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MATRIX METALLOPROTEINASES -21 AND -26 IN KERATINOCYTES AND IN KERATINOCYTE-DERIVED CANCERS

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To Juha, my companion and best friend, and

To Paula and Venla, our jumping joys and sunshines

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals:

I Ahokas K, Lohi J, Lohi H, Elomaa O, Karjalainen-Lindsberg M-L, Kere J, and Saarialho-Kere U. Matrix metalloproteinase-21, the human orthologue for XMMP, is expressed during fetal development and in cancer. *Gene* 301, 31-41, 2002.

II Ahokas K, Lohi J, Illman SA, Llano E, Elomaa O, Impola U, Karjalainen-Lindsberg M-L, and Saarialho-Kere U. Matrix metalloproteinase-21 is expressed epithelially during development and in cancer and is up-regulated by transforming growth factor-β1 in keratinocytes. *Lab Invest* 83, 1887-1899, 2003.

III Ahokas K, Skoog T, Suomela S, Jeskanen L, Impola U, Isaka K, and Saarialho-Kere U. Matrilysin-2 (Matrix metalloproteinase-26) is upregulated in keratinocytes during wound repair and early skin carcinogenesis. *J Invest Dermatol* 124, 849-856, 2005.

IV Ahokas K, Karjalainen-Lindsberg M-L, Sihvo E, Isaka K, Salo J, Saarialho-Kere U. MMP-21 and –26 are differentially expressed in esophageal squamous cell cancer. *Tumor Biology, in press* 2005.

ABBREVIATIONS

ADAM	adamalysin, a disintegrin and metalloproteinase
BCC	basal cell carcinoma
bFGF	basic fibroblast growth factor
BM	basement membrane
cDNA	complementary DNA
Coll	collagen
ECM	extracellular matrix
EGF	epidermal growth factor
ESCC	oesophageal squamous cell carcinoma
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IHC	immunohistochemistry
KC	keratinocyte
LN	laminin
MMP	matrix metalloproteinase
PMA	12-phorbol-13-myristate-acetate
RT-PCR	reverse transcriptase polymerase chain reaction
SCC	squamous cell carcinoma
TGF-β1	transforming growth factor beta 1
TIMP	tissue inhibitor of matrix metalloproteinases
TNF-α	tumour necrosis factor alpha
VEGF	vascular endothelial growth factor

Katja Ahokas, Matrix metalloproteinases -21 and -26 in keratinocytes and in keratinocyte-derived cancers. Department of Dermatology and Biomedicum Helsinki, Helsinki University Central Hospital and University of Helsinki.

ABSTRACT

The extracellular matrix (ECM) is a protein-rich network that provides information and structural support to the surrounding cells. Matrix metalloproteinases (MMPs) comprise a family of 23 human enzymes capable of degrading all components of the ECM. MMPs are essential for maintaining the indispensable balance between breakdown and production of ECM in physiological conditions, such as in reproductive processes and foetal development, but they can also create a neoplasm-favouring milieu where cells can permeate degraded basement membranes, where novel partners for interactions are revealed and where tumour-promoting cytokines and growth factors are made available.

We began this study by completing the human MMP family with MMP-21. As the roles of the novel MMPs are still rather enigmatic, we further wanted to define the expression profiles and inducing factors for the two most recent ones, MMP-21 and -26, in physiological and pathological conditions of the skin and oesophagus in order to clarify their roles in epithelial biology.

MMP-21 was not expressed under homeostatic conditions, during wound-healing processes or in hyperproliferative or dysplastic conditions of the adult skin or oesophagus. During foetal development its expression was tightly regulated in a time and space related fashion and was confined to epithelial structures. Cancer cells at the invasive front of grades II and III squamous cell cancers (SCCs) of the skin and oesophagus expressed MMP-21. In cultured keratinocytes, transforming growth factor beta 1 induced MMP-21 expression.

MMP-26 was not expressed in normal resting epithelium, but migrating keratinocytes induced its expression during acute wound healing. Cellular atypia and inflammation upregulated MMP-26 expression in pre-malignant cutaneous lesions, in invasive cancer cells in skin and oesophageal SCCs of grades I and II and in oesophageal dysplasia. Tumorigenic progression abolishes MMP-26 expression, with grade III SCCs being devoid of it.

Our research indicates that MMP-21 and MMP-26 play different roles in malignant transformation, and their expression profiles may help to identify patients needing more aggressive cancer treatment.

1. INTRODUCTION

As early as in 1889 Stephen Paget proposed his well-known hypothesis for pathogenesis of cancer, the "seed and soil" hypothesis, in which he claimed abnormal interactions between tumour cells and the tissue environment are needed for attachment of the tumour to occur, explaining the non-random pattern of metastasis (Paget, 1889). Many studies of xenografted tumours, single nucleotide mutations of tumour suppressors and oncogenes have forced researchers to revisit the hypothesis, as the microenvironment seems to determine the success of malignant processes (Fidler, 2002).

The research on matrix metalloproteinases (MMPs) began nearly half a century ago, when Gross and Lapière observed collagenolytic activity in a tadpole undergoing metamorphosis (Gross and Lapière, 1962). Since then, MMPs have been shown to be key players in the breakdown of barriers and supportive structures and in revealing novel cryptic sites of information needed for development and homeostasis of tissues as well as for establishment of many pathological conditions. Normal cells lack constitutive expression of MMPs, but their expression is upregulated in almost every type of human cancer (Egeblad and Werb, 2002). MMPs enzymatically degrade all components of the extracellular matrix (ECM). Balance between production and breakdown of ECM macromolecules is essential for all biological processes, and excessive proteolysis can cause or assist in the development of various pathological conditions, such as chronic inflammation, chronic wounds of the skin and intestine, arthritis, cardiovascular diseases and cancer.

The aim of this study was to identify novel members of the MMP family and to investigate their role in epithelial biology, determining whether they work for or against malignant processes.

2. REVIEW OF THE LITERATURE

2.1. STRUCTURE OF THE SKIN AND OESOPHAGEAL MUCOSA

The skin is composed of two layers, the outer layer being the *epidermis*, a stratified (containing multiple layers) squamous epithelium that overlays a layer of connective tissue, the *dermis* (Oikarinen and Tasanen-Määttä, 2003; McGrath et al., 2004) (Figure 1). The outer keratinized part of the epidermis functions as a barrier against drying, irradiation, mechanical, chemical or biological injury and foreign organisms. Barrier function is achieved by keratinocytes (KCs), which are organized in 10-20 layers with four levels of differentiation: basal, spinous, granular and cornified. Basal stem cells in the epidermis give rise to transiently amplifying KCs, which after early differentiation in the spinous layer, start the apoptotic machinery and via late and terminal differentiation lose their nuclei in the granular layer, eventually dying and cornifying forming the stratum corneum of the epidermis (Nemes and Steinert, 1999) (Figure 1). The time for a KC to undergo differentiation and cornification after division in the basal layer is 52-75 days in normal skin, but can be many times faster in hyperproliferative disorders, such as psoriasis. KCs are the most abundant cells in the epidermis, constituting 95% of epidermal cells. The epidermis also contains pigment-producing melanocytes, antigen-recognizing Langerhans cells and touch-sensitive neuroendocrine Merkel cells. The dermis consists of collagen, elastin, fibroblasts, blood vessels and adnexal structures (Oikarinen and Tasanen-Määttä, 2003).

The *oesophageal mucosa* is squamous and stratified in much the same way as the skin; the cells differ from skin KCs mainly by being non-keratinized, by having a

thicker proliferating basal layer and by expressing different keratin proteins (see Ross and Romrell, 1989; Rosai, 2004) (Figure 1).

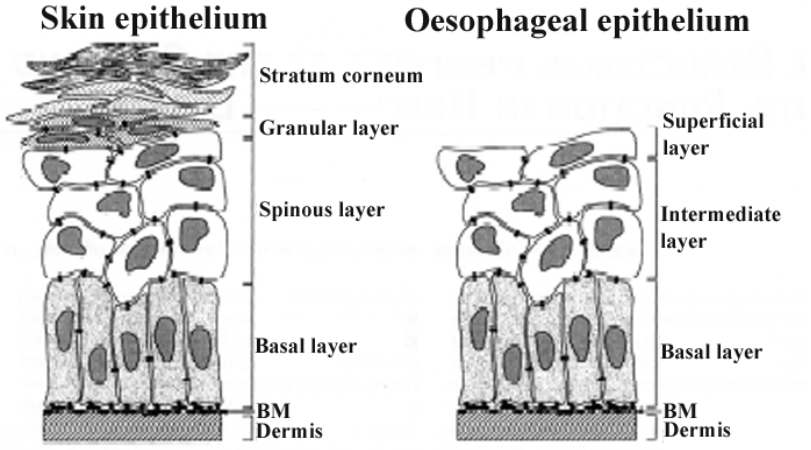

Figure 1. Schematic representation of skin and oesophageal epithelia (modified from Presland and Dale, 2000).

2.1.1. Skin basement membrane zone

The dermis and epidermis are compartmentalized, yet connected by basement membranes (BMs), which are self-assembling 50 to 100-nm-thick sheets of two networks linked by nidogen, the collagen (coll) IV and laminin (LN) networks. Covalently linked coll IV provide BMs with resistance to mechanical stress, whereas LN-polymers are essential for the adhesive properties of BMs (Yurchenko et al., 2004). BMs can control the shape, proliferation, differentiation, migration, gene expression and apoptosis of epithelial cells. Basal cells connect to the BMs via hemidesmosomal complexes (Figure 2). LN-5, a major component of anchoring filaments, is connected to the cell surface via association with $\alpha 6\beta 4$-integrin and extends to the basal lamina escorted by collagen XVII. LN-5 is unable to bind nidogen or coll IV, instead interconnecting with LN-6 or -7 to incorporate itself into nidogen and eventually coll IV (Timpl, 1996; Ghohestani et al., 2001).

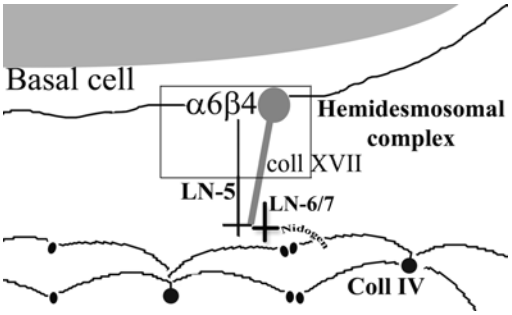

Figure 2. Schematic representation of the cutaneous basement membrane zone (modified from Ghohestani et al., 2001).

2.1.2. Extracellular matrix

All cells of the body are embedded in a tissue-specific extracellular matrix (ECM). Fibroblasts secrete components of dermal ECM, including collagens, elastin, laminins and other fibrous proteins embedded in networks of large glycoproteins. The ECM is a well-organized web of solid-state support for cells, it is a reservoir of bound cytokines and growth factors and it carries cryptic information that can be cleaved by proteinases to give rise to fragmented molecules with novel bioactivities. Adhesion of cells to each other is primarily a function of E-cadherin (=epithelial calcium-dependent adhesion molecule) and the associated catenin complex (α–, β– and γ–catenins and catenin-like p120ctn) (Beavon, 2000). As ECM is proteolysed, interactions between cells are lost and new interactions between cells and the surrounding matrix arise, promoting diverse cellular functions including migration, proliferation and differentiation. ECM remodelling is essential in many physiological conditions, including reproduction, wound healing, angiogenesis and foetal development, but it is also responsible for tissue destruction in such pathological conditions as chronic wounds, atherosclerosis, cancer invasion and metastasis (Timpl, 1996; Kalluri, 2003; Radisky and Bissell, 2004).

2.2. CUTANEOUS WOUND HEALING

Wounding is essentially a loss of integrity within a tissue, affecting the skin, mucosa or organs. The complex processes of healing aim at repair of the damage that occurs in overlapping phases of inflammation, proliferation, migration, matrix synthesis and contraction; repair usually takes 1-2 weeks (Martin, 1997) (Figure 3). As wounding disrupts the integrity of blood vessels, bleeding occurs, activating the blood coagulation system and rapidly leading to *clot formation*. The primary function of the fibrin clot is termination of bleeding (Hatz et al., 1994; Martin, 1997). The clot is transformed into *granulation tissue* as neutrophils, monocytes, plasma cells, mast cells, fibroblasts and endothelial cells invade the clot and fill it with blood vessels and a novel provisional matrix. Neutrophils and other granulocytes defend the wounded site against micro-organisms (Hatz et al., 1994; Martin, 1997). KCs surrounding the wounded area begin to migrate along the novel matrix between the clot remnants and granulation tissue within 3-6 h of injury (*epithelialization*). As KCs migrate, basal cells distal to them start proliferating. Once the wounded area is covered by a monolayer of KCs, the suprabasal KCs undergo a standard differentiation program, leading to reconstitution of the squamous stratified epithelium at the site of injury (Hatz et al, 1994; Martin, 1997). *Wound contraction* occurs next, with fibroblasts differentiating into myofibroblasts, which contract and bring the wound margins together. The *reparative phase* is the last stage in wound healing; fibroblasts in the dermis produce new connective tissue, i.e. collagen fibrils. As collagen matures, cell density and vascularization of the wound decrease and scar formation results (Figure 3).

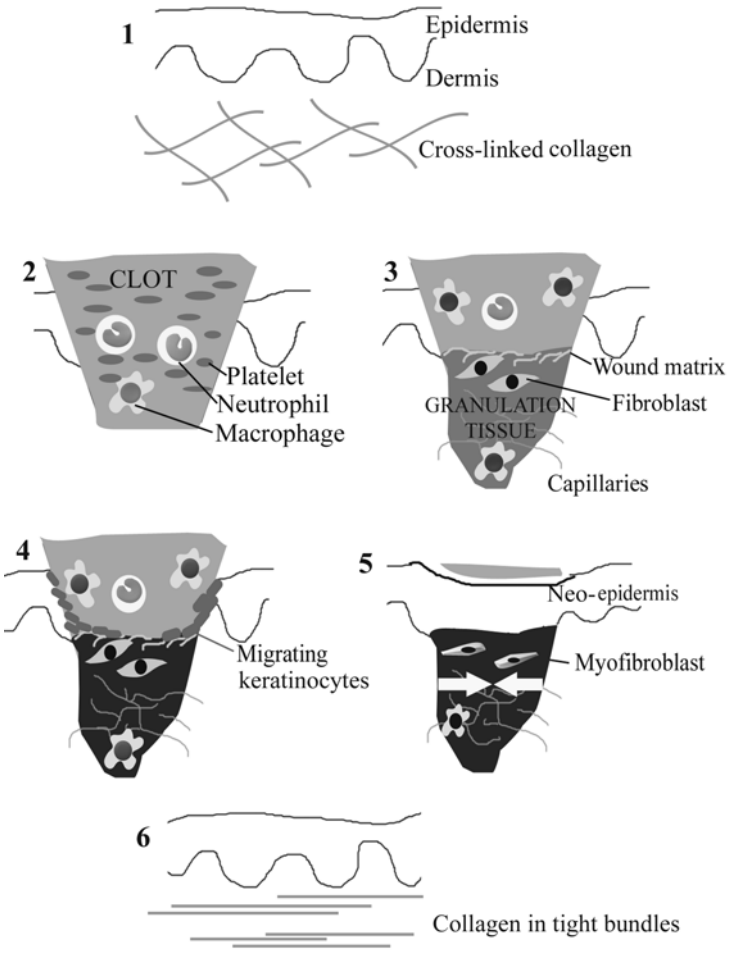

Figure 3. Schematic representation of wound healing. 1) Intact skin with collagen arranged in networks. 2) Clot formation and rapid filling with neutrophils and macrophages. 3) Fibroblasts and endothelial cells invade the clot and deposit the granulation tissue. 4) Simultaneously, keratinocytes begin to migrate along the wound matrix deposited by fibroblasts, and distal keratinocytes start proliferation. 5) Contact inhibition seizes the migration of keratinocytes and normal differentiation begins to form a neo-epidermis. At this time fibroblasts differentiate into myofibroblasts, which subsequently contract to bring the wound margins together. 6) Fibroblasts have remained in the granulation tissue and secreted novel extracellular matrix, whose collagens are in tight bundles instead of the network architecture in intact skin (modified from Werner and Grose, 2003).

Wound healing employs many processes also essential in tumorigenesis endothelial cell invasion in granulation tissue formation and angiogenesis in tumorigenesis, KC migration and proliferation in epithelialization and invasion and metastasis in tumour progression, and the fibrin-induced infiltration of endothelial cells, macrophages and fibroblasts during both wound healing and tumour progression (Dvorak, 1986).

2.3. CARCINOMAS

2.3.1. Tumorigenesis and invasion

Tumorigenesis is a complex multistep process involving genetic changes, selection of malignant cell populations and modifications in the environment that favour malignant progression (Hanahan and Weinberg, 2000) (Figure 4).

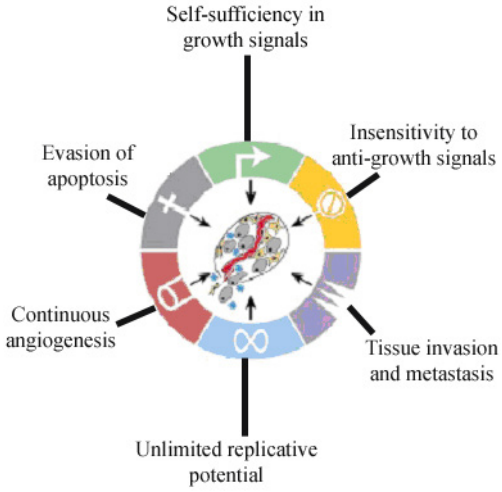

Figure 4. Malignant properties acquired by most cancers during their development (modified from Hanahan and Weinberg, 2000).

One of the first steps towards malignancy is the epithelial to mesenchymal transition (EMT), resulting in free movement of the formerly tightly bound epithelial cells. The ECM works as a barrier to free cell movement, and in order to migrate, cells need to activate their cytoskeletal motors for movement, modulate their cell-surface adhesion molecules for traction, cleave ECM to break the barrier and respond to chemoattractants for guidance of migration (Vu and Werb, 2000). A primary tumour can reach a maximum size of 1-2 mm without a blood supply. Angiogenesis results from hypoxia-induced expression of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), growth factors that promote endothelial cell recruitment (Wyke, 2000). Tumour neovasculature leaks more than normal

vasculature, thus being more efficient in delivery of oxygen to the tumour, but as its endothelial cells proliferate rapidly, they also respond better to anti-angiogenic therapies than the normal vasculature (Sternlicht et al., 1999). Metastatic processes are dependent on stromal cells, endothelial cells, the environment of the tumour cells and the tumour cells themselves. The processes are also dependent on interactions between these components and the balance between steps acting for and against metastasis (Hanahan and Weinberg, 2000). Stromal cells, infiltrating immune cells and tumour cells are all capable of synthesizing the MMPs necessary for invasion through the BMs, to lymph and blood vessels (Egeblad and Werb, 2002). Tumour infiltrates extravasate in a tissue-specific way according to the "seed and soil" hypothesis of cancer development, initially presented in 1889 (see Fidler, 2002).

2.3.2. Pre-malignant and malignant lesions of the skin

The most common skin cancer worldwide is *basal cell carcinoma (BCC)*. In Finland, 5000-5500 new cases of BCC are detected annually, accounting for approximately 22% of all new cancer cases (Finnish Cancer Registry, www.cancerregistry.fi). BCCs grow slowly, cause local skin destruction and rarely metastasize. They are believed to arise from transformed basal stem cells of the epidermis or hair follicles, and they occur in both a sporadic and an inherited fashion (Bäckvall et al., 2005). Histologically, BCCs can be divided into nodular, superficial and aggressive sclerosing (morpheaform) subtypes (Weedon, 2002). The genetic hallmark for BCCs is a loss-of-function mutation for *ptch*, a member of the sonic hedgehog-signalling pathway (Boukamp, 2005). BCCs are primarily treated by surgical removal of the tumour or cryotherapy with liquid nitrogen.

Squamous cell carcinoma (SCC) arises from an unidentified progenitor, possibly a stem cell, and is often the result of cumulative sun exposure. The 900 new

cases of SCCs a year account for 3% of all new cancer cases in Finland (Finnish Cancer Registry). SCCs grow quickly and the development is a step-wise process consisting of squamous cell dysplasia (actinic keratosis), severe dysplasia (carcinoma in situ), invasive SCC and metastasizing SCC (Bäckvall et al., 2005). Hot spot mutations in the *p53* gene are common among SCC patients, occurring early during tumorigenesis (Greenblatt et al., 1994). Transgenic animals with mutations in their p53 alleles have induced susceptibility to develop SCC in response to UV-irradiation, without changes in the latent period before tumour development (Li et al., 1995). A further mutation that occurs with progression of actinic keratosis to SCC is loss of heterogeneity in tumour suppressor $p16^{INK4a}$ (Mortier et al., 2002). As SCC is more frequent in immunosuppressed patients, "dormant" SCC precursors have been suggested to exist in normal skin and to be under control of the immune system (Boukamp, 2005). SCCs demand surgical removal of the tumours, with at least 5-mm margins of healthy skin.

Bowen's disease is a carcinoma in situ type of lesion that is characterized by cellular atypia and frequent mitoses. The BM remains intact and the tumour can be treated by surgical removal or by crytreatment with liquid nitrogen (Kane et al., 2004).

Keratoacanthoma (KA) is a rapidly growing benign squamous neoplasm, often present on sun-exposed skin. It develops rapidly (4-8 weeks) and can regress spontaneously after 3-6 months (Schwartz, 2004). KAs have less complex molecular and cytogenetic changes than SCCs, suggesting that they are genetically incomplete SCCs (Boukamp, 2005). As some KAs become aggressive carcinomas and the malignant behaviour is unpredicatble, all KAs are treated by surgical removal of the tumour (Kane et al., 2004; Schwartz, 2004).

2.3.3. Pre-malignant and malignant lesions of the oesophagus

Intraepithelial neoplasia of the oesophagus is frequently a precursory lesion of *oesophageal squamous cell carcinoma (ESCC)* in high-risk areas and is typically found adjacent to an invasive ESCC. It has cytological and architectural abnormalities and is characterized by a disorganized epithelium and cells with irregular and hyperchromatic nuclei and high mitotic activity. Dysplasia can be graded as low- or high-grade, depending on the severity of the atypical changes. In carcinoma in situ, the entire thickness of the epithelium presents with dysplasia, without evidence of maturing cells at the surface (Mandard et al., 1984).

ESCC is a highly malignant tumour located in the thoracic oesophagus. Approximately 200 new cases of ESCC are diagnosed annually in Finland, accounting for less than 1% of all new cancer cases (Finnish Cancer Registry). Behaviours resulting in chronic irritation and inflammation of the oesophagus, such as smoking and heavy drinking, are risk factors for ESCC (Launoy et al., 1997). At the time of initial diagnosis, the cancer often exhibits extensive local invasion and metastases to regional lymph nodes (Enzinger and Mayer, 2003). Surgery frequently provides only palliation of the symptoms. And the registered 5-year survival rates are around 10% (Hamilton and Aaltonen, 2000).

2.4. MATRIX METALLOPROTEINASES

Completion of the Human Genome Project has resulted in the discovery of 20 000-25 000 human genes, more than 2% of these being proteases or protease inhibitors, emphasizing the importance of regulated degradation of macromolecules in processes as diverse as reproduction, development, host defence, inflammatory diseases, neurological disorders and cancer (Puente et al., 2005). Matrix metalloproteinases (MMPs) are a family of 23 human endopeptidases capable of degrading all

components of the ECM, and the number of non-ECM substrates for MMPs is expanding continually (Overall and Lopéz-Otín, 2002). MMPs are usually absent from normal healthy tissue, but are detected in all repair and remodelling processes and in most cultured cell types (Parks et al., 2004). MMPs and their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs), participate in all processes of tumour development depicted in Figure 4, including loss of cell adhesion, escape from apoptosis, loss of cell growth control, alterations in responses to anti-growth signals and cleavage of barriers to invasion and metastasis.

Based on their structural features, MMPs are divided into five subgroups: collagenases, gelatinases, stromelysins, matrilysins and membrane-type MMPs (MT-MMPs) (Figure 5). Novel members of the family, such as MMP-19 (Pendás et al., 1997), - 23 (Velasco et al., 1999), and -28 (Lohi et al., 2001), often contain additional structural elements, making them unsuitable for any of the pre-existing subclasses. The basic structural units that an enzyme must have to be an MMP are the pro-domain with a Cys-switch for preservation of latency and the catalytic domain containing three histidines to bind the Zn^{2+} ion needed for activity. In addition, all but the matrilysins have a haemopexin domain. The pro-peptide release and thereby activation of MMPs can be achieved with proteinases (also MMPs acting on each other or autolytically) or with chaotropic agents (Vu and Werb, 2000).

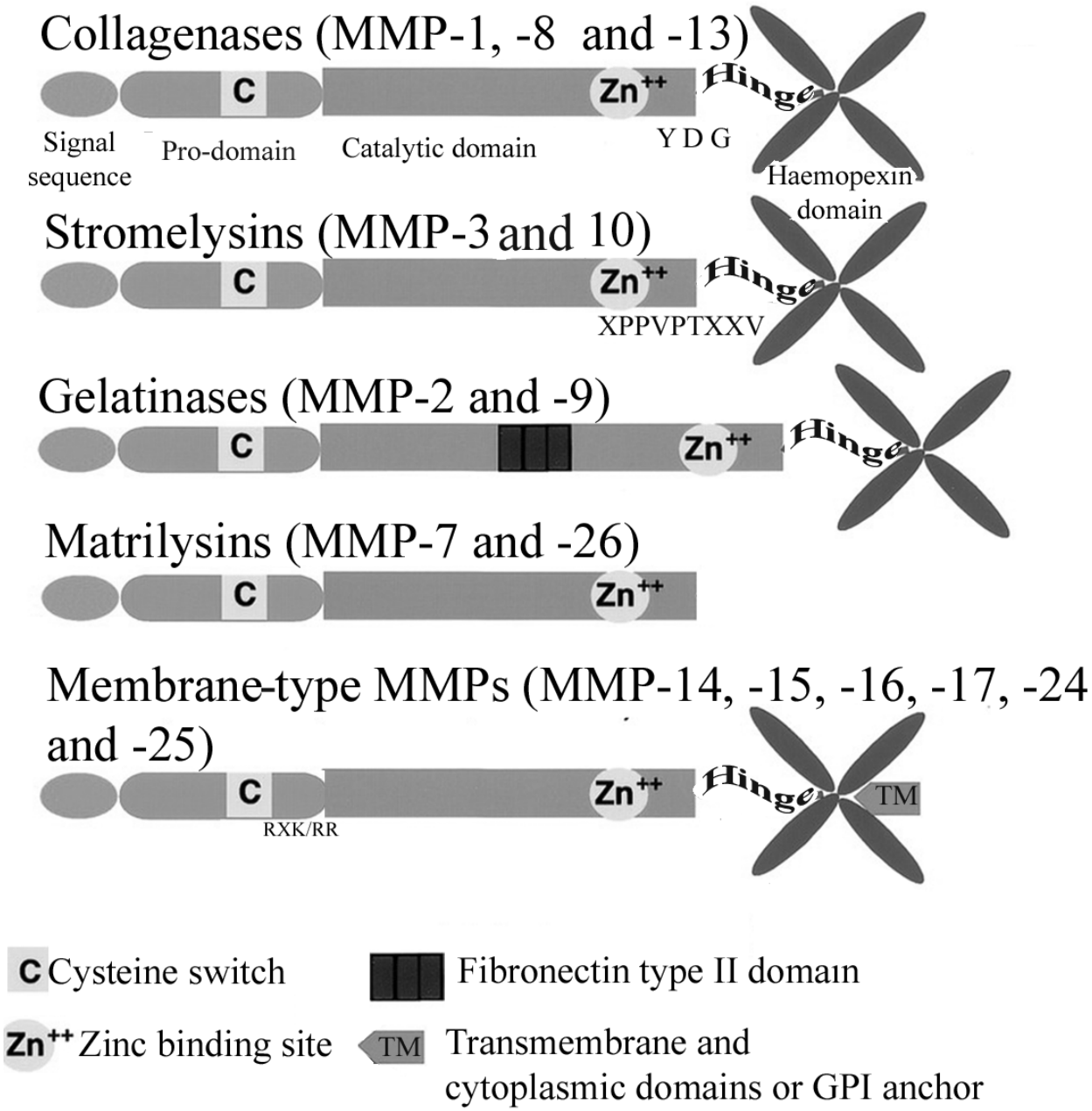

Figure 5. Structural subgroups of matrix metalloproteinases (modified from Vu and Werb, 2000).

MMP-1, -8 and -13 are collagenases with amino acids Tyr214, Asp235 and Gly237 (numbering in MMP-13) in their catalytic domain, enabling binding of the triple-helical structure of native fibrillar collagens in a way sensitive to proteolysis. Non-collagenous substrates for collagenases include aggregan and MMP-inhibitor α2-macroglobulin (Vu and Werb, 2000; Kerkelä and Saarialho-Kere, 2003) (Table 1).

MMP-2 and -9 are gelatinases with three fibronectin-like inserts in their catalytic domain to enable degradation of denatured collagens. Gelatinases cleave also the abundant BM protein collagen IV and release TNF-α and other growth factors from their precursors (Vu and Werb, 2000: Kerkelä and Saarialho-Kere, 2003) (Table 1).

MMP-3, -10 and -11 are stromelysins with the characteristic amino acid insert XPPVPTXXV in the C-terminal end of their catalytic domain to enable degradation of non-collagen ECM substrates such as E-cadherin, LN-1 and type IV collagen (for MMP-3) (Vu and Werb, 2000; Kerkelä and Saarialho-Kere, 2003) (Table 1).

MMP-7 and -26 are matrilysins, the smallest MMPs as they lack the haemopexin domain, making their substrate specificity exceptionally wide, including Fas-ligand, pro-α-defensins and macromolecules harbouring growth factors (Vu and Werb, 2000; Kerkelä and Saarialho-Kere, 2003) (Table 1).

MMP-14, -15, -16, -17, -24 and -25 are MT-MMPs. Their catalytic activities occur on cell surfaces, and they are bound to cell surface either by a transmembrane domain or by a specific glycosyl-phosphatidyl-inositol (GPI) anchor. MMP-14 has been shown to specifically activate MMP-2 (Vu and Werb, 2000; Kerkelä and Saarialho-Kere, 2003) (Table 1).

Other family members form the group of novel MMPs characterized by a furin cleavage site (MMP-21 and -28), novel inserts of, for example Ig-like motifs or unclassical Cys-switches (MMP-23A/B) in their pro-domain (Overall and Lopéz-Otín, 2002). Physiological substrates for many of the novel MMPs remain to be elucidated. Known substrates and chromosomal locations for all MMPs are listed in Table 1.

Table 1. Chromosomal locations and substrates for human matrix metalloproteinases (modified from Kerkelä and Saarialho-Kere, 2003; with additional data from Lohi et al., 2001; Marchenko et al., 2003; Sadowski et al., 2005).

MMP	Chromosomal Location	Substrates
MMP-1	11q22.3	Col I*, II*, III, VII, VIII, X, aggregan, MBP, serpins, α2M, perlecan, pro-TNF-α, IGFBP, α1P1, entactin/nidogen, vitronectin, tenascin, fibrinogen
MMP-2	16q13-q21	Col I, IV, V, VII, X, gelatin, FN, tenascin, fibrillin, osteonectin, entactin, aggregan, vitronectin, plasminogen, MBP, decorin, α2M, LN-5, IGFBP, pro-TNF-a, pro-TGF-β*, α1PI, chemokine ligands CCL* and CXCL*
MMP-3	11q22.3	Col III, IV*, V, VII, IX, X, elastin, FN, fibrillin, gelatin, aggregan, LN-1*, nidogen, osteonectin, decorin, α1PI, pro-TNF-α, MBP, E-cadherin*, β-catenin, IGFBP, fibrinogen, vitronectin, tenascin, plasminogen
MMP-7	11q21-q22	Col IV, elastin*, FN, LN-1, entactin, aggregan, vitronectin, versican, nidogen, tenascin, versican, α1PI, osteopontin, MBP, decorin, osteonectin, E-cadherin*, plasminogen, β4-integrin,proα-defensin*, Fas ligand*, pro-TNFα,syndecan-1*
MMP-8	11q22.3	Col I, II, III, aggregan, serpins,α2M, fibrinogen, mouse chemokine ligand CXCL*
MMP-9	20q11.2-q13.1	Col I, IV, V, VII, XI, XIV, XVII, elastin, FN, fibrillin, gelatin, osteonectin, aggregan, vitronectin, α1PI*, pro-TNF-α, MBP, IGFBP, α2M, plasminogen, TGF-β, decorin, pro-VEGF*, fibrin*
MMP-10	11q22.3	LN5γ2, Col III, IV, V, IX, X, XIV, elastin, FN, gelatin, aggregan, LN-1, nidogen
MMP-11	22q11.23	α1PI, IGFBP
MMP-12	11q22.3	Elastin, Col IV, FN, LN-1, gelatin, vitronectin, entactin, proteoglycan, heparan and chondroitin sulphates, pro-TNF-α, plasminogen, fibrillin, fibrinogen, α1PI
MMP-13	11q22.3	Col I*, II*, III, IV, IX, X, XIV, aggregan, fibrillin, FN, gelatin, LN-1, large tenascin C, osteonectin, serpins, PAI, fibrinogen
MMP-14	14q11-q12	Col I*, II*, III*, gelatin, FN, LN-1, LN5γ2*, vitronectin, aggregan, tenascin, nidogen, perlecan, fibrinogen/fibrin*, fibrillin, α1PI, α2M, CD44, pro-MMP-2*, syndecan-1*
MMP-15	16q13-q21	FN, LN-1, gelatin, aggregan, tenascin, nidogen, perlecan, vitronectin , fibrin*
MMP-16	8q21	Col III, FN, gelatin, LN1, aggregan, casein, vitronectin, α2M, α1PI, fibrin*, syndecan-1*
MMP-17	12q24.3	Gelatin, pro-TNF-α , fibrillin, FN
MMP-19	12q14	Col IV, gelatin, LN1, nidogen, tenascin, FN, aggregan, COMP, fibrinogen, LN5γ2
MMP-20	11q22.3	Amelogenin*, aggregan, COMP
MMP-21	10q26.2	α1-antitrypsin
MMP-23A/B	1p36.3	Aggregan, COMP, gelatin
MMP-24	20q11.2	Heparan and chondroitin sulphates, gelatin, FN
MMP-25	16p13.3	Col IV, gelatin, FN, fibrin, vitronectin,α1PI, LN1
MMP-26	11p15	Col IV, gelatin, FN, fibrin, α1PI, β-casein, TACE-substrate, pro-MMP-9
MMP-27	11q24	ND
MMP-28	17q11-q21.1	casein

FN, fibronectin; LN-1, laminin-1; LN5γ2, gamma-2-chain of laminin-5; Col, collagen; IGFBP, insulin-like growth factor binding protein; MBP, myelin binding protein; COMP, cartilage oligomeric matrix protein; PAI, plasmin activator inhibitor; TACE, TNF-α converting enzyme; α2M, α2-macroglobulin; α1PI, α1-proteinase inhibitor; ND, not determined; *, substrates verified by mutagenesis or overexpression studies (Parks et al., 2004).

Chromosomal locations from the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene).

2.4.1. MMP-26

MMP-26, also known as matrilysin-2 or endometase, is a recent addition to the MMP family. The gene for MMP-26 is located in the short arm of the chromosome 11, a site distinct from the cluster of classical MMPs in 11q21-23, and codes a 261-amino-acid-long enzyme with the signal sequence for secretion, the pro-domain and the catalytic domain. MMP-26 can undergo autocatalytic activation, an exceptional feature among MMPs (de Coignac et al., 2000; Park et al., 2000 and 2002; Uría and Lopéz-Otín, 2000).

In vitro substrates for MMP-26 include native coll IV, denaturated collagens I-IV, fibronectin, fibrinogen, vitronectin, α1-antitrypsin, α2-macroglobulin and insulin-like growth factor binding protein 1. Furthermore, MMP-26 cleaves pro-MMP-9 in a specific site to create gelatinase species with unusually stable activity. Proteins resistant to MMP-26 activity include LN5, tenascin C and native collagens I-III. MMP-26 can be inhibited by TIMP-2 and –4 (Marchenko et al, 2001 and 2002).

MMP-26 is expressed in the prostate and endometrium under physiological conditions and in the corresponding cancers. Other tissues expressing MMP-26 include the placenta, lungs, kidneys and uterus (Zhang et al, 2002; Isaka et al, 2003; Pilka et al, 2003; Tunuguntla et al, 2003; Zhao et al, 2003). MMP-26 seems to participate in the reproductive processes and in malignant processes.

2.4.2. Inhibition of MMPs

MMPs are powerful degrading enzymes of the ECM, and accordingly, their activities are tightly regulated at all production levels. Transcription and translation are controlled by cytokines, growth factors, matrix substances and other stimuli. Between

secretion and action, MMPs are kept inactive by disulphide bridging between Cys-residues in the pro-domain and in the catalytic centre. Once activated, MMPs can be inhibited by one of the four existing TIMPs, by the reversion-inducing cysteine-rich protein with Kazal motifs (RECK) (Noda et al., 2003), by attraction to scavenger receptors via the plasma protein α2-macroglobulin (Hojilla et al., 2003), by chemically modified tetracyclines and by bisphosphonates (see Vihinen et al., 2005) and by specially designed molecules, such as the patented CTT peptide (Björklund et al., 2004; Medina et al., 2005).

2.4.3. Expression of MMPs during tissue homeostasis

Most adult tissues are kept in a relatively dormant balance between cell death and renewal, with very low expression levels of MMPs. Wounding disrupts the balance and activates the reparative machinery to replace lost tissue and repair the wounded area. The steps in the process of wound healing (see Figure 3) necessitate the action of MMPs. Invasion of endothelial cells into the fibrin clot is dependent on MMP-14 (Hotary et al., 2002). Migrating KCs at the leading edge of the wound facilitate the migration by secreting MMP-1, -9 and -10 (Parks, 1998), whereas the proliferating basal KCs distal to the migrating tip express MMP-3, -19 and -28 (Parks, 1998; Saarialho-Kere et al., 2002; Hieta et al., 2003;).

The regulated proliferation, tissue contractions, foldings and migrations essential for proper wound healing are also essential for embryonic morphogenesis (Martin and Parkhurst, 2004). In addition, the reproductive processes necessitate extensive reorganization of the extracellular surroundings, whether during gestation, implantation of the embryo, parturition or cycling of the endometrium (see Vu and Werb, 2000). Dissection of developing murine tissues has revealed a rather ubiquitous expression of MMPs 14-16 and TIMPs, whereas abundant expression of MMP-2 is

restricted to the heart, lungs, kidneys and muscle; of MMP-19 to the lungs, liver and testes; of MMP-11 to the lungs, uterus and testes; and of MMP-23 to the reproductive tissues. MMP-26 lacks the murine counterpart, but its closest structural relative, MMP-7, has the highest expression levels in the uterus (Nuttall et al., 2004).

2.4.4. Expression profiles of MMPs in epithelial cancers of the skin and oesophagus

The ECM carries critical controlling information for cell adhesion, differentiation, proliferation and apoptosis, and accordingly, disruptions of the ECM increase tumorigenic potential (Hojilla et al., 2003). MMPs are located at the cell-ECM interface and have been proven to cause alterations in cell attachment to the ECM in cancer. They can also cleave molecules situated between the ECM and the intracellular cytoskeleton, thereby breaking cells' connections with neighbour surveillance (Mott and Werb, 2004). The significance of MMPs in tumour biology was highlighted by the metastatic potential of cancer cells being correlated with their degradative potential against BM collagen IV, today known to be cleaved by gelatinases MMP-2 and -9 (Liotta et al., 1980; Overall and Lopéz-Otín, 2002). Many of the members of the human MMP family were isolated from tumours or tumour cell lines, were upregulated by cytokines abundantly expressed during malignant transformation (Mueller and Fusenig, 2004) or were found to have binding sites for transcription factors that are often activated in malignant processes (Marchenko et al., 2002). Recent research has evidenced the role of MMPs in all stages of tumour progression, including initiation, growth, cell migration and angiogenesis, with individual MMPs having roles for or against tumour growth and invasion (Egeblad and Werb, 2002; Overall and Lopéz-Otín, 2002). The production of certain MMPs is an early event in tumour development. Immune cells produce angiogenic factors when

accumulating in the vicinity of neoplastic cells – even before the MMP-9-dependent angiogenic switch (Bergers et al, 2000; Kalluri, 2003).

In BCCs, the upregulation of MMP-1 in the stromal compartment is an early event, whereas epithelial expression of MMP-3, -7 and -10 occurs only in the most aggressive subtype, thus being associated with further acquisition of malignant properties (see Kerkelä and Saarialho-Kere, 2003).

In SCCs, the imbalance between MMPs and their inhibitors is well documented; high MMP levels correlate with more aggressive tumours (Johansson and Kähäri, 2000). Upregulation of MMP-1 is an early event, occurring at the stage of actinic keratosis, whereas epithelial expression of MMP-2, -3 and -10 requires further dedifferentiation, as they are absent from premalignant conditions preceeding SCC (Tsukifuji et al., 1999; Kerkelä et al., 2001).

Expression of MMP-7, a structural relative of MMP-26, correlates with recurrence and poor prognosis of ESCC patients, and co-expression of it and MMP-9 at the invasive front shortens survival significantly (Etoh et al., 2000; Tanioka et al., 2003), although MMP-9 expression alone does not correlate with poor prognosis (Yamashita et al., 2004).

An essential acquired malignant feature of SCCs is their tendency to metastasize and invade through BMs, and accordingly, SCCs frequently present with decreases in coll IV and increases in MMP-2 and -9 levels, as compared with the less malignant KC-derived cancers (Dumas et al., 1999; Samantaray et al., 2004).

Cancer cells stimulate host cells to express and secrete MMPs rather than producing MMPs themselves. Instead of being the ultimate cause of malignant behaviour, MMP overproduction is now seen as one of the responses of stromal cells to stimuli from tumour cells (Pavlaki and Zucker, 2003).

2.4.5. Transgenic studies for MMPs and TIMPs

Studies with transgenic animals have sometimes yielded contradictory roles for MMPs in tumour development. Several MMP knock-outs have shown reduced tumorigenesis of various cancer types such as intestinal tumours for MMP-7-null animals (Wilson et al., 1997), chemically induced fibrosarcomas for MMP-19-null animals (Pendás et al., 2004), chemically induced mutagenesis cancers for MMP-11-null animals (Masson et al., 1998) and melanomas for MMP-2- and MMP-9-null animals (Itoh et al., 1998 and 1999). Conversely, other MMP knock-out mice have manifested an increase in tumorigenesis, e.g. MMP-8-null male and MMP-3-null animals for chemically induced skin cancers (Balbín et al., 2003; McCawley et al., 2004). Our group has demonstrated that MMP-19 disappears upon dedifferentiation of human SCCs (Impola et al., 2003). Yet the MMP-19-null mouse shows a decrease in susceptibility to chemically induced tumours, contradicting the role of this MMP in protection from cancer (Pendás et al., 2004). TIMP-1 by virtue of its inhibitory effect on MMPs could easily be considered a protector against tumorigenesis. However, its overexpression in a skin cancer model promotes carcinogenesis in K14-HPV16 mice by potentiating keratinocyte proliferation (Rhee et al., 2004). Selection of appropriate methods for inactivating or potentiating a given gene to fight cancer in becoming increasingly complicated with studies uncovering new phenotypes of transgenic animals. Major published MMP- and TIMP-transgenic genotypes and phenotypes are listed in Table 2.

Table 2. MMP- and TIMP-transgenic mice and their phenotypes with references to original articles (modified from Vu and Werb, 2000).

Genotype	Phenotype	Reference
MMP-1 overexpression	Increased incidence of chemically induced skin tumours	D'Armiento et al., 1995
MMP-2 null	Reduced tumour-induced angiogenesis, delayed mammary gland development, decreased pancreatic tumour growth in RIP-Tag mice	Itoh et al., 1998; Bergers et al., 2000; Sternlicht and Werb, 2001
MMP-3 null	Impaired wound contraction, enhanced initial growth, aggressive behaviour in chemically induced SCC	Bullard et al., 1999; McCawley et al., 2004
MMP-3 overexpression	Increased incidence of mammary tumour development or reduced incidence of chemically induced mammary tumours	Witty et al., 1995; Sternlicht et al., 1999
MMP-7 null	Reduced innate intestinal immunity, impaired mucosal wound re-epithelialization, reduced intestinal tumorigenesis	Wilson et al., 1997; Dunsmore et al., 1998; Wilson et al., 1999
MMP-7 overexpression	Enhanced Neu-induced mammary tumorigenesis	Rudolph-Owen et al., 1998
MMP-8 null	Increased susceptibility to chemically induced skin tumours in male mice	Balbín et al., 2003
MMP-9 null	Delayed and reduced skin tumorigenesis, but more aggressive tumours, delayed bone development, reduced pancreatic tumorigenesis in RIP-Tag mice	Vu et al., 1998; Bergers et al., 2000; Coussens et al., 2000
MMP-10 overexpression	Disorganized migrating tip in wounds, aberrant LN-5 deposition	Krampert et al., 2004
MMP-11 null	Reduced susceptibility to chemically induced cancer of the mammary gland and ovary	Masson et al., 1998
MMP-12 null	Reduced susceptibility to smoking-induced emphysema	Hautamäki et al., 1997
MMP-13 null	Delayed bone development	Inada et al., 2004
MMP-9 and MMP-13 null	Abnormal bone formation	Stickens et al., 2004
MMP-14 null	Impaired skeletal development and angiogenesis, lethal by 3 weeks of age	Holmbeck et al., 1999
MMP-14 overexpression	Increased mammary hyperplasia, dysplasia, adenocarcinoma	Ha et al., 2001
MMP-19 null	Diet-induced obesity and reduced susceptibility to chemically induced skin cancer	Pendás et al., 2004
MMP-20 null	Abnormal tooth development and morphology	Caterina et al., 2002
TIMP-1 null	Increased lung injury after bleomycin exposure, abnormal menstrual cycling	Nothnick, 2000; Kim et al., 2005
TIMP-1 overexpression	Increased incidence of tumours in K14-HPV16 mice, decreased chemically induced mammary tumorigenesis, decreased liver carcinogenesis	Martin et al., 1996; Rhee et al., 2004; Yamazaki et al., 2004
TIMP-2 null	Deficient MMP-2 activation	Wang et al., 2000
TIMP-3 null	Impaired branching morphogenesis of the lungs, accelerated apoptosis in mammary glands, impaired lung function	Fata et al., 2001; Leco et al., 2001; Gill et al., 2003
RECK null	Embryonic lethality, reduced integrity of vasculature, neural tube and mesenchymal tissues	Noda et al., 2003

3. AIMS OF THE STUDY

The main goals of this research were to identify any remaining members of the MMP family and to characterize the roles of the two newest MMPs, MMP-21 and MMP-26, in epithelial biology, especially in malignant processes. The expression profiles of novel MMPs must be carefully examined in tissue turnover before MMP regulation or inhibition can be considered for therapeutic purposes. Specific aims of the study were as follows:

I) To complete the human MMP enzyme family with MMP-21 and to examine the role of MMP-21 in remodelling processes during foetal development, in normal tissues and in pathological conditions.

II) To identify factors in tumorigenic processes and in foetal development that induce MMP-21 production.

III) To characterize the biological role of MMP-26 and to determine regulatory factors for this MMP in skin biology.

IV) To disentangle the roles of MMP-21 and MMP-26 in oesophageal biology and to determine their regulatory factors in epithelial tumorigenesis of the oesophagus.

4. MATERIALS AND METHODS

4.1. COMPUTATIONAL SEQUENCE ANALYSIS (I)

To find new members of the MMP family, a TBLASTN (Altschul et al., 1997) search of non-redundant (nr) and high throughput genomic sequences (htgs) databases of the GenBank™ was performed using the peptide string XXXFXXXXKWXXXXITYXIXXYXXDLXXXXXDDAFARAFXXWSXVTPLXF XRXXXXXADIXIXFGXXEHGDGYPFDGKDGLLAHAFXPGXGXXGDXHFDD DELWXLGXGXVVLFLVAAHEFGHAXGLXHSXXPXALMXPXYXXTXXXXLX XDDXXGIXXLYGXXP, composed of the catalytic domains of human MMP-2 and -9 excluding the fibronectin repeats. To predict the coding exons from the genomic sequences, we used the Genscan Web Server of Massachusetts Institute of Technology (http://genes.mit.edu/GENSCAN.html) (Burge and Karlin, 1998). For multiple alignments of nucleotide and amino acid sequences, we used ClustalX and BoxShade server 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

The protein sequence of MMP-21 was used to predict the presence of a signal peptide for secretion (http://www.cbs.dtu.dk/services/SignalP/). GeneComposer (www.bioinfo.helsinki.fi) was applied to create a hydropathy plot, and on the basis of hydrophilic residues in the sequence, we selected a 16-meric peptide for generation of antibodies.

4.2. CELL CULTURES (II-IV)

Epithelial cell lines were used in our studies on MMP-21 and -26 expression and regulation. Human *primary KCs* were isolated and cultured as described previously (Impola et al., 2003). Murine *BALB/MK-2* KCs a gift from Dr. Stuart Aaronson (National Cancer Institute, Bethesda, MD, USA), were cultured in minimum essential

media (MEM) supplemented with 4 ng/ml epidermal growth factor (EGF). Immortalized human *HaCaT* KCs and subclones of increasing malignancy (HaCaT – immortalized, *A5* – tumourigenic, *II-4* – invasive and *RT3* – metastatic) were cultured in Dulbecco's modified Eagle's medium (DMEM) of 1 g/l of glucose supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 5% foetal calf serum (FCS), 100 U/ml penicillin and 50 µg/ml streptomycin. The *ras*-transfected clones were further given 200 µg/ml geneticin to maintain their selective pressure (Boukamp et al., 1988).

MCF7 cells, originally from mammary gland adenocarcinoma, and 293 cells, originally from a human foetal kidney, were grown in Eagle's modified MEM supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin and 50µg/ml streptomycin. A-431 cells, originally from an epidermoid carcinoma, were grown in DMEM of 4.5 g/l glucose supplemented with 4 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 50 µg/ml streptomycin.

KYSE-30 cells, originally from a well-differentiated ESCC (Shimada et al., 1992), were grown in 1:1 mixture of RPMI 1640 and Ham's F12 supplemented with 2 mM L-glutamine, 2% FCS, 100 U/ml penicillin and 50 µg/ml streptomycin. *OE21* cells, originally from a moderately differentiated ESCC (Rockett et al., 1997) were grown in RPMI 1640 supplemented with 2 mM L-glutamine, 10% FCS, 100 U/ml penicillin and 50 µg/ml streptomycin.

4.2.1. Cytokines, growth factors, matrices and cultures on chamber slides (II-IV)

To study the regulation of MMP-21 and MMP-26 expression, equal amounts of cells were plated on 6- or 24-well tissue culture plates. The next day, the cells were repeatedly washed with phosphate buffered saline (PBS) and incubated in serum-and-supplement-free medium for 18-24 hours. The cells were then treated with 12-

phorbol-13-myristate-acetate (PMA, 10 ng/ml, Sigma, St. Louis, MO, USA), epidermal growth factor (EGF, 10 ng/ml, Sigma), insulin-like growth factor (IGF, 10 ng/ml, Sigma), tumour necrosis factor alpha (TNF-α, 10 ng/ml, Sigma), transforming growth factor beta 1 (TGF-β1, 10 ng/ml, Sigma), interleukin-1 beta (IL-1β, 5 U/ml, Sigma), interleukin-6 (IL-6, 1 ng/ml, Sigma), dexamethasone (Dxm, 10-5M, Sigma), retinoic acid (RA, 10-6 M, Sigma) or oestrogen (10^{-6}-10^{-8}M, Sigma). To trigger the differentiation of primary KCs, the cells were grown in a high Ca^{2+} (1.8 mM) concentration. Stimulation was allowed to proceed for either 24 or 48 hours, after which the cells were lysed and total RNA was extracted using a RNeasy Mini-kit (Qiagen, Chatsworth, CA, USA) as instructed by the manufacturer.

4.2.2. Creation of cell lines stably expressing MMP-21 (II)

To study the localization of MMP-21 in cells, we transfected 293A cells with the full-length human MMP-21 construct in a pEFIRES-P expression vector (Hobbs et al., 1998) using a FuGENE 6 transfection reagent (Roche, Indianapolis, IN, USA). The next day, the cells were given a fresh complete culture medium, and one day after this a medium into which 1 μg/ml puromycin was added to serve as a selective agent. The concentration of puromycin was gradually increased up to 160 μg/ml, leaving only the fittest cell clones alive. As a negative control, we used the cells transfected with an empty pEFIRES-P vector, which were also given up to 160 μg/ml puromycin.

4.3. Extraction and Analysis of RNA

4.3.1. MMP-21 cloning and sequencing (I)

Polymerase chain reaction (PCR) analyses of MMP-21 gene expression were done using PCR-ready human multiple tissue cDNA (MTC) panels (Clontech, Palo Alto, CA, USA). The coding region of MMP-21 was assembled from several overlapping

PCR fragments, amplified by gene-specific primers designed for GENSCAN-predicted exons. The overlapping fragments were amplified by PCR from the first-strand human placenta cDNA (Ambion, Austin, TX, USA) by reverse transcriptase (RT-) PCR using Advantage-2 polymerase (Clontech). For PCR analyses, water was always used as a negative control and placenta as a positive control. PCR products were run in agarose gel electrophoresis, excised and purified for sequencing or subcloning in a TOPO-TA system (Invitrogen, Carlsbad, CA, USA) using a gel extraction kit from Qiagen.

4.3.2. Expression analysis by Northern blotting and PCR panels (I, II)

Human foetal, human 12-lane and human tumour multiple tissue northern (MTN) blots (Clontech) were hybridised with ^{32}P radiolabelled full-length cDNA of MMP-21 in UltraHyb (Ambion) at 42°C for 18 hours. After washes, the results were visualized by autoradiography.

Human I, II, foetal, tumor, cell line and mouse I MTC panels (Clontech) were screened for MMP-21 with primers producing a 1014-bp fragment of MMP-21 gene corresponding to nucleotides 725 – 1739 in the coding sequence. The PCR products were analysed on agarose gel, and their identity was verified by sequencing.

4.3.3. Real-time quantitative PCT (TaqMan™)(II-IV)

Total RNA (0.5 μg) was transcribed to cDNA with random hexamers or oligo-dTs using TaqMan reverse transcription reagents (Applied Biosystems, Warrington, UK) or a SuperScript™ III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA) and then used as a template for TaqMan analyses on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers used for MMP-21 amplification were forward primer 5'- GGGATCCATAATGCAACCAAA –3', reverse primer 5'-

ATCCCTCACAGGAGCCATACA –3' and the probe 5' – CAGACAGGAAAGCAAT – 3'; for MMP-26 amplification forward primer 5'-ACGTTGGATGGTCAGCTTCAGACACTGGATATA -3' reverse primer 5'-ACGTTGGATGGGTACATTATGGAGCTCTGATTC-3' and the probe 5'-AGATTGGGCATTCTTTGGGC-3'. For endogenous control, we used the 20x ready-to-use glyseraldehyde phosphate dehydrogenase (GAPDH) mix containing the primers and probe. The probes for MMP-21, -26 and -10 (as a control of the cell stimulations with HaCaTs) contained a FAM reporter dye attached to the 5' end and a TAMRA quencher at the 3' end.

4.4. Analysis of Proteins

4.4.1. Tissue samples

The use of archival tissue samples for these studies was approved by the Ethics Committees of the Departments of Dermatology, Surgery and Internal Medicine, Helsinki University Central Hospital, Helsinki, Finland.

Foetal samples (I, II). Samples were obtained from Oulu University Hospital. Foetal age was estimated by menstrual age and by macroscopic and histological examinations. All foetal material used for immunohistochemistry originated from medical abortions performed at 7, 8, 10, 12, 14 and 20 weeks of gestation (n=15).

Skin samples (I- III). These samples included archival specimens of dermatitis herpetiformis (n=3), psoriasis (n=3), lichen planus (n=3), seborrhoeic keratosis (n=3), normal skin (n=3), acute (n=4), and chronic wounds (n=5), SCC of the skin (n=21, grades I-III), BCC of the skin (n=8), and Bowen's disease (n=3) from the Department of Dermatology, Helsinki University Central Hospital.

Carcinomas other than skin and oesophageal samples (I, II). Colon (n=4) and ovarian carcinomas (n=4) were obtained from the Department of Pathology, Helsinki University Central Hospital.

Mouse tissues (II). Formalin-fixed, paraffin-embedded mouse foetal samples included 10-, 13- and 16-day-old embryos (n=15) and respective placentas of NMRI mice were from the Haartman Institute, University of Helsinki, Finland.

Precursor and malignant oesophageal lesions (IV). Formalin-fixed, paraffin-embedded specimens were collected from 33 patients diagnosed with ESCC at Helsinki University Central Hospital. Six tumours were well-differentiated, 19 moderately differentiated and 8 poorly differentiated ESCCs, according to the World Health Organization (WHO) classification (Hamilton and Aaltonen, 2000). In addition, 4 nodal metastases and 6 samples representing oesophageal dysplasia were stained.

4.4.2. Immunohistochemistry (I-IV)

To reveal antigens, the specimens were pretreated as indicated in Table 3, and endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 for 30 min. Immunostaining was done with the avidin-biotin-peroxidase complex technique (StreptABComplex/HRP Duet (Mouse/Rabbit) Kit, Dako A/S, Glostrup, Denmark), with the dilutions of the antibodies and pretreatments listed in Table 3. Diaminobenzidine (DAB) or aminoethylcarbazole (AEC, for cells cultured on slides) was used as a chromogenic substrate and Mayer's haematoxylin as a counterstain. For negative controls, we used normal rabbit IgG, preimmune sera or sera where antigen specific sites were blocked with excess antigenic peptide.

Table 3: Antibodies and pretreatments used.

Antibodies	Source	Dilution / pretreatment
MMP-7	Oncogene Research Products, Cambridge, MA	1:80 / 95°C water bath
MMP-9	Research Diagnostics, Flanders, NJ	1:50
MMP-21	Custom antisera or Triple Point Biologics, Portland, OR (RP3MMP21)	1:250 / trypsin
MMP-26	Gifts from K. Isaka and C. Lopéz-Otín	1:150 and 1:400
E-cadherin	Zymed Laboratories, South San Francisco, CA	1:200-1:400
Ki-67	Dako (A047)	1:200
Laminin-5 γ2 chain	Gift from K. Tryggvason	1:800
p16INK4	BD Pharmingen, San Diego, CA	1:350
β-catenin	Transduction Laboratories, Lexington, KY (C19220)	1:1000 / microwave oven

4.4.3. Antibodies for MMP-21 and Western blotting (I)

Based on hydropathy plotting, a LYENRNNRTRYGDPIQ peptide was synthesized corresponding to amino acids 366-382 in the haemopexin domain and used to raise antibodies in rabbits. Peptide synthesis and antibody production were purchased from Sigma Genosys (Rehovot, Israel). The specificity of the antibodies was confirmed by Western blotting, using a construct of a partial catalytic domain and a complete haemopexin domain in a pGEX-6P-3 expression vector. Three hundred nanograms of the solubilized fusion protein, produced in *Escherichia coli*, was diluted 1:1 in Laemmli reducing sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) with 5% β–mercaptoethanol. Denaturated proteins were separated in a 10% polyacrylamide gel and transferred to Hybond C-extra (Amersham, Buckinghamshire, UK) nitrocellulose membrane using standard protocols. Immunized rabbit antiserum was used as a primary antibody and peroxidase-conjugated anti-rabbit IgG as a secondary antibody. The protein bands were visualized by chemiluminescence using standard protocols. For a negative control, we used peptide-blocked antiserum as the primary antibody.

5. RESULTS

5.1. DISCOVERY AND STRUCTURE OF MMP-21

Search of the high throughput genomic sequences (HTGS) database with our peptide string resulted in more than 50 hits, among them AL360176 (accession number for the NCBI database of nucleotides), a 156-kb-long genomic sequence on chromosome 10. Of the nine predicted coding sequences by GENSCAN analysis, the protein translation of one contained characteristic domains of an MMP without a complete match to any known MMP. PCR analyses and sequencing confirmed an open reading frame for seven exons coding for a signal sequence for secretion, a pro-domain with a furin-recognition sequence and classical Cys-switch (PRCGVPD), a catalytic domain with a Zn^{2+}-binding site (VAVHEIGHVLGLPH) and a haemopexin domain. MMP-21 is a poor fit in the MMP subfamilies; it lacks the characteristic amino acids of collagenases and stromelysins, the fibronectin repeats of gelatinases and the membrane association of membrane-type MMPs (Figure 6). MMP-21 nucleotide sequences have deposited in GenBank™ under accession number AF331526.

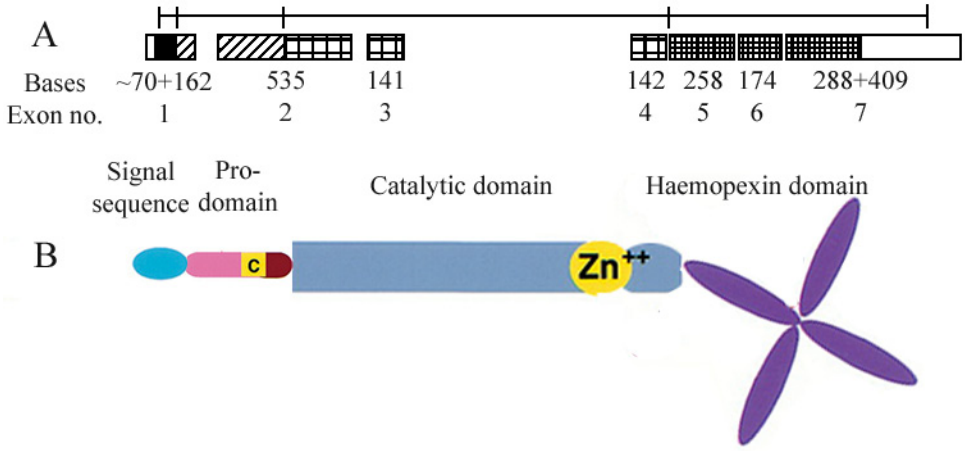

Figure 6. Structure of MMP-21 gene (A) and protein (B).

5.2. MMP-21 AND -26 IN PHYSIOLOGICAL CONDITIONS (I-III)

The MMP-21 protein was absent from adult human skin, and wound healing or hyperproliferation was unable to induce its expression, as evidenced by our negative results in chronic and acute wounds, Bowen's disease and psoriasis. Cultured human and murine KCs, by contrast, were positive for MMP-21 mRNA and protein. Inflammatory cells expressed MMP-21; as normal human leukocyte cDNA and neutrophils in a dermatitis herpetiformis sample were positive for MMP-21 mRNA and protein.

Expression levels for human MMP-21 were quite low, and by Northern blotting, we detected the 2.5-kb transcript only in foetal liver. At 16-32 weeks after gestation, the human brains, kidneys and liver expressed MMP-21 mRNA. In addition the foetal brains, kidneys, oesophagus, colon, ileum and bronchi expressed MMP-21 protein epithelially 7-20 weeks after gestation. In mice, MMP-21 mRNA expression begins 10 days post-conception, is most abundant by day 13, then declining until day 16, with parturition occurring on day 21. Foetal murine tissues positive for MMP-21 protein were the kidneys and the skin. Human and murine placentas were always positive for MMP-21 mRNA and protein.

MMP-26 protein was expressed by KCs of hair follicles in the anagen (hair growth) phase in otherwise negative adult human skin. Induction of MMP-26 occurred in migrating KCs upon acute or chronic wound healing, inflammation or microdisruptions of the BM. In acute wounds, MMP-26 co-localized with tumour suppressor p16 and with KC migration marker LN5γ2.

5.3. DIFFERENTIAL INDUCTION OF MMP-21 AND -26 EXPRESSION DURING MALIGNANT TRANSFORMATION OF CUTANEOUS AND OESOPHAGEAL EPITHELIA (I-IV)

MMP-21 mRNA was expressed in epithelial cancers of the colon, breast and prostate, and in the corresponding cell lines. MMP-21 protein was detected in epithelial cancer cells and leukocytes surrounding the cancer. The malignant squamous carcinoma cell line A-431 was positive for MMP-21 mRNA, but surprisingly, the *ras*-transformed HaCaT subclones revealed no correlation between MMP-21 mRNA levels and malignancy potential.

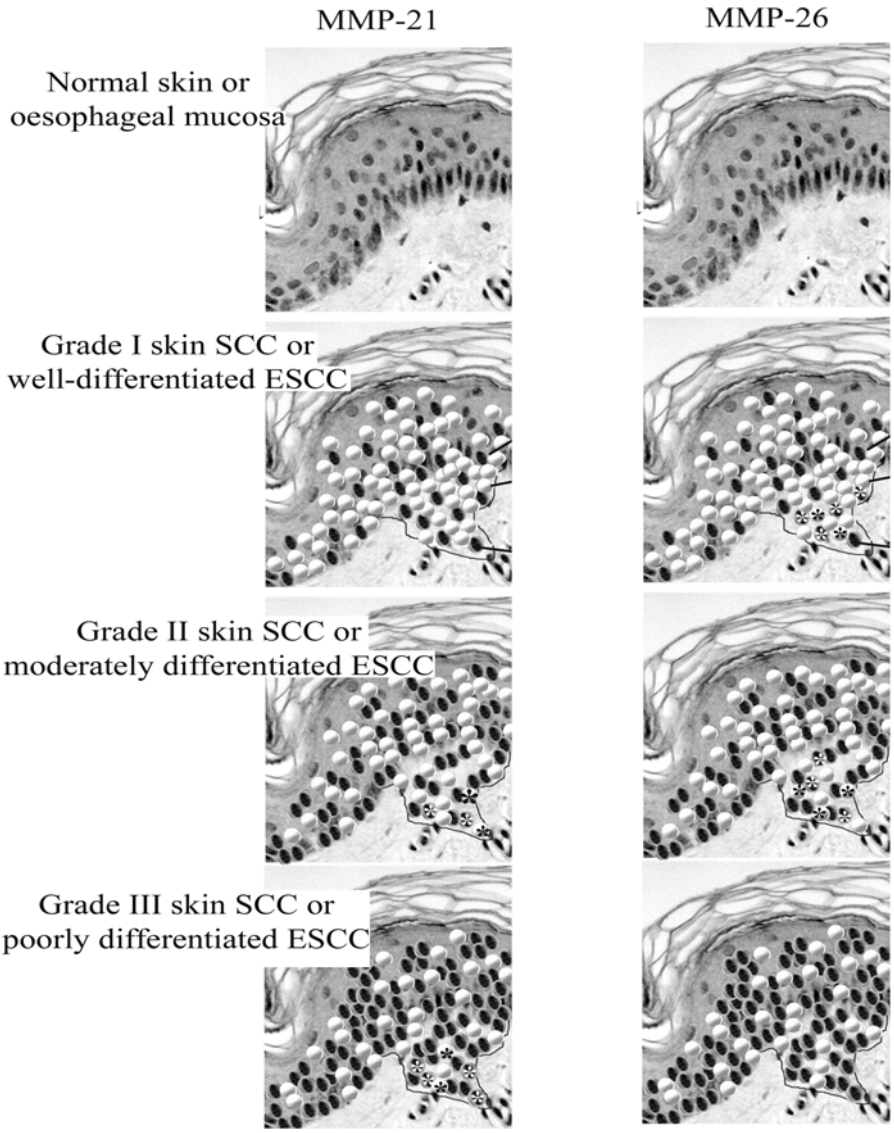

Figure 7. MMP-21 and -26 expression patterns during squamous cell carcinoma progression. White spheres depict differentiating epithelial cells and black spheres depict basal, undifferentiating cells. Asterisks on cells indicate MMP-21 or -26 expression.

Progressing SCCs of the skin and oesophagus were negative for MMP-21 protein until grade II was reached (Figure 7). Grades II and III SCCs of the skin and oesophagus demonstrated MMP-21 protein in invading epithelial cancer cells and surrounding neutrophils. A subset of the SCCs had MMP-21-positive islands of well-differentiated epithelial structures. No association of MMP-21 with MMP-7, transactivated β-catenin, proliferation marker Ki-67, KC migration marker LN5γ2 or apoptosis marker TUNEL was evident in SCCs, although MMP-21 was often found adjacent to apoptotic areas. In BCCs, only the aggressive, sclerosing subtype was positive for MMP-21 protein.

MMP-26 expression was induced, with skin and oesophageal mucosa developing malignant behaviour, already in KA of the skin and dysplasia of the oesophagus. In grade I and II SCCs, epithelial cancer cells expressed MMP-26 protein at the invasive front, but further dedifferentiation of the cancer abolished MMP-26 protein expression and grade III SCCs were devoid of it (Figure 7). Association of MMP-26 with p16 became less evident upon dedifferentiation, as they partially co-localized in grade I SCC, but in grade II SCC were merely detected in the same areas. MMP-26 protein co-localized with LN5γ2, demonstrating that MMP-26 is derived from migrating KCs. MMP-26 also co-localized with fragmented elastin in SCC samples. The proliferative marker Ki-67 was not associated with MMP-26 protein expression in SCCs.

Only occasional atypical cells of BCCs expressed MMP-26 protein in E-cadherin-negative areas, but the cancer islands of BCCs were always negative for MMP-26.

To mimic the auxiliary factors involved in tumorigenesis, human KCs were treated with seven different growth factors postulated to be important in dermal

carcinogenesis (EGF, bFGF, IGF, PMA, TNF−α, TGF−β1 and VEGF; for details, see Materials and methods): only TGF−β1 was able to increase MMP-21 mRNA levels up to 8-fold. To induce MMP-26 expression from KCs and ESCC cell lines, we expanded the selection of growth factors and matrices (see Materials and methods), but none of the factors in our system was able to induce MMP-21 mRNA expression from ESCC cells or MMP-26 mRNA expression from KCs or ESCC cells.

6. DISCUSSION

6.1. CHARACTERISTICS OF MMP-21 (I, II)

Most physiological substrates for MMP-21 remain unidentified, despite cleavage of α1-anti-trypsin being shown (Marchenko et al., 2003). In our zymographic analyses, we were unable to demonstrate gelatinolytic or caseinolytic activities of MMP-21. It will be intriguing to see where the physiological substrates place MMP-21, perhaps even at the beginning of a proteinolytic cascade regulating breakdown of biological structures, should it be able to activate other proteinases or release growth factors or novel epitopes from ECM macromolecules.

6.2. PHYSIOLOGICAL EXPRESSION OF MMP-21 AND -26 (I-III)

MMP-21 has counterparts in metamorphosing reptiles: XMMP in *Xenopus laevis* (Yang et al., 1997) and CyMMP in *Cynopps pyrrhogaster* (Suzuki et al., 2001). XMMP is undetectable in the blastula stage, induced in the gastrula stage, expressed in the neurula stage and then down regulated in the pretailbud stage of the embryo (Yang et al., 1997). CyMMP is similarly under tight space and time regulation during early newt development (Suzuki et al., 2001). Promoter studies of human and mouse MMP-21 have revealed several putative transcription factor binding sites essential in foetal development, including Tcf-4, Pax-2, -5, -8 and the Notch-motif (Marchenko et al., 2003). Tcf-4 is linked to the wnt-signalling pathway, essential for TGF–β1-regulated hair follicle development (Li et al., 2003). Pax-2 is involved in kidney development, Pax-5 in haematopoiesis and Pax-8 in thyroid development (Chi and Epstein, 2002). The Notch-motif participates in the development of the central nervous system (del Barco Barrantes et al., 1999). Detailed dissection of developing murine organs has further demonstrated MMP-21 mRNA expression in foetal lungs of

the mouse (Nuttall et al., 2004). These data and our results suggest that the expression pattern of MMP-21 is highly specific to the developmental stage, cell type and tissue type in higher vertebrate development. Furthermore, MMP-21 mRNA and protein are most prominent in the placenta, suggesting a role in embryo implantation and development, as previously shown for many other MMPs. Whether MMP-21 is needed for degradation of the provisional matrix, for invasion of cells or for branching morphogenesis is currently not known. The epithelial expression pattern makes it likely to be excluded from angiogenesis but enables its engagement in the development of skin and ductal structures of various organs.

MMP-21 is expressed in mRNA and protein levels in leukocytes, implying a role as a cytokine-regulated effector during innate immune responses or tissue injury. MMP-21 is also expressed in low levels in various adult mouse tissues as well as in the adult human brain, lung, testis, ovaries and colon, suggesting a role in normal tissue homeostasis or turnover. Additional studies with knock-out mice are crucial to identify the processes dependent on MMP-21 expression, whether during homeostasis, foetal development or cancer progression.

MMP-26 is absent from the normal intact epidermis but is produced in migrating KCs upon skin injury, similar to several other MMPs (see Saarialho-Kere et al., 2002). Our positive results for MMP-26 in chronic wounds agree with earlier data on MMP-1 and -10, which are also induced in KCs bordering normally healing or chronic wounds (Vaalamo et al., 1996). In lichen planus and keratoacanthomas, skin disorders with prominent inflammation, MMP-26 protein is detected in basal KCs above the inflammatory infiltrate. In psoriasis, another inflammatory skin disorder, MMP-26 protein is expressed at the top of dermal papillae, where the BM is though to suffer from microdisruptions.

MMP-2, -14 (Giannelli et al., 1997; Koshikawa et al., 2005), -8, -13 and -20 (Pirilä et al., 2003) have been shown to release the pro-migratory γ2 epitope from human or murine epidermal LN5, and recent additions to MMPs able to cleave LN5 include MMP-10 (Krampert et al., 2004) and -19 (Sadowski et al., 2005). MMP-26 co-localizes with the the LN5γ2-chain in chronic and acute wounds and in well-differentiated cancers. However, MMP-26 is unable to cleave LN5 (Marchenko et al., 2001).

Although MMP-26 has been detected in peripheral leukocytes, lymphocytes (Park et al., 2000; Nuttall et al., 2003), fibroblasts and macrophages of other tissues than the skin (Bar-Or et al., 2003; Marchenko et al., 2004), our study confirms its epithelial origin in the skin and oesophageal mucosa. Furthermore, MMP-26 has recently been found in epithelial corneal and conjunctival cells in vivo (Marchenko et al., 2004).

Inability of the growth factors and matrices used to induce MMP-21 and -26 mRNA expression excludes the participation of some pathways in their regulation. TNF–α is unable to increase MMP-21 or -26 mRNA levels from KCs and ESCC cells, suggesting that NF–κB is irrelevant in their regulation (Han et al., 2001). PMA induces the expression of MMPs from a variety of cells, but HaCaT cells have been reported to suffer from a loss of mitogen-activated protein kinase (MAPK)- mediated MMP-1 upregulation by PMA, even if EGF-stimulated MMP-1 induction is functional (Sudbeck et al. 1999). Primary KCs and ESCC cells are also unable to induce MMP-21 or -6 expression upon PMA stimulation, reflecting the independence of expression from PMA stimulation. HUVEC, MRC-5 and PC3 cells have previously been shown to induce MMP-26 expression upon PMA stimulation (Nuttall et al., 2003). Recently, MMP-26 expression was suggested to be oestrogen-dependent, as its

promoter contains an oestrogen-responsive element and MMP-26 is expressed by many oestrogen-dependent cell lines (Li et al., 2004). In our system, addition of oestrogen at physiological levels was unable to upregulate MMP-26 in ESCC cells. It is possible that in KCs and ESCC cells at least two stimulators, hypoxia, certain integrin interactions or co-culture studies employing fibroblasts, macrophages, neutrophils or other stromal components are needed for upregulation of MMP-21 and -26 or that actual in vivo events with cell-cell and cell-matrix interactions modifying MMP expression can not be recapitulated in simplistic cell culture models.

6.3. EXPRESSION OF MMP-21 AND -26 IN KERATINOCYTE-DERIVED CANCERS OF THE SKIN AND OESOPHAGUS (II-IV)

The Wnt- signalling pathway is commonly activated in epithelial carcinogenesis, often involving a loss of cell-cell contacts via E-cadherin and catenins. β–catenin then accumulates in the cytoplasm and in the nucleus, where it acts as a regulatory element for transcription of several genes (Seidensticker et al., 2000). The presence of the Tcf-4 binding site in the promoter of MMP-21 suggests its regulation to coincide with transactivation of β–catenin, although we were unable to detect an association between accumulated nuclear or cytoplasmic β–catenin and MMP-21 protein in skin, colon or oesophageal cancers. In ESCC, MMP-7 expression, together with nuclear β–catenin expression, correlates with tumour invasion to neighbouring tissues (Saeki et al., 2002), but in cutaneous and oesophageal SCCs, MMP-7 and -21 proteins were not co-localized in our material.

The induction of MMP-21 mRNA expression in KCs by TGF–β1 is intriguing, as TGF–β1 either up- or downregulates several MMPs, depending on cell type (Gold et al., 2000). TGF–β1 increases the mRNA levels of MMP-1, -10 and -9 in primary KCs (Rechardt et al., 2000; Han et al., 2001). In primary and immortalized

KCs, TGF−β1 inhibits growth and proliferation (Gold et al., 2000), whereas these effects disappear in the more malignant A-431 cells (Lange et al., 1999). Indeed, TGF−β1 has been shown to have a double role in epithelial tumorigenesis; at early stages, it induces an epithelial to mesenchymal transition (EMT) (Bhowmick et al., 2001), but it may also act as a tumour suppressor and, with malignant progression, it may promote tumorigenesis (Lange et al., 1999). Macrophages are also an important source for TGF−β1, which in turn stimulates MMP-production from tumour or stromal cells to aid in the invasive processes. Interestingly, increased expression of TGF−β isoforms has been observed in a variety of human carcinomas, including colon, breast and prostate cancers (Gorsch et al., 1992; Truong et al., 1993), with all tumours also expressing MMP-21.

The inability of TGF−β1 to increase expression levels of MMP-21 mRNA in ESCC cells may indicate the loss of TGF−β1 control of more malignant epithelial cells or that at the more advenaced stages, MMP-21 is no longer expressed in the cells. The induction of MMP-21 expression occurs early in melanocyte transformation, it disappears from the most malignant tumours and there is no correlation between MMP-21 expression and the existence of micrometastases (Kuivanen et al., 2005). MMP-21 mRNA levels are unaltered between malignant and non-malignant samples of the prostate (Riddick et al., 2005). Using foetal and cancer cell lines of low and high invasive potential, we created three cell lines that stably over-express MMP-21, changes in the invasive capacities and malignant properties of which remain to be studied.

MMP-21 was detected in neutrophils in skin and oesophageal SCCs. However, discrepant data exist on the role of neutrophils proteases in cancer; while loss of neutrophil MMP-8 confers increased susceptibility to skin tumours in mice (Balbín et

al., 2003), neutrophil MMP-9 promotes skin carcinogenesis (Coussens et al., 2000). Whether MMP-21 is a cause for or a result of inflammation and contact with the cancer remains to be elucidated; not all neutrophils in contact with cancer expressed MMP-21 in our material. MMP-21 contains a furin activation sequence, which makes furin inhibition a potential means of eliminating MMP-21 activity. Indeed, localization of furin at the invasive front of SCCs strongly resembles localization of MMP-21 (Lopéz De Cicco et al., 2004).

While our study was underway, Yamamoto et al. (2003) showed that MMP-26 expression correlated strongly with invasion depth, lymph node and distant metastasis and advanced TNM stage in ESCC. The essential role of MMP-26 in oesophageal tumorigenesis was further demonstrated to arise from activation of MMP-9. We also observed the involvement of MMP-26 in the tumorigenic processes, especially in early invasion through the BM and in the association with inflammation and apoptotic tumour islets. Loss of heterogeneity (LOH) for p16 is a frequent event in SCC progression (Boukamp, 2005). In the skin either inflammation or atypia was sufficient for MMP-26 induction in KCs, and MMP-26 was associated with early cutaneous tumorigenesis, often co-localizing with tumour suppressor p16. In well-differentiated SCCs of the skin and oesophagus, the expression patterns of MMP-26 resembled that of p16, and the disappearance of MMP-26 from the dedifferentiated, aggressive SCCs suggests the importance of MMP-26 in early cutaneous and oesophageal tumorigenesis, whereas other MMP members promote the invasive phenomena. Thus, lack of MMP-26 could be a marker for aggressive growth, since, like the novel MMPs -19 and -28, it is expressed already in pre-malignant lesions and disappears from the most dedifferentiated SCCs (Saarialho-Kere et al., 2002; Impola et al., 2003). This agrees with previous results on oral SCC (Impola et al., 2004) and with the data of

Zhao et al. (2004), who found higher levels of MMP-26 in ductal carcinoma in situ than in infiltrating breast carcinoma, suggesting that once the BM has been breached the cancer cells become less dependent on the activity of MMP-26. Furthermore, the tumour-to-normal tissue ratio of MMP-26 in both kidney tissue and endometrial carcinoma is considerably different from that of MMPs classically associated with malignancy, and MMP-26 is detected in many carcinomas and the corresponding normal tissues (Isaka et al., 2003; Marchenko et al., 2004). However, recent data show that epithelial and stromal cells of prostate cancers express MMP-26 and its upregulation correlates strongly with the Gleason score indicating aggressiveness of the tumour (Riddick et al., 2005).

In conclusion, our findings reveal that MMP-26 is important in the early steps of cutaneous and oesophageal tumorigenesis, while MMP-21 contributes to invasion, based on its presence in the invasion front. MMP-26 could serve as a marker for incipient invasion, aiding in identifying patients in need of more radical treatment for dysplastic and premalignant changes in the cutaneous and oesophageal epithelia.

7. CONCLUSIONS AND FUTURE PROSPECTS

In tissue homeostasis, the balance between production and breakdown of the extracellular matrix is crucial. ECM remodelling requires that old structures be degraded before new ones are built. In biological situations, one group of molecular bulldozers is the MMPs. Processes demanding extensive remodelling, such as foetal development or female reproductive cycles, are characterized by high expression levels of MMPs and TIMPs. Sometimes tissue degradation is extensive without apparent reason and can cause or assist in creating and maintaining pathological conditions, such as chronic wounds, rheumatoid arthritis, atherosclerosis or cancer. Numerous studies have proven MMPs to be important players in these processes. Here, we introduced a novel member of the MMP family of proteolytic enzymes, MMP-21, and examined its expression, along with another novel MMP, MMP-26, in SCCs of the skin and oesophagus. Furthermore, we studied the role of MMP-21 in human and murine development, and detected a restricted pattern of protein expression in selected epithelial structures of the kidney, skin and lungs. Expression of MMP-21 mRNA in cultured KCs was induced in response to TGF−β1.

MMP-21 contains all of the characteristic features of MMPs: a signal sequence for secretion, a pro-domain with a Cys-switch sequence for preservation of latency prior to activation, a furin-cleavage site, catalytic domain with binding sites for the Zn^{2+} ion needed for activity and the four-leafed haemopexin domain needed for substrate recognition and interactions with TIMPs. MMP-21 mRNA was expressed in human and murine adult and foetal tissues and in human cancer cell lines of epithelial origin. MMP-21 protein was detected in developing epithelial cells, in neutrophils and in aggressive subtypes of squamous and basal cell cancers. Normal

skin was devoid of MMP-21 protein both in adult humans and mice. We found no co-localization of MMP-21 with transactivated β–catenin or MMP-7 in cutaneous or oesophageal SCCs.

In normally healing wounds and in cutaneous SCCs of grades I and II, the migrating LN5γ2 positive KCs and the KCs of the epithelial tip of chronic wounds expressed MMP-26 protein, whereas MMP-21 was not associated with wound repair or KC migration. Both normal and developing skin was negative for MMP-26 protein, and it never co-localized with the proliferation marker Ki-67 in SCCs of the skin or oesophagus or in BCCs of the skin.

MMP-26 can activate MMP-9, and they were found in the same regions in normal wounds and cutaneous SCCs, but adjacent to each other rather than directly co-localizing. MMP-26 was induced by KC atypia and by BM microdisruptions even in benign skin lesions. It was associated with early carcinogenesis but disappeared from more aggressive tumours. MMP-26 mRNA was expressed only at low levels in cultured cells of the skin and oesophagus.

In conclusion, this study introduced the last member of the MMP-family, MMP-21, and its mRNA and protein expression in transformed epithelial cells of the skin and oesophagus. We showed for the first time that MMP-21 mRNA is upregulated in KCs by TGF–β1. Oesophageal SCC cell lines had lost the ability to respond to TGF–β1 by upregulating MMP-21 mRNA expression. MMP-21 is expressed at mRNA and protein levels during human and murine foetal development. We also illustrated for the first time the role of MMP-26 in skin biology; it is present in migrating KCs, co-localizes with tumour suppressor p16 and disappears from SCCs along with dedifferentiation.

Tumours select their locations based on the "seed and soil" hypothesis. The microenvironment must be optimal for cancerous growth to proceed, for blood supply to function and for dissection of tumour cells and their passage and attachment to distant sites to form metastases. MMPs do the work of processing the ECM and activating cancer-promoting cytokines, but, as the processes work in favour of all steps in the tumourigenic process, they also have adverse roles and hinder all phases of the transformation and subsequent malignant processes. To precisely address the significance of a given protein in epithelial biology, a transgenic gain or loss of function animal model would be useful. To our knowledge, an MMP-21-null or over-expressing mouse does not yet exist, and since MMP-26 lacks the murine counterpart, it is unlikely to be a target of knock-out or knock-in experiments. However, because human and mouse systems are not identical, care needs to be taken when interpreting the necessity of a protease in humans based on the results of its deletion in mice. The hallmarks of malignancy, the major causes for cancer-related mortality and morbidity and the main impediments to improved cures are invasion and metastasis. Regardless of the regulatory mechanisms, it is essential to determine whether MMP expression can be a predictive marker for metastasis, identify a target for therapeutic intervention or aid in choosing adjuvant therapy for patients needing a more radical treatment for cancer.

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