray. Briefly, the results showed that KGF clearly elevated the expression of a variety of genes involved in cell signaling and cell cycle regulation, as well as of genes involved in extracellular and intracellular maintenance (IV, Table 1). It was noted that the KGF-treatment elevated the expression of 11 genes that were specifically down-regulated (more than -2 fold) in SCC cells compared to normal keratinocytes, while five genes were found to have higher expression levels in SCC cell lines compared to normal keratinocytes (more than 2 fold), and were further stimulated by KGF treatment (IV, Table 1). The up-regulated genes included a panel of known target genes of extracellular signal-regulated kinase 1/2 (ERK1/2) signaling, including *ETV4, ETV5, DUSP4, DUSP5, IER3, PHLDA1* and *SPRY4*. Of these at least *SPRY4* (Sprouty homolog 4) (Tennis et al. 2010), *DUSP4* and *DUSP6* (dual-specificity phosphatases 4 and 6) (Furukawa et al., 2003; Sieben et al., 2005) and *PHLDA1* (Pleckstrin homology-like domain family A, member 1) (Neef et al., 2002), as well as upregulated *LRIG1* (Leucine-rich repeats and Ig-like domains 1) (Goldoni et al., 2007) have been reported to have tumor suppressor properties (IV, Table 1). On the other hand, suggesting tumor suppressive effects for KGF, 13 genes of the 18 genes down-regulated by KGF in SCC cells, were found to be up-regulated specifically in SCC cells compared to keratinocytes (more than 2.0 fold) (IV, Table 2). Ingenuity pathway analysis (IPA, Ingenuity Systems, www.ingenuity.com) of KGF-regulated genes in the microarray data of SCC cells identified a functional network related to ERK1/2 signaling (IV, Fig. 3).

Among the genes most down-regulated by KGF in SCC cells, there were four genes whose expression was specifically up-regulated in SCC cells compared to normal keratinocytes: *MMP13* (matrix metalloproteinase-13, collagenase-3), *MATN2* (matrilin 2), *CXCL10* (chemokine (C-X-C motif) ligand 10, IP-10) and *IGFBP3* (insulin-like growth factor binding protein 3). Due to their association with extracellular matrix homeostasis, regulation of angiogenesis and cancer progression and metastasis (Ala-aho et al., 2004; Bodnar et al., 2009; Jogie-Brahim et al., 2009; Mates et al., 2002; Zhong et al., 2008), the KFG induced expression and regulation of these genes were studied further. In addition, the up-regulation of the expression of tumor suppressor genes *DUSP4* and *DUSP6* was verified.

*Matrilin2, CXCL10, and MMP-7 are novel target genes down-regulated by KGF*

To verify the results of the global gene expression study, the levels of *MATN2, CXCL10, IGFBP3, DUSP4* and *DUSP6* expression were examined with quantitative real-time RT-PCR in 7 to 8 cutaneous SCC cell lines, HaCaT cells and normal keratinocytes treated with rKGF (10 ng/ml, 24 h). The expression of *MATN2*, *CXCL10* and *IGFBP3* mRNAs were undetectable or very low in normal keratinocytes. In accordance with the microarray results, the expression of *MATN2* was clearly up-regulated in SCC cell lines compared to keratinocytes. KGF significantly reduced *MATN2* mRNA expression in 6/7 KGFR-positive cell lines (t-test, n=3-4) (IV, Fig. 4A). *CXCL10* mRNA was up-regulated in 3/7 SCC cell lines and in HaCaT cells compared to keratinocytes. KGF-treatment significantly down-regulated the expression of *CXCL10* in 3/4 cell lines (IV, Fig. 4A). Finally, the expression of *IGFBP3* mRNA was increased in 6/7 SCC cell lines and in HaCaT cells compared to keratinocytes, and a significant reduction in the expression of *IGFBP3* mRNA by KGF was observed in all KGFR-positive cell lines (IV, Fig. 4A). The basal expression of *DUSP4* and *DUSP6* was clearly detectable in all cell cultures. A reduction in basal *DUSP4* mRNA levels was seen in 7/8 SCC cell lines, and in *DUSP6* mRNA levels in 4/8 SCC cell lines as compared to normal keratinocytes or HaCaT cells (IV, Fig. 4B). KGF markedly enhanced the expression of *DUSP4* and *DUSP6* in 5/6 and 6/6 KGFR-positive SCC cell lines, respectively. Both DUSPs were up-regulated also in keratinocytes and HaCaT cells (IV, Fig. 4B). As expected, KGF did not affect the gene expression of a KGFR-negative cell line, UT-SCC-111 (IV, Fig. 4, A and B).

The expression of matrix metalloproteinase-7 (MMP-7) is associated with the malignant transformation of epithelial cells of chronic cutaneous wounds to SCC (Impola et al., 2005; Kivisaari et al., 2010; Kivisaari et al., 2008). *MMP7* transcript was detected in 5/6 cutaneous SCC cell lines and in HaCaT cells by quantitative real-time RT-PCR (IV, Fig. 5C) and KGF-treatment induced a marked reduction in *MMP7* mRNA expression (34-65%) compared to untreated controls (IV, Fig. 5C). *MATN2, CXCL10* and *MMP7* have not previously been reported as target genes for KGF-treatment. The down-regulation of *IGFBP3* in human normal airway epithelial cells by KGF has been reported previously (Prince et al., 2001).

*KGF inhibits the expression of MMP-13*

MMP-13 is a wide spectrum endopeptidase capable of cleaving various non-matrix and matrix proteins including fibrillar collagens (Knäuper et al., 1996; II, Fig. 2B). An up-regulation of MMP-13 is detected in various types of cancers and the effects of MMP-13 on promoting cancer cell growth and metastasis as well as on cancer angiogenesis are well established (Ala-aho et al., 2004; Johansson et al., 1997a; Lederle et al., 2010). The DNA microarray study uncovered a strong repression of *MMP13* mRNA by KGF in cutaneous SCC cells (IV, Table 2). To verify the result, quantitative real-time RT-PCR was performed and six KGFR-positive skin SCC cell lines and HaCaT cells were analyzed for their *MMP13* mRNA expression after 24-h of KGF-treatment. The expression of *MMP13* was detected in 5/6 cutaneous SCC cell lines and in HaCaT cells (IV, Fig. 5A). KGF potently reduced *MMP13* expression in all of the SCC cell lines

47-94% in comparison with untreated samples (IV, Fig. 5A). As demonstrated by western immunoblotting, KGF-treatment of three cutaneous SCC cell lines and HaCaT cells also resulted in a marked reduction in the amount of secreted latent proMMP-13 protein into the cell culture media as compared to corresponding control samples, while the amount of proMMP-2 remained unaltered (IV, Fig. 5B).

*KGF inhibits the invasion of UT-SCC-7 and UT-SCC-12A cells*

MMP-13 promotes SCC cell invasion and growth *in vitro* and *in vivo* (Ala-aho et al., 2004). Since KGF was demonstrated to be an efficient suppressor of MMP-13 expression in cutaneous SCC cells, the effect of KGF-treatment on SCC cell invasion through collagen was examined. Here, KGF significantly decreased the invasion of UT-SCC-7 cells through collagen by about 60 % and also, to a lesser extent, the invasion of UT-SCC-12A cells (IV, Fig. 5D).

### 5.4.4 Inhibition of MMP-13 expression by KGF is mediated via ERK1/2 MAPK signaling

The signaling molecules activated in a selection of epithelial cells by KGF include mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase 1/2 (ERK1/2) and p38, NF-κB and phosphatidylinositol 3-kinase (Mehta et al., 2001; Niu et al., 2007; Uzan et al., 2009; Zeigler et al., 1999). Also in our gene expression data of KGF-treated SCC cells, the ingenuity pathway analysis identified a functional interaction network of ERK1/2 signaling (IV, Fig. 3). To assess the kinetics of the activation of MAPK pathways in cutaneous SCC cells by KGF, western immunoblotting was used to monitor the phosphorylation of ERK1/2 and p38 in UT-SCC-7 cells established from a metastasized skin SCC, in HaCaT cells representing immortalized but benign keratinocytes and in normal primary keratinocytes, at indicated time points after KGF-treatment. In HaCaT cells and in UT-SCC-7 cells a rapid induction of p-ERK1/2 signal by KGF was detected, and it was still strong at 24 h, and in HaCaT cells still after 48 h (IV, Fig. 6, A and B). The activation of ERK1/2 appeared to be stronger and more persistent in HaCaT cells compared to UT-SCC-7 cells. A slight enhancement in the phosphorylation of p38 (p-p38) was seen after 60 min and 120 min in HaCaT cells and UT-SCC-7 cells, respectively. The p-p38 signal was still detected at 24 h in both cell lines and at 48 h in HaCaT cells (IV, Fig. 6, A and B). Culturing HaCaT cells in serum-depleted medium for 72 h induced auto-phosphorylation of p38 to the level of cells treated with KGF for 48 h (IV, Fig. 6B). Rapid and transient phosphorylation of ERK1/2 was detected also in normal keratinocytes by KGF, but this appeared to be less prominent compared to HaCaT cells or SCC cells (IV, Fig. 6C). In summary, KGF induces the phosphorylation of ERK1/2 and p38 MAP-kinases in a similar manner in UT-SCC-7 and HaCaT cells, and it seems to be more persistent in HaCaT cells.

To test whether the ERK1/2 or p38 MAPK pathways are involved in the KGF induced down-regulation of MMP-13, the pathways were abrogated using the small

molecular inhibitors PD98059 (PD) and SB203580 (SB), respectively. In UT-SCC-7 cells and in HaCaT cells, treatment with PD, the inhibitor of MEK1, prior to KGF-stimulation, resulted in the inhibition of the down-regulation of *MMP13* mRNA expression by KGF (IV, Fig. 6, D and E). The expression of MMP-13 in transformed epithelial cells is mediated by the p38 MAPK pathway (Johansson et al., 2000). In accordance, while PD alone had no effect on *MMP13* expression, SB, an inhibitor of p-p38 activity, was able to effectively and significantly inhibit the expression of *MMP13* mRNA on its own, as shown for HaCaT cells (IV, Fig. 6E). The SB-induced suppression of *MMP13* expression decreased even further with a combinational treatment with KGF (IV, Fig. 6E).

Inhibition of KGF-induced down-regulation of MMP-13 by PD was evident also at the protein level, as indicated by immunoblotting for UT-SCC-7 cells. And SB in combination with KGF practically blocked the expression of MMP-13 (IV, Fig. 6F). The amount of secreted MMP-1 remained unaltered in the cell culture media after treating with KGF or small molecule inhibitors (IV, Fig. 6F).

To further elucidate the function of the ERK1/2 pathway in the down-regulation of MMP-13 in transformed epidermal cells, HaCaT cell series stably transfected with a Ha-*ras* viral oncogene was used. The malignancy in the HaCaT cell series increases in the order A5 (benign)<II4<RT3 (Boukamp et al., 1990; Fusenig and Boukamp, 1998). It was hypothesized that these cells should display increased endogenous ERK1/2-activation and thus, the levels of MMP-13 expression could be altered. Untreated II4 and RT3 cells displayed stronger phosphorylation of ERK1/2 compared to A5 cells (IV, Fig. 7A) and interestingly, the quantitative RT-PCR analysis revealed that the expression of *MMP13* mRNA decreased with increasing malignancy of the cell lines (IV, Fig. 7B). KGF efficiently down-regulated the expression of *MMP13* about 50% and PD (30 μM) inhibited this down-regulation, except in the most aggressive cell line (RT3), which expressed *MMP13* mRNA at a relatively low level (IV, Fig. 7B).

Finally, to further examine whether the constitutive activation of ERK1/2 alone would be enough to suppress *MMP13* expression, the ERK1/2-pathway was specifically activated in HaCaT cells by infection with a recombinant replication-deficient adenovirus RAdMEK1ca to induce the over-expression of a constitutively active MEK1 (mitogen activated protein kinase kinase 1) (IV, Fig. 8A). RAdMEK1ca-infected HaCaT cells showed a more than 70% decrease in *MMP13* mRNA levels as measured 48 h after RAdMEK1ca-infection, compared to cells infected with a control adenovirus, suggesting that ERK1/2-signaling is adequate for *MMP13* down-regulation (IV, Fig. 8B). As a conclusion, because KGF is not able to induce the down-regulation of *MMP13* expression if the activation of ERK1/2 is abolished by PD, it is proposed that ERK1/2-signaling is needed for the KGF-induced repression of MMP-13 expression. Moreover, the specific and sustained activation of ERK1/2 was sufficient to induce the down-regulation of *MMP13* mRNA in HaCaT cells. However, the fact that in Ha-*ras*-transfected HaCaT cells with endogenous high ERK1/2-activation, PD was not able to recover the *MMP13* expression to the level seen in the most benign forms off these cells, suggests that possibly the ERK1/2-pathway alone is not responsible for the down-regulation of MMP-13, but that other mechanisms must be involved.

### 5.4.5 Inhibition of KGF receptor expression by KGF is mediated via ERK1/2 MAPK signaling

KGF was shown to induce a functional negative feedback loop in skin SCC cells by enhancing expression of genes such as *DUSP4* and *DUSP6*, which negatively regulate activity of ERK1/2. To study the effects of KGF on the expression of its own receptor, KGFR, the levels of FGFR2 in SCC cells after KGF-treatment were examined. A clear reduction of FGFR2 protein levels in UT-SCC-7 and -12A cells and in HaCaT cells in response to 24-h KGF-stimulation further point to a functional negative feedback loop for KGF signaling, (IV, Fig. 6G). FGFR2-IIIb and –IIIc have been reported to be mutually exclusive (Warzecha et al., 2009), and here based on RT-PCR results (IV, Fig. 1B) FGFR2 is the epithelial splice variant IIIb (KGFR).

To study the function of the ERK1/2 MAPK pathway in the negative regulation of KGFR expression by KGF, the MAPK inhibitors PD98059 (PD; inhibitor of ERK1/2 activation) and SB203580 (SB; inhibitor of p38 activity) were used. As assessed by quantitative RT-PCR, the expression of KGFR mRNA was markedly down-regulated after a 24-h KGF-treatment in UT-SCC-7 cells (IV, Fig. 6H) and in HaCaT cells (IV, Fig. 6I). In both cells PD inhibited the down-regulation of KGFR by KGF indicating that the effect is mediated via the ERK1/2 signaling pathway (IV, Fig. 6, H and I). The inhibition of p38 by SB had, in turn, no effect in UT-SCC-7 cells (IV, Fig. 6H).

To further examine the role of ERK1/2 in the regulation of KGFR expression, we defined the expression levels of KGFR mRNA in a Ha-*ras*-transfected HaCaT cell series (A5, II4 and RT3). The expression level of KGFR mRNA negatively correlated with the increasing malignancy of the cells and the activation of ERK1/2, and KGF potently and significantly decreased KGFR expression in A5 and II4 cells (IV, Fig. 7C). In RT3 cells, the expression of KGFR was decreased to such a level that KGF did not reduce it further. Pre-treatment of cells with PD efficiently inhibited KGF-induced down-regulation of KGFR, and in malignant II4 cells the expression of KGFR could be recovered even to the level of A5 cells (IV, Fig. 7C). Moreover, although KGF was not able to repress KGFR expression in RT3 cells, PD significantly increased the level of KGFR expression (IV, Fig. 7C) suggesting strong dependency on ERK1/2-signaling. To test whether sustained ERK1/2-signaling alone would be sufficient for KGFR down-regulation, a quantitative real-time RT-PCR analysis for KGFR was performed for HaCaT cells, which were infected with RAdMEK1, and in which, as a consequence, ERK1/2-activation was increased (IV, Fig. 8A). The analysis showed a potent down-regulation (85%) of KGFR mRNA expression 24 h after the RAdMEK1ca-infection (IV, Fig. 8C), indicating a clear involvement of ERK1/2-signaling in the regulation of KGFR expression and suggesting that sustained ERK1/2 activation is enough for the down-regulation of KGFR expression.

# 6 DISCUSSION

## 6.1 WHY MMP-13?

It is well established that MMPs together with plasmin are pivotal for normal wound closure as demonstrated with WT and plasminogen-deficient mice treated with broad spectrum MMP inhibitors (Lund et al., 1999; Mirastschijski et al., 2010). MMP-13 is a collagenase and has been shown to be capable of cleaving, at least *in vitro,* fibrillar collagens of type I, II and III as well as gelatin, type IV collagen, fibronectin, large tenascin C and fibrillin-1, all of which are found in skin (Ashworth et al., 1999; Knäuper et al., 1997; Knäuper et al., 1996). In contrast to *e.g.* MMP-1, MMP-13 has also been shown to cleave type I collagen at the amino-terminal nonhelical telopeptides (Krane et al., 1996), which may be meaningful for collagen matrix remodeling and degradation *in vivo*. Moreover, MMP-13 may modulate inflammation by inactivating chemokines such as monocyte chemoattractant protein-3 (MCP-3) (McQuibban et al., 2002) and stromal cell-derived factor-1 (SDF-1, CXCL-12) (McQuibban et al., 2001). There is increasing evidence that also one of the major growth factors in skin wounds, TGF-β, can be activated by MMP-13 (D'Angelo et al., 2001; Hattori et al., 2009).

At the time when this thesis work began, the physiological expression of MMP-13 had been associated with fetal bone development and postnatal remodeling (Johansson et al., 1997b; Ståhle-Bäckdahl et al., 1997), and with gingival wound healing in fibroblasts in adults (Ravanti et al., 1999b). Also, the expression of *MMP13* mRNA in chronic skin ulcers by fibroblasts deeply imbedded in the wound tissue had just been shown, while in the same study normal adult skin wounds were found to be negative for *MMP13* mRNA (Vaalamo et al., 1997). As adult gingival wound healing shares several similarities with fetal cutaneous wounds at early gestation (Häkkinen et al., 2000) it was hypothesized that MMP-13 could also be found in the fibroblasts of fetal wounds. Moreover, while adult gingival wounds and early gestation human fetal skin wounds tend to heal without or only with minimal scarring, it was suggested that, since it is a collagenase, MMP-13 would function in the efficient collagen remodeling related to scarless healing.

Finally, the expression of MMP-13 had been linked to a variety of epithelial cancers (Airola et al., 1997; Etoh et al., 2000; Freije et al., 1994; Johansson et al., 1997a; Johansson et al., 1999) and particularly, high expression of MMP-13 was detected in cutaneous SCC tumor cells as well as in cancer stromal fibroblasts (Airola et al., 1997). Later, specific inhibition of MMP-13 expression in SCC cells was shown to abolish cancer cell invasion and tumor growth (Ala-aho et al., 2004). Since several features of wound healing, though abnormally regulated, are seen in cancer, (Schafer and Werner, 2008), studying the effect of MMP-13 on wound healing associated processes also provides information on the function of MMP-13 in cancer.

## 6.2 METHODOLOGICAL DISCUSSION

To examine the expression of MMP-13 in fetal cutaneous wounds during early gestation, we utilized a xenograft model, where fetal skin was grafted in a patch-like manner to the dorsal skin of a severe combined immunodeficient (SCID) mouse (I). This model makes it possible to examine human fetal tissue *in vivo* (Lorenz et al., 1992). However, it has been shown that actually, in contrast to subcutaneously implanted wounded fetal skin, the wounded fetal skin grafts used in the described model heal with a scar (Lorenz et al., 1992) possibly because of the presence of adult mouse ECM in the wound base granulation tissue. In our study (I), where wound healing was not followed for more than four days and the collagen composition was not specified, it is not known for sure, whether scarring occured in the wounds. However, at day 4 the wounds had already re-epithelialized and showed numerous hair follicles, suggesting scarless healing.

In this thesis, two strategies were used to study the role of MMP-13 in wound healing related events: 1) the adenoviral introduction of a MMP-13 transgene (Ala-aho et al., 2002) into human fibroblasts, which do not produce MMP-13 by definition, resulting in the upregulation of MMP-13 as compared to fibroblasts infected with a control adenovirus (II), and 2) the comparison MMP-13-deficient mice with WT mice, in which MMP-13 is the main interstitial collagenase (III).

The serotype 5 human adenovirus with genomic E1 and E3 deletions is a widely used gene transfer tool. It is a non-replicative DNA-virus and remains as episomal DNA in the target cell and induces transient and high level transgene expression (Campos and Barry, 2007). Recombinant adenoviruses are excellent tools for gene expression particularly *in vitro,* where they are not attacked by the host's immune system. However, adenoviral vectors are reported to affect target cell signaling by inducing the activation of the ERK1/2 MAPK and PI3K pathways upon cell entry and this way affecting *e.g.* chemokine production and cell survival (Flaherty et al., 2004; Liu et al., 2005). In this study, this fact was evaded using comparable amounts of control adenovirus constructs. Moreover, in original publication II, the activation of ERK1/2 and Akt was examined about 72 h after infection, whereas phosphorylation by adenoviral infection has been reported to take place up to 24 h post infection (Flaherty et al., 2004). Thus, it is quite unlikely that the adenoviral infection itself would have affected the results in terms of ERK1/2 and Akt activation in RAdMMP-13 infected fibroblasts in a 3D culture environment.

The effects of MMP-13 deficiency on granulation tissue formation were studied in an MMP-13 null mouse strain (Inada et al., 2004) (III). These mice contain a null mutation in the *Mmp13* gene resulting in the deletion of exon 5, which is critical for the proteolytic activity of MMP, and subsequent defects in translation. Tissue samples from these mice had a very low level of mutated *Mmp13* mRNA, and no detectable protein expression. Moreover, mutated MMP-13 protein was reported to be devoid of collagenase activity (Inada et al., 2004). Although the similar collagenase expression pattern in the wound epidermis in humans and mice suggests functional homology for human MMP-1 and mouse MMP-13, murine MMP-13 displays higher sequence homology (86%) with human MMP-13 (Freije et al., 1994). The MMP-13 null mouse

is an excellent model for studying MMP-13 activities in the events involved in granulation tissue formation *in vivo*. The data obtained from this model (III) can primarily be applied to human tissues, where MMP-13 is expressed, that is, to human gingival wounds (Ravanti et al., 1999b), human fetal early gestation skin wounds (I) and to several cancer types. Moreover, it could be speculated that some of the observations associated with MMP-13 activity could be extrapolated to the human collagenases MMP-1 and MMP-8 (collagenase-2) in, for instance, acute skin wounds (Nwomeh et al., 1998).

Granulation tissue formation in MMP-13 null mice was assessed by inducing tissue growth with a viscose cellulose sponge (VCS). The VCS implant is a widely used and commonly accepted model for studying the biology of granulation tissue especially in rats (Elenius et al., 1991; Niinikoski et al., 1971; Pajulo et al., 1996; Tommila et al., 2008). It allows the simple examination of different parameters such as granulation tissue growth, angiogenesis and inflammation specifically in granulation tissue, and it is extremely useful for *e.g.* RNA-extraction from granulation tissue excluding the adjacent intact dermis and epithelium. Obviously, the VCS induces an immunological reaction in the host tissue, which should be taken into account when comparing the resulting granulation tissue to granulation tissue induced purely by injury.

Since tissue fibroblasts live in the ECM providing a mechanical scaffold rich in bioactive components, 3D fibroblast culture models are much more compatible with *in vivo* systems compared to conventional 2D cell culture plastics (Cukierman et al., 2001). In this study, a mechanically unloaded 3D collagen culture model was used to study the biology of dermal fibroblasts (II, III). This type of 3D culture model is believed to resemble dermis (Grinnell and Petroll, 2010). In it, the migratory movement and cell contraction of fibroblast, regulated by a variety of factors, result in collagen translocation and gel contraction (Grinnell et al., 2003; Miron-Mendoza et al., 2008; Tamariz and Grinnell, 2002). Thus, the model provides data on the motogenic and contractile activity of cells associated with collagen reorganization. Moreover, in this model, as well as in conventional cell cultures, cellular events such as DNA-synthesis, apoptosis and many others can be quite easily examined. However, it should be kept in mind that the collagen products that were used in this study were extracted with pepsin digestion resulting in atelocollagen. The lack of telopeptides in the type I collagen triplehelix disrupts the cross-linking of molecules during fibrillogenesis. Thus, it is likely that the sliding of collagen molecules in an atelocollagen gel occurs easier than in a collagen gel prepared from telopeptide-containing collagen molecules, and naturally influences gel stiffness. That is, the effect of MMP-13 on collagen matrix remodeling is probably more obvious here *in vitro* compared to the *in vivo* situation. Also, the effects of the collagenolytic activity of MMP-13 on telopeptides (Krane et al., 1996) cannot be measured when using this model.

## 6.3 THE REGULATION OF MMP-13 EXPRESSION BY TGF-β AND KGF

MMP-13 is not expressed at significant levels by unstimulated skin fibroblasts in culture, but its expression is induced by contact with fibrillar type I collagen in a 3D

culture via α1β1 and α2β1 integrins through a mechanism involving p38 activity (Ravanti et al., 1999a; Vaalamo et al., 1997). Moreover, since *MMP13* mRNA was detected in fibroblasts imbedded deep in the collagen of chronic leg ulcers, but not in normal wound fibroblasts *in vivo* (Vaalamo et al., 1997), it is likely that the physiological expression of MMP-13 by skin fibroblasts is regulated in a more complex manner than purely by integrin-collagen ligation, possibly involving the composition of the ECM. In this study (I) we demonstrated that soluble growth factors such as TGF-β1 and –β3 are potential regulators of MMP-13 expression in fetal skin fibroblasts *in vitro*. This is in accordance with results showing MMP-13 induction by TGF-β in human gingival fibroblasts (Ravanti et al., 1999b). In both cases the positive signaling appears to be mediated via the p38 MAPK pathway (I and Ravanti et al., 1999b). However, specific activation of the p38 pathway alone is not enough for the induction of MMP-13 in these cells (I, Fig. 5 and Ravanti et al., 1999b). Interestingly, it was later shown for gingival fibroblasts, that the induction of MMP-13 expression by TGF-β actually requires cross-talk between two distinct signaling pathways, the p38 and Smad pathways, both activated by TGF-β in these cells (Leivonen et al., 2002; Ravanti et al., 1999b). Similar signaling mechanism may also hold true for fetal skin fibroblasts.

In contrast to neonatal fibroblasts, the ERK1/2 pathway was not activated by TGF-β in fetal skin fibroblasts (I) and it appears not to be in charge of regulating TGF-β induced MMP-13 expression in gingival fibroblasts (Ravanti et al., 1999b). However, integrin ligation with type I collagen in a 3D culture results in the activation of both the p38 and ERK1/2 pathways, and the upregulation of MMP-13 expression. Here, it is p38 MAPK that was shown to mediate MMP-13 upregulation and ERK1/2 was implicated in the negative regulation of MMP-13 expression in human dermal fibroblasts (Ravanti et al., 1999a).

In terms of MMP-13 expression, very similar regulatory systems seem to be used in transformed epidermal cells. In transformed keratinocytes and in SCC cells, the expression of MMP-13 is enhanced by TNFα and TGF-β (Johansson et al., 2000) and suppressed by interferon-γ (IFN-γ) (Ala-aho et al., 2000). Similarly to fetal fibroblasts, p38 MAPK activity appears to be absolutely required for the up-regulation of MMP-13 in transformed keratinocytes, whereas ERK1/2-activation did not play a marked role in the process (Johansson et al., 2000). In our study (IV), using transformed keratinocytes and skin SCC cell lines, p38 MAPK was shown to mediate a high endogenous expression of MMP-13. With equal importance, KGF was shown to induce a potent and long-lasting activation of ERK1/2, resulting in the down-regulation of MMP-13. In accordance with our results, the activation of ERK1/2 MAPK has also been reported to be responsible for the down-regulation of MMP-13 by IFN-γ (Ala-aho et al., 2000). Our results with activating adenovirus constructs also suggested that the activation of ERK1/2 pathway alone is sufficient for down-regulating MMP-13 expression (IV). It is possible that the strong enhancement of p38 activation by TGF-β counteracts the inhibitory effect of activated ERK1/2 in the aforementioned study with transformed keratinocytes (Johansson et al., 2000), especially in comparison to KGF, which only slightly induced the activation of the p38 pathway in transformed keratinocytes or SCC cells. Or it may be that the duration and the magnitude of the ERK1/2 signal determine

MMP-13 transcription. Taken together, the results of this thesis study strongly support the previous observations that p38 MAPK seems to be a key mediator of the positive regulation of MMP-13 expression, and ERK1/2 is responsible for the negative regulation of MMP-13 expression. This regulation appears to take place in fibroblasts as well as in transformed epidermal cells. In terms of MMP-13 expression, the similar response of fetal fibroblasts and gingival fibroblasts to TGF-β *in vitro*, which is distinct from the response seen in neonatal and adult fibroblasts, proposes a notable increase in the proteolytic capacity of these cells during wound healing, and may be related to enhanced matrix reorganization.

Finally, understanding the regulatory mechanisms behind the expression of MMP-13 in skin SCC cells *e.g.* by KGF, provides a window for modifying MMP-13 expression in these cells. Moreover, as KGF may have desirable effects on skin SCC cells, understanding the underlying signaling mechanisms brings up pivotal issues to be considered when planning cancer treatment.

## 6.4 THE FUNCTION OF MMP-13 IN WOUND HEALING – IMPLICATIONS FOR CANCER

In this study, the effect of MMP-13 on wound healing related events was examined by assessing the effects of adenovirally induced expression of MMP-13 on the phenotype of adult dermal fibroblasts cultured in a 3D collagen matrix (II). Moreover, the development of subcutaneous granulation tissue physically induced by a viscose cellulose sponge (VCS) implant was examined in MMP-13 knockout (KO) mouse strain and compared to wild type (WT) mice (III).

During cutaneous wound healing in mouse, MMP-13 expression is detected in the migrating keratinocyte tip at the wound edge and in stromal fibroblasts (Hattori et al., 2009; Madlener et al., 1998). Some expression by the wound endothelium has also been reported (Hattori et al., 2009). In the report by Hattori *et al*. *Mmp13* transcript expression was detected at day one post injury and continued for at least two weeks as determined by RT-PCR. Also in our study (III), based on DNA microarray results, *Mmp13* mRNA was detected in the granulation tissue at day 7, 14 and 21 time points showing its highest level of expression at day 14. Using MMP-13 deficient mouse strain, we identified MMP-13 as essential for normal development of VCS-induced granulation tissue. Granulation tissue growth was significantly delayed at day 21 in KO mice compared to WT. As assessed by an immunohistochemical analysis and morphometrical evaluation we noted delayed myofibroblast organization and altered pattern of blood vessels in KO granulation tissue. Global gene expression comparison and the functional analysis revealed differential regulation of the genes involved in cellular movement, inflammatory response, cellular growth and proliferation, cell death and cardiovascular system development and function, and identified several candidate genes to mediate different functions noted to be altered in KO granulation tissue. *In vitro* studies using KO mouse skin fibroblasts (MSF) suggested reduced motile and contractile activity of KO MSF as detected in a 3D collagen culture, and showed significantly reduced *Mmp3* expression in KO fibroblasts.

There are a few previous papers suggesting, that MMPs are involved in wound-associated induction and alignment of myofibroblasts (Bullard et al., 1999; Mirastschijski et al., 2004a; Mirastschijski et al., 2010). As there are three different strains of MMP-13 deficient mice published (Hartenstein et al., 2006;Hattori et al., 2009;Inada et al., 2004), two studies using the same strain of MMP-13 null mice were not able to detect any abnormalities in cutaneous wound healing (Hartenstein et al., 2006; Juncker-Jensen and Lund, 2011). In another wound healing study utilizing MMP-13 deficient mice, MMP-13 was implicated in the fibroblast-myofibroblast differentiation *in vivo* and *in vitro*, and this was suggested to influence via the activation and activity of TGF-β (Hattori et al., 2009). *In vivo* they quantified less myofibroblasts in MMP-13 KO granulation tissue after completion of re-epithelialization which took place at different times in control and KO mice.

In our study, we noted delayed orientation of myofibroblasts, which is likely to reflect their functionality. We did not observe significant difference in the appearance of αSMA positive myofibroblasts in MMP-13 KO granulation tissue at the 7d time point (III). In accordance, the global gene expression data analysis identified no significant differences in the genes involved in muscle contraction at 7d between the WT and KO tissues. However, it is not known whether an earlier time point would have shown differences. Clearly, our observations show that defective myofibroblast function in KO mice can be overcome, yet exhibiting delayed resolution of myofibroblasts. In a recent paper describing an application of broad spectrum MMP inhibitor to a mouse full thickness wound, very similar results were reported, including irregular organization of myofibroblasts and delayed myofibroblast resolution (Mirastschijski et al., 2010). In our study at 21d, persistent expression of myofibroblasts in the majority of KO granulation tissues was noted. The myofibroblasts were abundant at the areas in the proximity of the implant surface characterized with strong accumulation of fibrous ECM, and this was suggested to be due to impaired migration of cells into the VCS. Moreover, the gene expression data functional analysis also strongly suggested reduced apoptosis in KO at 21d. Thus, it may be that as a result of a certain level ECM maturation and stress-relaxation in WT, apoptosis of myofibroblasts is taking place, and does not occur in KO due to the lack of MMP-13 activity. Most probably, the alterations noted in the distribution of myofibroblasts at 21d are not responsible for delayed granulation tissue growth in KO but is rather a secondary effect of this.

Impaired motile activity of myofibroblasts was suggested to be involved in delayed granulation tissue development in KO mice. This is supported by the gene expression functional analysis which associated a set of differentially expressed genes with the biofunction migration of cells in high significance. Fibroblast-mediated contraction of a floating collagen gel is proposed to result from migratory movement of the cells which is dependent on the appropriate cell adhesion and cytoskeletal activity (Grinnell and Petroll, 2010). Using a 3D collagen culture model we showed that MMP-13 KO skin fibroblast showed markedly reduced collagen gel contraction compared to WT in the presence of FCS (III). This was further supported by our results showing increased collagen matrix remodeling by human adult dermal fibroblasts, which were manipulated to up-regulate MMP-13 (II). In both studies, MMP-13 expression was

associated with a marked stimulation of cell protrusions toward the matrix and toward other cells. The morphological changes were induced by TGF-β and FCS (also containing TGF-β) in WT but not in KO fibroblasts. Since the recombinant TGF-β protein that was used here is likely to be already in its active form, our result does not directly show that the activation of TGF-β by MMP-13 would be involved. Moreover, as morphological alterations were noted also in low serum, they could rather be explained by MMP-13 induced restricted matrix cleavage and subsequent alterations in cell-ECM interactions. It is also of note, that no clear signs of defected TGF-β signaling were noted *in vivo* with the gene expression data functional analysis of KO granulation tissues.

Supported by these *in vitro* findings (II, III), it could be assumed that MMP-13 also mediate remodeling of the wound ECM and cell migration *in vivo*. MMP-13 increases markedly intercellular contacts *in vitro* which on the other hand may partially explain the delayed collective assembly of myofibroblasts in VCS-induced granulation tissues (III). On the other hand it might even stimulate multicellular migration (Friedl and Wolf, 2010), which may well also play a role in cancer. The effects of MMP-13 deficiency on the fibroblast phenotype in 3D collagen were explored for the first time in this study.

VCS provides a valuable model for studying gene expression specifically in granulation tissue (III). As the difference in granulation tissue growth was developed during the third week after implantation, the most interesting time point for the gene expression profiling and the functional data analysis was day 14. The most obvious and systematic observation by the differential gene expression functional analysis at 14d was reduced inflammation KO tissues. One of the key molecules here appears to be IL-6, which was down-regulated in KO. IL-6 was involved also in other systems, which showed down-regulation in KO at 14d, such as proteolysis and angiogenesis. Thus, it would be of great interest to understand the mechanism behind the down-regulation of IL-6, and its cellular source. Providing one possible explanation is that MMPs, including MMP-13, are capable of modulating inflammation via processing of different chemokines and their receptors, which again may regulate the expression of IL-6 (Parks et al. 2004). Also, immunohistochemical studies are needed to study inflammatory reaction in KO and WT granulation tissues in detail. The possible role of MMP-13 in the regulation of immune reaction involved in wound healing is not previously reported, and is worth noticing, since inflammation is one of the main features also in cancer (Schäfer and Werner, 2008).

Another specifically interesting and multifunctional molecule coming up from global expression data is PDGF-D, which was downregulated in KO tissues at all the time points (III). Its function in the positive regulation of fibroblast proliferation, activation of macrophages and angiogenesis is reported (Uutela et al., 2004; Wang et al., 2007). Also CADM-1 and IFI202b were up-regulated in KO granulation tissues. These two molecules are implicated in the negative regulation of cell proliferation (Nowacki et al. 2008; Xin et al., 2003) In addition, the sets of other differentially expressed genes were associated with cellular growth and proliferation in high significance. Still, we were unable to get systematic support for our previous results on the enhancement of fibroblast viability or proliferation by MMP-13 from gene

expression data analysis. This may be related to the simplified culture environment in 3D collagen and the lack of products produced by other cells *in vivo*.

At 14d time point, also several genes involved in collagen metabolism and fibrillogenesis were down-regulated in KO suggesting reduced matrix turnover (III). The major ECM proteins in granulation tissue, collagen type I and fibronectin did not show significant differences in their expression. In contrast, gene expression analysis suggested reduced MMP-activity in MMP-13 KO granulation tissues, specifically the reduction of gelatinases MMP-2 and MMP-9, and MMP-3, which is implicated in wound contraction via mediating actin cytoskeleton abnormalities (Bullard et al., 1999). All these MMPs possess a wide substrate spectrum and they are able to activate variety of growth factors and other MMPs. Because the most prominent differences in MMP expression were noted at 14d characterized by similar granulation tissue growth, it is not likely that down-regulation of MMPs only would reflect the overall delay in tissue growth of KO mice. Thus, the result suggests that the proteolytic action of MMP-13 in mouse granulation tissue is potentiated by these MMPs involved in wound contraction and angiogenesis (Kato et al., 2001; Bullard et al., 1999; Vu et al., 1998). Possibly, MMP-9 deprivation is due to impaired inflammation (Inkinen et al., 2000; Okada et al., 1997).

Indeed, MMP-13 was also shown to affect blood vessel formation (III). Previously, MMP-13 has been implicated in the positive regulation of physiologic and pathologic angiogenesis (Hattori et al., 2009; Zigrino et al., 2009; Lederle et al. 2010). In accordance, in this study (III) the differential gene expression functional analysis predicted systematic down-regulation of the events related to angiogenesis at 14d and 21d time points, although statistical significance was not quite reached. However, the immunohistological and morphometrical evaluation of CD34 positive blood vessels actually showed significantly increased density of microvessels at 14d. Since the gene expression study is likely to reflect the outcome to possibly seen later in tissue, the immunohistological quantitation is not contradictory to gene expression data. Thus, predicted down-regulation of angiogenesis at mRNA level at 14d may reflect the lack of large vessels noted in KO tissues and which were abundantly present in WT at 21d. Together these results suggest that MMP-13 can mediate positive and negative regulation of different aspects of angiogenesis in mouse cutaneous granulation tissue.

## 6.5 THE ROLE OF MMP-13 IN SCARLESS WOUND HEALING

Suggesting that MMPs might be involved in scarless wound healing, an increased MMP to TIMP ratio, including MMP-13, has been reported for wounds in non-scarring early gestation rat skin compared to wounds in scarring skin in late gestation (Dang et al., 2003). Very recently, the scarless skin wound healing of athymic FOXN1-deficient nude mice was linked to increased levels of MMP-13 and MMP-9 (Gawronska-Kozak, 2011). In humans, scarlessly healing adult gingival wounds also display MMP-13 expression by fibroblasts (Ravanti et al., 1999b). In accordance, it was shown that fibroblasts in human fetal skin produce the MMP-13 protein during scarless wound healing *in vivo* (I). Thus, the presence of MMP-13 in variety of wounds showing

healing with minimal scarring suggests that MMP-13 may participate in scarless healing. The potent collagenolytic activity of MMP-13 and its suggested function in scarless wound repair have tempted researchers to hypothesize that MMP-13 augments dissolution of fibrotic conditions. For instance, the up-regulation of MMP-13 expression was reported to associate with the regression of keloid tissue (Kuo et al., 2005) and as produced by scar-associated macrophages, MMP-13 was implicated in the resolution of hepatic fibrosis in rats (Fallowfield et al., 2007). In the later study, the resolution of fibrosis was shown to be retarded in MMP-13 deficient mice finally providing direct evidence for the competence of MMP-13 in anti-fibrotic events. In accordance, adenovirally induced up-regulation of MMP-13 in rat liver enhanced the resolution of fibrosis (Endo et al., 2011). The concept of using collagenases in the treatment of fibrotic conditions is put into practice by using *Clostridium histolyticum* collagenase injections to reduce contractures in Dupuytren's disease (Hurst et al., 2009). Dupuytren's disease is a fibroproliferative disorder causing the flexion contracture of joints in the palm and fingers resulting in a hand function disability (Hurst et al., 2009).

In our study (I), TGF-β1 and TGF-β3 were both shown to stimulate MMP-13 expression by fetal skin fibroblasts *in vitro*. There is evidence that fetal skin has a higher expression ratio of TGF-β3 to TGF-β1 and -2 compared to adult skin (Hsu et al., 2001). In sharp contrast to TGF-β1 (Verrecchia and Mauviel, 2007), TGF-β3 has been proposed to be an anti-scarring agent in fetal skin wounds (Shah et al., 1995). Indeed, recombinant active TGF-β3 is currently going through clinical trials for reducing scarring in acute incisions (Bush et al., 2010). Whereas the level of TGF-β1 is very low in fetal skin and also during wound healing (Whitby and Ferguson, 1991), TGF-β3 predominates in fetal skin. The mechanisms of function for TGF-β3 in fetal skin include the down-regulation of collagen deposition (Shah et al., 1995). TGF-β3 is also likely to stimulate MMP-13 expression *in vivo* as suggested by our study (I). Moreover, since the level of TGF-β1 is low in fetal skin, the activation of TGF-β1 and possible subsequent enhancement of collagen synthesis and activation of myofibroblasts by MMP-13 may not play a significant role here. Instead, MMP-13 may also activate TGF-β3 thus promoting its action in fetal skin (Deng et al., 2000).

In this study, we demonstrated that MMP-13 markedly enhances the ability of fibroblasts to remodel a collagen matrix as assessed by collagen gel contraction (II). This was accompanied by alterations in the actin-cytoskeleton structure with undetectable αSMA incorporation, enhanced cell proliferation and escape from loose collagen gel -induced apoptosis (Tian et al., 2002). Since they took place in a low serum concentration and in the absence of exogenously added TGF-β, the phenomena were suggested to involve an altered collagen-integrin interaction resulting from restricted pericellular collagenolysis. This provides an alternative mechanism for the action of MMP-13 in fetal skin wounds in addition to the activation of TGF-β3. In this study (III) we also noted signs at the gene expression level of decreased inflammation in MMP-13 KO mice during the granulation tissue development. As inflammation is considered as a risk for the tissue fibrosis and scarring, this result is not supportive for MMP-13 as an anti-scarring agent. However, reduced inflammation in KO tissue was not yet studied further and more details about the cell distribution and temporal

expression should be understood to make any final conclusions about the activity of MMP-13 on the regulation of inflammation.

Together our findings show that MMP-13 is present in scarless fetal skin wounds and that MMP-13 expression enhances the collagen matrix reorganization capacity of fibroblasts associated with increased cell viability. Yet, until now, there appears to be no direct evidence showing that MMP-13 would stimulate scarless healing. In contrast, based on a wound healing study with MMP-13 deficient mice, where the wounded skin was followed for thirty days, the authors reported a similar distribution, mass and organization of collagen fibers in WT and MMP-13 deficient mice (Hartenstein et al., 2006). Maybe the best way to address this question would be to introduce MMP-13 into wounds which normally develop scars. This could be possible *in vivo* in mouse skin, which does not, however, form scars comparable to those in humans. Thus, an even better choice would be to use human skin xenografts, which do not express MMP-13 by definition.

## 6.6 THE EFFECT OF KGF ON THE BIOLOGY OF SKIN SCC CELLS

Questioning the role of KGF in epithelial carcinogenesis is rationalized by the facts that KGF is one of the key growth factors regulating the proliferation of normal epithelial cells (Rubin et al., 1989; Yi et al., 1994) and its expression is highly induced under specific conditions, that is in post natal tissue repair (Marchese et al., 1995; Werner et al., 1992). Since tumors can be thought of as wounds that do not heal (Dvorak, 1986), it would not be surprising if KGF has oncogenic functions under some circumstances similarly to many other growth factors (Grose and Dickson, 2005; Leivonen and Kähäri, 2007). Moreover, there is general interest for using KGF as a protective medicine for normal tissues during epithelial cancer treatment (Finch and Rubin, 2006).

The published data concerning the role of KGF in the pathogenesis of epithelial cancers are partially hard to interpret (Finch and Rubin, 2006). Yet, there is increasing evidence that the expression level of KGF and/or its receptor as well as the effect of KGF on cancer cells are highly dependent on the cancer type (Finch and Rubin, 2006). Today, there are several studies on the role of KGF in SCC of the head and neck (HN-SCC) (Brizel et al., 2008; Knerer et al., 1998; Ning et al., 1998) but virtually none for the more frequent cutaneous SCC (Finnish Cancer Registry, www.syoparekisteri.fi). Thus, in this study we explored the expression of KGFR and KGF in skin SCCs, and examined the effects of KGF-stimulation on skin SCC tumor cells (IV).

In this study (IV), we first showed that KGFR mRNA is typically expressed in skin SCC tumors *in vivo*. The expression levels appeared to be lower than in normal tissues. At the moment, there are only extremely limited data available describing the expression of KGFR either in skin SCC or in HN-SCC tumors (LaRochelle et al., 1995). Therefore, our observation is of special interest. It would be highly desirable to find out the expression pattern of the KGFR protein in cancer tissues. Unfortunately, the development of good antibodies specific for KGFR has proven to be a great

challenge, and today there are no commercially available antibodies against KGFR to be used for immunohistochemistry.

Parallel to the data received from SCC tumor samples, two of eight cutaneous SCC cell lines were found to be virtually negative for KGFR mRNA expression. Despite the fact that KGFR-positive skin SCC cell lines were responsive to KGF as assessed by the activation of ERK1/2-signaling and the negative feed-back regulation of KGFR expression, they did not show proliferative activity when treated with KGF. Similar observations are reported *e.g.* on HN-SCC cell lines (Ning et al., 1998), colorectal cancer cell lines (Otte et al., 2000) and hepatocellular carcinoma cells (Amann et al., 2010). This phenomenon has not been described for skin SCC cells before. We suggest that KGF:KGFR-induced ERK1/2-signaling is uncoupled from its mitogenic effects in skin SCC cells. Whereas ERK1/2 is activated in HaCaT cells and SCC cells in a similar manner, there must be something else that makes the difference between these benign and malignant cells, respectively. The intensity and duration of the KGF induced phosphorylation of ERK1/2 were found to be stronger and more persistent in HaCaT cells compared to UT-SCC-7 cells, which may explain the different mitogenic response. Also, the temporal expression of DUSP proteins should be examined in terms of clarifying the earlier dephosphorylation of ERK in SCC cells. We also propose that the expression of KGFR may be a disadvantage for SCC cells as will be discussed below, and lost in cancer cell evolution during cancer development. This was supported by the observation of reduced KGFR mRNA levels associated with increased malignancy of Ha-*ras*-transformed HaCaT cells. Similar data have been previously reported for *e.g.* a hepatocellular carcinoma (Amann et al., 2010). Interestingly, a switch from FGFR2-IIIb to FGFR1-IIIc expression was recently linked to the TGF-β-induced epithelial-mesenchymal transition (EMT) of mammary epithelial cells (Shirakihara et al., 2011), a phenomenon occurring also in many epithelial cancers (Kalluri and Weinberg, 2009). Whether the skin SCC cell lines reported here to be KGFR-negative have gone through EMT and acquired the ability to express other FGFRs instead of KGFR, remains to be determined.

Somewhat surprisingly, very few global gene expression analyses have been performed on cells to study the effects of KGF-treatment. In this study we explored the gene expression profile of KGF-treated skin SCC cell lines using a genome wide DNA microarray analysis (IV). Actually, when SCC cells were treated with KGF for 24 h, the resulting lists of up- or down-regulated genes were not very long. It is possible that examining gene expression in an additional shorter time point would have revealed more regulated genes as was the case *e.g.* in airway epithelial cells (Prince et al., 2001). Here we identified several up-regulated genes (*ETV4* and *-5*, *DUSP4* and *-6*, *IER3*, *PHLDA1* and *SPRY4*), which are known targets for ERK1/2-signaling, emphasizing signaling via ERK1/2 in KGF-mediated gene regulation. The up-regulated proteins DUSPs, PHLDA1, Sprouty 4 and LRIG1 have been previously implicated as tumor-suppressors (Goldoni et al., 2007; Neef et al., 2002; Okudela et al., 2009; Sieben et al., 2005; Tennis et al., 2010).

Among the genes most down-regulated by KGF in skin SCC cells we found several genes, which are highly up-regulated specifically in skin SCC cells compared to normal keratinocytes. Of these we identified two novel KGF-regulated target genes in

skin SCC cells, *Matrilin 2* and *CXCL10*. The third gene to be studied further, *IGFBP3*, was reported to behave in a similar way in human airway epithelial cells (Prince et al., 2001).

Matrilin 2 is a putative adaptor protein for ECM assembly (Piecha et al., 2002b). It is widely expressed, and in skin it localizes to the basement membrane zones of the epidermis and blood vessels (Piecha et al., 2002a; Szabo et al., 2008). The chemokine CXCL10 is a ligand for the chemokine receptor CXCR3. CXCL10 inhibits angiogenesis (Bodnar et al., 2009) suggesting an anti-malignant function by remodeling the tumor microenviroment. But it has also been associated with cancer promoting effects (Zipin-Roitman et al., 2007). IGFBP3 (insulin-like growth factor binding protein 3) acts via stabilizing insulin-like growth factors (IGFs) and affecting their affinity towards IGF receptors. It can also function separately from the IGF/IGFR axis (Jogie-Brahim et al., 2009). The role of matrilin 2 in cancer is largely uncharacterized, but there is an *in vitro* study showing that down-regulation of matrilin 2 associates with increased migration of HaCaT cells (Ichikawa et al., 2008). CXCL10 has both cancer promoting and suppressing properties (Bodnar et al., 2009; Zipin-Roitman et al., 2007) and IGFBP3 is largely described as a tumor-suppressor due to its function as a negative regulator of cell proliferation, and as a promoter of apoptosis (Jogie-Brahim et al., 2009; Mitsui et al., 2006). IGFBP3 has also been reported to be down-regulated in certain HN-SCC cancers (Papadimitrakopoulou et al., 2006; Rajah et al., 2002). Thus, it is not known why the genes for CXCL10 and especially IGFBP3 are highly up-regulated in skin SCC cells as compared to normal keratinocytes (IV, Table 2). Moreover, the KGF-induced up-regulated genes included several interferon-inducible genes (IV, Table 1). Although the specific genes appear to be different, the same effect was noted for airway epithelial cells (Prince et al., 2001). This may indicate that KGF can reduce inflammatory effects in SCC cells induced by interferons.

Finally, the gene most strongly down-regulated by KGF in skin SCC cells was *Mmp13*. Tempted by this observation we explored the KGF-induced regulation of also several other MMPs by quantitative RT-PCR, and identified *Mmp7* as another novel target gene for KGF, and to be strongly down-regulated too. Since MMP-13 is strongly associated with SCC growth and invasion (Ala-aho et al., 2004; Cazorla et al., 1998) and the expression of MMP-7 is associated with the malignant transformation of epidermal cells (Impola et al., 2005) and the regulation of cell motility (Noe et al., 2001), we hypothesized that KGF could also inhibit the invasion of skin SCC cell lines. Indeed, it was demonstrated that KGF does not promote skin SCC invasion, which rather appears to be suppressed by KGF.

Thus, several observations in this study (IV) support the role of KGF as a tumor suppressive factor for KGFR-positive skin SCC cells. As some genes negatively affected by KGF are also associated with anti-cancer functions, it is the net effect of KGF that accounts. This would be best examined *in vivo* or using sophisticated organotypic culture systems. Finally, cytoprotective effects of KGF on skin SCC cells were not studied here, and they are equally important with the cancer stimulatory effects when considering KGF as a drug for the side effects caused by skin SCC treatment.

# 7 SUMMARY AND CONCLUSIONS

In this thesis study, MMP-13 expression was detected in the fibroblasts of fetal wounds characterized by minimal scar formation. Subsequently, MMP-13 was shown to enhance collagen matrix reorganization and to increase the survival and proliferation of adult dermal fibroblasts in a nonstressed collagen matrix. It is proposed that restricted collagenolysis alters cell-ECM contacts and the formation of cell extensions, or it may be that MMP-13 modulates growth factors such as TGF-β, which again may affect cellular events in several ways. The results suggest that MMP-13 may increase cell viability and efficient collagen remodeling participating in scarless healing also in human early fetal wounds. In addition, a deficiency in murine MMP-13 was shown to inhibit or delay cellular activities, such as the collective assembly of myofibroblasts, inflammation, proteolysis and the formation of large vessels in subcutaneous granulation tissue. This indicates that MMP-13 either directly, or indirectly by modulating other factors, participates in several events involved in wound healing.

As MMP-13 activity and events such as cell migration, proliferation, myofibroblast activity, proteolysis, angiogenesis and inflammation are all key components of many cancers, these results may well also be used in cancer research. To my knowledge, in this thesis study, granulation tissue *per se* and as a whole excluding wound epidermis and remnants of adjacent normal tissue, and harvested at these particular time points, was subjected for microarray analysis for the first time. The data reveal specific gene expression signature for MMP-13 KO granulation tissues including the up-regulation of *Npy* and *Adamts4* and down-regulation of several MMPs, *Il6* and *Pdgfd* proposing new candidate genes as potential effectors of MMP-13-mediated processes.

The usage of KGF during epithelial cancer treatment is restricted due to unknown effects of KGF on cancer cells. Before this thesis study, virtually nothing was known about the effects of KGF on skin SCC cells. KGF was shown to up-regulate expression of a set of genes specifically downregulated in skin SCC cells compared to normal epidermal keratinocytes including genes with tumor suppressing properties (*SPRY4, DUSP4, DUSP6, LRIG1, PHLDA1)*. Moreover, many genes specifically up-regulated in SCC cells and associated with cancer progression, were downregulated by KGF (*MMP13, MMP7, MATN2, CXCL10, IGFBP3*). KGF reduced the invasion of SCC cells. The results support the use of KGF as a drug when treating skin SCC. Still more studies may be needed to verify the observations. In this study several novel target genes for KGF were identified in cutaneous SCC cells.

Taken together, the observations of this thesis work indicate a pivotal role for MMP-13 in cellular events such as myofibroblast activity, fibroblast-mediated collagen matrix reorganization, fibroblast viability and angiogenesis associated with wound healing and cancer, and that the cancer related expression of MMP-13 in SCC cells is negatively regulated by KGF. The results also suggest that MMP-13 expressed in human adult gingival and fetal skin wounds may participate in efficient dermal remodeling leading to scarless healing. The data obtained from this study may be useful when designing novel strategies for stimulating poorly healing wounds and treating fibrotic scars, or when developing targeted cancer therapy.

# 8 ACKNOWLEDGEMENTS

This work was carried out in MediCity Research Laboratory, the Department of Dermatology and Venerology, and the Department of Medical Biochemistry and Genetics, the University of Turku. The study was initiated at Turku Centre for Biotechnology, the University of Turku and Åbo Akademi. I thank the heads of MediCity Research Laboratory and Turku Centre for Biotechnology, Professor Sirpa Jalkanen and Professor Riitta Lahesmaa, respectively, for providing excellent facilities for the research. I am truly grateful to former head of the Department of Medical Biochemistry, Professor Jukka Finne for giving me the opportunity to get valuable teaching experience during this time.

My deepest gratitude is directed to my supervisor Professor Veli-Matti Kähäri, the professor of the Department of Dermatology and Venerology at the University of Turku and Turku University Hospital. I truly appreciate the fact that he gave me the time that was needed to complete this thesis. His guidance and persistency have been a great learning experience for me. I thank Turku Graduate School of Biosciences, TuBS, for financial support and for providing scientific and nonscientific events of excellent quality. Especially, I want to express my gratitude to the members of my supervisory committee, Professor Jyrki Heino and Professor Juha Peltonen for their interest in my work and fresh views concerning my research. Docent Kaisa Lehti and Professor Tuula Salo are warmly acknowledged for reviewing my thesis manuscript and providing excellent comments and suggestions for improving it.

This work would not have been done without great collaborators in Finland and abroad. I owe my sincere thanks to Laura Ravanti, Risto Ala-aho, Matti Laato, Olli Carpén, Varpu Marjomäki, Reidar Grénman, Sirkku and Juha Peltonen, Risto Penttinen, Jyrki Heino, Suvi Ruohonen, Eriika Savontaus, Timothy Crombleholme, Marco Foschi, Jiahuai Han, Andrew Baker, Masaki Inada and Stephen Krane for valuable co-operation. Moreover, I am grateful to the people in BTK, Medicity and Medisiina, Marjo Hakkarainen, Jouko Sandholm, Perttu Terho, Markku Saari, the staff of the Finnish Microarray and Sequencing Centre, Antti-Pekka Laine and Heidi Pakarinen for professional help with recombinant virus construction, microscopes, image analysis, microarray data analysis, computers and assistance in laboratory work. Thanks to the technical staff in the Medicity and BTK labs and offices for keeping everything up and running. I also highly acknowledge people who have provided me with valuable help through discussions on different scientific issues. It is fantastic to work in an place with an atmosphere like Medicity, where you can just go and ask for help.

I express my gratitude to the great and mighty MMP friends of mine, the former and the present ones, Atte Kivisaari, Pilvi Riihilä, Mehdi Farschian, Elina Siljamäki, Liisa Nissinen, Niina Reunanen, Janne Kallio, Melissa Junttila, Suvi-Katri Leivonen, Risto Ala-aho, Song-Ping Li and Matti Ahonen for so many wonderful moments in the lab and outside of it. Special thanks go to Sari Pitkänen and Johanna Markola-Wärn for indispensable technical assistance. You guys have really been the basal structure, something to rely on. Thanks to everyone for discussions and just for being such lovely

workmates. Thanks to the Incredible Integrins for convivial companionship and sharing so much, both literally and metaphorically. Moreover, I warmly remember the weeks that were spent with my assistant pals in LBK, especially Anne Jokilammi, Erika Ekholm, Vuokko Loimaranta and Saija Kiljunen. Teaching was actually fun and refreshing for a change!

I express my warmest appreciation to my dear friends, Harjavallan jengi, Saara, Rea, Minna, Riikka, Kata, Krisse and Johanna. Olen onnellinen, että olemme pystyneet pitämään porukan kasassa, ja enenevissä määrin, kaikki nämä vuodet. Kiitos siitä, että hilpeässä seurassanne olen saanut nauraa monen lisävuoden edestä ja nollata työasiat täysin. Thanks to Sanna and Pete for support and encouragement. I will never forget our trips to beautiful Saimaa or the Baltic region. I appreciate your friendship very much. Thanks to Reetta and Tapsa, Kolkkanen family and to Mäkilä family for lovely bbq parties, cruises and other great moments. I am grateful for your friendship.

My mother and father, Raili and Matti Ahonen, have given me the best support during my thesis project and in life by encouraging and letting me make my own choices. Kiitos äiti ja isä, että olette aina olleet tukenani ja sinnikkäästi olleet kiinnostuneet kuulemaan tutkimukseni vaiheista. Tuhannet kiitokset Ritvalle ja Santulle ystävyydestä ja erityisesti siitä, että Ritva on ollut niin mahtavasti käytettävissä lastenhoitoapuna. Valtavat kiitokset Pajusen perheelle lukuisista mukavista yhdessä vieteyistä päivistä ja illoista.

Finally, I want to thank my dear husband and best friend, Mikko. He has supported me and pushed me towards my goals. Mikko, jättikiitos korvaamattomasta tuestasi ja kannustuksestasi näinä väitöskirjavuosinani. Elämää ilman sinua on hankala kuvitella. Kiitos kaikesta yhdessä kokemastamme.

This thesis work has been financially supported by TuBS and the University of Turku, the Sigrid-Jusélius Foundation, the Cancer Research Foundation of Finland, the Southwestern Finland Cancer Societies, Turku University Hospital, the Finnish Cultural Foundation, the Maud Kuistila Foundation, the Orion-Farmos Research Foundation, the K. Albin Johansson Foundation, the Paulo Foundation and the Turku University Foundation.

Turku, March 2012

Mervi Toriseva

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