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Matrix metalloproteinases as biomarkers in premalignant and malignant tumors of the human skin

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Academic dissertation

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ABBREVIATIONS

AK	Actinic keratoses
atRA	All-*trans*-retinoid acid
BCC	Basal cell carcinoma
BD	Bowen's disease
BM	Basement membrane
CDK4	Cyclin dependent kinase 4
CDKN2A	Cyclin dependent kinase-inhibitor-2A
CyA	Cyclosporin
ECM	Extracellular matrix
EMPD	Extramammary Paget's disease
EMT	Epithelial-to-mesenchymal transition
ER-β	Estrogen receptor-β
FasL	Fas ligand
FN	Fibronectin
HB-EGF	Heparin binding epidermal growth factor
HD	Hemidesmosome
HPV	Human papilloma virus
IC	Immunocompetent
IS	Immunosuppressed
KA	Keratoacanthoma
KC	Keratinocyte
KO	Knock-out
LN-5	Laminin-5 (laminin 3,3,2)
MM	Malignant melanoma
MMPs	Matrix metalloproteinases
MMPIs	Matrix metalloproteinase inhibitors
NF-κB	Nuclear factor kappa B
NMSC	Non-melanoma skin cancer
OTR	Organ transplant recipients
$p16^{INK4A}$	Transcript of CDKN2A locus: cyclin-dependent kinase inhibitor 2A
RT-PCR	Reverse transcriptase polymerase chain reaction
SCC	Squamous cell carcinoma
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitors of metalloproteinases
TNF-α	Tumor necrosis factor-alpha
TN-C	Tenascin-C
UV	Ultraviolet

Tiina Kuivanen

Matrix metalloproteinases as biomarkers in premalignant and malignant tumors of the human skin

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ABSTRACT

The incidence of non-melanoma skin cancer is increasing worldwide. Basal cell carcinoma followed by squamous cell carcinoma and malignant melanoma are the most frequent skin tumors. Keratoacathoma is a benign tumor resembling both clinically and histologically squamous cell carcinomas. Extramammary Paget's disease is a rare intraepidermal adenocarcinoma, usually glandular in origin. Immunosuppressed patients have an increased risk of neoplasia, of which non-melanoma skin cancer is the most common. Matrix metalloproteinases (MMPs) are structurally related, zinc-dependent proteolytic enzymes that collectively are capable of degrading virtually all components of the extracellular matrix. MMPs can also process substrates distinct from extracellular matrix proteins and influence cell proliferation, differentiation, angiogenesis, and apoptosis. Under normal physiological conditions MMPs are expressed at low levels, but their expression is induced in diseases, like arthritis, atherosclerosis, blistering dermatoses, chronic wounds, periodontitis, and cancer. MMP activity is regulated by their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs).

In this study, the expression patterns of MMPs, TIMPs, and certain cancer-related molecules were investigated in premalignant and malignant lesions of the human skin. As methods were used immunohistochemisty, *in situ* hybridization, and reverse transcriptase polymerase chain reaction (RT-PCR) from the cell cultures. Our aim was to evaluate the expression pattern of MMPs in extramammary Paget's disease in order to find markers for more advanced tumors, as well as to shed light on the origin of this rare neoplasm. Novel MMP -21, -26, and -28 were studied in melanoma cell culture, in primary cutaneous melanomas, and their metastases. The MMP expression profile in keratoacanthomas and well-differentiated squamous cell carcinomas was analyzed to find markers to differentiate benign keratinocyte hyperproliferation from malignantly transformed cells. Squamous cell carcinomas of immunosuppressed organ transplant recipients were compared to squamous cell carcinomas of matched immunocompetent controls to investigate the factors explaining their more aggressive nature.

We found that MMP-7 and -19 proteins are abundant in extramammary Paget's disease and that their presence may predict an underlying adenocarcinoma in these patients. In melanomas, MMP-21 was upregulated in early phases of melanoma progression, but disappeared from the more aggressive tumors with lymph node metastases. The presence of MMP-13 in primary melanomas and lymph node metastases may relate to more aggressive disease. In keratoacanthomas, the expression of MMP-7 and -9 is rare and therefore should

raise a suspicion of well-differentiated squamous cell carcinomas. MMP-19 and p16 are expressed abundantly in keratoacanthomas, but they disappear from malignant squamous cell carcinomas and thus, could aid in differentiating between these two tumors. MMP-26 staining was significantly stronger in squamous cell carcinomas and Bowen's disease samples of organ transplant recipients and it may contribute to the more aggressive nature of squamous cell carcinomas in immunosuppressed patients. In addition, the staining for MMP-9 was significantly stronger in macrophages surrounding the tumors of the immunocompetent group and in neutrophils of those patients on cyclosporin medication.

In conclusion, based on our studies, MMP-7 and -19 might serve as biomarkers for more aggressive extramammary Paget's disease and MMP-21 for malignant transformation of melanocytes. MMP -7, -9, and -26 seem to play an important role in the pathobiology of keratinocyte derived keratoacanthomas, Bowen's diseases, and squamous cell carcinomas of immunocompetent as well as immunosuppressed patients, and could be interesting MMPs to investigate in future studies.

1. INTRODUCTION

The incidence of skin cancer is continuously rising. Basal cell carcinoma (BCC) followed by squamous cell carcinoma (SCC) and melanoma are the most frequent skin tumors. The role of matrix metalloproteinases (MMPs) in cancer has been intensely studied over the last two decades. The knowledge on MMPs in cancer initiation and growth has greatly benefited from the development of animal models and the use of transgenic and knock-out (KO) mice. The contribution of MMPs in cancer progression is complex and the evidence shows that members of the MMP family may promote or inhibit cancer development. Extracellular matrix (ECM) and stromal cells are important contributors to tumor growth and metastasis and recent studies have shown how stromal MMPs promote cancer progression. The bivalent role of MMPs and the timing of their administration may be the reason for rather disappointing results with synthetic MMP inhibitors in the treatment of various cancers in clinical trials. More research is needed to evaluate the specific functions of various MMPs in tissues in order to provide better targeted therapies. This study aimed to investigate the roles of MMPs in various benign and malignant skin tumors and to shed light on the pathobiology of these lesions. Knowing the precise functions of MMPs in tumors is important for the development of targeted therapies in various cancers.

2. REVIEW OF THE LITERATURE

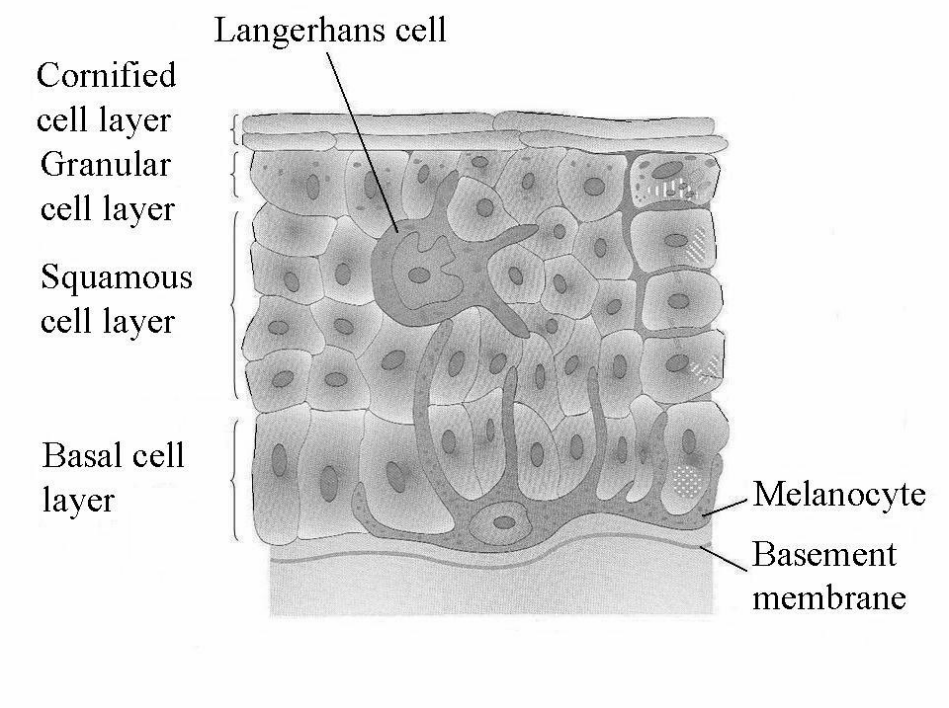
2.1. Structure of the skin

The histology of skin is exceptionally complex. Divided into two different but functionally interdependent layers (epidermis and dermis), the skin is composed of cells with many functions including mechanical- and photoprotection, immunosurveillance, nutrient metabolism, and repair (Murphy 1997). The cutaneous basement membrane (BM) zone separates these two distinct compartments and provides adhesion and a dynamic interface between them.

2.1.1. Epidermis

The epidermis is derived from the ectoderm in the developing embryo and consists of stratified epithelium, which is made up of cells known as keratinocytes. They compose 90% of the epidermal cells, with minority populations of Langerhans cells, melanocytes, and neuroendocrine (Merkel) cells (Khavari 2006). Keratinocytes are arranged into four layers: the basal, the squamous, the granular, and the cornified cell layers. Basal cells are responsible for the mitotic activity and are connected to each other and overlying spinous cells by specific intercellular junctions (Murphy 1997). Intercellular junctions are important for the integrity of the epidermis. Four kinds of cell-cell junctions have been described in the epidermis: desmosomes, adherens junctions, gap junctions, and tight junctions (see Hentula *et al*. 2001). At their base, basal cells are attached to the BM via hemidesmosomes. Keratinocytes move upwards to the surface of the skin and mature from basal cells to spinous, granular, and cornified cells (Figure 1). During differentiation keratinocytes change their shape, get filled with keratin and finally lose their nuclei preceding scaling. Melanocytes are pigment producing cells that are located among basal cells and provide protection against mutagenesis caused by ultraviolet (UV) light. Langerhans cells have antigen-presenting capacity and play an important role in contact sensitization, immunosurveillance against viral infections, and neoplasms of the skin. Merkel cells are present within the basal cell layer and their function is still unclear. They take part in mechanoreception or at least interact with neurons, but little is known about their interactions with other epidermal cells (Boulais and Misery 2007).

Figure 1. The layers of the epidermis.

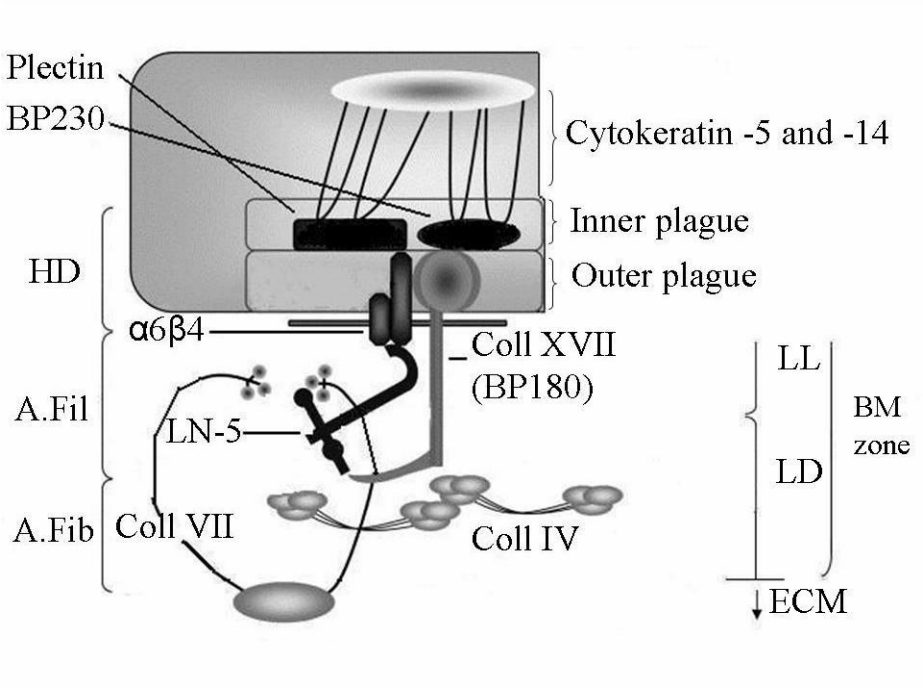


Modified from Oikarinen *et al*. 2003.

2.1.2. Basement membrane

The epidermal BM zone lies between the basal keratinocytes and the dermis. The BM has three main functions: 1) it attaches epithelial cells to the underlying extracellular matrix, 2) it acts as a permeability barrier, and 3) it controls cell organization and differentiation by mutual interactions between cell-surface receptors and molecules in the ECM (Masunaga 2006).
The basal keratinocytes contain small, electron-dense structures called hemidesmosomes (HDs) (McMillan *et al*. 2003). The cytoplasmic part of the HDs can be further divided into the inner and outer plaque (Figure 2). In the BM zone, the electron-lucent area along the basilar surface of keratinocytes is called lamina lucida and the electron-dense area is called lamina densa (Figure 2). Intermediate filaments, consisting of cytokeratins-5 and -14, are inserted into the inner plaque of the hemidesmosome. The transmembrane components of HDs include the integrin α6β4 receptor, which contains transmembrane α and β subunits, and a 180kD bullous pemphigoid antigen (also called collagen XVII), and also intracellular components 230kD bullous pemphigoid antigen and plectin. Anchoring filaments are located within the lamina lucida and connect hemidesmosomes to the lamina densa. Anchoring fibrils are fibrillar structures composed of type VII collagen and they attach the dermis to the BM. They originate at the lamina densa and extend into the dermis (Figure 2) (Masunaga 2006). Lamina densa is composed mainly of type IV collagen and laminin-5 (LN-5).

Figure 2. Epidermal BM zone and some of its proteins.



Abbreviations: α6β4, integrin α6β4; A. Fib, anchoring fibrils; A.Fil, anchoring filaments; BP, bullous pemphigoid antigen; BM, basement membrane; Coll, Collagen; ECM, extracellular matrix; HD, hemidesmosome; LD, lamina densa; LL, Lamina Lucida; LN-5, laminin-5. Modified from Schneider *et al*. 2007.

Laminin-5

Laminins are a family of large extracellular glycoproteins that are abundant in the BM zone and comprise three distinct chains that together assemble into the cross-shaped cruciform molecule (Figure 2) (see McMillan *et al*. 2003). To date, five α chains, three β chains and three γ chains have been discovered and as many as 16 different laminin isoforms with distinct functions have been identified (Marinkovich 2007). Laminins are involved in several important biological processes such as tissue remodeling, wound healing, and tumorigenesis. Laminin-5 (LN-5), also known as laminin 332, is highly upregulated in several types of epithelial tumors including cutaneous, oral, esophageal, laryngeal, tracheal, cervical, and colon carcinomas and its expression correlates positively with tumor invasiveness and poor prognosis (Marinkovich 2007). The secreted form of LN-5 contains 3 α-chains, 3 β-chains and 2 γ-chains. These chains can be proteolytically processed, for example, by plasminogen activators and MMPs. In particular, overexpression of the γ2-chain has been described as a marker of invasion in epithelial cancers (Sadowski *et al*. 2005). LN-5 interacts with two major epithelial intergrin receptors, α3β1 and α6β4, and stimulates migration of various cells, including tumor cells.

MMPs cleave LN-5: MMP-2 and MMP-14 cleave its γ2-chain in mice and MMP-19-dependent processing of the γ2-chains leads to the integrin switch favoring epithelial migration in cell lines. (Giannelli *et al*. 1997; Koshikawa *et al*. 2000; Sadowski *et al*. 2005). MMPs-3, -12, -13, and -20 are able to cleave LN-5 in human cell lines (Pirilä *et al*. 2003).

2.1.3. Dermis

The dermis was previously considered to be a cutaneous layer primarily responsible for taking care of the circulation that served to nourish the epidermis. Today we know that it is a dynamic microenvironment with several cells and matrix proteins with important functions. The dermis is derived from the embryonic mesoderm and divided into the subepithelial papillary dermis where collagen fibers are arranged as finely woven meshwork and the reticular dermis where collagen fibers are united into thick bundles. Fibroblasts, endothelial cells, macrophages, and mast cells are located in the dermis together with emigrant inflammatory cells, neutrophilic, eosinophilic, and basophilic granulocytes, lymphocytes, and plasma cells, derived from blood vessels (Murphy 1997). Hair follicles, sebaceous and sweat glands, and sensory nerves are all embedded in the dermis. The dermis consists primarily of type I and type III collagens, whereas type IV, VII, and XVII collagens are found in the dermo-epidermal junction (Uitto and Pulkkinen 1997).

2.1.4. Extracellular matrix

The interaction of cells with the ECM is critical for the normal development and function of organisms. Cellular growth and migration are critically dependent on turnover and remodelling of the ECM, which is regulated by a fine balance between the synthesis and degradation of ECM proteins. The ECM is a relatively stable structural material that lies under the epithelia and surrounds connective tissue cells. The main protein constituents of the ECM are collagenous and elastic fibres. The fibrillar collagens (types I, II, III, V, XI, XXIV, and XXVII) typically consist of three α-chains containing a variable number of Gly-X-Y repeats that form a tight triple-helix. The α-chains are secreted as precursors with N- and C-terminal propeptides that prevent them from folding prematurely (Ricard-Blum and Ruggiero 2005). The main collagens found in skin are type I, which gives strength, and type III, which gives flexibility to tissues. Various proteoglycans and glycoproteins are important components of the ECM (Wight *et al*. 1991). Proteoglycans (syndecan, versican, aggregan, decorin) consist of a core protein attached by numerous sulfated sugar chains, glycosaminoglycans (hyaluronan, chondroitin and dermatan sulphate, heparan sulphate, and keratan sulphate). Glycoproteins differ from proteoglycans in that their carbohydrate component constitutes a much smaller proportion of the ovarall molecule (Ruoslahti and Vaheri 1974; Cattaruzza and Perris 2006). Fibronectin (FN) is one of the most abundant glycoproteins in the ECM. It is prominent in the matrix of a variety of connective tissues and is most abundant during embryonic development and tissue remodeling, whereas malignantly

transformed cells tend to lack FN production (Amstrong and Amstrong 2000). Cell surface receptors, such as integrin receptors, link the cytoskeleton to the extracellular environment. Most of the components of the ECM are formed by fibroblasts, but also by keratinocytes and other stromal cells, or from the co-operative interactions between these two cell populations. ECM is now recognized as a major component regulating cell activity and is important in proper tissue development, adult tissue maintenance, wound healing, and oncogenesis (Ziober *et al*. 2006).

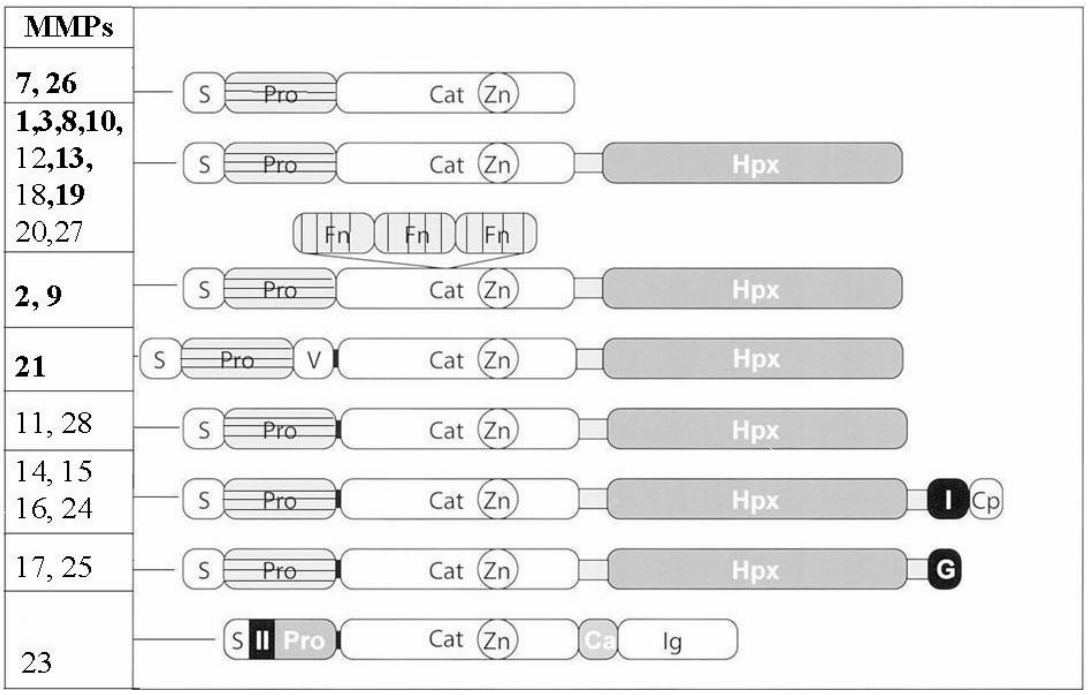
Tenascin-C

Tenascin-C (TN-C) is a star-shaped molecule that is capable of mediating both adhesive and anti-adhesive interactions, as well as binding to certain proteoglycans and FN. TN-C is temporarily upregulated in fetal development and absent or greatly reduced in adult tissues (Orend and Chiquet-Ehrismann 2006). Its expression is associated with morphogenetic events such as wound healing, inflammation, and tumorigenesis. In skin tumors, it is expressed both by transformed keratinocytes and stromal cells, and its expression is upregulated in actinic keratoses (AKs) and SCCs (Dang *et al*. 2006). The effects of TN-C interactions on cellular signalling are mainly unknown. TN-C and MMPs are often co-localized in areas of active tissue remodeling in pathologic conditions, suggesting reciprocal regulation (Kalembeyi *et al*. 2003) and several MMPs, such as MMP-1, -2, -3, -7, -8, -14, -15, and -19, can cleave TN-C *in vitro* (see Kerkelä and Saarialho-Kere 2003).

2.2. Matrix metalloproteinases

Matrix metalloproteinases (MMPs), 23 highly homologous human extracellular zinc-dependent endopeptidases, are known for their ability to cleave several ECM constituents as well as non-matrix proteins (Egeblad and Werb 2002) (Table 1). They mediate tumor angiogenesis, malignant conversion, proliferation, and apoptosis by degrading BMs, cell attachment proteins, and various matrix components, as well as by activating chemokines and growth factors (Egeblad and Werb 2002). MMPs are secreted or anchored to the cell surface, thereby confining their catalytic activity to membrane proteins or proteins within the secretory pathway or extracellular space. They comprise a large family of proteases sharing common structural and functional elements (Figure 3). All MMPs contain a signal peptide, an approximately 80 amino acids long prodomain with consensus sequence PRCXXPD, and a catalytic domain with three conserved histidines in the sequence HEXXHXXGXXH, which ligates zinc at the active center. In addition, MMPs have variable inserts such as a furin-cleavage site insert, fibronectin-like repeats, proline-rich hinge region, hemopexin like C-terminal domain and membrane insertion extension (Figure 3) (Woessner 1998; Ra and Parks 2007).

Figure 3. Structure and subclasses of vertebrate MMPs.



Modified from Visse and Nagase 2003. The domain structure of MMPs: S, signal peptide; Pro, propeptide; Cat, catalytic domain; Zn, active-site zinc; Hpx, hemopexin domain; Fn, fibronectin domain; V, vitronectin insert; I, type I transmembrane domain; II, type II transmembrane domain; G, GPI anchor; Cp, cytoplasmic domain; Ca, cysteine array region; Ig, IgG-like domain. Furin cleavage site marked as a black band between propeptide and catalytic domain.

The structural similarity suggests that MMPs arose by duplications of an ancestor gene. MMPs are secreted as inactive zymogens from inside the cell to the cell surface and into the extracellular environment where they are able to degrade both ECM and non-ECM proteins. Eight distinct structural classes of MMPs exist, which on the basis of their substrate specificity and function can be further divided into six distinct subfamilies of collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs (Figure 3) (Table 1).

Table 1. Common substrates of human matrix metalloproteinases investigated in this study

ENZYME	COMMON SUBSTRATES
Collegenase-1 (MMP-1)	Collagen I, II, III, VII, VIII, X, and XI, aggregan, gelatin, FN, nidogen, TN-C, vitronectin, fibrin, fibrinogen, casein, pro-TNFα, IL-1β, α1-PI, α2-M, proMMP-1, and -2
Collagenase-2 (MMP-8)	Collagen I, II, and III, aggregan, α1-PI, α2-M, fibrinogen, LN-5, TN-C, nidogen, proMMP-8
Collagenase-3 (MMP-13)	Collagen I-IV, IX, X, and XIV, aggregan, fibrillin, FN, gelatin, LN-1, TN-C, osteonectin, serpins, fibrinogen, pro-TNF-α, endostatin, α2-M, casein, proMMPs -9 and -13
Gelatinase A (MMP-2)	Collagen I, IV, V, VII, and X, gelatin, FN, TN-C, fibrillin, osteonectin, decorin, α2-M, LN-5, pro-IL-1β, pro-TNF-α, pro-TGF-β, α1-PI, proMMPs -1, -2, and -13
Gelatinase B (MMP-9)	Collagen I, IV, V, VII, and X, gelatin, FN, TN-C, fibrillin, osteonectin, decorin, α2-M, LN-5, pro-IL-1β, pro-TNF-α, pro-TGF-β, FGFR-1, α1-proteinase inhibitor, proMMPs -1, -2, and -13
Stromelysin-1 (MMP-3)	Collagen III, IV, V, VII, IX, and X, elastin, FN, fibrillin, gelatin, aggregan, LN-1, nidogen, osteonectin, decorin, TN-C, α1-PI, pro-TNFα, E-cadherin, fibrinogen, plasminogen, β-catenin, vitronectin, osteonectin, proMMPs -1, -2, -8, -9, and -13
Stromelysin-2 (MMP-10)	Collagen III, IV, V, IX, X, and XIV, LN-5, elastin, FN, gelatin, aggregan, LN-1, nidogen, fibrinogen, proMMPs -1, -2, -8, and -13
Matrilysin-1 (MMP-7)	Collagen IV, aggregan, elastin, nidogen, gelatin, FN, LN-1, entactin, TN-C, vitronectin, E-cadherin, pro-α-defensin, fibrinogen, α1-PI, pro-TNF-α, plasminogen, decorin, FasL, syndecan-1, HB-EGF, proMMPs -1, -2, and -9.
Matrilysin-2 (MMP-26)	Collagen IV, FN, gelatin, fibrinogen, α1-PI, β-casein, TNF-α-converting enzyme, proMMP-9
MMP-19	Collagen IV, FN, gelatin, LN-1, TN-C, aggregan, LN-5
MMP-21	α1-antitrypsin
Epilysin (MMP-28)	Casein

α1-PI, α1-proteinase inhibitor; α2-M, α2-macroglobulin. Modified from Sternlicht and Werb 2001; Kerkelä and Saarialho-Kere 2003; Folgueras *et al*. 2004; Sadowski *et al.* 2005; Nagase *et al*. 2006.

2.2.1. Collagenases (MMP-1, MMP-8 and MMP-13)

Collagenases are the principal secreted proteinases capable of cleaving types I, II, III, V, and IX collagens and they play a decisive role in remodelling and degradation of the ECM. Collagenases have a multidomain structure consisting of a signal peptide, a propeptide, a catalytic domain, a hinge region, and a hemopexin domain (Figure 3). They cleave fibrillar collagens at the specific site of the α-chain resulting in the generation of ¾ N-terminal and ¼ C-terminal fragments, which can be further degraded by other MMPs (Ala-aho and Kähäri 2005). The three collagenases are: collagenase-1 (MMP-1), collagenase-2 (MMP-8), and collagenase-3 (MMP-13). MMP-1 hydrolyzes type III collagen more rapidly than type I, while MMP-8 shows a slight preference for type I collagen (see Ala-aho and Kähäri 2005; Sternlicht and Werb 2001). Conversely, MMP-13 prefers type II collagen and hydrolyzes this collagen much more rapidly than MMP-1 or MMP-8 (Hasty *et al*. 1987; Knäuper *et al*. 1996a).

MMP-1

Human fibroblast collagenase (MMP-1) was the first vertebrate collagenase purified from the tail of a tadpole (Gross and Lapiere 1962) and cloned and sequenced as the first MMP cDNA from adult skin fibroblasts (see Goldberg *et al*. 1986). MMP-1 is expressed *in vitro* by several cells such as keratinocytes, fibroblasts, endothelial cells, monocytes, macrophages, hepatocytes, chondrocytes, and osteoblasts (Ala-aho and Kähäri 2005). MMP-1 is mainly upregulated during tissue remodelling including embryonic development, wound healing, and different types of malignant tumors, but is undetectable in resting tissues. It degrades collagens as well as other matrix molecules (Table 1) (Pardo and Selman 2005). MMP-1 is also able to cleave cell surface molecules and other non-matrix substrates, for example, antichymotrypsin, antitrypsin and tumor necrosis factor-α (TNF-α), making MMP-1 a multifunctional protein (McCawley and Matrisian 2001).

MMP-8

For a long time MMP-8, also called neutrophil collagenase, was considered to only be expressed in neutrophil precursors during late myeloid maturation, but it is now evident that it is expressed by a variety of cells at different stages of inflammation and in cancer (Balbín *et al*. 2003; Moilanen *et al*. 2002). It is expressed predominantly by polymorphonuclear leukocytes *in vivo*, and by other inflammatory cells, fibroblasts, migrating keratinocytes, and tumor cells in SCCs (Moilanen *et al*. 2002; Pirilä *et al*. 2007). In culture, MMP-8 is expressed by a variety of cells, including inflammatory cells, chondrocytes, fibroblasts, cutaneous keratinocytes, bronchial and oral epithelial cells, and endothelial cells (Van Lint and Libert 2006). The common substrates for MMP-8 are listed in Table 1 (Van Lint and Libert 2006; Pirilä *et al*. 2003).

MMP-13

MMP-13 plays an important role in forceful tissue remodeling as well as in pathological processes such as cancer and arthritis.

It was first cloned from a breast cancer cDNA library (Freije *et al*. 1994). It is produced as an inactive proenzyme and activation by cleavage of the N-terminal propeptide can be carried out by various compounds, including MMP -2, -3, and -14 (Knauper *et al*. 1996a; 1996b). Endothelial cells, fibroblasts, macrophages, epithelial cells, osteoblasts and chondrocytes synthesize MMP-13 by means of different stimuli (Vaalamo *et al*. 1997; Zaragoza *et al*. 2002; Ala-Aho and Kähäri 2005). MMP-13 cleaves fibrillar collagens and a large variety of ECM components in addition to inactivating chemokines and activating pro-transforming growth factor-β3 (TGF-β3) (Ala-Aho and Kähäri 2005) (Table 1).

2.2.2. Gelatinases (MMP-2 and MMP-9)

Interest in gelatinases (MMP-2 and –9) originates from their ability to break type IV collagen, found in the BM (Devarajan *et al*. 1992). They play an important role in angiogenesis as well as tumor invasion and metastasis, and have frequently been associated with poor prognosis (Coussens *et al*. 2000; Egeblad and Werb 2002). In addition to gelatin and type IV collagens, they are responsible for the final degradation of fibrillar collagens after initial cleavage by collagenases. Gelatinases are secreted as inactive pro-forms that are activated extracellularly (Woessner 1991). The FN domains within their catalytic domains are important for elastolytic activity (Shipley *et al*. 1996).

MMP-2

Unlike many other MMPs, MMP-2 is constitutively expressed by a wide range of cell types, including endothelial cells, macrophages and many malignant cells (Chakrabarti and Patel 2005). MMP-2 cleaves several ECM components, growth factors and also proMMP-1, -2, and -13 (Table 1) (McCawley and Matrisian 2001). MMP-2 is detected during cancer invasion (Egeblad and Werb 2002), but in MMP-2 knock-out (KO) mice reduced angiogenesis and tumor growth was detected (Itoh *et al*. 1998).

MMP-9

Constitutive expression of MMP-9 is restricted to neutrophils (Devarajan *et al*. 1992), but MMP-9 is detected in malignant transformation of various cells and is associated with tumor metastasis (Coussens *et al*. 2000; Egeblad and Werb 2002). Inflammatory stimulation can induce MMP-9 expression in several cells, including endothelial cells, macrophages, fibroblasts and mast cells (Coussens *et al*. 2000). MMP-9 participates in the angiogenic switch necessary for tumor development (Bergers *et al*. 2000), although other reports suggest anti-angiogenic effect (Heljäsvaara *et al*. 2005). MMP-9 mostly cleaves the same ECM components as MMP-2 (Table 1). In addition, however, MMP-9 can cleave several non-ECM components, including pro-TGF-β2, pro-interleukin (IL)-1β, cell-surface bound interleukin-2 receptor antagonist (IL-2Ra), plasminogen, α1-proteinase inhibitor and pro-TNF-α (McCawley and Matrisian 2001).

2.2.3. Matrilysins (MMP-7 and MMP-26)

Matrilysins (MMP-7 and MMP-26) are the smallest MMPs. They lack the C-terminal hemopexin domain common to other MMP family members, and they have markedly smaller molecular weights (Wilson and Matrisian 1996).

MMP-7

Matrilysin -1 (MMP-7), first discovered as an enzyme of the involuting rat uterus (Woessner and Taplin 1988), is secreted as a proenzyme and activated by endoproteinases and plasmin through proteolytic removal of the prodomain (Wilson and Matrisian 1996). It has wide-ranging substrate specificity against ECM components (Table 1) (Wilson and Matrisian 1996). MMP-7 activates intestinal crypt α-defensins, which are antimicrobial peptides participating in the innate immune system of the intestine (Wilson *et al*. 1999). It also activates latent forms of other MMPs (proMMP -1, -2, and -9) and plays an important role in ectodomain shedding of cell-surface molecules to promote inflammation and tumor invasion, such as TNF-α precursor (Gearing *et al*. 1995), Fas ligand (FasL) (Powell *et al*. 1999), heparin-binding epidermal growth factor (HB-EGF) (Yu *et al*. 2002), and E-cadherin (Nöe *et al*. 2001). MMP-7 is widely expressed by glandular epithelium in normal adult tissues and its upregulation is associated with cancers of epithelial origin (Kerkelä and Saarialho-Kere 2003), and more recently also with inflammation (Wielockx *et al*. 2004).

MMP-26

MMP-26 (Matrilysin-2) was recently cloned from fetal cDNA (Park *et al*. 2000; Uria and Lopez-Otin 2000). A unique PH81CGVPD cystein-switch distinguishes human MMP-26 from other MMPs (Marchenko *et al*. 2002) and leads to the unorthodox, autolytic mechanisms of the MMP-26 zymogen activation (Zhao *et al*. 2003). MMP-26 primarily accumulates in the intracellular milieu (Park *et al*. 2000; Uría and López-Otín 2000) and efficiently cleaves collagen IV, gelatin, FN, and vitronectin (Marchenko *et al*. 2002), and the non-ECM substrates fibrinogen, inactive serpin (Park *et al*. 2000), and proMMP-9 (Zhao *et al*. 2003). It is upregulated in dysplastic changes in prostatic tissue but downregulated in invasive cancer (Lee *et al*. 2006) and in a similar manner becomes downregulated during the spreading of ductal breast cancers (Zhao *et al*. 2004). Unlike many other MMPs, MMP-26 has an estrogen-response element in the promoter and can be induced in hormone regulated carcinomas (Li *et al*. 2004). It has been linked to inflammation and favorable prognosis in breast cancer patients, so it might also have anti-tumor properties (Strongin 2006).

2.2.4. Stromelysins

Stromelysins degrade various components of the ECM and activate collagenases via the proteolytic removal of a propeptide (Sternlicht *et al*. 2000). Stromelysin-1 (MMP-3) and -2 (MMP-10) are highly structurally and functionally related, whereas stromelysin-3 (MMP-11), in some classifications called stromelysin-like or other MMP, diverges significantly from stromelysin-1 and -2 in amino acid sequence and in enzymatic activity (Birkedal-Hansen 1995).

MMP-3 and MMP-10

MMP-3 was first cloned as a cancer-specific gene (Matrisian *et al*. 1985). It degrades ECM (Table 1), and also activates MMP-9, serpin-type serine proteinase inhibitors, and releases a number of cell-surface molecules, like E-cadherin, L-selectin, HB-EGF, and TNF-α (Sternlicht *et al*. 1999). MMP-3 is expressed by various cells including keratinocytes, fibroblasts and chondrocytes (Kerkelä and Saarialho-Kere 2003) and acts as a natural tumor promoter in mammary carcinogenesis (Sternlicht *et al*. 2000). MMP-10 was originally identified in an adenocarcinoma cDNA libary (Muller *et al*. 1988). It has a structure and substrate specificity similar to that of MMP-3, although with a lower proteolytic efficiency and is expressed in migrating keratinocytes, enterocytes and epithelial tumor cells (Vaalamo *et al*. 1998; Rechardt *et al*. 2000).

2.2.5. Other MMPs (MMP-19, -21, and -28)

MMP-19

MMP-19 was first cloned from human mammary gland (Cossins *et al*. 1996) and liver cDNA libraries (Pendas *et al*. 1997). On the basis of its structural characteristics, chromosomal location, and expression pattern it was proposed to be a member of a new MMP subfamily (Pendas *et al*. 1997). MMP-19 has an unusual expression pattern compared to other MMPs. It is detected in many normal adult tissues like placenta, lung, pancreas, ovary, spleen, intestine, and basal keratinocytes (Murphy *et al*. 1999). MMP-19 is upregulated in suprabasal and spinous layers in psoriasis, eczema and tinea, (Sadowski *et al*. 2003), but becomes downregulated in invasive carcinomas (Impola *et al*. 2005). Recent results in MMP-19-knock out (KO)- mice indicated that an increased early angiogenic response and increased tumor invasion were associated with MMP-19 deficiency (Jost *et al*. 2006). MMP-19 degrades components of the BM, connective tissue, and cartilage (Table 1) (Stracke *et al*. 2000).

MMP-21

MMP-21 is the last cloned human MMP (Ahokas *et al*. 2002; Marchenko *et al*. 2003). It has all the typical features of an MMP family member: pro-, catalytic-, and hemopexin domains with signal sequence. Additionally, MMP-21 has a furin activation sequence (Ahokas *et al*. 2002). It is most closely related to MMP-17, -23, and -25 (Ahokas *et al*. 2002). The only known physiological substrate for MMP-21 is α1-antitrypsin (Ahokas *et al*. 2002; Marchenko *et al*. 2003). Using reverse transcriptase polymerase chain reaction (RT-PCR), MMP-21 mRNA has been found in various normal adult tissues like the kidney, brain, lung, testis, ovary, and colon as well as several cancer cell lines (Ahokas *et al*. 2002). *In vivo*, it has been detected in BCCs and SCCs (Ahokas *et al*. 2002; 2003). MMP-21 is upregulated in primary keratinocyte culture by TGF-β1 (Ahokas *et al*. 2003) and is a putative target of β-catenin transactivation in promoter analysis (Marchenko *et al*. 2003).

MMP-28

MMP-28 (epilysin) was originally cloned from human keratinocyte and testis cDNA libraries (Lohi *et al*. 2001), and also from a lung cDNA library (Marchenko and Strongin 2001). The structure of epilysin consists of classical MMP domains: the signal sequence, pro-, catalytic-, and hemopexin domains followed by specific furin activation sequence and it's most closely related to MMP-19 (Lohi *et al*. 2001). MMP-28 is widely expressed in several normal and malignant tissues like the testis, lung, heart, and gastrointestinal tract as shown by RT-PCR (Lohi *et al*. 2001; Marchenko and Strongin 2001), but is downregulated in colon cancer (Bister *et al*. 2004). The presice function, physiological substrates, regulation, and specific epilysin-producing cells, except for keratinocytes, are still mostly unknown (Saarialho-Kere *et al*. 2002; Illman *et al*. 2006). Recombinant epilysin degraded casein in a zymography assay (Lohi *et al*. 2001) and it induced TGF-β1 mediated epithelial to mesenchymal transition (EMT), important in cancer progression particularly, in lung carcinoma cells (Illman *et al*. 2006).

2.3. Regulation of MMPs

2.3.1. Introduction for the regulation of MMPs

Formerly MMPs were thought to function mainly as enzymes that degraded structural components of the ECM. Recent reports, however, have demonstrated that MMPs act on non-matrix substrates as well, such as cytokines, chemokines, growth factors, cell surface receptors, and adhesion molecules (Chakraborti *et al*. 2003). MMPs can affect cell behavior in many ways: the cleavage products of MMPs signal in an autocrine or paracrine manner, they cleave intercellular junctions or the BM regulating epithelial tissue architecture and they activate the action of latent and deactivate the action of active signalling molecules (Ra and Parks 2007). Cell-ECM interactions trigger cellular signalling that promotes cell differentiation, migration, and mobilization, essential for normal cellular homeostasis. With the exceptions of MMP-2, -7, -19 and -28, which are constitutively expressed in normal

tissues, most MMPs are induced only in repair or remodelling processes or in response to various diseases or inflammation (Ra and Parks 2007). Regulation of MMP activities can be achieved at multiple levels: transcriptional and posttranscriptional regulation, proenzyme activation, compartmentalization, enzyme inactivation, and availability and affinity of substrates (Nagase and Woessner 1999).

2.3.2. Transcriptional and post-transcriptional regulation

Transcriptional regulation

MMP gene promoters have several *cis*-elements like TATA box, activator protein-1 (AP-1) binding site, polyoma enhancer A binding protein-3 (PEA3)-binding site, GC box, and nuclear factor (NF)-κB binding site that can regulate MMP gene expression by various *trans*-activators like AP-1, PEA3, Sp-1, β-catenin/T-cell factor-4 (Tcf-4), and NF-κB (Yan and Boyd 2007). MMPs have been recently sorted into three groups on the basis of the mechanism regulating their expression, which could be an important viewpoint in guiding the development of drugs (Yan and Boyd 2007). Group 1 contains the TATA box and AP-1 binding site (MMP-1, -3, -7, -9, -12, -13, -19, and -26), group 2 the TATA box without an AP-1 binding site (MMP-8, -11, and -21), and group 3 lack both AP-1 binding site and the TATA box (MMP-2, -14, and -28). All MMP promoters contain multiple elements, such as Sp-1 and NF-κB that either induce or repress gene expression. The presence of the AP-1 binding site in most MMPs sensitizes them alone or in cooperation with PEA3 to *trans*-activators and to a large variety of cytokines and growth factors (Chakraborti *et al*. 2003; Yan and Boyd 2007). The effect of specific growth factors varies in different MMP genes, for example the TGF-β1 suppresses the expression of MMP-1 and MMP-3, but induces the expression of MMP-13 and MMP-10 (Uria *et al*. 1998; Wilkins-Port and Higgins 2007). Nuclear NF-κB levels are regulated by an inflammatory cytokine-activated pathway (Mercurio and Manning 1999) and NF-κB-binding site, located in the human MMP-9 promoter, exposes MMP-9 gene to tumor necrosis factor-α (TNF-α). Functional polymorphisms in promoters derived from nucleoside insertions, substitutions, or microsatellite instability also regulate MMP gene expression by enhancing or reducing promoter activity (Ye 2000). For example, the invasiveness of cutaneous malignant melanoma is influenced by a variation in the MMP-1 gene promoter and a polymorphism at the MMP-3 promoter region may be associated with unfavorable prognosis in breast cancer patients (Ye *et al*. 2001; Ghilardi *et al*. 2002).

Post-transcriptional regulation

Post-transcriptional mechanisms are also involved in the control of MMP expression, for example TGF-β1 increases MMP-2 and -9 levels by extending the half-life of MMP mRNAs in human gingival fibroblasts (Overall *et al*. 1991) and induces MMP-11 expression by both inducing transcription and stabilizing the transcript in mouse osteoblasts and fibroblasts (Delany and Canalis 2001).

Several pharmacological reagents, including doxycycline, all-*trans* retinoic acid, 13-*cis*-retinoic acid, and 1α25-dihydroxyvitamin D(3), regulate MMP expression by affecting mRNA stability (Yan and Boyd 2007).

2.3.3. Proenzyme activation

Cysteine switch and proenzyme activation

MMPs are produced as secreted or membrane-bound proenzymes or zymogens, which become activated by removal of the NH_2-terminal propeptide (Nagase and Woessner 1999). The interaction of the conserved cysteine in the propeptide with the catalytic Zn^{2+} ion seals the catalytic activity and keeps the proMMP in an inactive state. A mechanism common to all MMPs is called the cystein-switch mechanism (Van Wart and Birkedahl-Hansen 1990). Once the propeptide is removed, the Zn^{2+} ion becomes available for the binding of a substrate due to a conformational change. MMPs are activated by proteolytic removal of the propeptide domain. This activation can be mediated by: 1) cleavage of a prodomain by proteases (furin, MMPs, plasmin), 2) reduction of free thiol by oxidants or by heavy metal ions, and disulphides, or 3) allosteric activation of zymogen (Van Wart and Birkedahl-Hansen 1990; Fu *et al*. 2008). In allosteric activation, the prodomain cleavage is not necessary for zymogen activation and only disruption of the zinc-thiol interaction is absolutely required (Fu *et al*. 2008). Furin is a serine protease located in intracellular *trans*-Golgi network and those MMPs (all MT-MMPs, MMP-11, and MMP-28) possessing a furin cleavage site are processed intracellularly before secretion (Thomas 2002; Nagase *et al*. 2006). Plasmin, generated from plasminogen through the action of urokinase-type plasminogen activator (uPA), can activate proMMP-1 and -3 (Nagase *et al*. 2006). *In vitro*, a number of MMPs can cleave the prodomain of other MMP zymogens leading to activation and it has been suggested that they mediate the final proteolytic step to produce a fully active enzyme (Nagase 1997). The relevance of this mode of activation, *in vivo*, is uncertain since MMPs might need other compounds to work with, such as serine proteinases, other MMPs, aspartate proteinases, or cysteine proteinases. For example, proMMP-2 activation requires cooperative action of both MMP-14 and TIMP-2 (Strongin *et al*. 1995; Ra and Parks 2007) and activated MMP-2 and -13 can both activate proMMP-9 (Fridman *et al*. 1995; Knäuper *et al*. 1997). Oxidants, such as hypochlorous acid (HOCl), a product of neutrophil myeloperoxidase, and hydroxyl radicals activate *in vitro* proMMP -1, -7, and -9 (Michaelis *et al*. 1992; Fu *et al*. 2001). The *in vivo* role of oxidants has not been established. Recent studies, however, indicate that the pericellular production of HOCl by phagocytes provides a physiological mechanism for regulating both the activation and inactivation of MMPs within an inflammatory setting (Fu *et al*. 2001; 2003).

Compartmentalization

Compartmentalization means where and how in the pericellular environment MMP is released and held and is important for regulating the specificity of proteolysis and the affinity of the enzyme-substrate interaction (Ra and Parks 2007). A significant overlap exists in the

substrates MMPs can cleave and specific enzymes cleave some substrates more efficiently than others (Sternlicht and Werb 2001). MMPs are secreted and anchored to the cell membrane, thereby targeting their catalytic activity to specific substrates within the pericellular space. MMPs are attached to cells via specific interaction to membrane proteins, and determining these anchors will lead to identifying activation mechanisms and pericellular substrates. In addition to membrane-bound MMPs, various specific cell-MMP interactions have been reported, such as the binding of MMP-2 to the αvβ3 integrin (Brooks *et al.* 1996), MMP-9 to CD44 (Yu and Stamenkovic 2000), and MMP-7 to surface proteoglycans (Yu and Woessner 2000).

2.3.4. Inhibition of MMPs

The proteolytic activity of MMPs is under tight control by specific inhibitors. In plasma, the general protease inhibitor α_2-macroglobulin is the predominant inhibitor, whereas the tissue inhibitors of metalloproteinases (TIMPs) are considered to be the main inhibitors in tissue. Direct endocytosis also plays a role in silencing of MMPs (Ra and Parks 2007).

Tissue inhibitors of MMPs

TIMPs comprise a family of four (TIMP-1, -2, -3, and -4) proteins that inhibit MMP activity by binding to the catalytic site of these enzymes in a 1:1 stoichiometric fashion. They have N- and C-terminal domain, which each have three conserved disulfide bonds (Nagase *et al*. 2006). The N-terminal domain folds wedge-like as an independent unit and slots into the active site cleft of MMP in a manner similar to that of the substrate (Visse and Nagase 2003; Nagase *et al*. 2006). TIMPs are multifunctional proteins that, in addition to their MMP inhibitory effect, can regulate apoptosis, inflammation and cell proliferation (Sternlicht *et al*. 2001). Their diminished or increased expression has been reported in various cancers, dependent on the tumor type (Chirco *et al*. 2006). Whereas TIMP-2 expression is constitutive and widely expressed throughout the body, TIMP-1, -3, and -4 expression is inducible and often tissue specific (Chirco *et al*. 2006). TIMP-1 can be resistant to apoptosis, whereas TIMP-2 and -3 promote cell death (Lambert *et al*. 2004). Unbalanced activities of MMPs and TIMPs are associated with pathological conditions. Individual TIMPs differ in their ability to inhibit various MMPs. TIMPs inhibit all MMPs tested so far *in vitro*, except TIMP-1 is a poor inhibitor for MT1,3,5-MMPs and MMP-19. The therapeutic use of TIMPs also in cancer treatment is still in its infancy, since TIMPs possess other important biological capacities.

Other natural MMP inhibitors and endocytosis

Several other known or suspected inhibitors of MMPs exist. An important role is played by α_2- macroglobulin in the irreversible clearance of MMPs in tissue fluids, while α_2-macroglobulin/MMP complexes are removed by scavenger receptor-mediated endocytosis of macrophages (Visse and Nagase 2003).

Protein subdomains are another recently recognized class of MMP inhibitors. The procollagen C-terminal proteinase enhancer protein (PCPE) functions as an MMP inhibitor *in vitro* (Mott *et al*. 2000) and RECK (reversion-inducing cystein rich protein with Kazal motifs), a membrane-bound glycoprotein, inhibits MMP-2, -9, and -14 (Oh *et al*. 2001). Inhibitors of MMP gene expression, like TGF-β1, glucocorticoid hormones, and vitamin A analogues (retinoids) can diminish transcription of MMPs (Egeblad and Werb 2002). MMPs are also regulated via their own proteolytic inactivation and physical clearance (Sternlicht *et al*. 2001). Little is known about the autoproteolysis of active MMPs, while endocytosis at the cell surface clearly could be used directly to silence MMPs. For example, MMP-13 is rapidly internalized after it binds to an MMP-13 specific receptor on various cell types via low density lipoprotein receptor-related protein (LRP) (Barmina *et al*. 1999).

Synthetic MMP inhibitors

Several agents have been developed to block the synthesis of MMPs (Egeblad and Werb 2002). Clinical trials using synthetic MMP inhibitors (MMPIs) have proved disappointing in cancer treatment and none of the developed drugs have passed yet (Fingleton 2008). The presence of a zinc-binding group in MMPIs is essential for chelating the catalytic Zn^{2+} ion. Several different groups of MMPIs have been developed and tested: peptidomimetic MMP inhibitors, non-peptidic inhibitors, tetracycline derivatives, and bisphosphonates (Vihinen and Kähäri 2005). They may inhibit tumor growth by developing a fibrotic capsule around the tumor, by stabilizing cell-cell contacts (Ho *et al*. 2001) and by inducing apoptosis (Nelson *et al*. 2000). Peptidomimetic MMPIs mimic the cleavage sites of MMP substrates and include Batimastat and Marimastat. Batimastat cannot be administered orally and was soon replaced in clinical trials by the oral analog, Marimastat, which inhibits the activity of MMP-1, -2, -3, -7, -9, and -12 and has shown improved survival in pancreatic and gastric carcinoma (Egeblad and Werb 2002; Vihinen and Kähäri 2005). The non-peptidomimetic MMPIs like Primomastat and BAY12-9566 have not yet proven to be beneficial in cancer treatment. Tetracycline derivatives like Metastat, which not only inhibit MMPs, but decrease their production, have shown benefit in patients with Kaposi's sarcoma (Vihinen *et al*. 2005). Bisphosphonates inhibit the enzymatic activity of MMPs and have shown beneficial, but also adverse, effects in several cancers (Vihinen *et al*. 2005). Various factors, like the lack of selectivity, mechanism of MMPI activity, trial design, and side effects have caused the failure of MMPIs in clinical trials (Egeblad and Werb 2002). In the future, it might be important to target MMPs early in cancer progression or their specific functions and to remember that they could also be used as adjuvant drugs in combination with other treatments. The identification of MMPs that favor the host instead of the tumor is an important aspect for future clinical drug trials (Lopez-Otin and Matrisian 2007).

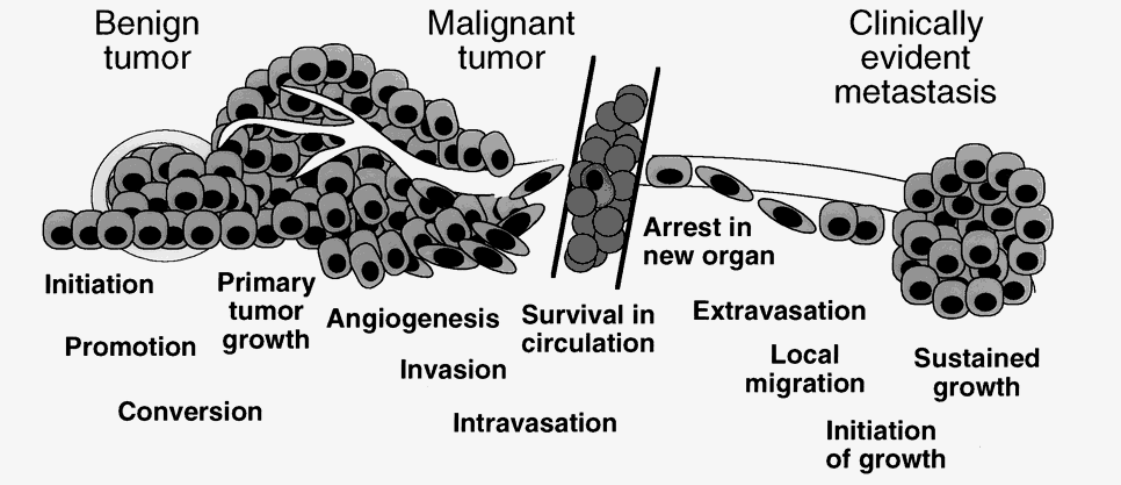
2.4. Cancer

2.4.1. Tumorigenesis and invasion

Tumor growth

Cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis. Cancer progression is a multistep process that includes various alterations in cellular physiology, such as production of autocrine growth signals, insensitivity to growth-inhibitory signals, escape from apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion, and metastasis (Hanahan and Weinberg 2000) (Figure 4). Each step is critical for the development of a tumor that can later metastasize. These steps are regulated through interactions of many genes, including MMPs. Mutations associated with the development of tumors alter structural and functional properties of proto-oncogenes and lead to activation of oncogenes, such as growth factors, growth factor receptors, signal transducers, transcription factors, and regulators of apoptosis (programed cell death) (Langley and Fidler 2007). The development of a malignant tumor requires both oncogene activation and tumor suppressor inactivation. For example, the tumor suppressor gene p53 has critical roles in cell-cycle arrest, apoptosis, cellular senescence and differentiation. Somatic p53 mutations occur in about half of all human cancers (Hollstein *et al*. 1996) and the inactivation of p53 leads to rapidly growing tumors containing few apoptotic cells (Symonds *et al*. 1994). The formation of new blood vessels (angiogenesis) is essential for tumor growth. The induction of angiogenesis is a consequence of an imbalance between multiple inhibitor and stimulator molecules and referred to as the angiogenic switch (Bergers and Benjamin 2003). The activators of angiogenesis include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and epidermal growth factor (EGF). The inhibitors of angiogenesis include thrombospondin, angiostatin, endostatin, and tumstatin (Bergers and Benjamin 2003). MMPs can contribute to tumor growth either directly, through processing of several growth factors such as TGF-β and fibroblast growth factor (FGF) (Peschon *et al*. 1998), or indirectly by regulating proliferative signals through integrins (Agrez *et al*. 1994). MMPs can regulate tumor growth by promoting or inhibiting apoptosis (Egeblad and Werb 2002), *e.g.* MMP-7 generates a soluble form of FasL (Powell *et al*. 1999). MMPs also contribute to angiogenic regulatory balance, MMP-9 acts by increasing the bioavailability of pro-angiogenic VEGF (Bergers *et al*. 2000). MMPs may also participate in the inhibition of neovascularization by converting plasminogen to angiostatin, which is another potent antiangiogenic protein (Cornelius *et al*. 1998). They also release fragments of ECM that are anti-angiogenic, like MMP-9 that releases a fragment from type IV collagen, called tumstatin (Martin and Matrisian 2007).

Figure 4. The cellular changes in tumor progression.



Modified from Nelson *et al*. 2000.

Invasion and metastasis

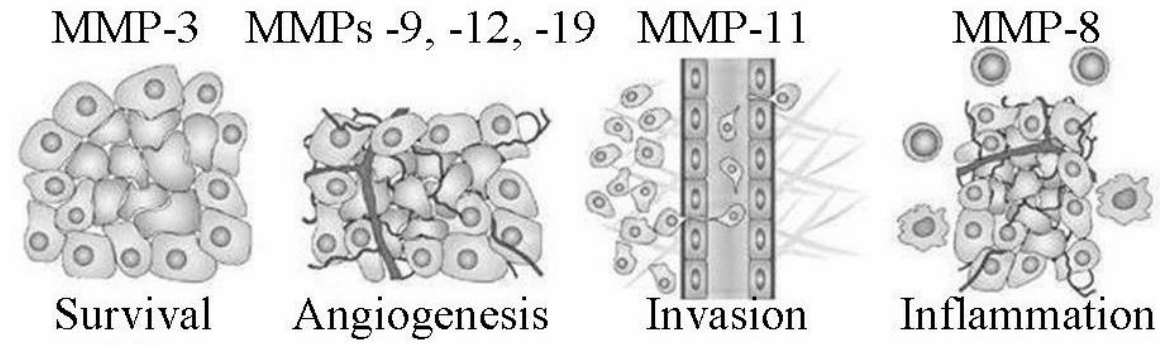
During invasion and metastasis cancer cells must complete sequential steps: 1) degrade the BM zone and penetrate into the interstitial stroma, 2) penetrate into blood and lymphatic vessels, and 3) invade from the bloodstream to target organs and undergo expansive growth (Woodhouse *et al.* 1997). Over 90 % of all human neoplasms arise in epithelia that are bound by an underlying BM. BM invasion is a critical determinant of malignancy in most epithelial cancers, including those of the skin, breast, prostate, lung, and kidney (Friedl and Wolf 2003). Cancer cell-migration is typically regulated by integrins, cell-cell adhesion molecules, and MMPs. Carcinoma cells shift the integrin expression present in normal epithelium to those intergrins (*e.g.* α6β4, αVβ3, αVβ6) that facilitate invasion and metastasis (Guo and Giancotti 2004). E-cadherin, a cell-to-cell adhesion molecule, acts as a tumor suppressor of invasion and metastasis, since its function is lost in a majority of epithelial cancers (Perl *et al*. 1998). Invasive tumor cells penetrate the BM zone using laminins and integrin receptors, and BM zone degrading enzymes, gelatinases (MMP -2 and - 9), which are all typically overexpressed in cancers with poor prognosis (Liotta *et al*. 1980; Sternlict and Werb 2001; Cukierman *et al*. 2001; Rabinovitz *et al*. 2001). TIMP-1 expression correlated both with suppression or inhibition of metastasis in experimental and spontaneous metastasis models (Khokha 1994; Watanabe *et al*. 1996). Extracellular matrix metalloproteinase inducer (EMMPRIN) is a cell surface glycoprotein and highly expressed on the surface of tumor cells and stimulates adjacent fibroblasts or tumor cells to produce matrix metalloproteinases. EMMPRIN has been reported to induce several MMPs, including MMP-1, -2, -3, -9, -14, and -15 (Nabeshima *et al*. 2006).

MMPs in tumor suppression

Recent studies have shown that several members of the MMP family (MMP -8, -12, and -26) provide a protective effect at different stages of cancer progression (Lopez-Otin and Matrisian 2007) (Figure 5). Other protumorigenic MMPs (-3, -9, -11, and -19) might function as protective enzymes in some specific situations (Figure 5). Animal models have provided new evidence for MMP research. In the vast majority of cases, MMP-null-animals have been made as constitutive knock-outs (KO), that is they are genetically deficient in the relevant enzyme. Surprisingly, only MMP14-KO-mice show a significant phenotype related to total lack of the enzyme (Holmbeck *et al*. 1999). To reveal aberrant phenotypes in other KO-mice they are challenged using wounding, tumor inoculation, phorbol myristate acetate (PMA) pretreatment, *etc*. Either disease induction or reduction of various types of cancer as well as inflammatory conditions predisposing to cancer has been observed in several of the MMP-KO-mice (Table 2). The incidence of skin tumors strongly increases in MMP-8 deficient mice and loss of MMP-8 causes profound abnormalities in the inflammatory response induced by carcinogens leading to sustained inflammation (Balbin *et al*. 2003). MMP-3 accelerates the rate of apoptosis, and in the skin of MMP-3 deficient mice, squamous cell carcinomas (SCCs) are less differentiated and grow faster (McCawley *et al*. 2004).

MMP-26 expression is strongly induced in different hormone-regulated carcinomas and associated with favorable clinical outcome in breast cancer patients (Savinov *et al*. 2006). Both MMP-8 and -26 might take part in an anti-tumor inflammatory response that contributes to better clinical prognosis in patients with certain cancers (Savinov *et al*. 2006). Other studies showed that human papilloma virus (HPV)-16-induced carcinomas in MMP9-null-mice are more aggressive and MMP-9 inversely correlates with liver metastasis in patients with colorectal cancer (Takeha *et al*. 1997; Coussens *et al*. 2000). The decreased suspectibility of MMP19-KO-mice to develop chemically induced skin tumors suggests that MMP-19 might promote tumor growth (Pendas *et al*. 2004), although MMP-19 has also been reported to inhibit tumoral angiogenesis (Jost *et al*. 2006). Table 2 shows several examples of MMPs with dual roles in cancer.

Figure 5. The protective roles of MMPs in tumor progression.



Modified from Lopez-Otin and Matrisian 2007.

Table 2. Cancer and inflammatory response phenotypes of KO or transgenic mice deficient of MMPs.

MMP-1 (TG)	Increased chemical skin carcinogenesis with MMP-1 overexpression
MMP-2 (KO)	Suppressed tumor-induced angiogenesis, decreased ductal invasion in the mammary gland, melanoma growth, and lung and pancreatic carcinoma metastasis, reduced skin tumor progression
MMP-3 (KO)	Less differentiated and faster growing SCCs, fewer chemically induced breast tumors
MMP-7 (KO)	Reduced intestinal and pancreatic neoplasia, reduced skin tumor progression
MMP-8 (KO)	Increased skin carcinogenesis, delayed neutrophil recruitment to dermis surrounding skin tumors
MMP-9 (KO)	Reduced skin and pancreatic carcinogenesis, reduced experimental metastasis, more aggressive HPV16-induced tumors, reduced angiogenesis
MMP-10 (KO)	Increased inflammation and increased mortality in response to infection or wounding
MMP-11 (KO)	Reduced mammary carcinogenesis, decreased tumor cell survival and growth, increased number of metastasis
MMP-12 (KO)	Increased lung tumor growth, increased angiogenesis
MMP-19 (KO)	Decreased susceptibility to chemically induced skin tumors, earlier onset of tumoral angiogenesis
MMP-28 (KO)	Increased inflammatory response

TG, transgenic; KO, knock-out; Based on Balbin *et al*. 2003; Folgueras *et al.* 2004; Pendas *et al*. 2004; Handsley and Edwards 2005; Overall and Kleifeld 2006; Lopez-Otin and Matrisian 2007; Page-McCaw *et al*. 2007; Fingleton 2008

2.4.2. Cancer and inflammation

The infiltration of leukocytes into solid tumors was noticed over a century ago and it has become evident that early inflammatory responses lie at the basis of neoplasms (Van Kempen 2006). In normal tissue injury the inflammatory response is downregulated upon re-epithelialization, but in cancer, however, it seems to be chronic, with persistent inflammatory cell recruitment and high levels of cytokines/chemokines (Mueller 2006). In animal models, mast cells, neutrophils, and macrophages are contributors to the progression of cancer (Coussens and Werb 2001). The same phenomenon is also detected in humans (Mueller 2006). Progression of malignant and invasive SCCs is associated with enhanced and persistent recruitment of neutrophils and macrophages. Mast cells, on the other hand, might

have an important role in the initial stages of tumor development (Mueller 2006). Neutrophils promote angiogenesis and tumor invasion by generating reactive oxygen and nitrogen, and secreting MMP-9 (Vosseler *et al*. 2005; Van Kempen *et al*. 2006). Macrophages are differentiated in the vicinity of tumors into tumor-associated macrophages (TAMs), which promote tumor progression by secreting pro-inflammatory cytokines, chemokines, as well as angiogenic factors and MMPs (Lewis and Pollard 2006). Inflammatory cell infiltration might be a protective sign in certain tissues or microenvironments (Takeha *et al*. 1997). The pro-inflammatory transcription factor NF-κB, links chronic inflammation and cancer by preventing apoptosis and by stimulating production of pro-inflammatory cytokines (Pikarsky *et al*. 2004). In neoplastic tissues, chronic availability of TNF-α has been associated with invasion and survival of neoplastic cells (Balkwill 2002). TNF-α null-mice could not develop benign or malignant skin tumors, thus, this pro-inflammatory cytokine could be important in the early stages of tumor promotion (Moore *et al*. 1999).

2.4.3. Premalignant lesions of the skin

Actinic keratoses (AK) and Bowen's disease (BD)

Actinic keratoses (AKs) are rough, erythematous, scaly patches on chronically sun-exposed skin. They are the most common premalignant lesions in humans, the prevalence varying from 11-25% of the population in the northern hemisphere and up to 50% of the population in Australia over the age of 40 years (Sober and Burstein 1995; Quaedvlieg *et al*. 2006). AKs may have three evolutionary possibilities: 1) spontaneous clearing, 2) persistence, and 3) progression into invasive SCC at the rate of 0.025-20% (Callen *et al*. 1997; Glogau 2000; Cassarino *et al*. 2006a). The risk factors for AKs include fair skin, excessive sun exposure, aging, outdoor work, immunosuppressive medication, and the clinical features of the lesion like induration, diameter, rapid enlargement, bleeding, erythema, and ulceration (Salasche 2000; Quaedvlieg *et al*. 2006). Several histopathological variants of AKs have been described, such as the pigmented, proliferative, atrophic bowenoid, acantholytic, or hyperplastic type. All are characterized by atypical keratinocyte proliferation involving the lower portions of the epidermis with overlying parakeratosis. Hyperchromaticity of nuclei and mitotic figures are present (Cockerell 2000; Cassarino *et al*. 2006a). Mutated p53 plays a key role in their pathogenesis (Leffell 2000). Some AKs (hypertrophic and proliferative) have been reported to transform at a higher rate to SCC, but any of the variants can potentially lead to any type of invasive SCC (Cassarino *et al*. 2006a).

Bowen's disease (BD) is an intraepidermal *in situ* SCC, which appears as a slowly progressive, scaly, sharply demarcated, erythematous plague on sun-exposed skin surfaces (Sober and Burstein 1995). Genital lesions with the same histology as BD include erythroplasia of Queyrat in males, vulvar intraepithelial neoplasia in females, and bowenoid papulosis in both sexes (Cox *et al*. 2007).

BD primarily affects older persons (Thestrup-Pedersen *et al*. 1988), and the etiological factors include chronic sun exposure, carcinogens *e.g.* arsenic, immunosuppression, HPV infection, male sex, and chronic injury (Kossard and Rosen 1992; Sober and Burstein 1995; Bordea *et al*. 2004; Cox *et al*. 2007). It may develop into invasive SCC in 3-5% of the patients (Sober and Burstein 1995). The clinical differential diagnosis includes psoriasis, basal cell carcinoma (BCC), nummular eczema, AK, and Paget's disease (Cassarino *et al*. 2006a). Histologically BD demonstrates full thickness intraepidermal keratinocyte atypia with mononuclear inflammatory infiltrate in the dermis (Sober and Burstein 1995). Recently, few morphological variants have been recognized, including clear cell, Pagetoid, and pigmented forms (Cassarino *et al*. 2006a).

Keratoacanthoma (KA)

Keratoacanthomas (KAs) are rapidly growing hyperkeratotic papules or nodules arising from follicles and pilosebaceous units. They resemble SCC, but rapid progression and frequent swift resolution distinguishes them from SCC (Schwartz 2004). KA tends to occur on areas most prone to sunlight exposure and more often in older patients (Mantegna and Iuculano 1995). Some cases are associated with genetic syndromes, like those of Ferguson-Smith, Muir-Torre, and Grzybowski (Weedon 2002a; Schwartz 2004; Ponti and Ponz de Leon 2005). Other risk factors include carcinogens *e.g.* tar, trauma, immunodeficiency, and HPV (Forslund *et al*. 2003; Schwartz 2004). Histologically KA is characterized by having a central keratin plug, basaloid layer in proliferating endophytic lobules, large cells with paler eosinophilic cytoplasm, lack of anaplasia, and a sharp outline between tumor nests and the stroma (Weedon 2002a). Some authors believe that KA is a well-differentiated variant of SCC representing a range of neoplastic activity with no clear distinction, while some consider the histological and cytological differentiation precise, sure, and possible (Strieth *et al*. 2002; Schwartz 2004; Boukamp 2005). Practically all these tumors are currently surgically excised (Goldberg *et al*. 2004). The expression of adhesion molecules, such as E-cadherin/β-catenin, and syndecan-1 have recently been reported to distinguish KA from SCC (Mukunyadzi *et al*. 2002; Papadavid *et al*. 2002). The staining pattern of KA for p53 resembles that of grade I SCC, but they show different chromosomal aberrations (Clausen *et al*. 2002).

2.4.4. Malignant lesions of the skin

Basal cell carcinoma (BCC)

The incidence of skin cancer is continuously rising in Finland. The total number of cases is about 8000/year. The most frequently encountered skin cancers are basal cell carcinoma (BCC), melanoma, and SCC accounting for 1800 cases/year (Cancer in Finland 2006). BCC is the most common cancer in individuals with fair skin and its incidence is continuously increasing (Diepgen and Mahler 2002). Although BCC is a malignant tumor, it is generally only locally invasive and rarely metastasizes (Spates *et al*. 2003). The development of BCC is linked to genetic factors, including the individual skin phototype (skin type I and II), as well as the cumulative exposure to solar ultraviolet B (UVB) radiation (Boukamp 2005).

Other risk factors include male sex, older age, chronic sun exposure, ionizing radiation, chemical carcinogens like arsenic and tars, and immunosuppression (Diepgen and Mahler 2002; Tilli *et al*. 2005). The majority of BCCs are sporadic tumors, while familial cases associated with certain hereditary syndromes, like Gorlin syndrome (nevoid basal cell carcinoma syndrome), are less common. At the molecular level, BCCs are characterized by aberrant activation of the hedgehog signaling pathway genes, usually due to mutations either in the Patched (ptch), sonic hedgehog (Shh), or smoothened (smo) genes (Athar *et al*. 2006). In addition, about half of the cases carry mutations in the p53 tumor suppressor gene, which are often UVB-associated C-->T transition mutations (Reifenberger *et al*. 2005). Clinically, BCCs may show a high degree of phenotypical variability and generally occur on sun-exposed areas such as the head and neck (Tilli *et al*. 2005). Histologically they are composed of uniform cells with darkly stained nuclei and they often form typical palisading structures (Brenn and McKee 2005). They can be classified into three different groups: superficial, nodular, and morpheaform (or sclerosing).

Squamous cell carcinoma (SCC)

Cutaneous squamous cell carcinoma (SCC) can be defined as a malignant proliferation of keratinocytes of the spinous layer of the epidermis. It is the second most common cancer among white individuals, accounting for 20% of all cutaneous malignancies (Bernstein *et al*. 1996), and associated, unlike BCC, with a substantial risk of metastasis (Rowe *et al*. 1992). The main risk factors are exposure to UV or ionizing radiation, chemical carcinogens, fair skin, chronically injured or diseased skin, ulcers, immunosuppression and potential HPV infection (Alam and Ratner 2001; Boukamp 2005). A recent study classified cutaneous SCCs as low malignant potential (SCCs arising in AK, verrucous and HPV-related, spindle cell, tricholemmal), intermediate malignant potential (acantholytic, lymphoepithelioma-like, Jadassohn tumor with invasion), high malignant potential (invasive BD, adenosquamous, malignant proliferating pilar, desmoplastic, de novo, arising in chronic conditions, radiation induced) and indeterminate malignant potential (clear cell, signet ring cell, papillary, pigmented, follicular, arising from adnexal cysts, squamoid eccrine ductal) (Cassarino *et al*. 2006a, 2006b). The most invasive SCCs occur on the head and neck and the five-year rate of metastasis is 5% (Rowe *et al*. 1992). Another study suggested large size of the tumor (>2cm), site of the tumor (lip, ear), rapid growth, immunosuppression, recurrence, and histologic features like deep (>4cm or Clark IV, V), poorly differentiated, spindle-cell or acantholytic tumor with perineural invasion as risk factors for metastasis (Alam and Ratner 2001). SCC usually arises as a sporadic tumor, but it is also encountered in several inherited disorders where they might appear as a result of increased genomic mutagenesis, including xeroderma pigmentosum, and dystrophic epidermolysis bullosa (Mallipeddi 2002; Cleaver 2005). Spontaneous SCC is associated with mutations in the p53 tumor suppressor gene, cyclin-dependent kinase inhibitor p16 ^{INK4}, and Ras oncogene (Boukamp 2005). Co-expression of oncogenic Ras with either cyclin dependent kinase 4 (CDK4) or NF-κB blockade produced highly invasive SCCs with downregulation of E-cadherin and upregulation of MMPs and vascular endothelial growth factor (VEGF) (Lazarov *et al*. 2002; Dajee *et al*. 2003).

Extramammary Paget's disease (EMPD)

Extramammary Paget's disease (EMPD) is an uncommon cutaneous neoplasm mostly affecting elderly people and localized in the apocrine-gland bearing body areas on the genital, anorectal, or axillary areas (Siesling *et al*. 2007). The skin lesions are well defined, slowly enlarging erythematous patches usually associated with itching and discomfort. The etiology of EMPD is unknown, but three main theories have been presented: 1) Paget cells arise from the pluripotential cells located in the epidermis; 2) dermal adenocarcinoma cells of apocrine duct origin migrate into the epidermis as Paget cells, or 3) Paget cells migrate into the epidermis from an underlying adenocarcinoma of adjacent organs (Pierie *et al*. 2003). The lesion is defined as an intra-epidermal neoplasm, and may be accompanied by an invasive adenocarcinoma or *in situ* adenocarcinoma of the apocrine glands. Visceral carcinomas, mostly adenoocarcinomas originating from the colorectum, prostate, breast, and extragenital skin can develop in EMPD patients (Siesling *et al*. 2007). Histological diagnostic confusion can arise between EMPD and malignant melanoma and atypical squamous disease. The glandular differentiation of EMPD is indicated by morphological appearances: the presence of intracellular mucin and positive immunohistochemical staining for glandular cytokeratins, such as CAM 5.2, epithelial membrane antigen (EMA), and carcinoembryonic antigen (CEA). The molecular events underlying EMPD differ from those of other epithelial malignancies: *e.g.* p53 mutations are often encountered in BCCs and SCCs, but not in EMPD (Takata *et al*. 1997). EMPD tumors, however, frequently show overexpression of the membrane associated receptor erbB2 (HER-2), a member of the human epidermal growth factor (EGF) family (Takata *et al*. 1999). To our knowledge, no studies have investigated the role of MMPs in EMPD. The recommended treatment of EMPD involves surgical excision, however, surgery is associated with a high rate of recurrence. The prognosis for *in situ* EMPD is good, but it may become invasive and metastasize with poor prognosis (Pierie *et al*. 2003). Probably due to its rarity, the role of MMPs in EMPD has not been investigated before.

Malignant melanoma (MM)

Malignant melanoma (MM) is a malignant tumor derived from melanin-producing melanocytes in the epidermis and its incidence has increased over the past several decades. Clinically melanomas are irregularly shaped asymmetrical lesions with various colors. They are classified into four subtypes: nodular, superficial spreading, lentigo maligna, and acral lentigous melanoma (Weedon 2002b). Both environmental factors and genetic predisposition are important in tumor development and progression (Benjamin *et al*. 2007). Some families have an incresed incidence of melanoma, mostly having a dysplastic nevus syndrome. Previous studies have located the gene of this syndrome to chromosome 1 (Bale *et al*. 1989). Epidemiological evidence supported by mouse models indicates that exposure to UV light, red or light-colored hair, fair skin, and the number of nevi is possible risk factors for melanoma.

The influence of UV light is debatable, however, because of an absence of UV signature mutations (Benjamin *et al*. 2007). The molecular pathogenesis of melanoma deviates from other skin cancers. Mutations affecting p53 or Ras oncogene appear to be rare or rather late events in melanoma progression, whereas activating mutations of the v-raf murine sarcoma viral oncogene homolog B1 (BRAF) and loss of a functional p16 ^{INK4} are detected in the majority of melanomas (Davies *et al*. 2002; Daniotti *et al*. 2004). The switch from E-cadherin to N-cadherin expression, which frees melanocytic cells from the control of keratinocytes, appears to be critical in melanoma progression (McGary *et al*. 2002). The majority of melanomas do not require immunohistochemistry to be diagnosed, however, spindle cell melanoma and metastatic melanoma reguire routine immunohistochemistry for confident and accurate diagnosis. The most freguently used antibody is the S-100 protein, and if additional stainings are needed, melanoma antigen Mart-1 and HMB-45 (gp100 melanosome-associated protein) (Carlson *et al*. 2005). The prognosis of melanoma has traditionally been based on histological criteria, such as tumor thickness (Breslow 1970), level of invasion (Clark *et al*. 1969), mitotic rate, increased nuclear volume, satellite deposits, and hemangiolymphatic invasion (Weedon 2002b). These criteria have been further supplemented by tumor ulceration and sentinel lymphadenectomy, which was created as a minimally invasive technique to provide regional lymph node staging information for patients at high risk for metastatic melanoma and it has become the most significant prognostic factor for patients with melanoma (Perrot *et al*. 2003). If not detected and removed early, MM is very aggressive and unresponsive to current therapeutic approaches (Benjamin *et al*. 2007).

2.4.5. Skin cancer in organ transplant recipients

The increased incidence of malignant tumors, of which non-melanoma skin cancer (NMSC) is the most common (Kyllönen *et al*. 2000), in organ transplant recipients (OTRs) has been ascribed to the immunosuppressive medication (Penn 1974). Other skin cancers such as Merkel cell cancer, sebaceous cancers and cutaneous lymphomas also occur at greater frequency in OTRs (Ho and Murphy 2008). Immunosuppressive drugs may accelerate the development of skin cancer by decreasing immunosurveillance or by being directly carcinogenic (Hojo *et al*. 1999; de Graaf *et al*. 2008). The level of immunosuppression is also significant in progression of malignant skin tumors in OTRs (Jensen *et al*. 1999). The most frequently encountered skin cancers in OTR are SCCs followed by BCCs. However, the ratio of SCC to BCC (1:4) noted in immunocompetent patients is reversed in OTRs (Bouwes Bavinck *et al*. 1996; Stockfleth *et al*. 2001). The overall incidence of malignancy after renal transplantation has been reported as being 3 to 5 times higher compared to the general population (Birkeland *et al*. 2000; Peto 2001), but SCC occurs 65-250 times as frequently, BCC increases up to 10-fold, and melanoma 2-5-fold (Euvrard *et al*. 2003; Le Mire *et al*. 2006). The incidence of skin cancer in OTRs rises with the: 1) duration and intensity of immunosuppressive drugs, 2) UV exposure, and 3) age at the time of transplantation (Bouwes Bavinck *et al*. 1996; Bordea *et al*. 2004; Cassarino *et al*. 2006b).

Cumulative UV exposure, in particular pre-transplantation, can hasten the onset of SCC post-transplantation in patients transplanted after the age of 50 (Ulrich and Stockfleth 2007). The relationship between tumorigenesis and immunosuppression is not fully understood. Some speculate that depletion of natural killer cells, which play an important role in the host's defence against malignancy, might have an impact on tumorigenesis in OTRs (Farag *et al*. 2003). As in the general population several risk factors, such as fair-skin, UV exposure, p53 mutation, and HPV infection, also play a role in the development of skin cancer in OTRs (Bouwes Bavinck *et al*. 1996; Espana *et al*. 2000; Meyer *et al*. 2003). Immunosuppressed patients have a higher HPV prevalence rate of up to 90% in SCCs than immunocompetent patients, and the same HPV types can be detected in different lesions of one patient (Berhout *et al*. 2000; Harwood *et al*. 2000; Pfister 2003). A portion of OTR-related SCCs have a tendency to be aggressive in behavior and metastasize in 5-8% of patients (Martinez *et al*. 2003). Recent studies suggest that OTRs developing large numbers of skin cancers may benefit from retinoid chemoprevention (DiGiovanna 2001).

2.4.6. MMPs and TIMPs in skin cancer

The expression and activation of MMPs is upregulated in almost all human cancers, in which they regulate central processes, like cancer cell proliferation, differentiation, apoptosis, migration, angiogenesis, and the function of the immune system (Folgueras *et al*. 2004). MMPs are expressed in tumor cells as well as stromal cells especially macrophages, fibroblasts, mast cells, and endothelial cells. Interestingly, recent studies have also demonstrated that several MMPs, such as MMP -8, -12, and -26 provide a protective effect in different stages of cancer progression and even MMP-3 and -19 might also function as protective enzymes in specific situations (McCawley *et al*. 2004; Lopez-Otin and Matrisian 2007).

MMPs and TIMPs in non-melanoma skin cancer (NMSC)

MMP-1 is frequently expressed in SCCs of the head and neck (Rosenthal and Matrisian 2006) and its upregulation in AKs has been associated with the early events in SCC development (Tsukifuji *et al*. 1999). Microarray analysis shows upregulation of MMP-1 in SCCs (Nindl *et al*. 2006). MMP-1 is expressed by epithelial and stromal cells of BCCs, in which it is the major collagenolytic MMP (Varani *et al*. 2000; Yucel *et al*. 2005; Boyd *et al*. 2008). MMP-8 is expressed in head and neck SCCs (Moilanen *et al*. 2002), and patient serum levels correlate positively with the tumor stage (Kuropkat *et al*. 2004). MMP-8 might serve as a protective marker in tumors, however, because loss of MMP-8 enhances rather than reduces skin carcinogenesis in male KO-mice (Balbin *et al*. 2003). MMP-8 has not been detected in BCCs except for occasional neutrophils (Varani *et al*. 2000; Boyd *et al*. 2008). MMP-13 expression is specific for transformed keratinocytes and not detected in, *e.g.* migrating keratinocytes, and is abundant in cutaneous SCCs (Airola *et al*. 1997; Johansson *et al*. 1997). In cancer cells, the constitutive expression of MMP-13 has been detected in 85.7% of SCCs of the head and neck and in 52.2% of malignant melanomas (Leeman *et al*. 2002).

Suppression of MMP-13 in human SCCs reduces tumor growth (Ala-aho *et al*. 2002). Expression of MMP-13 has also been detected in tumor cells of BCCs (Airola *et al*. 1997).
High activity of MMP-2 and -9 correlates with the invasiveness of oral SCC (Ikebe *et al*. 1999), but there are conflicting reports on the expression of MMP-2 and -9 in BCC (Varani *et al*. 2000; O'Grady *et al*. 2007; Boyd *et al*. 2008). MMP-2 and MMP-9 expression was more extensive in the stroma of SCC than of BCC or BD (O'Grady *et al*. 2007). In culture, MMP-2 is constitutively expressed by fibroblasts and melanoma cells, but barely by keratinocytes and BCC cells (Chen *et al*. 2006). In SCCs, infiltration by mast cells and activation of MMP-9 coincides with the angiogenic switch in premalignant lesions (Coussens *et al*. 1999). Transgenic mice lacking MMP-9 show reduced keratinocyte hyperproliferation at all neoplastic stages and a decreased incidence of invasive SCCs (Coussens *et al*. 2000). MMP-9 expression is upregulated in SCCs of immunosuppressed patients, as demonstrated by microarray expression profiling (Nindl *et al*. 2006).

MMP-7 is produced by cancer cells in SCCs and aggressive BCCs (Karelina *et al*. 1994). The expression of MMP-7 is common in oral SCC (Impola *et al*. 2004) and its epithelial expression provides a diagnostic clue for distinguishing SCCs from pseudoepitheliomatous hyperplasia in chronic wounds (Impola *et al*. 2005). MMP-26 protein is produced by cancer cells in grade I and II SCCs, but seems to disappear from poorly differentiated tumors, whereas tumor cells of BCCs are devoid of this enzyme (Ahokas *et al*. 2005).

MMP-10 expression does not correlate with the invasive behaviour of SCCs (Kerkelä *et al*. 2001). In BCCs, MMP-10 mRNA is expressed in tumor as well as stromal cells (Kerkelä *et al*. 2001; Boyd *et al*. 2008). Upregulation of MMP-10 may correlate to aggressiveness of BCC, since it is expressed most abundantly in the aggressive sclerosing subtype (Kerkelä *et al*. 2001). MMP-3 is detected mostly in stromal cells in BCCs and SCCs (Kerkelä *et al*. 2001). SCCs of MMP3-KO-mice are less differentiated and grow faster, suggesting a protective role for MMP-3 (McCawley *et al*. 2004). MT1-MMP is expressed in both tumor and stromal cells in SCCs, but only by stromal cells in BCCs (Kerkelä *et al*. 2001). Elevated MT1-MMP expression in SCC of the head and neck has been shown to positively correlate with an aggressive pattern of invasion, poor survival, and lymph node metastasis (Rosenthal and Matrisian 2006).

MMP-19 is detected in keratinocytes under normal quiescent conditions and downregulated in invasive SCCs and BCCs (Impola *et al*. 2003; 2005), and it might participate in the slowing down the angiogenic process in SCC (Jost *et al*. 2006). MMP19-KO-mice, however, have less chemically induced skin carcinomas (Pendas *et al*. 2004). MMP-21 has been detected in BCCs and poorly differentiated SCCs (Ahokas *et al*. 2003; 2005). MMP-28 is expressed in oral SCCs, but not in premalignant lesions (Lin *et al*. 2006). MMP-28, however, was not detected in the invading cancer cell nests of sclerosing BCCs or SCCs of various grades (Saarialho-Kere *et al*. 2002).

In cutaneous and oral SCCs, TIMP expression is detected in tumor as well as stromal cells (Kerkelä and Saarialho-Kere 2003). In a skin carcinogenesis model of HPV16 mice overexpressing TIMP-1, it inhibited the activity of gelatinases in tumor stroma but enhanced tumorigenicity and did not inhibit malignant progression or development of metastasis (Rhee *et al*. 2004). Novel studies have reported that TIMP-1 expression is greater in the stroma of BCC than that of SCC or BD and that TIMP-2 expression was higher in the stroma of SCC than of BD (O'Grady *et al*. 2007; Boyd *et al*. 2008). TIMP-3 is present in tumor cells of infiltrative BCCs and in surrounding stromal cells in SCCs (Airola *et al*. 1998). TIMP-1 and -3 are clearly upregulated during invasion of oral SCC (Sutinen *et al*. 1998). Recent murine studies show that TIMP-3 functions to inhibit metastatic dissemination of diverse cancer cells to multiple organs (Cruz-Munoz *et al*. 2006).

MMPs and TIMPs in melanoma

Various MMPs contribute to melanoma cell invasion. In MM, the most abundant expression of MMP-1 is observed by the peritumoral stromal cells, but also within the tumor by intratumoral endothelial cells (Johansson *et al*. 1997). Melanoma patients with MMP-1 or MMP-3 positive metastases had a significantly shorter disease-free survival time compared to patients with MMP-1 negative metastases (Nikkola *et al*. 2002). In invasive MM cell lines, MMP-1 synthesis is induced in fibroblasts by interleukin (IL)-1α and fibroblast growth factor (FGF)-mediated mechanisms (Löffek *et al*. 2005). Knockdown of MMP-1 in MM does not affect primary tumor growth, but significantly inhibits the overall collagenase activity of the tumors and prevents MM metastasis (Blackburn *et al*. 2007). MMP-13 is associated with aggressive MMs (Airola *et al*. 1999; Corte *et al*. 2005).

In cutaneous MM, stromal cells are the main source of MMP-2 (Hoffman *et al*. 2005). The expression of MT1-MMP and TIMP-2 and the activation of MMP-2 are correlated with tumor progression in human MM (Hoffman *et al*. 2000). MT1-MMP expression in melanoma cell lines has been linked to activation of proMMP2 and increased tumor formation (Sounni *et al*. 2002), and its upregulation promotes melanoma invasion into Matrigel (Iida *et al*. 2004). In melanocytic lesions, MMP-9 is variably expressed in radial, but not in the vertical growth phase, and de novo expression seems associated with early invasion (van den Oord 1997). High serum levels of MMP-1 and -9 were associated with rapid progression in patients with metastatic melanoma (Nikkola *et al*. 2005). MMP-7 is moderately expressed in primary cutaneous MM and strongly expressed in metastatic MM, but not in common nevi (Kawasaki *et al*. 2007).

TIMP-1 and -3 are abundantly expressed in invasive MM, but the expression of TIMP-2 diminishes with malignant progression of MM (Airola *et al*. 1999). TIMP-3 regulates invasion and survival of malignant cells and could be used in adenovirus-mediated gene therapy of malignant melanoma (Ahonen *et al*. 1998).

3. AIMS OF THE STUDY

The principal aim of this study was to investigate the role of several MMPs and other cancer-related proteins in precancerous lesions and different forms of skin cancer to find biomarkers for differentiating more aggressive tumors from slower growing ones. Since stromal cells produce various MMPs and play an important role in cancer progression, we included the analysis of MMPs in stromal cells. SCCs of patients under immunosuppression are more aggressive than those of immunocompetent patients and our interest was to explore if differences in MMP or TIMP expression patterns occur in immunosuppressed compared to immunocompetent patients. A key to develop more effective MMP inhibitors or other drugs against cancer is to identify the critical proteases involved in cancer initiation of aggressive tumors and in the formation of metastases in order to target them or their signal transduction pathways. In addition, when this doctoral thesis was started the biology of the novel MMP -19, -21, -26, and -28 was unknown in the skin and we aimed to further define their expression and functions in normal skin and tumorigenesis.

The specific aims of the work were:

I) To investigate whether MMP -1, -2, -3, -7, -9, -13, and -19 are expressed in EMPD, whether these MMPs assist in the invasion of Paget's cells, and whether their expression pattern reveals secondary EMPD.

II) To study the expression pattern of MMP-21, -26, and -28 in melanoma cells in vivo and in culture and to correlate the results with lymph node status as determined by sentinel node biopsies.

III) To investigate whether the expression of MMPs can differentiate KAs from well-differentiated SCCs, and if differential expression of cancer-related molecules known to be involved in the early skin carcinogenesis, like LN-5 and p16, can be found between these two entities.

IV) To study whether the profile of epithelial or stromal MMP or TIMP expression could explain why SCCs behave more aggressively in patients under immunosuppression compared to immunocompetent patients.

4. MATERIALS AND METHODS

4.1. Tissue samples (I-IV)

All tissue samples were paraffin-embedded, formalin-fixed histological skin biopsies of tumors obtained from Helsinki University Central Hospital (I-IV). The diagnoses were confirmed by an experienced dermatopathologist in each study. All studies were approved by the corresponding Hospital Ethical committee.

I) Extramammary Paget's disease (EMPD)

Histological material was obtained from 21 EMPD patients (9 males and 12 females) treated during the years 1990-2000. The tumors were located on the vulva and perineum of the females, and in scrotum, penis, and perineum of the males. The average age at diagnosis was 70 years. Six of the patients had an underlying adenocarcinoma of an adjacent organ. Tumors were classified into three groups: 1) epidermal (Paget's cells only in the epidermis), 2) micro-invasive (unclear, inflamed basement membrane zone and thickened epidermis), and 3) invasive (clear tumor in the dermis). Five specimens of mammary Paget's disease were used as controls.

II) Melanoma

The melanoma specimens were obtained from 63 cases (10 in situ melanomas and 53 invasive melanomas of 21 females and 32 males, of which all invasive melanoma patients had undergone lymphatic mapping and sentinel node biopsies during the years 2001-2003 (27 samples with no metastasis and 26 samples with nodal micrometastases). Five nevi, seven sentinel node positive, and 12 sentinel node negative biopsies obtained from these patients were also analyzed. The average age at diagnosis was 65 years. Clark's classification was used to determine the invasion level (mean 3.7 mm) and Breslow's classification to measure the tumor thickness (mean 3.1 mm).

III) Keratoacanthoma

Samples were obtained from 31 keratoacanthomas (KAs) of 11 males and 20 females and 15 grade I (well-differentiated) squamous cell carcinomas (SCCs). The average age at diagnosis was 69 years. Twenty of the KAs were located on the face and the rest on limbs or elsewhere on the body. Three of the KA samples were from patients that had undergone organ transplantation. KAs were characterized histologically by their structure, number of atypical cells (0-2), neovascularization (0-3), and inflammation (0-6).

IV) SCCs

SCC samples were acquired from 20 immunosuppressed renal transplant recipients (2 females and 18 males with mean age of 62 years) and 20 immunocompetent controls (11 females and 9 males with mean age of 79 years) so that the location and histology of the samples were matched. The tumors were histologically divided into 1) well-differentiated, 2) moderately

differentiated, and 3) poorly differentiated (Brenn and McKee 2005). Histological changes suggestive of HPV infection (keratinocytes with coarse keratohyaline granules in the upper layers of the acanthotic epidermis next to the malignant SCC areas, but not in the tumor cells themselves) were also recorded (Brenn and McKee 2005). Nine matched pairs of BD samples as an example of early stage cutaneous SCC, were also studied.

4.2. Immunohistochemistry (I-IV)

Immunostaining of the sections was performed by the avidin-biotin-peroxidase complex technique using the Vectastain ABC Kit (Vector laboratories, Inc, Burlingame, CA, USA), DAKO Kit (DAKOStreptABComplex/HRP Duet, Mouse/Rabbit, Glostrup, Denmark), or by the antibodypolymer detection technique (PowerVision Poly-HRP IHC Kit, ImmunoVision Technologies Co, Brisbane, CA, USA). Diaminobenzidine (DAB) or aminoethylcarbatzole (AEC) were used as chromogenic substrates and Mayer hematoxylin as counterstain, as described in detail in Saarialho-Kere *et al*. (1993). Samples were deparaffinized, dehydrated, and endogenous peroxidase was blocked by incubation with 3% H_2O_2 for 10 minutes at room temperature. If necessary, sections were pre-treated with 10 mg/ml trypsin, incubated in citrate (pH 6) or ethylenediaminetetraacetic acid (EDTA) (pH 9) buffer in a 95°C water bath or in a microwave oven. Primary antibodies (Table 3) were incubated for 1-2 hours at 37°C or overnight at 4°C in a humified chamber. Controls were performed from unimmunized animals.

4.3. In situ hybridization (I, III)

The production and specificity of the antisense MMP-1, -3, -7, -10, and -13 cRNA probes have been previously described (Saarialho-Kere *et al*. 1994; 1996; Vaalamo *et al*. 1997). *In situ* hydridization was performed on 5 µm sections using ^{35}S-labeled probes. Sections were hybridized overnight at 50-55°C after which slides were washed under stringent conditions and treated with RNAase A to remove unhybridized probe. After 20-50 days of autoradiographic exposure, the photographic emulsion was developed and the slides were stained with hematoxylin and eosin. Samples previously positive for each antisense probe were used as positive controls.

Table 3. Antibodies used in immunohistochemistry

ANTIBODY	SOURCE	DILUTION/PRETREATMENT
MMP-1	IM35L, Oncogene, San Diego, CA, USA	1:500/Trypsin
MMP-2	IM33L, Oncogene	1:75/Trypsin
MMP-7	IM40L, Oncogene	1:100/95°C water bath in citrate buffer
MMP-8	IM38L, Oncogene	1:20/Trypsin
MMP-9	GE-213, Research Diagnostics, Flanders, NJ, USA	1:100/Trypsin
MMP-9	MS-569, Neomarkers, Fremont, USA	1:100/Trypsin
MMP-10	NCL-MMP10, Novocastra, Newcastle, UK	1:250/Trypsin
MMP-13	MS-825, Neomarkers	1:10/95°C water bath in citrate buffer
MMP-19	RDI-MMP19abR, Research Diagnostics	1:70/ No pretreatment
MMP-21	RP3MMP-21, Triple Point Biologics, Portland, OR, USA	1:70/ No pretreatment
MMP-26	Prof Keiichi Isaka	1:150/95°C water bath in citrate buffer
MMP-28	Dr. Jouko Lohi	1:800/microwave in EDTA buffer
TIMP-1	IM63L, Oncogene	1:100/95°C water bath in citrate buffer
TIMP-3	IM43L, Oncogene	1:400/95°C water bath in citrate buffer
Laminin-5	Prof Karl Tryggvason	1:800/Trypsin
Tenascin-C	MAB143DB7, Biohit, Helsinki, Finland	1:2000/Trypsin
$p16^{INK4}$	G-175-405, BD Biosciences, San Jose, CA, USA	1:350/95°C water bath in citrate buffer

4.4. Cell cultures (II)

Commercial melanoma cell lines Bowes, G361, WM852, WM164, and WM165 were cultivated in Dulbecco's modified Eagle's medium/F-12 (D-MEM/F-12) (Gibco BRL, Gaithersburg, MD, USA), supplemented with 10% heat in activated fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, 50 mg/ml streptomycin, 0.1 µg/ml cholera toxin, and 3 ng/ml fibroblast growth factor-2 (FGF-2) at 37°C in humified atmosphere. The medium was changed twice a week.

4.5. Polymerase chain reaction and mRNA analysis (II)

Total cellular RNA from cultured cells was extracted using Rneasy Miniprep-kit (Qiagen, Chasworth, CA) according to manufacturer's instructions. RNA was then reverse transcribed to complementary DNA (cDNA) with Taqman^{TM} Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA) with random hexamers and used in a 1:5 dilution as a template for conventional RT-PCR. As primers we used T21F (forward nucleotides) and T21R (reverse nucleotides) for human MMP-21 (Sigma Genosys, Cambridge, U.K) as previously described (Ahokas *et al*. 2003). TaqMan PCR primers for human MMP-26 were purchased as a ready-to-use 20x reaction mix and the sequences are property of the vendor (Applied Biosystems). MMP-28 primers (Sigma Genosys, Cambridge, U.K) and probes (PE Biosystems, Warrington, U.K), described in more detail in our previous work (Saarialho-Kere *et al*. 2002), were designed using computer program Primer Express (Applied Biosystems). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The PCR for MMP -21, -26, and -28 was performed by conventional PCR, in separate reactions. The primers were used in a final concentration of 200 nM each (T21F, T21R, GAPDH forward, and GAPDH reverse), 400 µM of each nucleotide, 0.5 µl Advantage-2, 5.5 mM $MgCl_2$, and 3 µl of cDNA-template in a reaction volume of 25 µl. The PCR was started with an initial denaturation of 3 min at 94ºC, followed by a total of 40 cycles of 30 sec denaturation at 94ºC, 30 sec of annealing at 60ºC, and 30 sec of elongation at 72ºC with a final elongation of 10 min at 72ºC. Finally, PCR products were analyzed in a 2.5% agarose gel and stained with 5 ng/ml of ethidium bromide and visualized under ultraviolet light.

4.6. Statistical methods (II, III, IV)

Unpaired two-tailed t-test, chi-square test and Mann-Whitney U tests were performed to investigate the significance of results in Studies II, III and IV with SPSS 13.0 for Windows. A p value under 0.05 was considered as significant.

5. RESULTS AND DISCUSSION

MMPs are produced abundantly by various cells, but their functions or substrates *in vivo* are still often unknown. MMPs are rarely produced constitutively, but in response to specific signals. In order to understand their role in skin tumors it is necessary to study samples that reflect physiological and disease processes as they occur *in vivo* instead of relying merely on animal models that can differ in oncogenes or expression patterns of MMPs from humans. Immunohistochemistry and *in situ* hybridization are suitable methods for studying localizations of MMPs in tissues. RT-PCR, which shows cytosolic and nuclear mRNA, can be used to study gene expression. We investigated the expressions of various MMPs as well as a few tumor related proteins in precancerous lesions, *in situ* carcinomas and invasive tumors using immunohistochemistry, *in situ* hybridization and RT-PCR to find markers for detailed categorization of these tumors and to shed more light on the pathobiology of these lesions. Due to the different regulatory levels of MMP activity, it was convenient to use different methods to provide the most comprehensive view of the tissue events. Immunohistochemical assays, however, cannot discriminate between inactive zymogens and active enzymes. Additional studies using, for example, *in situ* zymography or western blotting, could have been done to further validate these results.

5.1. MMP-7 and MMP-19 are expressed by Paget's cells in extramammary Paget's disease (I).

EMPD is a rare adenocarcinoma of the apocrine skin and molecular events underlying EMPD differ from other epithelial malignancies. Since the contribution of MMPs to the biological behavior of EMPD has not been previously investigated, we studied a large panel of proteases in EMPD to gain more knowledge about the nature of these rare tumors (Table 4). Among the MMPs we investigated, positive staining for MMP-7 was detected in Paget's cells in 10/27 tumors. MMP-7 has many known functions in inflammation, apoptosis, proliferation, invasion, and angiogenesis, and it is widely expressed in epithelial and mesenchymal tumors associated with poor prognosis (Ii *et al*. 2006). The origin of EMPD is still unclear, but it may be the secretory cells of apocrine glands. Interestingly, MMP-7 is one of the few MMP family members expressed in glandular epithelium and detected during tumor progression in benign and malignant tumors arising from glandular epithelium (Wilson and Matrisian 1996; Nelson *et al*. 2000). The majority of MMPs are expressed by stromal cells, but some MMPs, including MMP-7, are expressed by tumor cells themselves, indicating that MMP-7 is expressed in a tumor-associated fashion. In our study it was detected in several of the EMPD tumors, which supports this theory of tumor-associated expression. Thus, the production of MMP-7 in tumor cells in EMPD could be a biological marker of an aggressive phenotype and used to guide therapeutic interventions. Since EMPD is a rare disease and large cohorts are difficult to obtain, however, these results should be confirmed by other studies. MMP-7 mediates cleavage of the E-cadherin ectodomain, resulting in disruption of the

E-cadherin/catenin complex (Noë *et al*. 2001). E-cadherin was recently shown to be downregulated in EMPD (Liu *et al*. 2007), which might be a consequence of MMP-7 overexpression. Accumulation of β-catenin coordinates upregulation of the MMP-7 gene transcription in intestinal adenocarcinomas (Crawford *et al*. 1999), but neither cytoplastic nor nuclear accumulation of β-catenin has been observed in EMPD (Takata *et al*. 1999).

We detected MMP-19 in Paget's cells in 12/26 samples. The physiologic function of MMP-19 is still mostly unknown, but it is typically expressed in the basal layer of healthy interfollicular epidermis and upregulated in suprabasal and spinous layers in psoriasis, eczema, cutaneous wounds, and tinea (Sadowski *et al*. 2003; Suomela *et al*. 2003; Impola *et al*. 2003). MMP-19 has also been detected in sweat glands of normal skin, but it is downregulated in malignant breast cancer cells and SCCs (Djonov *et al*. 2001; Impola *et al*. 2003). It is suppressed in human keratinocyte cell lines at high calcium concentrations and calcium-regulation occurs through E-cadherin mediated cell-cell contacts (Sadowski *et al*. 2003). Unlike in cutaneous SCCs, where MMP-19 is downregulated in malignant cells (Impola *et al*. 2003), we found positive staining for MMP-19 in malignant cells in several EMPD samples. This could indicate that the origin of the tumor plays a significant role in MMP expression.

5.2. MMP-7 and MMP-19 can predict an underlying carcinoma in extramammary Paget's disease (I).

MMP overexpression in the samples with underlying visceral malignancy was also of interest. We could find 3/7 MMP-7 positive specimens and 4/6 MMP-19 positive specimens with underlying malignancy. If the trend would become stronger with a larger cohort, these MMPs could aid in guiding the therapy and further investigations and also serve as prognostic factors in EMPD. The principal treatment of EMPD is surgical excision with local recurrences up to 30-60% (Pierie *et al*. 2003) and patients with recurrent EMPD might benefit from adjuvant therapies. Tumors strongly overexpressing certain MMPs could respond to agents interfering with MMP-signaling pathways, like patients having HER2 gene mutation may benefit from HER2 targeting immunotherapy (Tanskanen *et al*. 2003). According to our results, the patients with overexpression of MMP-7 and -19 in Paget's cells had had a variable number of operations and recurrences. No trend was observed between the expression of certain MMPs and malignant nature of the EMPD, so we could not find proper candidates for MMPI therapy.

5.3. MMP-2 and MMP-3 are not detected in Paget cells (I).

For the important functions that MMP-1, -2, -3, -9, and -13 have in tumorigenesis, we wanted to investigate their expression in EMPD as well (Egeblad and Werb 2002; Ala-aho and Kähäri 2005). Interestingly, MMP-2 was expressed occasionally by fibroblasts, but both MMP-2 and MMP-3 were absent in the malignant cells of EMPD samples. In addition, other MMPs tightly linked to epidermal transformation, MMP-1 and -13, were not overexpressed either.

MMP-9 was expressed in the surgical specimen of only one patient, who had undergone 10 operations due to aggressive disease, and in two lymph node metastases of different patients. MMP-9 positive neutrophils and macrophages were detected in several samples, but their presence did not correlate with the number of operations or underlying malignancy.

Previous studies have shown that MMP-2 is expressed by various types of adult cells under normal physiological conditions, especially fibroblasts, and it is a potent proteolytic enzyme with a major role in the digestion of BM type IV collagen, which is an important mechanism for vascular invasion and metastasis (Kähäri and Saarialho-Kere 1999). Upregulation of MMP-2 is a common feature seen in invasive carcinomas of different organs (Kähäri and Saarialho-Kere 1999). The expression of MMP-3 is upregulated by growth factors, like HB-EGF (Suzuki *et al*. 1997), and it is detected in a wide variety of tumor cell types. The upregulation of MMP-3 correlates with the progression and metastasis of breast, skin, and colon tumors (Sternlicht *et al*. 1999; Inuzuka *et al*. 2000; Kerkelä *et al*. 2001). Recent studies have shown its protective role in skin cancer. SCCs in MMP3-null mice are less differentiated and grow faster and MMP-3 expression accelerates the rate of apoptosis in transformed cells (McCawley *et al*. 2004). Interestingly, the wild-type p53, which is usually encountered in EMPD (Takata *et al*. 1997) downregulates the transcription of MMP-1, -2, and -13 (Overall and Lopez-Otin 2002; Ala-aho *et al*. 2002). It is possible that the lack of overexpression of these proteases contributes to the fact that a subset of EMPD tumors can behave in an indolent manner (Parker *et al*. 2000).

5.4. LN-5 is expressed in 50% of EMPD lesions while staining for TN-C is faint (I)

Since LN-5 is known to be proteolytically processed by several MMPs included in our study, we wanted to evaluate the staining pattern of LN-5 in our EMPD samples. Eleven of 20 samples showed LN-5 positivity in Paget cells and by growing invasiveness of the tumor, LN-5 positivity decreased. In a few of the samples, only keratinocytes had positive immunostaining for LN-5, because re-epithelializing cells are known to produce LN-5 as well as keratinocytes that are located on a disrupted BM (Giannelli *et al*. 1997; Koshikawa *et al*. 2000). The tumors with underlying visceral carcinoma did not differ from others in their LN-5 staining. Thus, LN-5 positivity does not serve as a marker for underlying adenocarcinoma.

LN-5 is considered to be a potential marker for carcinoma invasion (Lohi 2001) and the processing of γ2-chain has been shown to trigger epithelial cell migration (Giannelli *et al*. 1997; Koshikawa *et al*. 2000). Co-localization of LN-5 and MMP-19 was recently found at the invading front of well-differentiated SCCs and suggested that MMP-19-dependent processing of the γ2-chains leads to the integrin switch favoring epithelial cell migration (Sadowski *et al*. 2005). We could not find co-localization of LN-5 and MMP-19. On the contrary, most samples overexpressing MMP-19 were negative for LN-5. When comparing the LN-5 expression with other MMPs, we also found no association. Our results are in

agreement with previous data suggesting that molecular events differ in EMPD compared to other cutaneous malignancies.

Since TN-C is upregulated in transformed keratinocytes, as well as stromal cells in premalignant and malignant skin lesions (Dang *et al*. 2006) and as it is cleaved by several MMPs, like MMP-2, -7, -13, and -19 *in vitro* (Table 1), we immunostained for TN-C in EMPD samples. The staining was faint and seen only at the border of EMPD lesions and no co-localization or co-expression of TN-C with the MMPs studied occured. According to our results, the weak staining for TN-C, compared to many other tumor types, may contribute to the slow growth of Paget's lesions.

5.5. MMP-21 is upregulated at early stages of melanoma progression, but disappears with more aggressive phenotype (II).

Since MMP-21 is expressed in a subset of BCCs and SCCs *in vivo* (Ahokas et al, 2002; 2003), we wanted to investigate its expression in melanoma cells *in vivo* and in culture, as well as to correlate these results with lymph node status. In our study, MMP-21 protein staining was detected in invasive melanoma cells in 29/53 samples and in 6/10 *in situ* melanomas. Its expression was more intensive in melanoma samples without micrometastases: 18/27 of the cases without nodal invasion and 11/26 with nodal invasion were positive for MMP-21. We could not detect any connection between MMP-21 expression and ulceration. MMP-21 was also expressed in fibroblasts surrounding melanoma islands, agreeing with the results of Skoog *et al*. (2006), but we didn't observe positive staining for MMP-21 in endothelial cells or keratinocytes. MMP-21 staining was not detected in 19 metastatic lymph node samples. To study how MMP-21 behaves in benign melanocytic cells, we stained five additional samples of compound nevi for MMP-21. They all turned out negative. Since MMP-21 expression seemed to weaken with nodal invasion, we performed the Mann-Whitney U test comparing MMP-21 expression in grade II-III tumors versus grade IV-V tumors irrespective of lymph node status and found that MMP-21 expression was significantly stronger in well-differentiated melanomas (mean 1.0 vs. 0.57, $p=0.04$). Our results indicate that upregulation of MMP-21 is an early event in melanoma progression. While melanoma progresses, MMP-21 expression weakens and is finally absent from nodal metastases. This is supported by the fact that *in situ* melanomas showed positive staining for MMP-21, but all nevi samples turned out negative. Thus, MMP-21 could serve as a marker of malignant transformation in melanocytes. Larger cohorts, however, are needed to confirm these results. Furthermore, immunohistochemical approach cannot differentiate between latent and active MMP-21, which would need conventional or *in situ* zymography, but would lead to difficulties in determining the cells of origin on tissue level.

Similar results on the expression profile of MMP-21 in pancreatic adenocarcinoma were recently published from our group: MMP-21 was expressed in well-differentiated tumors, but diminished from the poorly differentiated ones (Bister *et al*. 2007). In contrast to this, expression of MMP-21 was associated with invasion in esophageal SCCs, but localized to

well-differentiated areas of the tumors (Ahokas *et al*. 2006) and with aggressive subtypes of BCCs and SCCs (Ahokas *et al*. 2003). This suggests that in certain cancers and cancer cell lines MMP-21 may, indeed, promote invasion. Interestingly, MMP-21 is upregulated in keratinocytes and downregulated in fibroblasts in cell culture by TGF-β1 (Ahokas *et al*. 2003; Skoog *et al*. 2006), which is also known to modulate melanoma growth and survival (Berking *et al*. 2001). Thus, TGF-β might also be a potential candidate to upregulate MMP-21 in melanoma cells *in vivo*. In promoter analyses, MMP-21 is a putative target of β-catenin transactivation (Marchenko *et al*. 2003). Indeed, alterations of β-catenin pathway have been reported in melanoma (Kielhorn *et al*. 2003). Our group recently published that MMP-21 expression was induced in pancreatic cell lines by EGF (Bister *et al*. 2007). A discrepancy, however, in the results of the role of EGF in MM exists (Shahbazi *et al*. 2002; McCarron *et al*. 2003). MMP-21 is induced in monocytic cells and in keratinocytes by all-*trans*-retinoic acid (atRA) (Skoog *et al*. 2006; Skoog *et al*. unpublished) and putative retinoid receptor binding sites have been detected in its promoter (Skoog *et al*. 2006). Interestingly, cultured melanoma cells treated with atRA induced cellular growth inhibition on both primary and metastatic melanoma cell lines (Zhang *et al*. 2003). Although the primary tumor cells were relatively more susceptible to this effect as compared to the matched metastatic melanoma cells, the study suggested the possibility to utilize atRA in the treatment of early human MM. If atRA induces MMP-21 expression in cells, it might have a protective role in the early phases of melanoma progression. MMP-21-KO-mice could give valuable data in further studies on the role of MMP-21 in MM.

5.6. MMP-26 is not detected in melanoma cells while MMP-28 is present occasionally in melanomas (II).

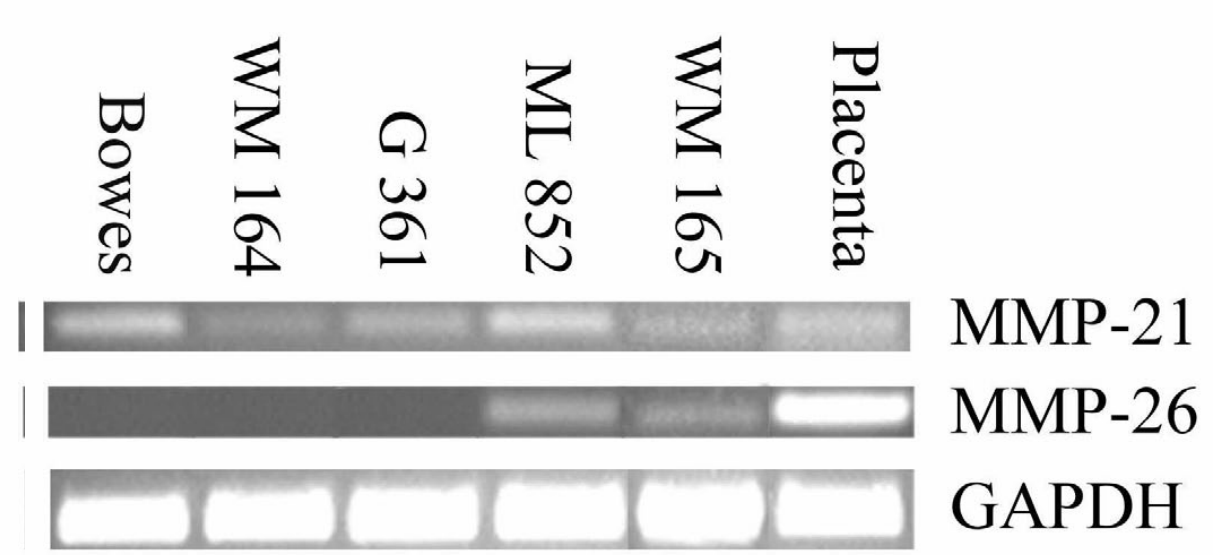
Twenty-one melanoma samples and 5 nevus samples were immunostained for MMP-26. They were all negative except for fibroblasts surrounding the tumor in a few of the samples and for the occasional basal migrating keratinocytes bordering ulcerations. We stained 36 invasive melanoma and 10 in situ melanoma samples for MMP-28 and found positive immunostaining in only five of them.

MMP-26, a recently cloned MMP, has been associated with early stages of tumor progression (Lee *et al*. 2006; Ahokas *et al*. 2005), but conflicting studies reporting its upregulation in invasive tumors, such as pancreatic, ovarian, and esophageal cancers exist (Yamamoto *et al*. 2004; Ripley *et al*. 2006; Bister *et al*. 2007). MMP-26 has been detected in benign skin lesions, like sarcoid granulomas and granuloma annulare (Skoog *et al*. 2006). According to our results MMP-26 does not seem to have an important role in the malignant transformation of melanocytes or even in benign melanocytic lesions. Greater estrogen receptor-β (ER-β) expression in severely dysplastic nevi and lentigo malignas compared with thick nodular MMs with greater Breslow depth was reported recently and investigators stated that estrogen may play a role in MM (Schmidt *et al*. 2006). The presence of a MMP-26-mediated intracellular regulatory pathway targets ER-ß in hormone-regulated malignancies (Savinov *et al*. 2006). If ER-β plays a significant role in MM and MMP-26 is important in the regulatory

pathway of ER-β, one would assume we would have found more positive staining for MMP-26 in our *in vivo* MM or nevus samples. We detected MMP-26 in a few fibroblasts surrounding melanoma islands, which is in agreement with the reported expression of MMP-26 by corneal fibroblasts (Marchenko *et al.* 2004). This all may be related to the contribution of host-derived proteases in tumor progression.

The precise role of MMP-28 in cutaneous biology is still unknown, but it is expressed in various carcinomas, including ovarian, colon, and pancreatic adenocarcinoma (Marchenko and Strongin 2001) and also by proliferative keratinocytes during wound repair (Lohi *et al*. 2001; Saarialho-Kere *et al*. 2002) as well as in intestinal inflammatory conditions (Bister *et al*. 2004). MMP-28 can induce EMT and cell invasion through a TGF-β1-dependent mechanism, suggesting novel biological roles for this enzyme in the induction of carcinogenesis (Illman *et al*. 2006). Since most of the *in vivo* samples in our study (41/46) were negative for MMP-28, it does not seem to be important in the pathogenesis of MM.

Figure 6. The mRNA expression of MMP-21 and -26 by RT-PCR in different cell lines.



5.7. MMP-21 is expressed in melanoma cells in culture, but detection of MMP-26 and -28 in melanoma cell lines is insignificant (II).

We examined five melanoma cell lines (Bowes, G361, WM852, WM164, and WM165) to study whether melanoma cells are able to express any mRNAs of the three novel MMPs in culture. MMP-21 mRNA was expressed by RT-PCR in Bowes and ML852 melanoma lines

while lower levels were apparent in WM164, WM165, and G361 cells (Figure 6). MMP-26 mRNA was detected only at low levels in ML852 and WM165 cells (Figure 6). A very low level of MMP-28 mRNA was detected in WM165 cells, while all other cell lines turned out negative (data not shown). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control and placenta as a positive control (Figure 6). The expression of MMP-21 in melanoma cell lines strengthened our hypothesis that MMP-21 could serve as a marker of malignant transformation in melanocytes. This is further supported by our negative findings in metastatic sentinel nodes. A larger cohort of various stages of melanoma would be required to finally confirm this, however.

We could detect *in vitro* two melanoma cells lines expressing MMP-26, a phenomenon not found in tissue samples. Such conflicting results in MMP studies are often observed as the amount of MMP proteins may be so low in tissues that it is undetectable by immunohistochemical staining *in vivo,* but generally expressed in cancer cell lines *in vitro* (Kerkelä and Saarialho-Kere 2003). Alternatively, cell-matrix and cell-cell interactions may up- or downregulate MMPs *in vivo*. While our study was in progress, another study reported high expression of MMP-26 by several melanoma cell lines in culture using immunohistochemistry and its correlation with estrogen dependency (Li *et al*. 2004). Our negative results on MMP-26 and MMP-28 expression in G361 cell line are identical to results published by Nuttall *et al*. (2003).

We detected only very low levels of MMP-28 in one cell line, though it was detected in a few melanoma samples *in vivo*. This further supported our conclusion that MMP-28 does not play a significant role in MM. So, as previously suggested (Bister *et al*. 2004), it may have a more vital function in tissue homeostasis rather than in tumor progression.

5.8. The presence of MMP-13 positive cells in melanoma and its micrometastases may correlate to more aggressive disease (II).

We found positive MMP-13 staining in melanoma cells of 39/53 samples of invasive melanoma, from which 19/27 specimens were without micrometastases and 20/26 had micrometastases. All metastatic sentinel lymph nodes expressed MMP-13, whereas MMP-13 was not detected in any of the non-metastatic lymph nodes. MMP-13 is a powerful and potentially destructive proteinase with strictly controlled expression under normal physiologic conditions (Ala-aho and Kähäri 2005). Studies that our group and others have published previously indicate that MMP-13 expression is induced in melanoma progression (Airola *et al*. 1999; Nikkola *et al*. 2002). High expression of MMP-13, however, is not associated with survival parameters (Nikkola *et al*. 2002). According to our results MMP-13 is upregulated in aggressive metastatic melanoma and associated with nodal micrometastases, agreeing with previous knowledge about the nature of this MMP.

5.9. The presence of MMP-7 and -9 in the epithelial pushing border of KAs is rare and should raise a suspicion of SCC (III).

We found positive staining for MMP-7 protein in epithelial cells of 4/31 KAs and 9/15 well-differentiated SCCs. The staining intensity for MMP-7 was clearly lower in KAs than in SCCs and agrees with previous findings (Impola *et al*. 2005). Thus, this MMP might be valuable in differentiating these closely related tumors. Neovascularization was observed in all MMP-7 positive KA samples, but the presence of MMP-7 did not associate with keratinocyte atypia or inflammation. The literature, expresses a lot of controversy about the question of whether KA is a variant of SCC or a unique benign lesion. Although most of the lesions that fit the diagnosis of KA behave in a predictably benign manner, occasional aggressive behavior, including metastases, has been described (Yus *et al*. 2000; Gottfarstein-Maruani *et al*. 2003; Schwartz 2004). MMP-7 has been detected in malignantly transformed keratinocytes (Karelina *et al*. 1994; Impola *et al*. 2005) and contributes to the initiation of EMT by cleavage of E-cadherin (Noë *et al*. 2001; Van Kempen *et al*. 2002). The presence of MMP-7 in angiogenic endothelial cells in various cancer types suggests that endothelial cell-derived MMPs are involved in neo-angiogenesis (Sier *et al*. 2007). Another study suggested that the VEGF-mediated angiogenic switch of fibroblasts is regulated by MMP-7 from cancer cells (Ito *et al*. 2007). Our results are congruent with these findings, since neovascularization occurred in all MMP-7 positive samples. MMP-7 has been upregulated in inflammation in earlier studies (Parks *et al*. 2004; Wielockx *et al*. 2004), but we could not find a connection between MMP-7 and histological grading of inflammation.

The expression of MMP-9 was detected in 5/31 KAs and 8/15 SCCs in a small number of epithelial cells at the pushing border. Thus, the expression of MMP-9 was rare in epithelial cells in KAs, but it was clearly upregulated in SCCs. MMP-9 was, however, detected in inflammatory cells in several KAs. In 22/31 KAs, MMP-9 was produced only by macrophages and neutrophils. Epithelial MMP-9 expression did not associate with keratinocyte atypia, angiogenesis, or inflammation in KAs. Our results agree with previous studies suggesting that MMP-9 might serve as a prognostic marker for more aggressive tumors, whereas its absence from epithelial cells may serve as a prognostic marker of non-invasive SCC (Kobayashi *et al*. 1996; Impola *et al*. 2004). Neutrophils, macrophages, and mast cells are the predominant source of MMP-9 in a mouse model of multistage SCC, rather than the neoplastic cells themselves (Coussens *et al*. 1999). In humans, high stromal MMP-9 expression correlated with a more advanced disease in ovarian cancer (Sillanpää *et al*. 2007). Whether the stromal positivity for MMP-9 is a result of the inflammatory response around the tumors rather than specific tumor activation is still unknown. Induction of angiogenesis involves MMP-9, which is upregulated in angiogenic islets and tumors, rendering VEGF more available to its receptors (Bergers *et al*. 2000). We could not find, however, an association between epithelial MMP-9 expression and angiogenesis. No connection was noted between MMP-9 expressing stromal cells and angiogenesis in KAs either (unpublished data).

5.10. The loss of MMP-19 and p16 from KA could aid in distinguishing between well-differentiated SCC and KA (III).

MMP-19 was generally present in epithelial keratinocytes at the pushing border of KAs, but only in 6/15 SCCs. In SCCs, MMP-19 was expressed by hyperproliferative epidermis, but generally disappeared from the invasive cancer cell nests. Indeed, our group and others have previously demonstrated that MMP-19 is absent from the invasive cancer cell nests of well-differentiated SCCs (Impola *et al*. 2005; Sadowski *et al*. 2005). Thus, the down-regulation or loss of MMP-19 might serve as a prognostic marker for malignant transformation in KAs. MMP-19 is upregulated in the hyperproliferative areas of keratinocytes in psoriasis (Suomela *et al*. 2003), which might explain its strong expression in rapidly growing, hyperproliferative, KAs.

Cyclin-dependent kinase inhibitor $p16^{INK4}$ (p16) plays an important role in inhibition of the cell cycle by specifically blocking the cyclin-dependent kinase 4 (CDK4) from phosphorylating the retinoblastoma protein (Boukamp 2005). The contribution of p16 to skin cancer development is controversial (Hodges and Smoller 2002; Salama *et al*. 2003). In our study, p16 was detected in hyperproliferating areas of KAs independent of the number of atypic keratinocytes or the degree of inflammation. Some specimens, however, clearly had fewer p16 positive cells than others. P16 expression was stronger in MMP-13 negative samples. In SCCs, p16 was mostly absent from the invasive cancer cell nests and only 7/15 samples had occasional p16 positive cells at the invasive front.

P16 expression is activated in cells in response to BM degradation/invasion and is detected in normal migrating keratinocytes as well as in chronic wounds (Natarajan *et al*. 2003; Ahokas *et al*. 2005). In our study, upregulation of p16 in Kas, however, was not uniform. This might be due to differences in UV exposure (Hashemi *et al*. 2003) or the nature of our KA samples. Burnworth *et al*. (2007) developed a skin carcinogenesis model, where p16 was strongly upregulated in KAs, but detected only in a few SCCs, whereas p16 was absent from invasive areas of SCCs (Impola *et al*. 2005). MMP-13, a marker of malignant transformation of keratinocytes (Ala-Aho and Kähäri 2005), was also negative in the KAs with p16 positive staining. According to our results, p16 might have a protective role in KA and its downregulation might serve as a warning sign for a more aggressive nature of KA, agreeing with previous reports from our group and others (Impola *et al*. 2005; Burnworth *et al*. 2007).

5.11. The staining for MMP-2, MMP-8, MMP-10, MMP-13 or LN-5 does not assist in differentiating KAs from well-differentiated SCCs (III).

MMP-2 protein was not detected in the epithelium of KAs or SCCs, but was abundantly expressed by fibroblasts in all samples. MMP-2 is expressed by various types of cells, *e.g.* fibroblasts as well as tumor cells, and it is a powerful proteolytic enzyme with a major role in the digestion of BM type IV collagen (Devarajan *et al*. 1992). The role of MMP-2 in cutaneous SCC is, however, unclear. Using different antibodies than ours, two groups reported positive MMP-2 staining in keratinocytes of a subset of SCCs (Fundyler *et al*. 2004;

O'Grady *et al*. 2007), but another group only found positive MMP-2 staining in fibroblasts surrounding SCCs, agreeing with our results (Tsukifuji *et al*. 1999). These discrepancies may be due to, *e.g.,* different antibodies used.

MMP-10 was expressed in the basal epithelial cells of the pushing border in 28/31 KAs and in the basal epithelium of cancer cell nests in all SCCs. MMP-10, also known as stromelysin-2, degrades multiple components of the ECM and is overexpressed in various cancers (Mathew *et al*. 2002; Cho *et al*. 2004). MMP-10 expression was not, however, associated with invasion and metastasis in cutaneous SCC, but rather reflected inflammation and matrix remodelling associated with tumor growth (Kerkelä *et al*. 2001). UV light is known to induce the expression of MMP-10 in cultured keratinocytes (Dazard *et al*. 2003; Ramos *et al*. 2004) and may account for the abundant expression of MMP-10 in KAs. Strong upregulation of MMP-10 in the epithelium may contribute to excessive degradation of type IV collagen, FN, and nidogen in the BM zone (Bister *et al*. 2007). Thus, elevated MMP-10 expression may play a role to the aberrant matrix degradation seen in KA patients. Co-stimulation of cultured keratinocytes with transforming growth factor-β1 (TGF-β1) and epidermal growth factor (EGF) leading to epithelial-to-mesenchymal-transition (EMT) correlates strongly with increased expression of MMP-10 (Wilkins-Port and Higgins 2007). In the future, studies on the interaction of MMP-10 with TGF-β1 or EGF receptor in different tumors and the mechanism of their putative interaction could be valuable. On the basis of our results, MMP-10 protein expression was increased in benign KAs as well as malignant SCCs. Since most of the KAs and all SCCs stained positively for MMP-10, however, it does not appear to be a significant marker in differentiating KAs from SCCs.

MMP-13 was expressed by basal keratinocytes in 16/31 KAs and 11/15 SCCs. No correlation of MMP-13 expression with atypical cells in KAs was noted, but a trend existed for more abundant angiogenesis in MMP-13 positive samples. MMP-13 expression is induced in forceful tissue remodeling and malignant transformation of keratinocytes (Johansson *et al*. 1997). No expression of MMP-13, however, is noted in premalignant tumors in human skin (Airola *et al*. 1997), or by normal epidermal keratinocytes in culture or *in vivo* (Johansson *et al*. 1997; Vaalamo *et al*. 1997). Interestingly, this powerful MMP was expressed in half of the benign KAs studied. Our results suggest that at least a subset of KAs cannot be classified as benign. We could not find differences in the positive staining of MMP-13 between KAs and SCCs. Thus, immunostaining for MMP-13 does not assist in distinquishing KAs from well-differentiated SCCs.

Epithelial expression for MMP-8 was detected in 3/30 KAs, but only MMP-8 positive neutrophils were seen in 5/15 SCCs. MMP-8 positive neutrophils detected in 14/32 KAs usually localized in the upper parts of the tumors rather than near the pushing epithelial border. The staining of MMP-8 in neutrophils was also more abundant in KA samples. All MMP-8 positive KAs had neovascularization. MMP-8 is mainly expressed by neutrophils, but relatively low sporadic MMP-8 expression has also been reported in oral SCCs (Moilanen *et al*. 2002). The *in vivo* function and biologic role of MMP-8 is, however, mostly unknown.

According to our previous report (Impola *et al*. 2005), MMP-8 is not detected in tumor cells of well-differentiated SCCs, but mostly in neutrophils. MMP-8 positive staining was observed in keratinocytes, however, in few of the KA samples. A recent study showed that increased inflammation in the absense of MMP-8 in KO-mice delayed wound healing. Therefore, MMP-8 might be necessary for the wound healing process to be properly completed (Gutiérrez-Fernández *et al*. 2007). MMP-8 was suggested to have protective functions in cancer due to the processing of inflammatory mediators which contribute to the host antitumor defence system (Balbin *et al*. 2003). Another study demonstrated that the TIMP-resistant form of MMP-8 activity expressed on the surface of human polymorphonuclear leukocytes is likely to contribute in important ways to its anti-inflammatory and interstitial collagen-degrading activities (Owen *et al*. 2004). Indeed, MMP-8 positive neutrophils in KAs might serve as a protective factor and also function in resolution of KA. We could find neovascularization in all MMP-8 positive samples, which might reflect the on-going process of resolution or matrix remodelling where blood supply is needed.

To our knowledge, this is the first study examining LN-5 protein expression in KA. The number of keratinocytes at the pushing border with cytoplasmic staining for LN-5 in this study was variable. LN-5 is a major adhesion protein of the cutaneous BM and involved in cell substrate attachment of keratinocytes, which is important for keratinocyte migration during epidermal wound healing as well as in cancer (Pyke *et al*. 1994; Sadowski *et al*. 2005). Several MMPs are known to process LN-5γ2 *in vitro* (Pirilä *et al*. 2003) and indeed, we could find a trend for stronger LN-5 expression in MMP-13 positive samples. MMP-10 enhances cell migration in wound healing and participates in the remodeling of LN-5 in keratinocytes (Salmela *et al*. 2004; Krampert *et al*. 2004). Interestingly, in our study LN-5 positive keratinocytes co-localized with MMP-10 positive cells at the pushing border in most samples. According to our study, however, LN-5 is not valuable in differentiating KA from SCC, since it is widely detected in both KA and SCC (Airola *et al*. 1997; Kerkelä *et al*. 2001).

5.12. MMP-26 may contribute to the more aggressive behavior of SCCs in organ transplant recipients (IV).

In the final study we compared the expression of MMPs in SCCs of the immunosuppressed (IS) and immunocompetent (IC) patients. Positive staining for MMP-26 was detected in cancer cells at the invasive front in 17/20 IS and in 8/20 IC SCCs. MMP-26 was detected in basal keratinocytes in 8/9 BD samples in IS patients, and in 3/9 tumors of the IC patients. MMP-26 expression was significantly stronger in cancer cells at the invasive front of SCCs ($p= 0.01$) and in epithelial cells of BD samples ($p=0.04$) of IS patients. MMP-26 has been implicated in keratinocyte migration during cutaneous wound repair and in the early stages of skin carcinogenesis (Ahokas *et al*. 2005). It is very tightly regulated as none of the common cytokines/growth factors are able to induce its expression in keratinocyte culture (Ahokas *et al*. 2005). The function of MMP-26 cannot be investigated using mouse models since the MMP-26 gene is not present in rodents (Uría and López-Otín 2000). Therefore, additional studies on MMP-26 protein using human tissues are necessary in elucidating its role. These

results show a significant difference in the MMP-26 staining of malignant keratinocytes between IS and IC patients. MMP-26 may function to promote inflammation (Li *et al*. 2004) and this may influence the more aggressive phenotype of the SCCs in IS patients. In addition, MMP-26 is known to activate MMP-9, one of the most critical MMPs in the growth of SCC (Mueller 2006). In cutaneous wounds and well- and moderately differentiated SCCs MMP-26 colocalizes with LN-5 and may be regulated by this matrix protein (Ahokas *et al*. 2003). MMP-26 may also be needed to degrade FN or type IV collagen when cancer cells initially penetrate the cutaneous BM (Ahokas *et al*. 2003). Another study suggested that estrogen-induced MMP-26 has a functional role in inflammation through proteolysis of the cytoplasmic estrogen receptor-β and it also has anti-tumor properties in several hormone-regulated malignancies (Savinov *et al*. 2006). The function and role of MMP-26, however, might be different in hormone-regulated cancers compared to skin cancer.

When patients were pooled into two groups irrespective of immune status, those using cyclosporin and those not, MMP-26 expression was significantly more intense in patients using cyclosporin ($p=0.04$). This agrees with recent Affymetrix data on a SCC cell line demonstrating differential regulation of various MMPs after treatment with cyclosporin A in culture (Tiu *et al*. 2006). Neither cyclosporin nor estradiol, however, is able to stimulate MMP-26 mRNA expression in HaCaT keratinocyte cell cultures (Saarialho-Kere and Skoog, unpublished data). All, except for two, of our immunosuppressed patients used azathioprine medication. Thus, when patients were pooled into two groups, those patients using azathioprine and those not, results were similar with results comparing IS and IC groups. The contribution of immunosuppressive medication to malignancy is complex and unclear. Jensen *et al*. (1999) noted that kidney transplant recipients using cyclosporin and prednisolone had a higher risk of NMSC than those taking azathioprine and prednisolone. In groups with the triple-drug regimens including cyclosporin, azathioprine, and corticosteroids, however, the skin cancer incidence was the highest (Jensen *et al*. 1999). Oral steroid use is associated with an increased risk of SCCs in several studies (Karagas *et al*. 2001; Patel *et al*. 2007). A recent study demonstrated that calcineurin inhibitor monotherapy reduces the risk for SCCs after kidney transplantation compared with bi or tritherapy (Abou *et al*. 2007). While the high incidence of neoplasm and its aggressive progression are thought to be due to the resulting impairment of the OTRs immune-surveillance system, azathioprine and cyclosporin might induce the risk of SCC independently of immunosuppression (Hojo *et al*. 1999; de Graaf *et al*. 2008). Another study compared the risk of skin cancer with different drug combinations and concluded that immunosuppression *per se* is responsible for the increased skin cancer risk and that this is independent of the agent used (Bouwes Bavinck *et al*. 1996). Although cyclosporin did not induce MMP-26 expression *in vitro*, this would be an interesting aspect to investigate *in vivo* in the future, like the regulation of cancer-related MMPs in epithelial cell cultures treated with immunosuppressive drugs. Affymetrix analysis could be used in comparing tumor samples of IC and IS patients or cell lines overexpressing MMP-26 and corresponding vector controls to understand the mechanism of immunesuppression-mediated tumorigenesis.

5.13. MMP-9 is induced in macrophages surrounding SCCs of the IC patients and in stromal neutrophils of the patients using cyclosporin (IV).

We detected MMP-9 in epithelial cells at the pushing border in 8/20 IS and 5/20 IC SCCs, with no differences between the two groups. In BDs, however, MMP-9 was only detected in a small number of epithelial cells in one sample of an IS patient. MMP-9 was also produced by neutrophils and macrophages in several samples. Its production in neutrophils tended to be more abundant in SCCs of the IS group, although in macrophages it was expressed more in the IC group. The differences, however, were not statistically significant between these groups. Due to the trends observed, we stained five additional pairs of SCCs and found that MMP-9 protein was significantly more prevalent in macrophages surrounding SCCs of the IC group ($p=0.02$).

These results on the expression of MMP-9 in tumor cells, as well as inflammatory cells of SCCs, are consistent with previous studies (Chebassier *et al*. 2002; Impola *et al*. 2005). Increased staining for MMP-9 has frequently been associated with cutaneous tumor progression and metastasis (Coussens *et al*. 2000; Egeblad and Werb 2002) and was also detected in SCCs of post-transplant patients by microarray expression profiling (Nindl *et al*. 2006). A recent study on mice suggests that TNF-α regulates epithelial MMP-9 expression during tumor promotion and TNF-α stimulated keratinocyte migration occurs via an MMP-9-dependent pathway (Scott *et al*. 2004). Interestingly, we found no statistically significant differences in MMP-9 expression between the IS and IC groups in tumor cells, which agrees with the results of a previous study done on SCCs of immunosuppressed patients (Chebassier *et al*. 2002). Tumor-associated inflammatory cells, neutrophils, macrophages and mast cells, are the major providers of MMP-9 in a skin carcinogenesis model (Coussens *et al*. 2000). MMP-9 participates in the angiogenic switch necessary for tumor development (Bergers *et al*. 2000). Infiltration of tumor-associated macrophages (TAMs) is a key process during cancer development in various cancer types (Tlsty and Coussens 2006). Indeed, induction of cervical carcinoma in K14-HPV16 mice is markedly reduced when mice are systemically treated with an amino-bisphosphonate that acts on MMP-9-expressing macrophages (Giraudo *et al*. 2004). A recent study suggested that MMP-9 from neutrophils is released as a TIMP-free zymogen and is readily available for activation, and therefore may serve as a unique proangiogenic molecule at the sites of physiologic and tumor angiogenesis (Ardi *et al*. 2007). The studies on the role of MMP-9 expressing macrophages show contradictory results on its role in tumor progression (Takeha *et al*. 1997; Giraudo *et al*. 2004). In liver cancer MMP-9 expressing macrophages were associated with better prognosis while cervical carcinogenesis was markedly suppressed after inactivating MMP-9 expressing macrophages. High stromal MMP-9 expression correlated with an advanced stage of the tumor and short disease-related survival in epithelial ovarian cancer (Sillanpää *et al*. 2007). Only few reports have investigated the expression of MMPs in skin cancers of the OTRs (Chebassier *et al*. 2002; Boyd *et al*. 2008). Boyd *et al*. (2008) reported that MMP-9 was detected more frequently in stromal macrophages in the BCCs of IC patients agreeing with our results. MMP-9 expressing

macrophages may have a protective role in IC SCCs that may be related to the role of macrophages in tumor regression (Takeha *et al*. 1997).

When immunosuppressed patients were pooled into two groups based on cyclosporin medication irrespective of the immune status, MMP-9 expression was significantly stronger in stromal neutrophils of the SCCs in patients using cyclosporin ($p=0.04$). A trend for more abundant staining of MMP-9 in neutrophils of the IS group and patients using cyclosporin may explain the behavior of SCCs in IS patients, since neutrophils promote angiogenesis and tumor invasion by secreting MMP-9 (Mueller 2006).

When the two patient groups were pooled irrespective of immune status and the immunostaining was compared for individual MMPs between well-differentiated and moderately-poorly differentiated SCCs, the only significant difference among MMPs studied noted was that MMP-9 expression increased in cancer cells of poorly differentiated tumors ($p=0.03$). MMP-9 induction is likely to be relevant for tumor development, because mice lacking MMP-9 show reduced keratinocyte hyperproliferation and a decreased incidence of invasive tumors during skin carcinogenesis (Coussens *et al*. 2000).

When the two patient groups were pooled irrespective of immune status and immunostaining was compared for individual MMPs between SCC samples with signs of HPV infection and those without, the only significant difference noted was that MMP-9 expression increased significantly in HPV-associated SCCs ($p=0.03$). Although it is widely accepted that certain high-risk HPV types play a central role in cervical cancer (Walboomers *et al*. 1999), the role of HPV in the development of cutaneous SCCs remains controversial. Strong evidence implicates HPV in the pathogenesis of cutaneous SCCs in immunosuppressed individuals (Harwood *et al*. 2000). OTRs also develop large numbers of warts, primarily on sun-exposed skin, that carry a high risk of subsequent malignant transformation to cutaneous SCCs (Harwood *et al*. 2000). The mechanism by which HPV causes oncogenesis in cutaneous SCC development is unclear. Skin tumors of HPV8 transgenic mice had elevated levels of proMMP-9 (Akgül *et al*. 2006), agreeing with our results that staining for MMP-9 was more intense in SCCs with signs of HPV infection.

5.14. Expression of MMP-1, -7, -8, and -13 does not differ in SCCs of IS and control patients (IV).

In healthy adult tissues, the levels of MMP-1 are usually low, but it is frequently activated in SCC of the head and neck as well as various other tumors often associated with poor prognosis. MMP-1 mRNA expression has been shown in epithelial cells within tumor islands but also with in the fibrous connective tissue adjacent to the tumor (Pardo and Selman 2005; Rosenthal and Matrisian 2006). In our study, positive staining for MMP-1 was detected in keratinocytes at the invasive front in 8/20 IS and in 12/20 IC SCCs and stromal staining for MMP-1 was detected in fibroblasts and macrophages. No statistical differences occured between these groups in epithelial or stromal staining. MMP-1 was expressed in epithelial

cells in 7/18 BD tumors with no statistically significant differences between the groups. Previous studies are congruent with our results (Pardo and Selman 2005; Rosenthal and Matrisian 2006). Tsukifuji *et al*. (1999) suggested that MMP-1 expression could be an early event in the development of SCC. We could not, however, find differences in the expression of MMP-1 in grade I vs grade II-IV SCCs or between BDs and SCCs. Another study from our group detected more MMP-1 staining in stromal macrophages in the BCCs of immunocompetent patients (Boyd *et al*. 2008), but we could not find the same phenomenon in our SCC samples.

We could detect positive MMP-8 staining in keratinocytes at the invasive front in 6/20 IS and in 3/20 IC SCCs. A trend existed for stronger MMP-8 expression in the IS SCC group (p= 0.15). The differences, however, were not statistically significant. The production of MMP-8 by stromal neutrophils was detected in four IS SCCs and in one IC SCC mostly in tumors that also expressed MMP-8 in cancer cells (p=0.15). MMP-8 was detected epithelially in 7/18 BD samples. Thus, we detected MMP-8 in transformed keratinocytes of SCCs and BD lesions as well as in stromal neutrophils. The expression of MMP-8 has been detected in tumor cells in SCCs of the head and neck, but not in cutaneous SCCs (Moilanen *et al*. 2002; Impola *et al*. 2005). Our results are mostly congruent with previous findings (Moilanen *et al*. 2002; Impola *et al*. 2005). Inhibiting MMP-8 promotes tumor formation and spreading in mice (Balbin *et al*. 2003). MMP-8 degrades type I collagen which is essential for cancer cell spread (Moilanen *et al*. 2002). In our study, we could not find significant differences in MMP-8 expression between the IS and IC groups. Overall, the positive staining for MMP-8 in SCCs was not strong (6/20 IS and 3/20 IC patients). Positive staining for MMP-8, however, was already detected in several of the BD samples (7/18). MMP-8 may, indeed, have a protective function in cancer (Balbin *et al*. 2003).

High MMP-13 expression level is detected in several invasive tumors often associated with poor prognosis and suppression of MMP-13 in human SCCs reduces tumor growth (Ala-Aho *et al*. 2002). In our study, epithelial staining for MMP-13 was detected in 12/20 IS and in 10/20 IC SCCs agreeing with previous reports, and it was expressed in fibroblasts, endothelial cells and giant cells in some of the samples. No statistical differences in epithelial or stromal expression occured between the two groups. MMP-13 was detected in epithelial cells in 8/18 BD specimens with no significant differences between the groups. Surprisingly, the expression of MMP-13 by tumor cells at the invasive front or by stromal or endothelial cells in SCC as well as BD was not significantly different in the post-transplant group compared to the immunocompetent.

MMP-7 was detected in the epithelial keratinocytes in 8/20 IS and in 11/20 IC SCCs and positive stromal staining for MMP-7 was detected in few macrophages and giant cells in both IS and IC SCCs agreeing with previous reports. Epithelial expression of MMP-7 was detected in 7/18 BD samples with no significant differences between the groups. We could not find any differences in staining for MMP-7 between the IS and IC groups. Overexpression of MMP-7 is predominantly associated with epithelial malignant cells as well as normal adult

glandular epithelium (Kerkelä and Saarialho-Kere 2003). It also plays an important role in ectodomain shedding of cell-surface molecules, such as epidermal growth factor receptor (EGFR) (Mimori *et al*. 2004), heparin binding epidermal growth factor (HB-EGF) (Yu and Woessner 2000), Fasl (Powell *et al*. 1999) and E-cadherin (Noë *et al*. 2001) to promote invasion and angiogenesis. Interestingly, it does not seem to play a significant role in the pathobiology of NMSC of OTRs.

5.15. Expression of TIMPs-1 and -3 is not altered in tumors of IS patients

Epithelial staining for TIMP-1 at the invasive front was detected in 5/20 IS and 3/20 IC SCCs, with no differences between the IS and IC groups. In cutaneous and oral SCCs, TIMP expression is detected in tumor as well as stromal cells (Kerkelä and Saarialho-Kere 2003). TIMPs have both pro- and antineoplastic effects during cancer progression (Mannello and Gazzanelli 2001). In a skin carcinogenesis model of HPV16 mice overexpressing TIMP-1, it inhibited activity of gelatinases in tumor stroma but enhanced tumorigenity and did not inhibit malignant progression or development of metastasis (Rhee *et al*. 2004). Retinoid acid decreases the activity of several MMPs in UVB-irradiated skin (Fisher *et al*. 1996), and is associated with upregulation of TIMP-1 (Schroen and Brinckerhoff 1996). Recent results from our group reported that TIMP-1 expression was stronger in macrophages surrounding BCCs of the immuncompetent patients (Boyd *et al*. 2008). In our study, however, stromal staining for TIMP-1 in fibroblasts and macrophages was also detected, but the results did not differ between the IS and IC groups.

TIMP-3 was expressed in keratinocytes at the invasive front in 18/20 IS SCCs and 14/20 IC SCCs with no significant differences between the two groups. TIMP-3 is a multifunctional protein tightly bound to the ECM. It inhibits TNF-α converting enzyme and induces apoptosis through the stabilization of TNF-α receptors on the cell surface (Mannello and Gazzanelli 2001). Adenoviral expression of TIMP-3 inhibits SCC tumor growth more potently than p53 adenovirus in mice (Ahonen *et al*. 2002). Interestingly, using *in situ* hybridization as analyzing method previous studies have found TIMP-3 expression in infiltrative tumor cells of BCCs, but only surrounding stromal cells in SCCs (Airola *et al*. 1998; Sutinen *et al*. 1998). Because of these abilities TIMP-3 has, we wanted to see if its expression is different in IS group compared to IC group. No significant differences between the two groups were noted, however, although it was expressed in keratinocytes at the invasive front in the majority of SCCs in both groups.

Table 4. Summary of MMPs investigated in this thesis.

	KA e	**BD e**	**SCC e**	**MM e**	**EMPD e**
MMP-1	n.d.	+	+	n.d.	+
MMP-2	-	n.d.	-	n.d.	-
MMP-3	n.d.	n.d.	n.d.	n.d.	-
MMP-7	+	+	+	n.d.	+
MMP-8	+	+	+	n.d.	n.d.
MMP-9	+	+	+	n.d.	+
MMP-10	+	n.d.	+	n.d.	n.d.
MMP-13	+	+	+	+	+
MMP-19	+	n.d.	+	n.d.	+
MMP-21	n.d.	n.d.	n.d.	+	n.d.
MMP-26	n.d.	+	+	-	n.d.
MMP-28	n.d.	n.d.	n.d.	+	n.d.

-, no staining; +, positive staining; n.d., not determined; e, epithelial cancer cell staining; KA, keratoacanthoma; BD, bowen's disease; SCC, squamous cell carcinoma; MM, malignant melanoma; EMPD, extramammary Paget's disease

6. CONCLUSION

Cutaneous cancer is the most common human malignant disease and over 50% of all neoplasms arise in the skin. Lifelong immunosuppression in OTRs increases the risk of NMSC leading to substantial morbidity and mortality in these patients. MMPs are associated with many types and stages of cancer in numerous studies. This study aimed to investigate the roles of MMPs in various benign and malignant skin tumors *in vivo* and to shed light to the pathobiology of these lesions.

This is the first study on MMPs in EMPD. We found, among the several MMPs studied, expression of MMP-7 and -19 in Paget cells in EMPD. Their presence might predict an underlying adenocarcinoma in these patients. Since this tumor is very rare, however, stainings with larger patient cohorts would be valuable in the future. Furthermore, expression of MMP-7 and -19 supports the theory that Paget cells originate from dermal adenocarcinoma cells of apocrine duct origin. Unlike in most cancers, upregulation of classical MMPs is not a general feature in EMPD, which may associate with the rather benign clinical behavior of a subgroup of EMPD tumors.

This study was the first to compare MMP expression in primary melanomas and their sentinel nodes. In MM, MMP-21 was upregulated in the early phases of malignant progression, but disappeared from the more aggressive lesions and nodal micrometastases. In conclusion, MMP-21 might serve as a protective MMP in MM. A murine MMP-21-knock-out model would be needed to examine this hypothesis further. MMP-13 was detected in the more aggressive MMs as well as lymph node metastases agreeing with previous studies. Thus, MMP-13 might serve as a marker for more aggressive tumors.

Adhesion molecules and the degree of angiogenesis have been previously studied to differentiate KAs from well-differentiated SCCs. We were the first to compare their protein profiles and observed that positive staining for MMP-7 and -9 in the epithelial pushing border should raise a suspicion of malignant conversion to SCC. The expression of MMP-19 and p16 were abundant in KAs, but disappeared from SCCs, suggesting that lack of MMP-19 and p16 in clinical KAs could indicate that KAs are turning into SCCs. Frequent expression of the transformation-specific MMP-13 in KAs supports their treatment by excision, as a subgroup of them are already incomplete SCCs.

Differences in the inflammatory cell profile, adhesion molecules, or the profile of proteases or their inhibitors might contribute to the exceptionally aggressive behavior of cutaneous SCCs in OTRs. In SCCs and BDs of the IS patients, positive staining was found significantly more often for MMP-26 than in those of control patients. MMP-26 expression was also significantly stronger in patients using cyclosporin. According to previous studies, MMP-26 may function to promote inflammation or to activate MMP-9 and this may influence the more aggressive phenotype of the SCCs in IS patients. Since MMP-26 is not present in rodents,

more studies in human tissues are needed to specify its role in cancer progression, particularly as it is known that the progression sequence for cutaneous cancers may vary between the human disease and its corresponding mouse models. Expression of MMP-9 was significantly stronger in macrophages surrounding SCCs of the IC patients being understandable as tumor-associated macrophages may have a protective role in progression of SCCs possibly through participation in the host-response reaction provoked by the cancer. On the contrary, when our two patient groups were pooled irrespective of immune status, MMP-9 staining in neutrophils of patients using cyclosporin was significantly more abundant. MMP-9 expression in tumor cells was also upregulated in less differentiated SCCs and in SCCs with histological signs of HPV infection. MMP-9 expressing neutrophils have been associated with tumor angiogenesis and progression in previous studies. Thus, they may have an important function in tumor progression in OTRs using cyclosporin. Surprisingly, classical cancer-related MMPs, such as MMP-1 and -13, did not differ in their expression between IS and IC groups, nor did we observe diminished expression of TIMP -1 or -3 in immunosuppressed patients.

MMPs have an important role in tumor progression. Recent studies have revealed, however, that some of them might also provide protective effects in different stages of cancer progression or in certain cancer types. The future challenges in MMP research are to increase our understanding of the relevant *in vivo* substrates for specific MMPs. The actual role of an individual MMP in tumor progression and in different cancers is relevant for targeting the therapies more precisely. Expression of certain MMPs could also be used as prognostic markers in planning of treatment strategies or adjuvant therapies.

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