

Jarkko Korpi

COLLAGENASE-2 (MATRIX
METALLOPROTEINASE-8)
IN TONGUE SQUAMOUS
CELL CARCINOMA, BONE
OSTEOSARCOMA, AND
WOUND REPAIR

FACULTY OF MEDICINE,
INSTITUTE OF DENTISTRY, DEPARTMENT OF ORAL AND MAXILLOFACIAL SURGERY,
DEPARTMENT OF DIAGNOSTICS AND ORAL MEDICINE,
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REPAIR**

Academic dissertation to be presented with the assent of the Faculty of Medicine of the University of Oulu for public defence in Auditorium I of the Institute of Dentistry (Aapistie 3), on 13 February 2010, at 12 noon

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Abstract

Degradation of extracellular matrix (ECM) and basement membrane (BM) are required both in normal physiological conditions such as wound healing and in pathological tissue remodelling such as chronic ulcers and cancers. Matrix metalloproteinases (MMPs) are an enzyme family, which can cleave most ECM and BM components. They are associated with physiological and pathological processes but their exact roles are still largely unknown.

The expression of MMP-8 and MMP-26 in acute and chronic human cutaneous wounds using histological and cell culture methods were investigated. MMP-8 was expressed in epithelial cells, neutrophils, and other inflammatory cells especially in chronic ulcers while in acute wounds MMP-8 expression was weak or absent. MMP-26 was temporarily present in acute wounds while it was strongly expressed in close vicinity to the BM in multiple cell types of most chronic ulcers. *In vitro* keratinocyte wound assay showed that MMP-8 and -26 were expressed in migrating cells.

Bone formation, collagen metabolism, and inflammation in MMP8^{-/-} mice tooth extraction wounds and also periapical lesion formation were analysed. No differences between wild type or MMP-8-deficient mice in the new bone area or periapical lesion size were found. However, type III procollagen production was increased and inflammatory cell influx was decreased in MMP8^{-/-} mice. In addition, Fas ligand (FasL) production was increased in mandibular alveolar mucosa but decreased in alveolar bone of MMP-8 deficient mice. MMP-8 was also found to cleave FasL *in vitro*.

A total of 90 human mobile tongue squamous cell carcinoma (SCC) samples were collected. Bryne's malignancy scores, thickness of the SCCs, expression of microvessel density (CD31 and factor VIII), cyclooxygenase-2 (COX-2), the laminin-5 (currently termed laminin-332) γ 2-chain, integrin $\alpha\beta$ 6, estrogen receptor- α (ER- α), estrogen receptor- β (ER- β), and MMPs (-2, -7, -8, -9, -20, and -28) were analysed. The high expression of MMP-8 was associated with a better prognosis for the patients, particularly in females. In addition, tongue carcinoma formation in MMP8^{-/-} mice was investigated. Tongue SCC developed more often in MMP8^{-/-} female mice than wild type littermates. In addition, MMP-8 can cleave ER- α and - β and estrogen can induce MMP-8 production *in vitro*.

A total of 22 biopsies, 10 resection sections, and three lung metastases of 25 osteosarcoma patients samples were stained with MMP-2, -8, -13, -26, and tissue inhibitor of metalloproteinase-1 (TIMP-1) using immunohistological methods. Expression of these markers was mostly present in sarcoma cells but MMP-8 was not present in lung metastases. In resection sections, chemotherapy altered MMP-2, -8, and -13 expressions compared to biopsies. However, an association between the expression and prognosis of osteosarcoma patients could not be found.

In conclusion, MMP-8 seems to be an estrogen-related protective factor in tongue SCC and can regulate ECM and BM components and inflammation during wound healing. Further studies are needed to evaluate the exact function especially of MMP-8 in human osteosarcoma.

Keywords: carcinoma; squamous cell, matrix metalloproteinases, osteosarcoma, survival, wound healing

“To my family”

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Oulu, December 2009

Jarkko Korpi

Abbreviations

ABC	avidin-biotin enzyme-complex
ANOVA	analysis of variance
APECED	autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
AP-1	activator protein-1
BAG-1	apoptosis regulator Bag1
BAX	apoptosis regulator Bax
BCL-2	apoptosis regulator Bcl2, B-cell leukemia/lymphoma 2
BM	basement membrane
cDNA	complementary DNA
CD31	platelet/endothelial cell adhesion molecule-1
CLSM	confocal laser scanning microscope
CMT	chemically modified tetracycline
COOH	carboxyterminal
COX-2	cyclooxygenase-2
CO ₂	carbon dioxide
CT	computed tomography
C1	human oral squamous cell carcinoma cell line
CTT	CTTHWGFTLC peptide
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EGF	epidermal growth factor
EGF-R	epithelial growth factor receptor
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
Ets-1	C ets 1 protein, p54
FasL	fas ligand
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HSC-3	human tongue squamous cell carcinoma cell line
HE	hematoxylin-eosin
HMK	human oral mucosal keratinocyte
HRP	horseradish peroxidase
IL	interleukin

IFN	interferon
KC	keratinocyte-derived chemokine
kDa	kilodalton
KGF	keratinocyte growth factor
Ki-67	antigen identified by monoclonal antibody Ki-67
LIX	lipopolysaccharide-induced CXC chemokine
MMP	matrix metalloproteinase
MMPI	matrix metalloproteinase inhibitor
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MPO	neutrophil-specific myeloperoxidase
MT-MMP	membrane-type matrix metalloproteinase
MVD	microvascular density
NO	nitric oxide
NOS	nitric oxide synthase
PBS	phosphate-buffered saline
PF	platelet factor
PDGF	platelet-derived growth factor
PMN	polymorphonuclear
pQCT	peripheral quantitative computerized tomographic
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SCC	squamous cell carcinoma
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TNF	tumour necrosis factor
TNM	tumour node metastasis
VEGF	vascular endothelial growth factor
ICTP	carboxyterminal telopeptide of type I collagen
IIINTP	aminoterminal telopeptide of type III collagen
4NQO	4-Nitroquinoline-N-oxide

List of original articles

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

- I Pirilä E*, Korpi JT*, Korkiamäki T, Jahkola T, Gutierrez-Fernandez A, Lopez-Otin C, Saarialho-Kere U, Salo T & Sorsa T (2007) Collagenase-2 (MMP-8) and matrilysin-2 (MMP-26) expression in human wounds of different etiologies. *Wound Repair Regen* 15: 47–57.
- II Korpi JT, Kervinen V, Mäklin H, Väänänen A, Lahtinen M, Läärä E, Ristimäki A, Thomas G, Ylipalosaari M, Åström P, Lopez-Otin C, Sorsa T, Kantola S, Pirilä E & Salo T (2008) Collagenase-2 (matrix metalloproteinase-8) plays a protective role in tongue cancer. *Br J Cancer* 98: 766–775.
- III Korpi JT, Åström P, Lehtonen N, Tjäderhane L, Kallio-Pulkkinen S, Siponen M, Sorsa T, Pirilä E & Salo T (2009) Healing of extraction sockets in collagenase-2 (matrix metalloproteinase-8)-deficient mice. *Eur J Oral Sci* 117: 248–254.
- IV Korpi JT, Hagström J, Lehtonen N, Parkkinen J, Sorsa T, Salo T & Laitinen M Expression of matrix metalloproteinases -2, -8, -13, -26, and tissue inhibitors of metalloproteinase-1 in human osteosarcoma. Brief report. Manuscript.

*The first two authors contributed equally to this work.

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1 Introduction

Controlled proteolytic processing of the extracellular matrix (ECM) and basement membrane (BM) environments is critical for physiological processes, such as wound healing and bone remodelling. On the other hand, disturbance in proteolytic processing might lead to pathological tissue destruction in several diseases for example chronic ulcers and malignancies (Visse & Nagase 2003, Parks *et al.* 2004).

Matrix metalloproteinases (MMPs) are an enzyme family, which can collectively degrade almost all components of the ECM and BM. They also participate in apoptosis, angiogenesis, release of growth-promoting signals, and regulation of immune responses (Visse & Nagase 2003, Stamenkovic 2003, Parks *et al.* 2004). They are divided into the subfamilies of collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), matrilysins (MMP-7 and -26), membrane type (MT-)MMPs (MMP-14, -15, -16, -17, -24, and -25), and other MMPs (MMP-12, -19, -20, -21, -23, -27, and -28) (Nagase & Woessner 1999, Vu & Werb 2000, Visse & Nagase 2003, Stamenkovic 2003, Parks *et al.* 2004).

In general, expression of MMPs is associated with a poor prognosis but recently, several MMPs have been shown to have a protective effect in pathological processes (Witty *et al.* 1995, Kerkelä *et al.* 2002, Balbin *et al.* 2003, Agarwal *et al.* 2003, Montel *et al.* 2004, McCawley *et al.* 2004, Decock *et al.* 2007, Gutierrez-Fernandez *et al.* 2008). However, the exact functions of each MMP in pathological processes are not known.

In this work, the expression of MMP-8 and MMP-26 was studied in acute and chronic cutaneous wounds. Moreover, the role of MMP-8 in tooth extraction wounds and periapical lesions was analysed. The role of microvessel density (CD31 and factor VIII), cyclooxygenase-2 (COX-2), the laminin-5 γ 2-chain, integrin α v β 6, estrogen receptor- α (ER- α), estrogen receptor- β (ER- β), and MMPs (-2, -7, -8, -9, -20, and -28) in tongue cancer were studied by comparing immunohistochemical stainings with clinicopathological variables. The role of MMP-8 deficiency in mice *in vivo* tongue carcinogenesis was studied. Finally, the expression and prognostic value of MMP-2, -8, -13, -26, and tissue inhibitor of metalloproteinase-1 (TIMP-1) was studied in human osteosarcoma.

2 Review of the literature

2.1 Extracellular matrix components in wound healing

Wound healing is a cascade which can be divided into three phases: inflammation, cell proliferation, and remodelling. Tissue injury initiates an interactive process where migrating inflammatory cells, keratinocytes, endothelial cells, fibroblasts, regulatory factors, and adhesive matrix substrates communicate with each other within the context of the surrounding extracellular matrix. ECM components form a scaffold for the migrating cells and regulate the activity of growth factors and cytokines during wound healing (Broughton *et al.* 2006). The removal of a tooth initiates the same phases as in skin and mucosal wound healing. However, in addition to the other cell types, osteoblasts, and osteoclasts are involved in reconstruction and remodelling of the damaged ossified tissue (Lin *et al.* 1994, Witte & Barbul 1997).

Fibrillar collagens are the major molecules of the connective tissues. Type I collagen gives mechanical strength and type III collagen allows flexibility in tissues. Type I collagen is the major collagen in most tissues comprising approximately 70% of the total collagens and type III collagen is the second most common collagen in tissues. The molecular form of collagens consists of three polypeptide chains (α -chains). The chains are coiled into a left-handed helix with about 18 amino acids per turn and these chains are twisted into a right-handed super-helix. Each α -chain consists of repetitive Gly-X-Y-sequences (Prockop & Kivirikko 1995). Type I and III collagens are synthesised as procollagen molecules by fibroblasts. These procollagens have large propeptide extensions at both the aminoterminal (NH₂) and carboxyterminal (COOH) ends. Upon secretion, both propeptides are cleaved off by various proteases, resulting in a mature collagen molecule with short non-helical telopeptides at both ends. These telopeptides stabilise the collagen fibres increasing the tensile strength of tissue (Eyre *et al.* 1984, Engel & Prockop 1991).

The carboxyterminal telopeptide of type I collagen (ICTP) is one of these telopeptides. It has been shown to be an indicator of type I collagen degradation and can be used as a marker of bone resorption (Risteli *et al.* 1993). ICTP fragments are mainly generated by various MMPs, which are activated under pathological conditions (Sassi *et al.* 2000). In addition, the aminoterminal telopeptide of type III collagen (IIINTP) is found in old, fully matured type III

collagen fibres (Bode *et al.* 2000). The amount of both ICTP and HIINTP are found to be lower in malignant than in benign tissues (Kauppila *et al.* 1999).

Type IV collagen is a component of the basement membrane whereas type VII collagen anchors fibrils to the epithelium. Elastic fibres participate in tissue elasticity (Prockop & Kivirikko 1995, Kielty *et al.* 2002). Proteoglycans are a family of macromolecules (Gallo 2000) which are composed of a core protein linked to one or more glycosaminoglycan molecules. Glycosaminoglycans fill most of the extracellular space and allow mechanical support to the tissue (Gandhi & Mancera 2008).

The ECM includes several adhesive components such as fibronectin and vitronectin but there are also various proteins that have anti-adhesive functions such as tenascin-C (Mackie & Tucker 1999, Schwartz *et al.* 1999, Pankov & Yamada 2002). ECM components and cells communicate through cell surface receptors such as integrins (Hynes 2002). Fibrin is not a component of the normal ECM but it is a crucial component for blood clot formation during wound healing (Mosesson *et al.* 2001).

2.1.1 Cutaneous wound healing

Inflammation phase

After injury, inflammation is a protective response to the host from infection and tissue damage. The known signs of inflammation are redness, heat, pain, swelling, and also loss of function at the site of the wound (Manicone & McGuire 2008). The inflammatory phase can be divided into hemostasis and inflammation immediately upon injury through days 4–6. Immediately after wounding blood vessels vasoconstrict and activate the clotting cascade. The blood clot consists of fibrin, collagen, platelets, thrombin, and fibronectin and releases cytokines and growth factors initiating the inflammatory phase (Witte & Barbul 1997). The clot provides a scaffold for the migrating cells such as neutrophils, monocytes, fibroblasts, and endothelial cells. After clot formation, cellular signals initiate neutrophil influx into the wounded area through interleukin-1 (IL-1), tumour necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β), platelet factor-4 (PF4), and bacterial products. Neutrophils begin the bacteria and cellular debris clearing processes in the injured area. Neutrophils release proteases such as MMPs and serine proteases which can cleave extracellular matrix components in

the wound area. Thereafter, the tissue and blood monocytes are transformed into macrophages (2–4 days) initiating the proliferation phase (Broughton *et al.* 2006) (Fig 1).

Cell proliferation phase

Epithelialisation, angiogenesis, granulation tissue formation, and collagen formation are the main events during the cell proliferation phase (days 4 to 14). Platelets and macrophages produce epidermal growth factor (EGF) and transforming growth factor- α (TGF- α) stimulating epithelial proliferation and chemotaxis (Grotendorst *et al.* 1989, Lawrence & Diegelmann 1994). Epithelialisation in the injured area begins when inflammatory cytokines (IL-1 and TNF- α) increase keratinocyte growth factor (KGF) gene expression in fibroblasts leading to keratinocyte migration (Smola *et al.* 1993). In the proliferation phase fibroblasts and endothelial cells are the predominant cells. Hypoxia induces nitric oxide (NO) synthesis by endothelial cells. The increased concentration of NO stimulates vascular endothelial growth factor (VEGF) formation mainly by wound keratinocytes, leading to new blood vessel formation, capillary tubules vasodilatation and also protects new tissue from the toxic effects caused by ischemia (Witte & Barbul 2002, Goldman 2004). Thereafter, granulation tissue formation begins when fibroblasts migrate into the wounded area induced by platelet-derived growth factor (PDGF) and EGF. Fibroblasts initiate collagen synthesis and also transform into myofibroblasts inducing wound contraction by TGF- β (Desmouliere *et al.* 1993, Ehrlich & Krummel 1996). A provisional matrix formation begins through synthesis of type III collagen, glycosaminoglycans, and fibronectin (Pierce *et al.* 1991). TNF- α induces integrin expression which can act as an anchor in the provisional matrix (Gailit *et al.* 1996). TGF- β secretion peaks during the proliferation phase causing fibroblasts to synthesise type I collagen and also decreasing MMP production. In addition, formation of tissue inhibitors of metalloproteinases (TIMPs) and cell adhesion proteins increases in the wounded tissue (Goldman 2004).

Remodelling phase

Remodelling is probably the most important and longest phase of wound healing, lasting from day 8 to 1 year. Some use the term maturation phase. In this phase, the provisional matrix is replaced and neovascularisation recedes. The uninjured

skin consists of 80 to 90 percent of type I collagen and 10 to 20 percent of type III collagen but in the granulation tissue, the amount of type III collagen is 30 percent and decreases to 10 percent in the mature scar (Ehrlich & Krummel 1996). Several MMPs remodel the ECM and regulate TGF- β , PDGF, IL-1, and EGF concentrations during the remodelling phase (Henry & Garner 2003). Despite a long remodelling phase, the wound will never reach the level of normal fibrillar collagens found in uninjured tissue and at 3 months (and beyond) scar tissue gains approximately 80 percent of the wound strength of uninjured tissue (Broughton *et al.* 2006). However, post-natal skin wounds, foetal skin wounds, and adult oral mucosal wounds have the unique ability to regenerate without or with minimal scar formation (Toriseva & Kähäri 2009).

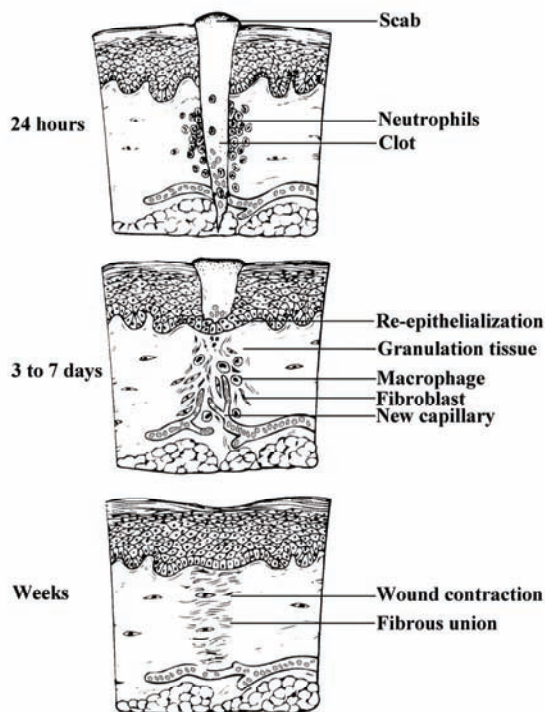


Fig. 1. Steps in cutaneous wound healing. Modified from (Kumar *et al.* 2003).

2.1.2 Chronic cutaneous wounds

Wound healing is a highly controlled co-operation of various cells, cytokines, growth factors, and proteolytic enzymes and their inhibitors. If this process is disturbed, normal wound healing fails and results in a chronic, non-healing ulcer. The overall prevalence of chronic venous leg ulcer patients ranges from 0.06% to 2% and is responsible for more than half of all leg ulcerations (Valencia *et al.* 2001). Chronic venous insufficiency, arterial disease, and diabetic neuropathy are the common causes of chronic leg ulcers (Baker *et al.* 1992). In addition, vasculitis, malignancies, bacterial infections, pressure in the lower extremities, and pyoderma gangrenosum may cause chronic leg ulcers (Menke *et al.* 2007).

Several mechanisms have been demonstrated to be a part of poor wound healing. Depending on the aetiology of ulcers, elevated numbers of macrophages, neutrophils, plasma cells, B-lymphocytes, T-lymphocytes, and mast cells accumulate in the wound area (Loots *et al.* 1998, Huttunen *et al.* 2000). Migration of keratinocytes may fail across the wound bed although cell proliferation may not be impaired (Adair 1977, Andriessen *et al.* 1995). Excessive protease activity may interfere with cell adhesion necessary for normal wound closure (Grinnell *et al.* 1992). Hypoxia in the wound may cause fibrosis formation (Ferguson & Leigh 1998). Cytokines such as IL-1, IL-6, and TNF- α are significantly upregulated in non-healing leg ulcers (Tregrove *et al.* 2000). A disturbed balance between MMPs and TIMPs has been shown to be associated with chronic wound pathogenesis (Toriseva & Kähäri 2009). In addition, synthetic broad-spectrum MMP inhibitors are shown to disturb normal wound closure and contraction (Mirastschijski *et al.* 2004). In chronic leg ulcers, development of squamous cell carcinoma (SCC) is increased (Baldursson *et al.* 1993, Baldursson *et al.* 1999, Impola *et al.* 2005).

2.1.3 Tooth extraction socket wound healing

Tooth extraction socket wound healing proceeds through the same phases as skin or mucosal wound healing. However, cellular and molecular events are not fully understood.

After the removal of a tooth, the socket fills with extravasated blood followed by the formation of a blood clot that seals the socket from the oral environment (Amler 1969, Lin *et al.* 1994). The blood clot that forms as a result of skin and mucosal wounds is primarily composed of the same components and similarly

initiates cell migration, growth factors, and cytokines release and bacterial digestion (Lin *et al.* 1994, Witte & Barbul 1997). After a few days, fibrinolysis begins to degrade the blood clot and the proliferation of residual periodontal cells form granulation tissue in the socket (Lin *et al.* 1994, Araujo *et al.* 1997). Residual periodontal ligament fibroblasts can produce type I and III collagens (Limeback & Sodek 1979). By the end of 1–2 weeks the extraction socket is filled with a vascular network consisting of connective tissue and inflammatory cells (Cardaropoli *et al.* 2003). By 4–6 weeks, most of the socket is covered by epithelium and at 4–6 months after the extraction the cortical bone from the crest and the walls of the socket are resorbed by osteoclasts and new trabecular bone begins to be involved. The origin of osteogenic tissue may be from the residual periodontal fibroblasts differentiating into osteoblasts (Lin *et al.* 1994, Kuru *et al.* 1999), periosteum, bone marrow (Friedenstein *et al.* 1987) or vascular pericytes (Schor *et al.* 1995, Doherty *et al.* 1998). The remodelling of the extraction socket is slow, the large amounts of mineralised tissue forms within six months. By 6–12 months, the newly synthesised mineralised tissue diminishes; the socket fills with woven bone and is later replaced by lamellar bone and marrow (Kingsmill 1999, Trombelli *et al.* 2008). Previous studies have shown a distinct role for MMPs (-2, -9, and -13) in tooth extraction wound healing (Devlin 2000, Silva *et al.* 2001, Zecchin *et al.* 2005, Accorsi-Mendonca *et al.* 2008).

2.2 Tooth apical periodontitis

Dental pulp contains the blood vessels, connective tissue, and nerves. Pulpitis is an inflammation or infection of the dental pulp resulting from bacterial effects, thermal injury, mechanical damage or chemical irritation. Tooth apical periodontitis is the most common consequence of untreated pulpitis. In addition, severe diseases such as apical abscesses, radicular cysts or osteomyelitis may form in untreated conditions of periodontitis or pulpitis (Neville *et al.* 2002).

In tooth apical periodontitis, inflamed granulation tissue (infiltrated with lymphocytes, polymorphonuclear [PMN] leukocytes, monocytes/macrophages, plasma cells, and eosinophils) is surrounded by a fibrous connective tissue. Inflammatory cells phagocyte and recruit other cells (monocyte/macrophage cells, plasma cells, fibroblasts, epithelial, and endothelial cells, PMN leukocytes, osteoblasts, and osteoclasts) to produce bone resorptive cytokines such as IL-1 α , IL-1 β , TNF- α , and IL-6. The activation of osteoclasts causes degradation of bone in the root apex area (Artese *et al.* 1991, Barkhordar *et al.* 1992, Miller *et al.*

1996a, Miller *et al.* 1996b, Marton & Kiss 2000). Expression of proteolytic enzymes such as MMPs can be stimulated by various cytokines and participate in the destructive process of apical periodontitis (Barkhordar 1987, Takahashi 1998, Wahlgren *et al.* 2001, Wahlgren *et al.* 2002).

2.3 Squamous cell carcinoma of the tongue

2.3.1 Incidence of tongue cancer

Of all tumours, five percent occur in the head and neck region and half of those occur in the oral cavity. In 2000, 615,000 new oral cavity tumours were reported worldwide and of those 300,000 were primary oral cavity squamous cell carcinomas. Cancer of the tongue, the leading type of oral SCCs, covers approximately one third of intraoral carcinoma diagnoses (Moller 1989, Regezi & Sciubba 1993). The incidence of tongue cancer in males ranges from 0.4–9.4/100,000 persons per annum, with the highest rates reported in Brazil, France, and in certain parts of India, being 7.4–9.4/100,000. The lowest rates occur in Northern Europe where the incidence varies between 0.4–1.0/100,000. Generally, females have a lower incidence of tongue cancer than males (Parkin 2002). In Finland, the incidence of tongue cancer is rising continuously with the current incidences being 1.3/100,000 in males and 1.1/100,000 in females (Finnish Cancer Registry 2007). The same trend is seen worldwide. The World Health Organization predicts a continuing increase in the number of cases of oral carcinoma per annum (Macfarlane *et al.* 1994).

2.3.2 Risk factors of tongue cancer

An important aetiological risk factor for oral cancers, including tongue cancer is tobacco smoking. Alcohol consumption is also an important contributing factor and these two factors together have a clearly synergistic dose-dependent effect of oral SCC development (LaVecchia *et al.* 1997, Moreno-Lopez *et al.* 2000, Hunter *et al.* 2005, Gillison 2007, Hashibe *et al.* 2007). Other aetiological risk factors are chewing of betel quid or areca nuts (Chen *et al.* 2008b), infection with human papilloma virus (Herrero *et al.* 2003, Hansson *et al.* 2005, Gillison 2007), poor oral hygiene (Moreno-Lopez *et al.* 2000, Guneri *et al.* 2005, Rosenquist *et al.* 2005), and large amounts of alcohol in mouthwashes (Winn *et al.* 2001). The

local application of Swedish moist snuff seems not to show risk for oral cancer (Luo *et al.* 2007). Premalignant lesions such as leukoplakia (2–6%) (Silverman *et al.* 1984, Schepman *et al.* 1998, Cowan *et al.* 2001) and erythroplakia (>90%) (Shafer & Waldron 1975, Mashberg & Feldman 1988, Mashberg & Samit 1995) are shown to be the risk for malignant transformation. In addition, oral lichen planus patients have a higher risk (0.4–4%) of developing oral cancer (Silverman *et al.* 1985, Holmstrup *et al.* 1988, Barnard *et al.* 1993). Several previous studies have described that a genetic predisposition increases the risk for oral cancer (Copper *et al.* 1995, Foulkes *et al.* 1996) but in contrast with a study by Goldstein *et al.* (1994) there is at most a weak correlation between familiar aggregation and oral cancer. In the literature, there are some heritable disorders which are shown to be associated with an increased risk for oral cancers such as Fanconi anemia (Kutler *et al.* 2003) and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (Rautemaa *et al.* 2007). On the other hand, diets rich in fresh fruits, vegetables, and olive oil are well-established to be associated with a lower risk for oral cancer (LaVecchia *et al.* 1997, Franceschi *et al.* 1999, Guneri *et al.* 2005, Pavia *et al.* 2006, Boeing *et al.* 2006).

2.3.3 Tongue cancer diagnosis and prognostic factors

Patients with a minor tongue cancer are often asymptomatic but symptoms are common with advanced local invasion. Typically SCC presents with a persistent mass, nodule or indurated ulcer (red lesions, or mixed red and white lesions) (Bsoul *et al.* 2005). Swelling, pain, bleeding, difficulty in mouth opening, chewing, swallowing, speech, and enlargement of cervical lymph nodes may be detected. The patients with ulcerative lesions have a worse prognosis than those with exophytic lesions (Asakage *et al.* 1998). The biopsy from the clinically most suspicious area confirms the diagnosis and some radiological techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) are used for staging of the primary tumour, regional lymph nodes, and metastasis (Tumour Node Metastasis [TNM] classification) (Table 1) (Johnson *et al.* 2005). The 5-year survival rate for oral cancer remains at approximately 50 percent without significant improvements, despite advanced surgery or radiation therapy (Sano & Myers 2007).

Table 1. TNM classification of carcinomas of the oral cavity (modified from Sobin & Wittekind 2002).

T	Primary tumour
TX	Primary tumour cannot assessed
T0	No evidence of primary tumour
Tis	Pre-invasive carcinoma (carcinoma in situ)
T1	Tumour 2 cm or less in greatest dimension
T2	Tumour more than 2 cm but not more than 4 cm in greatest dimension
T3	Tumour more than 4 cm in greatest dimension
T4a	Tumour invades through cortical bone, into deep/extrinsic muscle of tongue, maxillary sinus, or skin of face
T4b	Tumour invades masticator space, pterygoid plates, or skull base; or encases internal carotid artery
N	Regional lymph nodes (cervical nodes)
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
N2a	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3	Metastasis in a lymph node more than 6 cm in greatest dimension
M	Distant metastasis
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Stage grouping			
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1, T2	N1	M0
	T3	N0, N1	M0
Stage IVA	T1, T2, T3	N2	M0
	T4a	N0, N1, N2	M0
Stage IVB	Any T	N3	M0
	T4b	Any N	M0
Stage IVC	Any T	Any N	M1

The TNM stage of the tumour (Sobin & Wittekind 2002) is the most important prognostic factor of tongue SCC (Nathanson *et al.* 1989, Hiratsuka *et al.* 1997, Asakage *et al.* 1998, Silveira *et al.* 2007). Survival rates of stages I–II are approximately 80% whereas in stages III–IV they drop to 21%. The survival rate of patients with cervical lymph node metastasis is 52% whereas the extracapsular spread of cervical lymph node metastasis decreases the survival rate to only 28% (Kademani 2007).

The grades of SCC in the oral cavity can be categorised into: well-differentiated (Grade 1), moderately differentiated (Grade 2), and poorly differentiated (Grade 3). In well-differentiated tumours, keratinisation of cells and few mitotic figures can be seen. In moderately differentiated tumours, nuclear and cellular pleomorphism, mitotic figures, and abnormal cell shapes can be seen compared to well-differentiated tumours but keratinisation and intracellular bridges of cells are decreased. In poorly differentiated tumours, mitotic activity, atypical mitoses, cellular/nuclear pleomorphism, and multinucleated cells are seen. Keratinisation and intracellular bridges can not be seen. Well and moderately differentiated tumours can be classified as low-grade and poorly differentiated tumours as high-grade tumours (Pindborg *et al.* 1987).

Several other grading classifications have been introduced of which the Bryne's malignancy score analysis is most well-known. The malignancy score is based on five morphological features: 1) the degree of keratinisation, 2) the nuclear polymorphism, 3) the number of mitoses, 4) the pattern of invasion, and 5) the lymphoplasmacytic infiltration. The total score is summarised where each gives a score (1–4) (Bryne *et al.* 1992). The total score has been demonstrated to be a significant prognostic factor in oral cancer (Bryne *et al.* 1989, Bryne *et al.* 1992, Piffko *et al.* 1997, Gluckman *et al.* 1997).

Several studies have shown the association between high microvessel density and metastasis in tongue, oral, and head and neck region cancers (Gasparini *et al.* 1993, Williams *et al.* 1994, Shpitzer *et al.* 1996) but there are also several studies which have opposite results (Leedy *et al.* 1994, Gleich *et al.* 1996, Janot *et al.* 1996, Gluckman *et al.* 1997, Gleich *et al.* 1997, Hogmo *et al.* 1999).

The primary tumour thickness has been shown to be a prognostic factor for survival of tongue SCC (O-charoenrat *et al.* 2003), but in contrast Teixeira *et al.* (1996) found that tumour thickness does not correlate with survival of tongue SCC.

There are also large variations in the relevance of different prognostic histological markers in oral cancer. Schliephake (2003) classified twenty-nine

possible molecular markers in four groups according to their functions: 1) Enhancement of tumour growth (EGF, EGF-receptor, Cyclins [A, B₁, D₁, E], Proliferation cell nuclear antigen, Ki-67/MIB, Argyrophilic nucleolar organiser-region associated proteins, skp2, bcl2/BAG-1, Heat shock proteins [-27 and -70], and Telomerase), 2) Tumour suppression and anti-tumour response (Retinoblastoma protein, Cyclin dependent kinase inhibitors [p15, p16, p21, and p27], p53, Bax, Fas/Fas-ligand, ζ-chains, and Dendritic cells S100/p55), 3) Angiogenesis (VEGF/VEGF-receptor, Nitric oxide synthase type II, and Platelet-derived endothelial cell growth factor), 4) Tumour invasion and metastatic potential (MMPs, Cathepsins, Integrins, Cadherins/Catenins, Desmoplakin/plakoglobin, and Ets-1).

COX-2 has been shown to be upregulated in premalignant and malignant lesions in the oral cavity and also in the head and neck area. The presence or absence of COX-2 have been shown to be prognostic factors for patients but recent studies also found no prognostic value of COX-2 expression (Chan *et al.* 1999, Itoh *et al.* 2003, Lim *et al.* 2004, Atula *et al.* 2006, Pannone *et al.* 2007, Sakurai *et al.* 2007, Soland *et al.* 2008).

The cell adhesion molecule, integrin αvβ6 has been found to be expressed either normally, overexpressed, focal or extensive loss expressed in oral SCC. The β6 subunit may increase cell migration, invasion, and MMP production in malignant tissue (Lyons 2007). In addition, the laminin-5γ2-chain has been noticed to be an important basement membrane protein which was found to be a predictor of poor prognosis in oral SCC (Ono *et al.* 1999, Katoh *et al.* 2002, Gasparoni *et al.* 2007).

2.4 Osteosarcoma

2.4.1 Incidence of osteosarcoma

The most frequent primary bone tumour are osteosarcoma, Ewing sarcoma, and malignant fibrous histiocytoma of bone. Osteosarcoma represents 0.2% of all malignant tumours and its incidence is 0.3/100,000 persons per annum (Campanacci 1999). It occurs at any age but its peak incidence is between 15–25 years of age with a male prevalence (ratio 1.5:1) (Picci 2007). It occurs mostly at the metaphysis of long tubular bones (femur, tibia, pelvis, ribs, and upper arm)

but about 10% of osteosarcomas are located in the mandible or maxilla (Wanebo *et al.* 1992).

2.4.2 Risk factors of osteosarcoma

The aetiology of osteosarcoma is unknown. Ionising radiation is implicated as the cause of 2% of osteosarcomas (Finkel *et al.* 1976). Genetic background is associated with an increased risk of developing osteosarcoma. The strongest genetic predisposition is found in patients with retinoblastoma (Huvos 1991). In addition, 3–4% of children with osteosarcoma carry a mutation in p53 (Mcintyre *et al.* 1994). Several other conditions have been associated with osteosarcoma such as Paget's disease, chronic osteomyelitis, osteochondroma, enchondroma, fibrous dysplasia (Skubitz & D'Adamo 2007), Li-Fraumeni syndrome, Rothmund-Thomson syndrome, RAPADILINO syndrome, and Werner syndrome (Fuchs *et al.* 2004). In addition, tumour site, size, grade, and patient age are demonstrated to be risk factors in osteosarcoma (Davis *et al.* 1994), as well as, altered levels of alkaline phosphatase (Bramer *et al.* 2005), telomerase (Ulaner *et al.* 2003), and P-glycoprotein (Pakos & Ioannidis 2003).

2.4.3 Osteosarcoma diagnosis and prognostic factors

Patients with osteosarcoma usually have nonspecific clinical symptoms but the most common symptoms are pain and soft tissue swelling. In addition, weight loss, pallor, and fever can be present. In paediatric patients, over 15% develop a pathological fracture. Biopsy confirms the diagnosis and some radiological techniques such as CT, MRI, angiography, and dynamic bone scintigraphy can be used to stage the disease (Picci 2007). Conventional radiographs show bone destruction with periosteal reaction and soft tissue extension. Osteosarcoma has many histological subtypes which have in common the presence of proliferating malignant mesenchymal stem cells together with the production of osteoid or bone matrix (Benayahu *et al.* 2002, Marina *et al.* 2004). In general, treatment includes neoadjuvant chemotherapy and surgery. Today, limb salvage surgery is the main operation in over three-quarters of the patients. The 5-year survival rate of 20% in 1970 has changed to the current 70% because of efficient neoadjuvant chemotherapy (Spina *et al.* 1998, Stock *et al.* 2000, Gisselsson *et al.* 2002).

2.5 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases are a group of Zn^{2+} -containing (hence, the prefix 'metallo') endopeptidases which can degrade ECM components and BM macromolecules. Currently, 23 different MMPs have been identified and characterised in humans (Visse & Nagase 2003, Parks *et al.* 2004). MMPs are associated with many physiological conditions such as embryonic development, organ morphogenesis, ovulation, nerve growth, angiogenesis, inflammatory cell function, apoptosis, bone remodelling, and wound healing as well as pathological processes including cancer, arthritides, periodontal diseases, infection diseases, and chronic wound healing. In addition, MMPs are important regulators of various proinflammatory cytokines, chemokines, and growth factors (Nagase & Woessner 1999, Vu & Werb 2000, Visse & Nagase 2003, Stamenkovic 2003, Parks *et al.* 2004).

MMPs consist of a single polypeptide (20–100 kiloDalton [kDa]) and can be subdivided into subgroups based on their structure (Fig 2) or based on their substrate specificity (Table 2): collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), matrilysins (MMP-7 and -26), membrane-type (MT-)MMPs (MMP-14, -15, -16, -17, -24, and -25), and other MMPs (MMP-12, -19, -20, -21, -23, -27, and -28) (Nagase *et al.* 2006). In general, MMPs consist of a signal peptide at the amino terminal end followed by a propeptide domain, a zinc-containing catalytic domain, and a hemopexin domain at the carboxyl terminal end (Fig 2). The NH₂-terminal signal sequence is cleaved off directing MMP synthesis to the endoplasmic reticulum and the pro-domain preserves its latency until it is removed. The hemopexin domain is linked to the catalytic domain to determine substrate specificity. MMP-2 and MMP-9 have fibronectin type II-resembling repeats within their catalytic domain. Membrane-bound MMPs are anchored to the cell surface via a transmembrane component with a cytoplasmic tail or via a glycosylphosphatidyl inositol anchor. The structure of MMP-23 is unique, containing an NH₂-terminal anchoring signal peptide, a cysteine array, and an immunoglobulin-like domain (Fig 2) (Cawston & Wilson 2006).

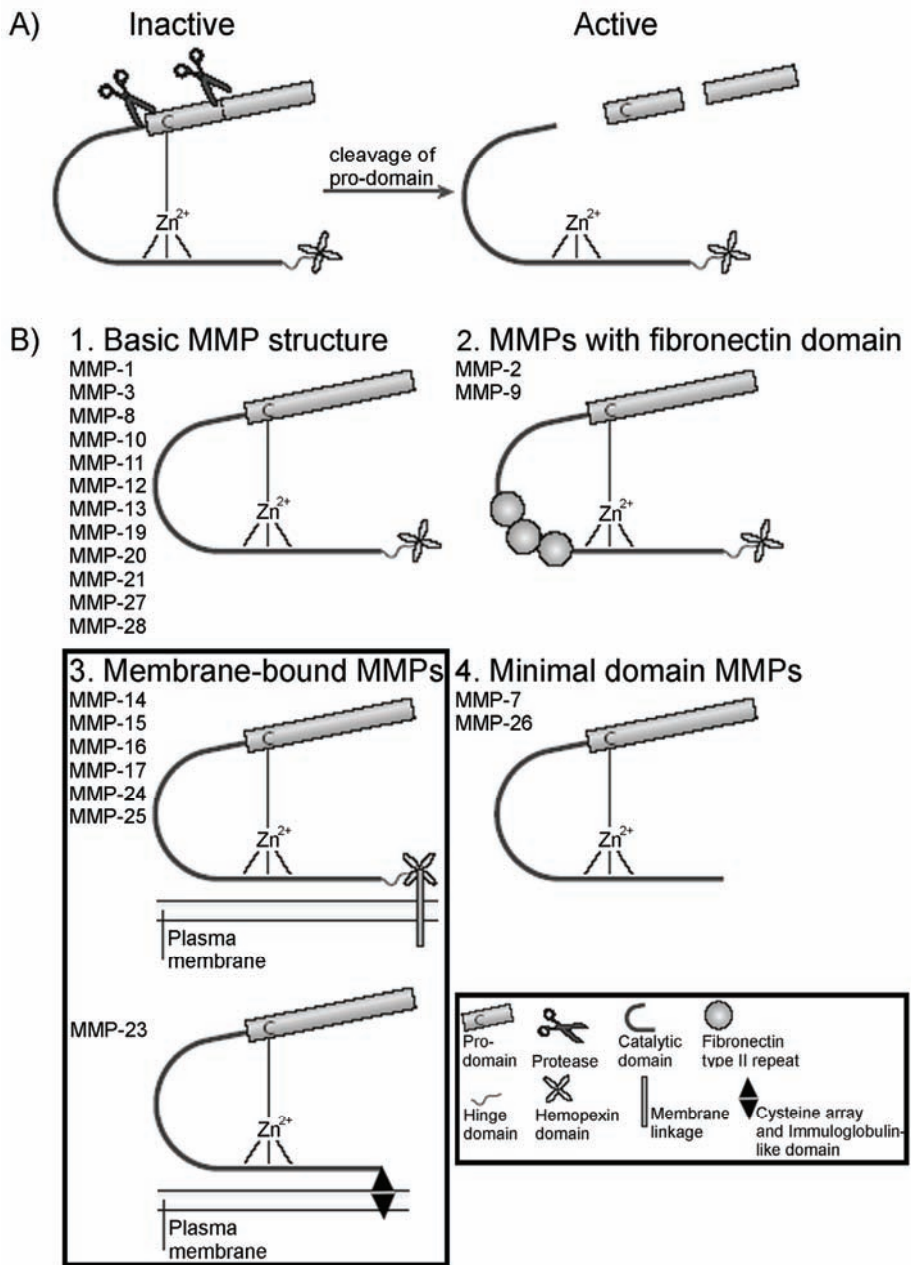


Fig. 2. The structure and activation of human MMPs. Modified from (Egeblad & Werb 2002, Visse & Nagase 2003, Page-McCaw *et al.* 2007).

Table 2. Human MMP classification and main substrates.

Enzyme subclass	Common name(s)	Main substrates
MMP		
Collagenases		
MMP-1	Collagenase-1, interstitial collagenase, fibroblast collagenase	Collagen III>I>II, VII, VIII, X, XI, gelatin, entactin, perlecan, laminin, casein, pro-MMP-1, proMMP-2, proMMP-9
MMP-8	Collagenase-2, neutrophil collagenase	Collagen I>II>III, VII, X, gelatin, entactin, aggrecan, tenascin, pro-MMP-8
MMP-13	Collagenase-3	Collagen II>III>I, VII, X, XVIII, gelatin, entactin, tenascin, aggrecan
Gelatinases		
MMP-2	Gelatinase A, 72 kDa gelatinase / type IV collagenase	Gelatin, collagen I, III, IV, V, VII, X, XI, elastin, fibrinogen, laminin, aggrecan, vitronectin, decorin, plasminogen
MMP-9	Gelatinase B, 92 kDa gelatinase / type IV collagenase, type V collagenase	Gelatin, collagen I, IV, V, VII, X, XI, XVIII, elastin, laminin, fibronectin, vitronectin, proMMP-2, proMMP-9
Stromelysins		
MMP-3	Stromelysin-1, transin, proteoglycanase, collagenase activating protein (CAP)	Aggrecan, laminin, gelatin, fibronectin, collagen III, IV, V, IX, X, XI, XVIII
MMP-10	Stromelysin-2, transin-2	Collagen I, III, IV, IV, gelatin, elastin, proMMP-1,-8 and -10
MMP-11	Stromelysin-3	Fibronectin, laminin, aggrecan, gelatin
Matrilysins		
MMP-7	Matrilysin-1, putative metalloprotease (PUMP-1), matrin	Fibronectin, laminin, gelatin, aggrecan, collagen I, IV, V, IX, X, XI, XVIII, Fas ligand
MMP-26	Matrilysin-2	Collagen IV, gelatin, proMMP-9
MT-MMPs		
MMP-14	MT1-MMP	Collagen I, II, III, gelatin, laminin, aggrecan, proMMP-2, -13
MMP-15	MT2-MMP	Proteoglycans, proMMP-2
MMP-16	MT3-MMP	Collagen III, fibronectin, proMMP-2
MMP-17	MT4-MMP	Gelatin, fibrinogen, proMMP-2
MMP-24	MT5-MMP	Fibronectin, gelatin, proMMP-2
MMP-25	MT6-MMP, Leukolysin	Collagen IV, gelatin, proMMP-2, -9
Other MMPs		
MMP-12	Macrophage elastase, metalloelastase	Elastin, collagen I, IV, fibronectin, laminin, proteoglycans, fibrinogen
MMP-19	Matrix metalloproteinase RASI-1	Collagen I, IV, gelatin, laminin, tenascin
MMP-20	Enamelysin	Amelogenin, aggrecan, laminin
MMP-21	-	Gelatin
MMP-23	Cysteine array (CA-) MMP	Gelatin
MMP-27	-	Not known
MMP-28	Epilysin	Casein

Reproduced from (Sternlicht & Werb 2001, Chakraborti *et al.* 2003, Nagase *et al.* 2006)

2.5.1 Regulation of MMPs

MMPs are expressed at low levels in normal conditions, but during inflammation their expression increases (Birkedal-Hansen 1995). MMPs are tightly regulated at several different stages: transcriptionally, post-transcriptionally, controlled at the protein level via their activators, inhibitors, and/or cell surface location. MMP expression can be up- or down-regulated by numerous stimulatory and initiatory factors such as phorbol esters, integrin-derived signals, extracellular matrix proteins, cell stress, changes in cell shape, cytokines, and growth factors. The effect can vary depending on the cell type and the same factor can have opposite effects on distinct MMPs. Many of these stimuli can activate the expression of the two proto-oncogene families (*c-jun* and *c-fos*), which heterodimerise and bind the activator protein-1 (AP-1) sites in various MMP gene promoter areas and activate transcription of the corresponding MMP gene, excluding MMP-2 (Sternlicht & Werb 2001). MMPs are secreted as proenzymes, stored in zymogen granules, and activated by serine proteases (trypsin and plasmin), other MMPs, microbial proteases or other factors such as oxygen-derived free radicals (Sternlicht & Werb 2001, Chakraborti *et al.* 2003). In addition, MMPs can be activated at the cell surface via cell surface receptors such as MMP-2 in the MMP-2/TIMP-2/MT1-MMP complex (Nagase 1997). Most MMPs are endocytosed (clearance) by binding to α -macroglobulin (Sottrup-Jensen & Birkedal-Hansen 1989). Thrombospondin-2 has also been demonstrated to be a part of MMP clearance (Yang *et al.* 2000).

2.5.2 Inhibition of MMPs

Endogenous inhibitors

Tissue inhibitors of metalloproteinases (TIMPs) are mainly responsible for MMP inhibitory activity (Sternlicht & Werb 2001, Chakraborti *et al.* 2003). The TIMP family (21–29 kDa) contains four endogenous protease inhibitors: TIMPs 1–4. All activated MMPs can be inhibited by TIMPs forming stoichiometric 1:1 complexes but TIMPs differ in their affinities to each MMPs. TIMP-1, -2, and -4 are known to be present in soluble forms and TIMP-3 is sequestered to the extracellular matrix. TIMPs can also prevent proMMP activation. However, TIMPs also have independent functions stimulating cell growth and proliferation, inhibiting angiogenesis, and promoting or suppressing apoptosis (Mannello &

Gazzanelli 2001, Visse & Nagase 2003). Previous reports have demonstrated that overexpression of TIMPs reduces tumour growth, invasion, and metastasis but contradictory findings have also been published (Duffy *et al.* 2008). MMPs can be regulated by various other endogenous inhibitors such as α_2 -macroglobulin, the major plasma inhibitor of MMPs or be self-regulated by their own proteolytic inactivation mechanisms (Hahn-Dantona *et al.* 2001).

Synthetic inhibitors

Synthetic MMP inhibitors (MMPIs) offer possibilities to regulate MMPs in different MMP-related diseases (Overall & Lopez-Otin 2002). Peptidomimetic MMPIs (Batimastat, Marimastat, GM6001, CT1746, and KB-R7785) can competitively and reversibly bind to the active sites of MMPs and inhibit a broad spectrum of various MMPs. Non-peptidomimetic MMPIs (Prinomastat, BMS275291, BAY12-9566, Ro 32-3555, and CGS 27023A) are designed to be more selective against specific MMPs. However, most of these MMPIs have been demonstrated to be ineffective or cause serious side effects during clinical or pre-clinical trials (Pavlaki & Zucker 2003, Peterson 2004).

Tetracyclines are antibiotics which can inhibit the synthesis and the activity of collagenases, gelatinases, and MMP-12 (Golub *et al.* 1994). Chemically modified tetracyclines (CMTs) have been modified from tetracyclines by removing the 4-dimethylamino group required for antibacterial activity. To date, at least 10 different CMTs have been developed with different MMP-inhibitory specificity and potency properties. CMT-8 and CMT-3 are effective MMP inhibitors for collagenases. A tetracycline analog (Periostat) is the only licensed synthetic MMPI being used for treatment of periodontitis (Golub *et al.* 1994, Overall & Lopez-Otin 2002, Pavlaki & Zucker 2003, Peterson 2004).

Bisphosphonates are the standard treatment for, *e.g.* metastatic bone diseases, osteoporosis, and Paget's disease (Woo *et al.* 2006). They affect the zinc-coordinating properties of MMPs and effectively inhibit activity of a wide spectrum of MMPs (MMP-1, -2, -3, -8, -9, -12, -13, -14, and -20) (Teronen *et al.* 1999, Heikkilä *et al.* 2002, Heikkilä *et al.* 2003).

There are also other MMPIs such as a synthetic cyclic decapeptide (CTTHWGFTLC, CTT peptide) which can selectively inhibit MMP-2 and MMP-9 but not MMP-8, -13 or -14. In cell and animal experiments, this peptide has the ability to target tumours without toxic side effects (Koivunen *et al.* 1999). Moreover, it can reduce invasion and intravasation of human tongue cancer cells

(Heikkilä *et al.* 2006). Recently, Suojanen *et al.* (2009) published the findings of an MT1-MMP-specific synthetic cyclic decapeptide, which also inhibited cell invasion *in vitro* and tumour spreading *in vivo*. In addition, the promising results with refractory renal cell carcinoma in phase II trial has been found using a MMPI called Neovastat. It has been extracted from cartilage tissue and can selectively inhibit MMP-2, -9, -12, and bind to VEGF receptor causing inhibition of angiogenesis (Batist *et al.* 2002).

2.5.3 Collagenase-2 and -3 (MMP-8 and -13)

The collagenase family consists of the three human collagenases-1, -2, and -3. All collagenases can cleave the native fibrillar collagens I, II, and III at a specific site producing $\frac{3}{4}$ N-terminal and $\frac{1}{4}$ C-terminal triple helical fragments but they can also degrade other extracellular molecules and soluble proteins (Table 2). The hemopexin domain structure is crucial for the ability of collagenases to degrade the triple helix of collagens (Nagase *et al.* 2006).

Collagenase-2 (MMP-8, neutrophil collagenase) was first described in the 1960s and later cloned from the peripheral leukocytes of a patient with chronic granulocytic leukemia (Hasty *et al.* 1987, Hasty *et al.* 1990, Devarajan *et al.* 1991). MMP-8 is mainly synthesised in PMN leukocytes before the cells leave the bone marrow and is stored as a latent enzyme (proMMP-8) in specific intracellular granules (Hasty *et al.* 1987, Hasty *et al.* 1990). However, MMP-8 can also be detected in a broad range of various other tissues and cells. It cleaves a wide range of different substrates (Table 2). MMP-8 cleaves native fibrillar type I collagen more efficiently than type III collagen (Hasty *et al.* 1987).

PMN leukocyte-type MMP-8 is secreted in a latent 75–80 kDa form and cleaved to a 65 kDa active form (Ding *et al.* 1996, Ding *et al.* 1997), and a latent form of non-PMN leukocyte-type MMP-8 (55 kDa) is cleaved to a 45 kDa active form (Moilanen *et al.* 2002, Moilanen *et al.* 2003). The high molecular form of MMP-8 (>100kDa) is most likely a complex with its endogenous inhibitors such as α_2 -macroglobulin, TIMPs or a result of dimerisation (Ingman *et al.* 1996, Chen *et al.* 1998) and the low molecular form of MMP-8 (<30kDa) is probably a degradation fragment (Apajalahti *et al.* 2003). Latent MMP-8 can be activated in response to reactive oxygen species (Saari *et al.* 1990), human trypsinogen-2 (Moilanen *et al.* 2003), MT1-MMP (Holopainen *et al.* 2003), MMP-3 (Knauper *et al.* 1993), several cytokines (Fuchs *et al.* 2004), and bacterial proteases (Sorsa *et al.* 1992). Non-PMN leukocyte-type MMP-8 has been detected from rheumatoid

synovial fibroblasts and endothelial cells (Hanemaaijer *et al.* 1997), gingival sulcular epithelial cells (Tervahartiala *et al.* 2000), oral carcinoma cells (Moilanen *et al.* 2002, Moilanen *et al.* 2003), plasma cells (Wahlgren *et al.* 2001), odontoblasts (Palosaari *et al.* 2000), melanoma cells (Giambernardi *et al.* 1998), leukemia cells (Kim *et al.* 2001), chondrocytes in rheumatoid arthritic and osteoarthritic lesions (Chubinskaya *et al.* 1999), migratory and proliferating keratinocytes, fibroblasts and inflammatory cells of healing wounds (Pirilä *et al.* 2001), human atheroma cells (Herman *et al.* 2001), breast cancer cells (Agarwal *et al.* 2003), and bronchial epithelial cells (Prikk *et al.* 2001).

Collagenase-3 (MMP-13) was first identified from human breast carcinoma (Freije *et al.* 1994). MMP-13 is expressed in many different cell types such as endothelial cells, fibroblasts, macrophages, epithelial cells, osteoblasts, and chondrocytes, though under normal physiological conditions, it is not expressed in most adult human tissues (Vaalamo *et al.* 1997, Zaragoza *et al.* 2002, Ala-Aho & Kähäri 2005). MMP-13 has a wide substrate specificity (Table 2). MMP-13 is predicted to have a role in tumour invasion and metastasis and is also involved in bone metabolism and homeostasis (Leeman *et al.* 2002).

2.5.4 Gelatinase A and B (MMP-2 and -9)

MMP-2 was first identified and purified from a malignant murine sarcoma cell line (Salo *et al.* 1983). MMP-2 is secreted in a latent 72 kDa form and converted to 59–62 kDa forms during activation (Birkedal-Hansen *et al.* 1993). The main activation process is assumed to be a complex where proMMP-2 binds to TIMP-2 and this complex binds to the active site of MT1-MMP with a 1:1:1 stoichiometric ratio (Ra & Parks 2007). MMP-2 can also autoactivate itself by formation of smaller activation products (Bergmann *et al.* 1995). The main substrate specificity is shown in table 2. MMP-2 is typically expressed in mesenchymal cells (Birkedal-Hansen *et al.* 1993) but its expression is also associated with many different malignant cell types (Huhtala *et al.* 1991, Birkedal-Hansen *et al.* 1993, Kähäri & Saarialho-Kere 1999).

MMP-9 was first identified from human macrophages (Vartio *et al.* 1982) and later cloned from transformed lung fibroblasts (Wilhelm *et al.* 1989). The latent form of MMP-9 (92kDa) can be secreted and activated to several sizes: 63- and 77–82-kDa forms (Sorsa *et al.* 1997, Duncan *et al.* 1998). In addition, dimeric forms of proMMP-9 (220 kDa) can be found (Triebel *et al.* 1992). Trypsin-2 together with MMP-3 are probably the most efficient activators of MMP-9 (Sorsa

et al. 1997, Cuzner & Opdenakker 1999). The main substrate specificity is shown in table 2.

MMP-9 has been found in many different cell types such as neutrophils, monocytes, dendritic cells, lymphocytes, endothelial cells, epithelial cells, and osteoblasts (Opdenakker *et al.* 2001). MMP-9 expression is shown to be important in tumour progression and metastasis, in addition to being involved in wound healing and inflammatory diseases (Sternlicht & Werb 2001).

2.5.5 Matrilysin-1 and -2 (MMP-7 and -26)

MMP-7 and -26 are the smallest MMPs. MMP-7 was first isolated from a mixed tumour library (Wilson & Matrisian 1996). Plasmin and MMP-3 can effectively activate MMP-7 (Nagase & Woessner 1999, Lijnen 2001). MMP-7 can degrade a large variety of substrates (Table 2) and is expressed in various tissues including, for example, tumours of the esophagus, stomach, colon, liver, pancreas, lung, skin, breast, prostate, and head and neck (Li *et al.* 2006).

MMP-26 was first cloned from a foetus (de Coignac *et al.* 2000), placenta (Uria & Lopez-Otin 2000), and endometrial tumour cDNAs (Park *et al.* 2000). MMP-26 (28 kDa) can autocatalytically activate itself (Uria & Lopez-Otin 2000, de Coignac *et al.* 2000, Park *et al.* 2000) and also be a part of the proMMP-9 activation process (Visse & Nagase 2003). MMP-26 digests a number of ECM components (Table 2) and is expressed by various cells including tumours such as those of the skin, prostate, and breast (Uria & Lopez-Otin 2000, Visse & Nagase 2003, Kuivanen *et al.* 2009). In addition, MMP-26 can cleave ER- β and regulate the estrogen signalling pathway (Savinov *et al.* 2006).

2.5.6 Other MMPs (MMP-20 and -28)

MMP-20 was first found in odontoblasts within newly formed enamel (Bartlett *et al.* 1998). Later it was detected in squamous cell carcinoma of tongue, odontogenic tumours, placenta, and tooth pulp (Väänänen *et al.* 2001, Väänänen *et al.* 2004). The main substrates of MMP-20 are listed in table 2.

MMP-28 (59 kDa) was first cloned from the testis and keratinocyte cDNA libraries. It is expressed at high levels in the developing germ cells of the testis (Lohi *et al.* 2001) but it is also found in lung and in a variety of tumours (Marchenko & Strongin 2001).

2.6 MMPs in wound healing

Wound healing is a strictly controlled event where a great variety of MMPs are found to be expressed spatially and temporarily by different cell types (Parks 1999). The successful interaction of these cell types and matrix components with proteinases (e.g. MMPs) and other biological factors (e.g. cytokines and TIMPs) enables the normal wound healing process. In a chronic wound healing process, disturbed interaction of these factors may be responsible for the delayed healing (Wlaschek *et al.* 1997, Trengove *et al.* 1999). In addition, bacteria colonisation is shown to increase expression of several MMPs in the wound area (Kanangat *et al.* 2006).

MMP-1, -3, and -10 are consistently expressed by migrating keratinocytes in both acute and chronic ulcers (Inoue *et al.* 1995, Ashcroft *et al.* 1997, Pilcher *et al.* 1998, Saarialho-Kere 1998, Pilcher *et al.* 1999). When re-epithelisation is completed, MMP-1 expression diminishes (Sudbeck *et al.* 1997). MMP-1 and MMP-3 are also expressed by stromal cells but the expressions are greater in chronic ulcers compared to acute wounds (Saarialho-Kere 1998). MMP-13 is abundantly expressed by stromal cells but undetected in keratinocytes. Expression of MMP-13 by fibroblasts is usually not detected in acute wounds but is high in chronic wounds (Vaalamo *et al.* 1997, Ravanti *et al.* 1999a). In contrast, gingival and foetal fibroblasts express MMP-13 during normal wound healing which can repair without or minimal scar formation (Ravanti *et al.* 1999b, Ravanti *et al.* 2001). MMP13^{-/-} mice do not show defects in skin wound healing compared to wild-type littermates, probably due to elevated expression of MMP-8 (Hartenstein *et al.* 2006) but Hattori *et al.* (2009) showed a delay in wound closure and re-epithelialisation in MMP13^{-/-} mice with larger sized wounds. In addition, MMP-13 is found to be expressed at sites of new bone formation in tooth extraction sockets (Devlin 2000).

Expression of MMP-8 is known to be increased in non-healing chronic dermal ulcers compared to normally healing wounds (Nwomeh *et al.* 1999). In addition, MMP-8 levels are decreased in patients with diabetic foot ulcers whose wounds heal well compared to the poor healers group (Muller *et al.* 2008). In animal experiments, MMP8^{-/-} mice showed a delay in skin wound closure and re-epithelialisation in acute wounds. During the first 48 h after wounding, lack of MMP-8 causes a delay in inflammatory cell (PMN leukocytes) recruitment to the wound site and up-regulation of MMP-9. It has been found that MMP-8 and MMP-9 can form specific complexes *in vivo*. An altered inflammatory response in

MMP8^{-/-} mice is postulated to be the reason for defects in wound closure and re-epithelialisation (Gutierrez-Fernandez *et al.* 2007). MMP-8 can cleave lipopolysaccharide-induced CXC chemokine (LIX) (Balbin *et al.* 2003, Tester *et al.* 2007) which is known to be involved in chemotaxis of PMN leukocytes (Rovai *et al.* 1998). In addition, proteolytically processed LIX has more chemotactic activity on neutrophils than the full-length form (Wuyts *et al.* 1999, Tester *et al.* 2007).

The gelatinases (MMP-2 and -9) are expressed and regulated differentially during wound healing. MMP-2 is known to be expressed by fibroblasts in both resting and healing skin and oral mucosa. MMP-2 can also be detected in the leading epidermal wound edge (Oikarinen *et al.* 1993, Salo *et al.* 1994) regenerating epidermis (Madlener *et al.* 1998). MMP-9 is expressed in basal and suprabasal keratinocytes in non-wounded oral mucosa (Salo *et al.* 1994) but it is not detected in normal skin (Pyke *et al.* 1992) or chronic cutaneous wounds (Saarialho-Kere *et al.* 1993). In murine models, MMP-9 is expressed in migrating keratinocytes in acute skin wounds (Okada *et al.* 1997, Madlener 1998, Lund *et al.* 1999). Previous studies demonstrated that MMP9^{-/-} mice have enhanced wound healing (Mohan *et al.* 2002) but in larger size wounds MMP-9 deficiency delays wound closure and re-epithelialisation (Hattori *et al.* 2009). Thus, MMP-2 seems to regulate long-term wound remodelling but MMP-9 is involved in wound re-epithelialisation. In tooth extraction sockets, MMP-2 and -9 expressions are also increased (Silva *et al.* 2001, Zecchin *et al.* 2005, Accorsi-Mendonca *et al.* 2008).

Matrilysin-2 (MMP-26) is expressed in the epithelial tip during acute and chronic cutaneous wound healing but not in intact epidermis (Ahokas *et al.* 2005).

2.7 MMPs in periapical lesions

Untreated bacterial infection in the dental pulp tissue leads to a destructive inflammatory reaction in the tooth periradicular area as a consequence of tooth periapical lesion formation. Various MMPs (-1, -2, -3, -9, -8, and -13) are expressed in the inflamed dental pulp tissues (Wahlgren *et al.* 2001, Shin *et al.* 2002, Belmar *et al.* 2008) and tooth periapical lesions (Wahlgren *et al.* 2001, Wahlgren *et al.* 2002). MMPs are also known to regulate osteoclast access to the bone resorption site (Blavier & Delaisse 1995, Inui *et al.* 1999). In addition, MMP-8 has been demonstrated to be present in the dental pulp and tooth periapical lesions where the successful root canal treatment decreases MMP-8

levels in the periapical exudates (Wahlgren *et al.* 2002). However, inhibition of MMPs by CMT-3 accelerates the growth of periapical lesion (Tjäderhane *et al.* 2007).

2.8 MMPs in cancer

MMPs are present in almost all human cancers and are known to regulate tumour invasion and growth. It was first thought that MMPs were being expressed by tumour cells but even more often they are expressed by surrounding stromal cells and inflammatory cells. Increased expression of MMPs has traditionally been thought to be associated with poor prognosis but recent studies have also shown that several MMPs (-3, -8, -12, -19, and -26) provide a protective role in cancer progression, metastasis, and prognosis (Witty *et al.* 1995, Kerkelä *et al.* 2002, Balbin *et al.* 2003, Agarwal *et al.* 2003, Impola *et al.* 2003, Montel *et al.* 2004, McCawley *et al.* 2004, Savinov *et al.* 2006, Decock *et al.* 2007, Gutierrez-Fernandez *et al.* 2008).

MMPs can promote tumour growth by processing of several growth factors such as fibroblast growth factor (FGF) and TGF- β (Whitelock *et al.* 1996, Imai *et al.* 1997). MMPs regulate cell proliferation indirectly through integrins (Agrez *et al.* 1994) and also regulate apoptosis such as MMP-7 by generating a soluble form of the death protein, Fas Ligand (FasL) (Powell *et al.* 1999). MMPs have been found to process a wide variety of cancer progression target proteins including structural ECM components, cell adhesion molecules, receptors, chemokines, and growth factors (Table 2). In tumour angiogenesis, MMPs cleave ECM and BM components as well as different substrates allowing endothelial cells to invade and form new blood vessels. Depending upon which substrates are being processed by specific MMPs, angiogenesis can also be impaired by generating angiogenesis inhibitors such as angiostatin and endostatin (Folkman 2004). In addition, MMPs may have a central role in the epithelial-mesenchymal transition (EMT), where migrating epithelial cells transform into fibroblastoid cells. EMT has been shown to be crucial for cancer progression to metastasis (Roy *et al.* 2009).

MMPs in tongue cancer

Various MMPs have been shown to be expressed in oral carcinoma cells and surrounding mesenchymal cells (Sorsa *et al.* 2004) but no specific member of the MMPs is found to be responsible for oral carcinoma progression.

MMP-2 protein and mRNA expressions have been found to be elevated in oral SCC compared to normal mucosa (Sutinen *et al.* 1998). In tongue SCC patients, increased MMP-2 expression has been found to be associated with an advanced clinical stage, and patients with low expression have a significantly longer disease-free survival (Yoshizaki *et al.* 2001). Patients with oral SCC highly express MMP-9 protein and mRNA in malignant tissue when compared to normal mucosa (Sutinen *et al.* 1998). MMP-9 mRNA expression is elevated in the oral dysplasias that progressed to oral cancer compared with those dysplasias that did not progress (Jordan *et al.* 2004). In addition, high MMP-9 expression may be used as a marker in the malignant transformation mainly in tongue lichen planus (Chen *et al.* 2008a). In tongue and also head and neck SCCs, MMP-9 immunoreactivity has been shown to correlate with a poor prognosis (Juarez *et al.* 1993, Kawamata *et al.* 1998, Nyberg *et al.* 2002, Ruokolainen *et al.* 2004, Ruokolainen *et al.* 2005) but in contrast Kim *et al.* (2006) found no association between the MMP-9 expression and prognosis of tongue SCC.

MMP-7 protein and mRNA are present in normal oral mucosa, oral SCC, and metastatic lymph nodes. In carcinoma tissues, MMP-7 expression is found to be increased compared to normal tissue (Muller *et al.* 1991, Birkedal-Hansen *et al.* 2000, O-charoenrat *et al.* 2001, Impola *et al.* 2004). Increased expression of MMP-7 is associated with dysplastic or carcinomatous changes of the buccal and tongue samples (Tilakaratne *et al.* 2009).

Cao & Li (2006) investigated oral SCC patients that mainly had tongue SCCs and found that MMP-1 polymorphism increases the risk of oral SCC. In addition, Shimizu *et al.* (2008) proved that MMP-1 polymorphism predicted a poor prognosis in tongue SCC. However, Tilakaratne *et al.* (2009) found that MMP-1 may not be involved in the early stages of buccal or tongue cancer formations. MMP-8 protein and mRNA are expressed in head and neck SCC including tongue SCC in a wide variety of cell types (Moilanen *et al.* 2002). Higher MMP-8 protein expression levels have been detected in dissected oral SCC cells compared to normal epithelial cells (Shimada *et al.* 2000). Elevated expression of MMP-13 is associated with a higher risk of head and neck cancer including tongue carcinoma progression or recurrence (Luukkaa *et al.* 2006) and invasion

capacity (Johansson *et al.* 1997). Lin *et al.* (2006) investigated the role of MMP-28 in oral SCC and found a weak correlation between positive immunostaining and better prognosis.

MMPs in osteosarcoma

Only a few studies have investigated the expression and function of various MMPs in human osteosarcoma. MMP-9 expression has been demonstrated in osteosarcoma (Himmelstein *et al.* 1998, Foukas *et al.* 2002, Uchibori *et al.* 2006). Correlation between invasiveness and MMP-9 has been found in cultured osteosarcoma cells (Kawashima *et al.* 1994, Kido *et al.* 1999, Bjornland *et al.* 2005). MMP-9 expression has been found to be a significant prognostic factor for the development of metastatic disease and disease-free survival in osteosarcoma patients (Foukas *et al.* 2002) and MMP-9 positively stained tumour cells are decreased after chemotherapy (Himmelstein *et al.* 1998). In contrast, Uchibori *et al.* (2006) found no correlation between expression of MMP-9 and prognosis.

Osteosarcoma cell lines express MMP-2 and MMP-14 (Heikkilä *et al.* 2003). Inhibition of MMP-2 reduces local spread and invasion of osteosarcoma cell lines (Cheng *et al.* 2004). However, there is no correlation with poor prognosis and MMP-2 expression but overexpression of MMP-14 significantly correlated with poor prognosis (Uchibori *et al.* 2006).

3 Aims of the present study

The main purpose of this study was to clarify the role of MMPs (-2, -7, -8, -9, -13, -20, -26, and -28) and other cancer and wound healing-related proteins in soft and hard tissue wound healing and malignant processes. Information on the specific function and expression of these factors in tissue healing processes and malignant progression may lead to better understanding and eventually better diagnostics and treatment modalities for patients.

The specific aims of this study were:

1. To evaluate the expression of MMP-8 and MMP-26 in human acute and chronic cutaneous wounds.
2. To examine the prognostic and predictive value of clinical and immunohistochemical markers including microvessel density (CD31 and factor VIII), cyclooxygenase-2 (COX-2), the laminin-5 (currently termed laminin-332) γ 2-chain, integrin α v β 6, estrogen receptor- α (ER- α), estrogen receptor- β (ER- β), and MMPs (-2, -7, -8, -9, -20, and -28) in human tongue SCC and to evaluate the role of MMP-8 deficiency in mice *in vivo* tongue carcinogenesis.
3. To investigate the role of MMP-8 in tooth extraction wound healing and periapical inflammation.
4. To evaluate the expression and prognostic value of MMP-2, -8, -13, -26, and TIMP-1 in human osteosarcoma.

4 Materials and methods

The detailed methods and reagents used appear in papers I–IV.

4.1 Patients (I, II, IV)

Table 3. Demographic characteristics of patients.

Study	I	II	IV
Patients, total (n)	16	90	25
Sex (male-female ratio)			
Male	6	47	13
Female	10	43	12
Age at the time of diagnosis			
Mean (years)	68		27
Median (years)		62	
Year of diagnosis	1998–2001	1981–2001	1976–2003
Samples (I)			
Venous leg ulcers	4	-	-
Diabetic leg ulcers	6	-	-
Vasculitis leg ulcers	2	-	-
Acute wounds	4	-	-
(controls)			
Samples (II)			
Tongue squamous cell carcinomas	-	90	-
Samples (IV)			
Biopsies	-	-	22
Resection sections	-	-	10
Metastases	-	-	3

4.2 Animals (II, III)

MMP8^{-/-} (knock-out) mice were produced in a C57BL/6 mice background and were born and weaned without any developmental anomalies in Carlot Lopez-Otin's lab in Spain as previously described (Balbin *et al.* 2003). All mice were bred and maintained in a barrier facility at the University of Oulu.

4.2.1 Induction of tongue squamous cell carcinoma (II)

Forty-seven wild-type and MMP8^{-/-} mice (13–16 weeks of age) were treated with tongue SCC-inducing chemical 4-Nitroquinoline-N-oxide (4NQO, Sigma, MO, USA) (Steidler & Reade 1984, Gannot *et al.* 2004) dissolved in propylene glycol (concentration 10 mg/ml). 4NQO was applied to the left dorsal side of the tongues 3 times per week for 12 weeks and mice were sacrificed by CO₂ inhalation and cervical dislocation at 55 weeks.

4.2.2 Tooth extraction procedures (III)

Sixty MMP8^{-/-} and wild-type male mice aged 30 days were anaesthetised by subcutaneous injection of 0.1 ml/10mg body weight of fentanyl 0.315 mg/ml, fluanisone 10 mg/ml in sterile water 1:1 / midazolam 5 mg/ml in sterile water 1:1. The second maxillary molars were removed and a subcutaneous dose of buprenorphine (1.5 µg/mouse) was given. Animals were sacrificed at day 4 and 7 after tooth extractions.

Eight MMP8^{-/-} and wild-type male mice aged 30 days were anaesthetised. The right side mandible molars were removed. At day 4, mice were sacrificed and jaw bones and alveolar mucosa were dissected and frozen in liquid nitrogen. Thereafter, samples were used to determine different cytokines (RayBio® Mouse inflammation antibody array 1.1., CA, USA), neutrophil-specific myeloperoxidase (MPO) (Hycult Biotechnology, Netherland) levels, and for Western blotting, and gelatin and reverse zymographies as described in chapter 4.5.

4.2.3 Periapical lesion induction (III)

Forty MMP8^{-/-} and wild-type male mice aged 30 days were anaesthetised. The mandible first molars' pulp chambers were opened by using a dental burs (Komet ISO 806 104; Komet Group, Germany). The exposed pulp chambers ensured microbial infection. At day 21, mice were sacrificed and the skulls were prepared and scanned with a peripheral quantitative computerized tomographic (pQCT) (Stratec XCT 960A, Norland Stratec Medizintechnik GmbH, Germany) device.

4.3 Cell experimentals (I, II)

4.3.1 *In vitro* scratch wound assay (I)

The human oral mucosal keratinocytes (HMKs) cell lines have been generated by removal of healthy gingival mucosa and then cultured according to a previously described method (Salo *et al.* 1994). Briefly, the HMK cells were cultured (100,000) and seeded in fibronectin (Sigma, MO, USA) coated Lab-Tek™ chamber slides (Nunc, Denmark). The cells were grown to confluence and allowed to attach overnight. The HMK cells were scratched once per well with a 5 ml plastic pipet tip (Biohit, Finland). The cells were washed and scratched areas were recoated with fibronectin (10 mg/ml in medium). The chambers were incubated with or without the following antibodies: MMP-26 antibody (1:50) (Uria & Lopez-Otin 2000), 10 mM broad-spectrum MMP inhibitor GM6001 (Ryss Laboratories, Union City, CA, USA in DMSO), 100 mM MMP-2 and -9 specific inhibitor CTTHWGFTLC-peptide (CTT) (Koivunen *et al.* 1999), or 10 mg/ml rabbit IgG (Vector Laboratories, Burlingame, CA, USA) for 62 hours. Control samples for the GM6001 were incubated with an equal amount of DMSO alone and control samples for the CTT-peptide were incubated with 100 mM scrambled sequence peptide. The cells were then fixed with 10% trichloric acid, stained with 0.1% crystal violet, and photographed with a phase-contrast microscope.

4.3.2 C1 and HSC-3 cell cultures (II)

Human oral squamous cell carcinoma cell line (C1) (Ylipalosaari *et al.* 2005) was plated onto 13-mm glass coverslips and incubated for 24 hours. Then the cells were rinsed in PBS and fixed in 10% formalin and stained as described in chapter 4.5.2.

Highly malignant tongue squamous cell carcinoma cells (HSC-3) (Japan Health Science Resources Bank, JRCB 0623, Japan) were cultured as previously described (Moilanen *et al.* 2003) with or without 10 nM estrogen (1, 3, 5 (10)-estradien-3 17 β -diol Steraloids Inc., RI, USA) overnight. Conditioned media was collected and used for Western blotting and RT-PCR.

4.4 Methods of RNA analysis (I, II)

4.4.1 *In situ hybridization (I)*

The protocol has previously been described (Pirilä *et al.* 2001). A 95 bp *SphI*-fragment of human MMP-8 cDNA (Hasty *et al.* 1990) and a 995 bp *SmaI*-fragment of human MMP-26 cDNA (Uria & Lopez-Otin 2000) were transcribed to digoxigenin-11-UTP-labeled sense and antisense cRNA-probes following the manufacturer's instructions (Roche GmbH, Germany). Briefly, the cutaneous wound samples were proteolysed in 0.2 M HCL and then in 0.5 mg/ml proteinase K-treatment (Finnzymes, Helsinki, Finland). The reaction was blocked by 100 mM glycine/PBS and acetylation was done with 0.25–0.5% acetic anhydride in 100 mM triethanolamine. Tissue sections were dehydrated and then prehybridised. The samples were covered with hybridization buffer (400–800 ng/ml digoxigenin-labelled antisense or sense cRNA probe) overnight. The samples were washed and the digoxigenin label was detected with alkaline phosphatase-conjugated anti-digoxigenin Fab-fragments (Boehringer Mannheim GmbH) and Fast Red tablets as chromogen. The tissue samples were counterstained with Mayer's hematoxylin (Merck KGaA, Germany) and analysed with a light microscope.

4.4.2 *RT-PCR (II)*

Total RNA was isolated from cultured HSC-3 cells (Japan Health Science Resources Bank, JRCB 0623, Japan) by the Trizol method (Gibco BRL Life, Technologies Inc., Denmark). Briefly, RT-PCR was carried out by using random decamers (Ambion Europe Ltd., Cambridgeshire, UK) and for PCR, the previously described MMP-8 primers (a band of 352 bp) (Moilanen *et al.* 2002) and β -actin primers (Ambion Europe Ltd., UK) were used. PCR products were analysed by standard agarose gel electrophoresis with 10 μ g/ml ethidiumbromide.

4.5 Methods of protein analysis (I-IV)

4.5.1 Immunohistochemical stainings (I-IV)

Immunohistochemical stainings were performed as previously described (Pirilä *et al.* 2001). Sections were pretreated and incubated in 0.3% H₂O₂ in methanol to block the endogenous peroxidase activity and washed. The non-specific binding was blocked with normal goat serum (polyclonal) or horse serum (monoclonal). Afterwards the sections were incubated with the primary antibodies (Table 4) overnight. Then the sections were incubated with non-immune rabbit (polyclonal) or non-immune mouse (monoclonal) IgGs and with biotinylated secondary antibody solution (Vector Laboratories, CA, USA). After washes the sections were covered with Vectastain Elite avidin-biotin enzyme-complex (ABC) (Vector Laboratories, CA, USA). Thereafter the tissue sections were stained with diaminobenzidine (Sigma, MO, USA) or 3-amino-9-ethylcarbazole (Sigma, MO, USA) and counterstained with Mayer's haematoxylin (Merck KGaA, Germany).

Table 4. List of antibodies used in immunohistochemical stainings.

Antibody	Source	Dilution/Concentration	Original publication
Monoclonals			
MMP-2	Suomen bioanalytiikka, SBA, Turku, Finland	1:2000	II
MMP-7	Calbiochem, San Diego, CA, USA	1:80	II
MMP-13	Calbiochem, San Diego, CA, USA	1:20	IV
Integrin α v β 6	(Weidner <i>et al.</i> 1991) generously provided by Biogen Idec, Inc, MA, USA	6.2G2 at 0.5 μ g/ml	II
COX-2	Cayman Chemical, Ann Arbor, MI, USA	1 μ g/ml	II
CD-31	Dako, Glostrup, Denmark	1:30	II
Polyclonals			
MMP-2	Suomen bioanalytiikka, SBA, Turku, Finland	1:1000	IV
MMP-8	(Hanemaaijer <i>et al.</i> 1997)	1:400, 1:200	I, II, IV
MMP-9	Neomarkers, Fremont, CA, USA	1:1000	II
MMP-20	(Väänänen <i>et al.</i> 2001)	1:1000	II
MMP-26	(Uria & Lopez-Otin 2000)	1:5000, 1:2500	I, IV
MMP-28	(Lohi <i>et al.</i> 2001)	1:500	II
TIMP-1	(Chemicon, Temecula, CA, USA)	1:250	IV
Laminin-5 γ 2-chain	(Pyke <i>et al.</i> 1995)	1:1000	II
Factor-VIII	Dako, Glostrup, Denmark	1:800	II
SP-32	(Risteli <i>et al.</i> 1988)	1:1500	III
ER- α	MC-20, Santa Cruz Biotechnology Inc., CA, USA	1:100	II
ER- β	Ab-24, Lab Vision, CA, USA	1:500	II

4.5.2 Immunofluorescent stainings (I, II)

The scratch wound assays using HMK cells were done as described in chapter 4.3.1. At days 1–3 after wounding slides were fixed in ethanol. The cells were incubated with normal goat serum in 2% bovine serum albumin and then with rabbit anti-MMP-26 antibody (1:1000) (Uria & Lopez-Otin 2000), anti-MMP-8 (1:200) (Hanemaaijer *et al.* 1997) or rabbit IgG overnight. Thereafter, FITC-conjugated anti-rabbit secondary antibody was applied and visualised using a confocal laser scanning microscopy (CLSM) (LSM 510, Carl Zeiss Inc., Germany).

Fixed C1 cells (Chapter 4.3.2) were stained with actin and MMP-8 antibodies as previously described (Moilanen *et al.* 2003). Bound MMP-8 antibody was detected with Alexa 488-conjugated anti-rabbit secondary antibody (1:200 dilution, Invitrogen Ltd., UK) and actin was detected with TRITC-conjugated phalloidin (5 ng/ml, Sigma, CA, USA) using CLSM (LSM 510, Carl Zeiss Inc., Germany).

4.5.3 Gelatin and reverse zymographies (I, III)

The gelatinases were studied by gelatin zymography using 10% SDS-PAGE gels containing 1 mg/ml 2-methoxy-2,4 dephenyl-3(2H) furanone-labeled gelatin (Invitrogen, CA, USA) (Mäkelä *et al.* 1994). The total protein contents of the tissue extracts were measured by a Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins from HMK cell culture media (I) and jaw bone and alveolar mucosa samples (III) were incubated with zymography buffer overnight. The degradation of gelatin was visualised under UV light and the proteins were stained with 0.5% Coomassie blue R-250.

The jaw bone and alveolar mucosa tissue extracts (III) were used to analyze the presence of TIMP-1 by reverse zymography. The samples and 200 ng recombinant TIMP-1 (control) were electrophoresed in 12% SDS-PAGE with 1 mg/ml gelatin and 100 ng/ml human recombinant MMP-9 (Calbiochem, NJ, USA) as described earlier (Satta *et al.* 2003).

4.5.4 In vitro cleavage assays (II, III)

3.1 µg human recombinant ER- α , 4.1 µg ER- β (Invitrogen, CA, USA), and 10 µg FasL (Calbiochem, NJ, USA) were incubated with human recombinant MMP-8

(Chemicon International, Inc, CA, USA) using different enzyme/substrate ratios as described in papers II and III. The reaction was carried out in an incubation buffer with or without MMP inhibitor GM6001, 10 μ M (Ryss Laboratories, CA, USA) for 22 hours and stopped by boiling in 4 x electrophoresis sample buffer. The reaction substances were then separated by SDS-PAGE. ER- α , ER- β , and FasL were detected by Western blotting.

4.5.5 Western immunoblotting (II, III)

HSC-3 cell (Japan Health Science Resources Bank, JRCB 0623, Japan) culture medium (II), tissue extracts from jaw bone and alveolar mucosa (III), and the samples from the cleavage assays (II, III) were separated by SDS-PAGE gel electrophoresis. The samples were then electrotransferred to nitrocellulose membranes (Millipore, MA, USA). Non-specific antibody bindings were blocked with 5% non-fat dry milk and incubated with antibodies recognising MMP-8 (Santa Cruz Biotechnology Inc, CA, USA) (II), ICTP (III), IIINTP (III) (Bode *et al.* 1999), ER- α (MC-20, Santa Cruz Biotechnology Inc., CA, USA) or with ER- β (Ab-24, Lab Vision, CA, USA) overnight. After washing and incubation with secondary antibodies (Dako A/S, Glostrup, Denmark) the membranes were incubated with ABCComplex/HRP (Dako A/S, Glostrup, Denmark). The proteins were then visualised with ECL Western blotting detection reagent and exposed to hyperfilm-ECL (Amersham Biosciences, NJ, USA).

4.5.6 Cytokine array analysis (III)

Jaw bone and alveolar mucosa samples from four wild-type and MMP8^{-/-} mice were pooled into four samples. The samples were minced in liquid nitrogen and dissolved in buffer. The protein concentrations were quantified using a DC Protein Assay-kit (BioRad, CA, USA). Totally 200 μ g protein from jaw bone and 100 μ g from alveolar mucosa were used. Membranes were coated with 40 specific inflammation factor antibodies (RayBio[®] Mouse inflammation antibody array 1.1., CA, USA) and probed with isolated protein samples. The antibody array was performed according to the manufacturer's instructions. Briefly, the membranes were incubated with the blocking buffer and then with samples. After washing, samples were incubated with biotin-conjugated antibodies overnight and then incubated with HRP-labelled streptavidin. After washing, proteins were visualised using detection buffers and exposed to an X-ray film (Hyperfilm ECL;

Amersham Biosciences, NJ, USA). The films were digitised and spot intensities were analysed (ScanAnalyze 2.50; Eisen Lab). The background staining and the intensities of positive controls on each membrane were used to obtain relative protein levels. The differences (%) in each of the 40 protein levels between MMP8^{-/-} mice to wild-type mice were calculated.

4.5.7 Neutrophil specific myeloperoxidase (MPO) enzyme assay (III)

The same pooled jaw bone and alveolar mucosa samples were used as described in chapter 4.5.6. The amounts of MPO enzyme were measured by using the mouse MPO enzyme-linked immunosorbent assay (ELISA) kit (Hycult Biotechnology, Netherland). Briefly, the tissue samples were incubated with standards in coated wells. Biotinylated tracer antibody was then added and allowed to bind to the antibody-MPO-complex. Streptavidin-peroxidase was allowed to bind to the tracer antibody and the substrate was added. The process was stopped with citric acid and the absorbance was measured at 450 nm. The amounts of MPO in each sample were determined based on the given standard curve.

4.6 Evaluations of samples (II-IV)

The detailed evaluations appear in the individual papers II–IV. All tissue samples were fixed in 10% formalin, paraffin embedded, cut, and stained with hematoxylin-eosin (HE), and with immunohistochemical methods. Samples were analysed under a light microscope at least twice by two to three blinded researchers without the knowledge of the clinical information (II, IV) or genetic backgrounds of the mice (II, III). To ensure the reliability of the analysis, the sample areas and parameters were clearly defined. After the first analysis, each researcher compared the results with an expert pathologist and repeated the analysis.

Tongue squamous cell carcinoma patient samples (II)

The tumour grade according to the classification (Pindborg *et al.* 1987) and TNM stage of the tumour (Sobin & Wittekind 2002) were collected. The Bryne malignancy score analysis (Bryne *et al.* 1992) and the thickness of the SCC tumour samples were determined.

Microvascular density (MVD) evaluation was done as described previously (Weidner *et al.* 1991) with slight modifications. Briefly, the vascularisation was visualised by using factor VIII and CD31 antibodies. The most highly vascularised areas ('hot spots') were selected from three different areas: 1) inside carcinoma islands 2) carcinoma marginals and 3) at the edge of the normal mesenchymal tissue. The highly vascularised areas were counted and divided into groups: 1) slight, 2) moderate, and 3) abundant MVD.

COX-2 positively stained cells of the tumour were classified as grades: 0–3 (<1% to >50%). Laminin-5 γ 2-chain expression was divided into negative (N = no staining within cancer cells), positive (P = cytoplasmic staining within cancer cells), and basement membrane (BM = the tumour nest periphery was partly or circumferentially stained) areas. Then different parts of tumour areas were categorised as grades: 1–5 (<20% to 80–100%). N and P areas were also evaluated grades: 1–4 (< 25% to 75–100%) of all tumours cells.

The levels of α v β 6-integrin expression were determined as follows: 0 = no positive, 1 = slight positive, 2 = medium positive, and 3 = strong positive in tumour cells. The positively stained tumour cells were categorised as grades: 0–4 (<1% to 76–100%). The intensity value and the category of positively stained cells was summarised (the score = 0–7).

Expression of MMP-2, -7, -8, -9, -20, and -28 was analysed respectively as described previously (Bachmeier *et al.* 2000). Briefly, the positively stained tumour cells were classified as follows: 0: <1 cell, score 1: 1–25 cells, score 2: 25–50 cells, score 3: 50–75 cells, and score 4: >75 cells. The intensity of staining was classified as follows: 0 = no positive, score 1 = weak positive, score 2 = moderate positive, and score 3 = strong positive. Positively stained MMP-8 and MMP-9 in cancer and inflammatory cells were counted separately whereas positively stained MMP-2 and MMP-7 were counted only from cancer cells. No grading score was used for inflammatory cell amount. Analysis of MMP-20 was excluded due to the small number of stained tumour cells. The total score was evaluated by multiplying the mean values of positively stained cells and staining intensity.

The intensity of ER- β expression was classified as follows: 0 = no positive, 1 = slight positive, 2 = medium positive, and 3 = strong positive in tumour cells and also in inflammatory cells.

Human osteosarcoma samples (IV)

The staining of MMP-2, -8, -13, -26, and TIMP-1 was classified as follows: 0 = no positive and 1 = positive. The positively stained samples were categorised as follows: 1 = slight positive, 2 = moderate positive, and 3 = strong positive.

Mice tongue cancer samples (II)

Lesions were graded into three classes, 1 = normal epithelium without any carcinogenic changes (*no change*), 2 = various (mild, moderate, severe) epithelial dysplasias (*dysplasia, including four dysplastic papillomas*), and 3 = SCC (*cancer*).

Tooth extraction socket and periapical lesion samples (III)

The areas of newly formed bone (%) were quantified using a 35000 μm^2 size square as a pattern in distal extraction sockets / the newly formed bone (μm^2). Inflammatory cell count from the highest density area ('hot spot') from jaw bone and alveolar mucosa was counted. The intensity of type III procollagen expression was evaluated and classified as follows: 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining in tooth extraction sockets. The pulpal exposure periapical lesion areas (mm^2) from the first molar distal roots were evaluated.

4.7 Statistical analysis (II-IV)

All statistics were computed using SPSS software or the R environment. T-test or one-way ANOVA were used in analysing the differences between groups. P-values less than 0.05 were considered significant.

Lifetime analyses were conducted using Kaplan-Meier analysis and Log-rank. The Cox proportional hazards regression model was used to analyse the relative hazards of death from tongue SCC. Computing odds ratios were used to analyse the association between various markers in tongue SCC patients. The function `twoby2` in the package `Epi`, version 0.7.0 was used to analyse differences in the proportion of developing dysplasia or cancer between the $\text{MMP8}^{-/-}$ and the wild-type mice.

4.8 Ethical considerations (I-IV)

The human studies were approved by the Ethics Committees of the Department of Dermatology and Surgery, Helsinki University Central Hospital, Finland (11/1998) (I), the Ethical Committee of the Faculty of Medicine, University of Oulu, Finland (18/2003) (II), and the Ethical Committee of the Pirkanmaa Hospital District, Finland (1/2001) (IV). The animal experiments were approved by the Animal Care and Use Committee, University of Oulu, Finland (037/04) (II) and (006/05, 046/06), (III).

5 Results

5.1 MMP-8 and -26 expression varies in acute and chronic human wounds (I)

MMP-8 mRNA and protein expression in acute wounds was weak or absent. In the chronic wounds, MMP-8 mRNA and protein was mainly expressed in neutrophils and inflammatory cells (macrophages or plasma cells) within the granulation tissue (I, Fig 1). MMP-8 protein expression was found in fibroblasts in three of 12 chronic ulcers and MMP-8 mRNA expression was detected in fibroblasts in four of 12 chronic wounds. In addition, MMP-8 protein was found in epithelium in three of 12 chronic ulcers and MMP-8 mRNA in epithelium in 7 of 12 chronic wounds (I, Table 2).

In the acute wounds, MMP-26 protein was predominantly expressed in the extracellular compartment in close vicinity to the epithelium BM area at day 1 but reduced at day 2 and was undetectable at day 3. Similar to the acute wounds, MMP-26 protein expression was detected in the BM area in the chronic ulcers (10/12) while the deeper granulation tissue layers stained negative. MMP-26 protein expression was difficult to localise to any specific cell type due to the extracellular reticular staining pattern. Some macrophage cells might express MMP-26 protein but areas with neutrophil clusters and fibroblast cells stained negative. In chronic ulcers, MMP-26 mRNA expression was detected away from the wound tip in basal and suprabasal epithelium (3/12) and inflammatory cells (2/12) (I, Fig 2, Table 2).

5.2 MMP-8 and MMP-26 are differentially expressed in migrating human oral mucosal keratinocytes *in vitro* (I)

MMP-8 expression was weak in resting unwounded cells and could not be detected after wounding at day 1. Interestingly, MMP-8 expression readily increased at the wound margin at day 3. MMP-26 expression was detected in resting unwounded HMK cells and after wounding at day 1. In addition, MMP-26 expression increased 3 days after wounding (I, Fig 5).

5.3 Addition of MMP-26 causes disorganised re-epithelialisation in migrating human oral mucosal keratinocytes *in vitro* (I)

The culture wells were coated with fibronectin, which is known to be a substrate for MMP-26 and also indirectly via MMP-26 activation of MMP-9. In uncoated controls HMK re-epithelialisation was slower than in fibronectin-coated wells. Fibronectin coated HMK cells formed an uniform, single-cell epithelial layer at day 3 after wounding but addition of MMP-26 antibody to the cell culture medium resulted in atypical cell morphology with multicell islands at day 3 after wounding. HMK cell re-epithelialisation was fully inhibited using the broad-spectrum MMP-inhibitor (GM6001) but an MMP-2 and -9 selective inhibitor (CTT-peptide) did not have any effect on re-epithelialisation. MMP-2 and -9 production or activation was not changed by addition of MMP-26 antibody as shown by gelatin zymography (I, Fig 3,4).

5.4 MMP-8 seems to be a protective factor in human tongue SCC (II)

Ninety eligible tongue SCC patients (47 males, 43 females) were studied. The overall five-year mortality of tongue SCC was 23%. Older patients with a more advanced TNM-stage, and/or high Bryne malignancy score had higher case fatalities but the tumour thickness was not a significant factor in patients' outcome (II, Table 1).

MMP-2, -7, -8, -9, -20, -28, COX-2, laminin-5 γ 2-chain, $\alpha\beta$ 6-integrin, ER- β or microvascular density were analysed by immunohistochemical methods but the only statistically significant factor for the prognosis of tongue SCC patients was MMP-8 (II, Table 2 and 3). Lack of MMP-8 expression in tongue SCC cancer cells was linked with a higher relative SCC mortality rate compared to MMP-8 positive cancer cells, giving 3.70 (95% CI 1.04–12.5) by the proportional hazards model when adjusted for age, sex, and TNM-stage. In addition, Cox's regression analysis showed that MMP-8 was the only factor which came up when age, gender, and TNM-stage were the main variables. By Kaplan-Meier survival analyses, the mortality of SCC patients lacking MMP-8 expression increased over time (II, Fig 1). In addition, the female tongue SCC patients with positive MMP-8 expression showed a tendency to have a better prognosis than male SCC patients with positive MMP-8 expression, but this finding was not statistically significant.

The associations of MMP-8 with other clinical or histological factors were not statistical significant.

5.5 MMP-8 deficiency increases tongue SCC incidence in mice (II)

Chemically induced (4NQO) tongue SCC in mice (23 MMP8^{-/-} and 24 wild-type) was studied to test the hypothesis that MMP-8 has a protective function in tongue SCC.

Incidence of either dysplasia or carcinoma was 67 percent higher in MMP8^{-/-} female mice compared to the wild-type littermates (83 vs 17%; 95% CI for the difference in proportions: +21 to +85 percentage points) but in the MMP8^{-/-} male mice the difference was only 20 percentage points higher (45 vs 25%; 95% CI for the difference in proportions: -21 to +55 percentage points). Female MMP8^{-/-} mice (6/12) had a statistically higher susceptibility of developing tongue SCC than wild-type littermates (0/12) and dysplasia incidence was higher in female MMP8^{-/-} mice (4/12) than in wild-type mice (2/12). Interestingly, MMP8^{-/-} or wild-type male mice had no statistically significant difference in carcinoma development between the groups (II, Table 4).

5.6 Estrogen induces MMP-8 expression in tongue carcinoma cells *in vitro* (II)

MMP-8 expression was mainly visualised in carcinoma cell (SCC C1) membranes and subcellular granules using confocal immunofluorescence (II, Fig 3A). MMP-8 protein was found in the molecular form of 75 kDa species in tongue SCC cells (HSC-3) by Western blotting. MMP-8 (75 kDa) expression was found to be induced by the addition of estrogen to the cell culture (II, Fig 3C). To verify this observation, RT-PCR was used to study MMP-8 mRNA expression. MMP-8 mRNA expression was undetectable in resting cultured cells but addition of estrogen to the cell culture induced MMP-8 mRNA expression (II, Fig 3B).

5.7 Estrogen receptors are expressed in tongue SCC samples and cleaved by MMP-8 *in vitro* (II)

Using immunohistochemical methods, ER- α and ER- β were found to be expressed in human and also mouse tongue SCC samples (II, Fig 4). ER- β expression in inflammatory cells showed a weak correlation to have a better

prognosis in human tongue SCC patients but the difference was not statistically significant. In an *in vitro* cleavage study, purified recombinant MMP-8 was found to cleave purified recombinant ER- α (66 kDa) dose dependently to the products of 44 and 26 kDa, but only minor cleavage of monomeric ER- β (53 kDa) by MMP-8 was found. Cleavage increased the approximate molecular weight forms of 20 kDa and 45 kDa and decreased dimeric and higher molecular weight forms of ER- β after incubation with MMP-8 as detected by Western blotting with MMP-8. Incubation of samples with the broad-spectrum MMP inhibitor (GM6001), vanished ERs cleavage by MMP-8 (II, Fig 5).

5.8 MMP-8 does not affect new trabecular bone formation or periapical lesion areas but changes collagen metabolism in tooth extraction socket wounds (III)

New trabecular bone formation in the second maxillary molar tooth extraction sockets at day four and seven did not show statistically significant differences between MMP8^{-/-} and wild-type mice. At four days after second maxillary molar extraction, the granulation tissue was heavily stained by type III procollagen revealing well organised newly synthesised fibres particularly at the trabecular bone surfaces. There was significantly ($p < 0.05$) more type III procollagen in MMP8^{-/-} mice as compared to wild-type in four-day-old tooth extraction sockets, but not in seven-day-old sockets (III, Fig 1).

Degradation of the carboxyterminal telopeptide of type I collagen (ICTP) and the aminoterminal telopeptide of type III collagen (IIINTP) were compared in MMP8^{-/-} and wild-type mice jaw bone and alveolar mucosa tissue extract samples collected four days after teeth extractions by Western blotting. The wild-type jaw bone and alveolar mucosa samples contained only minor amounts of 89 kDa ICTP and 91 kDa IIINTP fractions whereas in MMP8^{-/-} mice band intensities were stronger (III, Fig 4 A and B).

The periapical space in healthy first molar distal roots represented a normal ligament area in the histological and radiological analyses. The periapical lesion areas after pulp exposure to microbial infection for three weeks could be identified with both histological and radiographical methods but there were no difference between MMP8^{-/-} and wild-type mice periapical lesion areas (III, Fig 2).

5.9 Expression of proMMP-9 is decreased in MMP8^{-/-} mice alveolar mucosa compared to wild-type littermates in teeth extraction sockets wounds (III)

Gelatinolytic activity was detected in both MMP8^{-/-} and wild-type mice jaw bone and alveolar mucosa samples at day four. The amount of proMMP-9 (92 kDa) in bone was twice as high compared to alveolar mucosa samples in both groups. There was significantly less proMMP-9 in alveolar mucosa in MMP8^{-/-} mice compared to wild-type littermates but the same difference was not observed in jaw bone samples (III, Fig 4 C).

TIMP-1 activity was also detected in all MMP8^{-/-} and wild-type mice jaw bone and alveolar mucosa samples four days after teeth extractions and expression was higher in mucosa than in jaw bone. There was no difference in the amount of TIMP-1 between the MMP8^{-/-} and wild-type groups (III, Fig 4 D).

5.10 Cytokine expressions and inflammatory cell count are changed in MMP8^{-/-} and wild-type mice teeth extraction sockets wounds (III)

Table 5. The cytokine profile at day four in MMP8^{-/-} mice teeth extraction sockets wounds when compared to wild-type mice.

Jaw bone		Alveolar mucosa	
Increased expression	Decreased expression	Increased expression	Decreased expression
IFN- γ (140%)	FasL (-58.2%)	FasL (128.6%)	IL-6 (-45.2%)
GM-CSF (70.5%)	IL-6 (-51.6%)	IL-1 β (76.9%)	MIP-1 γ (-42.5%)
KC (59.6%)	Fractalkine (-50.7%)		MIP-1 α (-41.5%)
	IL-3 (-50%)		LIX (-33.7%)
	GCSF (-43.3%)		
	LIX (-12.2%)		

Since the cytokine array analysis showed that lack of MMP-8 changes the amount of FasL both in jaw bone and alveolar mucosa, we performed an *in vitro* cleavage assay to test whether FasL is a substrate for MMP-8. MMP-8 cleaved FasL *in vitro* producing a cleavage product of approximate 15–25 kDa in molecular weight. When the ligand was incubated alone, the FasL antibody recognised molecular forms of approximately 37–42 kDa representing the monomeric form, 79 kDa representing the dimeric form, 143 kDa representing the tetrameric form and a complex form of 204 kDa. When FasL was incubated with MMP-8, the

ligand tetramer could not be detected anymore and the amount of the ligand dimer was also strongly diminished. However, MMP-8 processing also produced a 64 kDa form of FasL which could be a dimer lacking the 15–25 kDa fragment (III, Fig 3 E).

Total inflammatory cell count showed a tendency to be decreased in the MMP8^{-/-} mice jaw bone and alveolar mucosa but this difference was not statistically significant. The neutrophil influx was clearly delayed in MMP8^{-/-} mice compared to controls at four days after teeth extractions both in jaw bone and alveolar mucosal tissues as measured with the MPO assay (III, Fig 3 C and D).

5.11 MMP-2, -8, -13, -26, and TIMP-1 are differentially expressed in human osteosarcoma (IV)

MMP-2 was strongly expressed in 86% of the 22 biopsies, moderately expressed in 50% of the ten resection specimens, and all the three lung metastases. MMP-8 immunoreactivity was detected in resection specimens in 60% and in 50% of biopsies. Immunostaining was not found in lung metastases. MMP-13 was predominantly expressed in sarcoma cells and altogether collagenases (MMP-8 and -13) immunoreactivities were detected more often in resection specimens (60%) than in biopsies (41%). Whereas, MMP-26 was present in all biopsies and metastases and in most resection specimens (80%). TIMP-1 was expressed in some biopsies, resection specimens and lung metastases (IV, Table 2, Fig 1).

Response to chemotherapy appeared to affect the immunoreactivities of MMP-2 and collagenases (MMP-8 and -13) in osteosarcoma. The patients with moderate or poor response expressed more MMP-2 compared to the cases with good to excellent responses. In addition, MMP-13 was positively stained in all the patients with a poor response. However, MMP-2, -8, -13, -26 or TIMP-1 immunoreactivities were not associated with disease-specific survival, but the late cases had a slight tendency to result in a better disease-specific survival ($p = 0.077$).

6 Discussion

6.1 Role of MMP-8 and MMP-26 in wounds (I, III)

Wound healing is a complex process where various cells, cytokines, growth factors, and proteolytic enzymes and their inhibitors are strictly regulated. MMP-8 is mainly synthesised by neutrophils (Hasty *et al.* 1987, Hasty *et al.* 1990) but there are also other cellular sources of MMP-8 such as keratinocytes (Tervahartiala *et al.* 2000, Pirilä *et al.* 2001), fibroblasts (Hanemaaijer *et al.* 1997), and carcinoma cells (Moilanen *et al.* 2002). MMP-8 can effectively degrade various ECM and BM components and interestingly it alters the inflammatory response (Balbin *et al.* 2003, Gutierrez-Fernandez *et al.* 2007).

MMP-8 protein and mRNA expressions were weak in human acute cutaneous wounds but in chronic non-healing ulcers expressions were clearly increased. The study is in line with previous findings where MMP-8 expression levels were found to be increased in diabetic wounds (Lobmann *et al.* 2002), and in unbalanced diabetes MMP-8 activity is clearly increased in gingival tissue and oral fluids (Ryan *et al.* 1999). In addition, MMP-8 expression is increased in gingival epithelium and neutrophils in chronic periodontitis (Tervahartiala *et al.* 2000). In line with the clinical observations, MMP-8 expression was minimal in *in vitro* mucosal keratinocyte wounds at day one, but increased at day three on.

The role of MMP-26 in acute and chronic cutaneous wounds was investigated. In contrast to MMP-8, MMP-26 was predominantly expressed in the ECM stroma in close vicinity to the BM area in acute wounds at day 1 but vanished thereafter. In chronic leg ulcers it was present in the same area in almost all samples. MMP-26 mRNA was present in three of 12 samples in both suprabasal and basal epithelial cells and in inflammatory cells in two samples. Consistently, MMP-26 expression is detected under the BM zone, but not in keratinocytes in inflamed colon tissue (Bister *et al.* 2004). However, Ahokas *et al.* (2005) showed, using another antibody, that MMP-26 is expressed in keratinocytes in normally healing wounds from day 1 to day 9. However, MMP-26 has been found to be expressed in various cell types (Uria & Lopez-Otin 2000, Visse & Nagase 2003). These differences can be explained by the specificity in the antibodies used or the differences in tissue type. These findings demonstrated that the source of MMP-26 may be from multiple cell types in wound healing. In addition, a distinct function of MMP-26 in cell proliferation and cell-cell adhesion

in vitro was found. Incubation of migrating HMK cells with MMP-26 antibody resulted in multicell islands and an atypical cellular morphology. MMP-26 is shown to activate proMMP-9 (Visse & Nagase 2003). However, blocking of MMP-26 did not change MMP-9 activation or synthesis in this study. In addition, Ahokas *et al.* (2005) did not find colocalisation between MMP-26 and MMP-9 in cutaneous wound samples. However, MMP-26 may activate proMMP-9 but MMP-9 is probably compensated by other activators such as MMP-8 if necessary. Taken together, MMP-26 seems to be strictly regulated both spatially and temporally and participates in cell proliferation and adhesion. MMP-26 may therefore be an important factor in wound healing but further studies are needed to evaluate the exact *in vivo* function.

LIX was shown to be a substrate for MMP-8 (Balbin *et al.* 2003, Tester *et al.* 2007) and to participate in PMN leukocyte chemotaxis (Rovai *et al.* 1998). In addition, the processed form of LIX has more chemotactic activity on neutrophils than the full-length form (Wuyts *et al.* 1999, Tester *et al.* 2007). In the present study, there was less LIX expression in MMP8^{-/-} mice in the alveolar mucosa of the tooth extraction socket. This may explain the finding that neutrophil and inflammatory cell infiltration was delayed in the MMP8^{-/-} mice. In the antibody arrays either processed, full-length or both forms of LIX are detected in the samples. Therefore, the current study can only speculate about the exact function of the LIX processed by MMP-8 in tooth extraction socket wound healing.

Interestingly it was found that, FasL levels were increased in alveolar mucosa but decreased in jaw bone of MMP8^{-/-} mice compared to wild type mice, and that FasL is a substrate for MMP-8. This may also explain the difference in MMP8^{-/-} mice neutrophil recruitment. FasL can be detected in membrane bound (mFasL) and also in the soluble forms (sFasL) where mFasL induce neutrophil apoptosis and sFasL neutrophil chemotaxis. The discrepancy between alveolar mucosa and jaw bone demonstrate spatial and temporal processing of FasL by MMP-8. The exact function of processed FasL in tooth extraction socket healing needs to be studied further.

Recently, Gutierrez-Fernandez *et al.* (2007) demonstrated a delay in wound closure and re-epithelialisation in MMP8^{-/-} mice skin wounds. In this study, there was no difference in new trabecular bone formation in MMP8^{-/-} mice tooth extraction socket wound healing. However, the amount of type III procollagen was increased after 4 days in MMP8^{-/-} mice as compared to wild type littermates. This difference was no longer seen at day 7, most probably due to compensation by other MMPs. Similar to MMP-13-deficient mice wounds, MMP-8 expression

is increased probably due to compensation of MMP-13 by MMP-8. These mice do not have any alteration in cutaneous wounds (Hartenstein *et al.* 2006) but a recent study by Hattori *et al.* (2009) showed a significant delay in wound closure and re-epithelialisation in MMP-13-deficient mice using larger size skin wounds and mice of a different age mice compared to a previous study. MMP-9 expression was shown to be increased in MMP8^{-/-} mice skin wound tissue (Gutierrez-Fernandez *et al.* 2007) but the present study showed the opposite observation in tooth extraction wounds. This compensation may actually benefit the healing of MMP8^{-/-} mice tooth extraction wounds because Mohan *et al.* (2002) showed that lack of MMP-9 may accelerate the healing process of skin and cornea enhancing the epithelial cell proliferation. However, a delay in wound closure and re-epithelialisation was found in larger-sized cutaneous wounds in MMP-9 deficiency mice (Hattori *et al.* 2009).

In bone fractures, MMP-8 expression is present at day one but decreases thereafter (Itagaki *et al.* 2008). Henle *et al.* (2005) have previously shown that MMP-8 expression is not altered during normal bone repair whereas in non-healing bone fractures MMP-8 expression was increased. This is probably due to increased inflammation. In line with their findings, the loss of MMP-8 did not affect the new bone formation in mice tooth sockets. Various MMPs have been shown to regulate osteoclast cell function in periapical lesions (Blavier & Delaisse 1995, Inui *et al.* 1999) and levels of MMP-8 in periapical exudates may decrease during successful root canal treatment (Wahlgren *et al.* 2002). However, the present study found no difference in MMP8^{-/-} mice tooth periapical lesions compared to wild type littermates. These findings together demonstrate that MMP-8 may not have a very crucial role in bone formation.

Various proteases including MMPs are regulated spatially and temporally in all phases of physiological wound healing. A large amount of proteases and excessive inflammation is assumed to be the reason for chronic ulcer formation. However, it is not quite clear why certain MMPs are down- or up-regulated in a chronic environment. In acute wounds, specific treatment is not necessary except for accurate surgical management but in chronic ulcers decreasing the necrotic tissue and protease burden by surgical debridement is essential to adjust the wound back into an acute phase. Another method might be to deliver different types of specific molecule inhibitors or protein vehicles to the site of injury achieving target inhibition or expression. Gutierrez-Fernandez *et al.* (2007) showed a delay in MMP-8 deficiency mice skin wound closure. It is clear that MMP-8 regulates the inflammatory cell influx, cytokines, ECM, and BM

components, especially the amount of type I and III collagens thus leading the felicitous healing together with other wound healing-related components. MMP-8 may increase inflammatory cell influx in the chronic wound area enhancing wound clearance and disproportionately degrading the ECM and BM components but the exact functions of overexpressed MMP-8 or MMP-26 in chronic ulcers are unknown. Large spectrum MMP inhibitors have been clinically tested for the treatment of cancer or arthritis (Nagase *et al.* 2006). It is possible that some individual MMP has a protective and beneficial role in various diseases such as in chronic wound repair. The limited knowledge of the function of individual MMPs may be one explanation as to why large spectrum MMPis have failed in clinical trials.

6.2 Potential prognostic markers in tongue squamous cell carcinoma and osteosarcoma (II, IV)

The 5-year survival rate in tongue SCC is approximately 50% (Sano & Myers 2007). In this study, the 5-year overall mortality was only 23% because of very effective treatment. Further analysis showed that patients (less than 70 years old) with lower clinical stages (TNM I and II) and a lower (5–10) Bryne’s malignancy score had the longest survival as previously described by Piffko *et al.* (1997) and Kantola *et al.* (2000). In addition, thickness of the tongue SCC was not associated with poor prognosis in this or a previous study (Teixeira *et al.* 1996). However, O-charoenrat *et al.* (2003) found a strong predictive value of cervical metastasis and poor prognosis when tumour thickness exceeded 5 mm.

The present study investigated a wide variety of possible new and previously proposed immunohistological prognostic markers including microvessel density (CD31 and factor VIII), cyclooxygenase-2 (COX-2), the laminin-5 γ 2-chain, integrin α v β 6, ER- α , ER- β , and MMPs (-2, -7, -8, -9, -20, and -28) for tongue SCC. However, MMP-8 was the only statistically significant factor in prognosis of tongue SCC. Patients with high MMP-8 immunostaining had a better survival. In addition, there was also a tendency for this to be more prominent in female tongue SCC patients compared to male SCC patients. Consistent with these findings, a strong statistical support was found that MMP8^{-/-} female mice developed dysplasias and tongue cancer more often than wild type littermates, and deficiency of MMP-8 increased tongue SCC formation in both genders compared to control mice. Interestingly, male MMP8^{-/-} mice have skin tumours more often than female MMP8^{-/-} mice or wild type littermates (Balbin *et al.*

2003). In addition, MMP-8 can inhibit breast cancer metastasis correlating with better prognosis (Decock *et al.* 2007, Gutierrez-Fernandez *et al.* 2008) and can be a tumour suppressor and an early recognisable serum marker in human melanoma (Vihinen *et al.* 2008, Palavalli *et al.* 2009). In contrast, MMP-8 expression is associated with poor prognosis in ovarian cancer (Stadlmann *et al.* 2003). This discrepancy is probably due to different functions of MMP-8 in various tissues and therefore in various cancers.

Shimada *et al.* (2000) demonstrated that MMP-8 is expressed in oral SCC cells. Later, Owen *et al.* (2004) showed that MMP-8 is cell-membrane associated in activated PMN leukocytes. In the present study, MMP-8 was also cell-membrane associated in cultured oral SCC cells. In addition, estrogen can induce MMP-8 mRNA and protein expression in cultured tongue SCC cells. Välimaa *et al.* (2004) demonstrated that ER- α is expressed in oral mucosa. The current study found that ER- α and - β are produced in carcinoma and inflammatory cells in tongue SCC samples. Törnwall *et al.* (1999) and Ulziibat *et al.* (2006) have showed that ER- β is expressed by purified and oviductal intraepithelial lymphocytes. Interestingly, in this study ER- β in inflammatory cells correlated weakly with better survival in tongue SCC. ERs have been used as a target for breast cancer treatments (Savinov *et al.* 2006). In a previous study, estrogen was found to stimulate MMP-26 gene expression through ERs (Li *et al.* 2004) but there was an inverse correlation between the amounts of MMP-26 and intact ER- β because MMP-26 was found to cleave ER- β . This estrogen signalling pathway is postulated to be associated with an antitumorigenic effect in breast carcinoma (Savinov *et al.* 2006). By *in vitro* studies, MMP-8 can cleave ER- α and - β . These findings partly explain the role of MMP-8 in estrogen and ERs signalling pathway during tumour development and may account for the protective role of MMP-8 in tongue squamous cell carcinoma, especially in females (Fig 3).

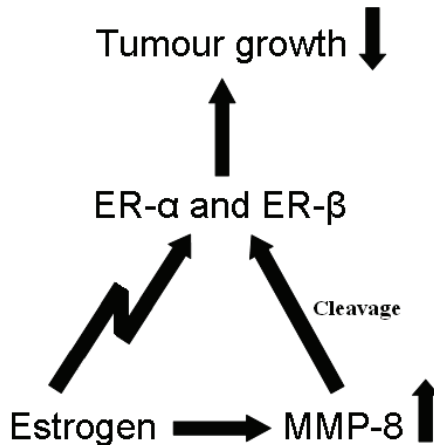


Fig. 3. Role of MMP-8 in estrogen and estrogen receptors (ERs) signalling pathway in tongue cancer. Estrogen induces MMP-8 expression whereas high production of MMP-8 can cleave ERs inhibiting the function of estrogen in tumour growth especially in females.

Using human osteosarcoma biopsy, resection, and lung metastases samples we investigated several previously proposed and possible new immunohistological prognostic markers, which included MMP-2, -8, -13, -26, and TIMP-1. MMP-2 was strongly expressed in most of the biopsy samples but after chemotherapy MMP-2 was present only in 50% of resection samples and staining intensity diminished. In spite of an effective chemotherapy influence of MMP-2, the present study was in line with Uchibori *et al.* (2006), where there was no correlation between MMP-2 expression and disease-free survival. In addition, MMP-13 and MMP-26 are expressed in osteosarcoma samples, but those factors were not related to survival. The current study demonstrated for the first time, that MMP-8 was expressed in osteosarcoma cells excluding lung metastases. However, there was no association between MMP-8 expression and disease-free survival. Due to the relative small number of patients these results should be interpreted with caution. Furthermore, a larger number of patients is needed to study the exact function of these markers in human osteosarcoma.

Individual MMPs have multiple roles in the various cancer types and stages and almost all MMPs are present in normal physiological homeostasis in the body. Thus, the use of MMP inhibitors or inducers to treat tumours is challenging. It is even more difficult when the primary tumour has entered circulation and has been disseminated to the secondary sites. However, inhibition or induction of

specific MMPs could be useful targets for therapeutic purposes in various states of cancer progression. In tongue SCC, MMP-8 seems to be an estrogen-regulated anti-tumourigenic factor which may further be used as a prognostic marker. In addition, it may be used as a target for the design of a specific drug which induces MMP-8 production in tongue cancer tissue and/or metastasis.

6.3 Future perspectives in wound repair and cancer studies using the MMP-8 knockout mice model

At least fourteen different MMP knockout mice and some double knockout mice have been generated (Page-McCaw *et al.* 2007). Human and mouse genes share a high homology offering valuable information about the function of the genes in biological systems and how a similar gene may cause or contribute to various diseases in humans. In addition, knockout mice offer the possibility to test or develop various drugs or other therapies in cancer and wound models. However, there may be some limitations to the use of knockout models, like p53. This gene is associated with more than half of human cancers in various tissues but p53 knockout mice develop tumours in different tissues than humans. Previous studies have demonstrated that p53 deletion is quite rare in humans and is more frequently targeted by a mutation (Zhang *et al.* 2002). However, the current study showed that use of MMP-8 knockout mice in a tongue cancer model resulted in results in line with the human tongue cancer study. Thus, the MMP-8 knockout model seems to be a reliable method to study cancer progression and also wound repair.

7 Summary and conclusions

The results have led to the following conclusions:

1. MMP-8 and -26 are distinctly expressed in acute and chronic wounds. MMP-8 is predominantly present in chronic wounds, whereas MMP-26 is temporarily present in acute wounds but is expressed in chronic wounds. In addition, MMP-26 participates in cell proliferation and adhesion, thus being an important regulatory factor in wound repair. Gutierrez-Fernandez *et al.* (2007) showed a delay in skin wound healing in MMP-8 deficiency mice. Thus, increased expression of MMP-8 in chronic wounds may benefit the healing process but the exact function needs to be studied further.
2. Estrogen-related MMP-8 seems to be a protective anti-tumourigenic factor in human and mice tongue SCC. In the future, MMP-8 may be used as a specific marker or target for a drug which induces its production.
3. MMP8^{-/-} does not affect newly processed alveolar bone formation in the tooth extraction socket or tooth periapical lesion size. However, MMP-8 deficiency changes collagen metabolism, neutrophil recruitment, the amount of MMP-9 and various cytokines in the tooth extraction socket. The current study showed that MMP-8 has no crucial role in mandibular alveolar bone formation or periapical lesion size but in the acute phase wounds, MMP-8 is important for regulating inflammation and collagen metabolism.
4. MMP-2, -8, -13, and -26, and TIMP-1 were found in human osteosarcoma samples. However, these markers seem not to be related to the disease-free survival of human osteosarcoma patients. This preliminary study gives a direction for further studies to evaluate the exact functions of MMP-2, -8, -13, -26, or TIMP-1 but a larger body of research material is needed to evaluate the prognostic power of these markers in human osteosarcoma.

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Original publications

- I Pirilä E, Korpi JT, Korkiamäki T, Jahkola T, Gutierrez-Fernandez A, Lopez-Otin C, Saarialho-Kere U, Salo T & Sorsa T (2007) Collagenase-2 (MMP-8) and matrilysin-2 (MMP-26) expression in human wounds of different etiologies. *Wound Repair Regen* 15: 47–57.
- II Korpi JT, Kervinen V, Mäklin H, Väänänen A, Lahtinen M, Läärä E, Ristimäki A, Thomas G, Ylipalosaari M, Åström P, Lopez-Otin C, Sorsa T, Kantola S, Pirilä E & Salo T (2008) Collagenase-2 (matrix metalloproteinase-8) plays a protective role in tongue cancer. *Br J Cancer* 98: 766–775.
- III Korpi JT, Åström P, Lehtonen N, Tjäderhane L, Kallio-Pulkkinen S, Siponen M, Sorsa T, Pirilä E & Salo T (2009) Healing of extraction sockets in collagenase-2 (matrix metalloproteinase-8)-deficient mice. *Eur J Oral Sci* 117: 248–254.
- IV Korpi JT, Hagström J, Lehtonen N, Parkkinen J, Sorsa T, Salo T & Laitinen M Expression of matrix metalloproteinases -2, -8, -13, -26, and tissue inhibitors of metalloproteinase-1 in human osteosarcoma. Brief report. Manuscript.

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