Trypsin – MMP – Claudin cascades in the modulation of tongue SCC microenvironment

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Academic Dissertation

To be presented, with the permission of Faculty of Medicine, University of Helsinki, for public examination in main auditorium of the Institute of Dentistry

On August 31st 2012 at 12 noon
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ISBN 978-952-10-8068-5 (PDF)  
ISSN 1457-8433  
Helsinki University Print  
Helsinki 2012
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*Publication IV has been included in the thesis of Ibrahim Bello.*

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM</td>
<td>a disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>APMA</td>
<td>4-aminophenylmercuric acetate</td>
</tr>
<tr>
<td>ASN</td>
<td>asparagine</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CRV</td>
<td>CRVYGPYLLC</td>
</tr>
<tr>
<td>CTT</td>
<td>CTTHWGFTLC</td>
</tr>
<tr>
<td>CTT2</td>
<td>GRENYHGCTTHWGFTLC</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>FHHN</td>
<td>familial hypomagnesemia with hypercalciuria and nephrocalcinosis</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HNSCC</td>
<td>head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HSC-3</td>
<td>human squamous cell carcinoma cell line</td>
</tr>
<tr>
<td>ICD</td>
<td>International Classification of Diseases</td>
</tr>
<tr>
<td>JAM</td>
<td>junctional adhesive molecule</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LCA</td>
<td>leucocyte common antigen</td>
</tr>
<tr>
<td>LEU</td>
<td>leucine</td>
</tr>
<tr>
<td>LYS</td>
<td>lysine</td>
</tr>
<tr>
<td>MAGI</td>
<td>membrane-associated guanylate kinase</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>membrane type metalloproteinase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MMPi</td>
<td>matrix metalloproteinase inhibitor</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MUPP1</td>
<td>multi-PDZ domain protein 1</td>
</tr>
<tr>
<td>NISCH</td>
<td>Neonatal ichthyosis–sclerosing cholangitis</td>
</tr>
<tr>
<td>OSCC</td>
<td>oral squamous cell carcinoma</td>
</tr>
<tr>
<td>PAR-2</td>
<td>proteinase activated receptor-2</td>
</tr>
<tr>
<td>Peptide G</td>
<td>GACFSIAHECGA</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PPC</td>
<td>CGYGRFSPPC</td>
</tr>
<tr>
<td>PRSS</td>
<td>protease serine</td>
</tr>
<tr>
<td>RECK</td>
<td>reversion inducing cysteine rich protein with Kazai motif</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
</tbody>
</table>
RT-PCR  reverse transcription polymerase chain reaction  
SEM  standard error of the mean  
SER  serine  
SCC  squamous cell carcinoma  
SD  standard deviation  
SDS  sodium dodecyl sulfate  
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis  
TAT  tumour-associated trypsin  
TATI  tumour-associated trypsin inhibitor  
TFPI-2  tissue factor pathway inhibitor  
TGF-β  transforming growth factor beta  
TIMP  tissue inhibitors of metalloproteinase  
TJ  tight junction  
TNF-α  tumour-necrosis factor-α  
TNM  tumour lymph nodes metastasis  
TRY2-HSC-2  trypsin-2 over-expressing human squamous cell carcinoma cell line  
UICC  Union of International Cancer Control  
VEGF  vascular endothelial growth factor  
WHO  World Health Organization  
ZO  zonula occludens  
ZONAB  zonula occludens–associated nucleic acid binding protein
ABSTRACT

Epithelial neoplasm is considered malignant when it has penetrated the basement membrane and invaded the surrounding tissues. In this process, carcinoma cells lose their cohesion, and carcinoma and stromal cells produce extracellular matrix and various proteinases that can cleave basement membrane. Proteinases enterokinase, tumor associated trypsin-2, and the matrix metalloproteinase-9 (MMP-9) activate cascade, seem to play important roles in the aggressive behavior of oral squamous cell carcinoma (OSCC). Trypsin-2 and MMP-9, the expression of which has been previously connected to malignancy, are revealed here, by confocal microscopy and immunohistochemistry, to co-localize in intracellular vesicles in OSCC; which seems to be specific to epithelial tumors, but not those of mesenchymal origin. Furthermore, trypsin-2 increases the invasion of OSCC cells in vitro and in vivo by processes including the changing of the expression pattern of tight junction proteins claudin-1 and -7 and activating MT1-MMP, which is known to play an important role in carcinogenesis.

When it became evident that MMPs played a role in cancer progression, the invention of their broad-spectrum inhibitors commenced. Unfortunately, in clinical trials these new drugs were disappointing, due to many side effects that are associated with the physiological functions of MMPs. Specific MMP inhibitors, however, represent new promising tools against cancer. Increased expression of gelatinase MMP-9 has been shown in many cancers. The antigelatinolytic CTT peptide was demonstrated here to inhibit the growth of primary OSCC xenograft tumors, but it did not have any effect on tumor cell spread in mice. The combination therapy of antigelatinolytic peptides and proMMP-9 targeting peptide vs. CTT alone did not provide any significant benefits for treatment. Nonetheless, since CTT is capable of targeting tumor vasculature, when fused with green fluorescent protein GFP it might become a useful tool in tumor imaging pre- and perioperatively.

During carcinogenesis epithelial cells lose their polarity, and changes in the expression of cell junction proteins are seen. These events are typical for
epithelial-mesenchymal transition where epithelial cells lose their typical traits and acquire mesenchymal characteristics. Expression analysis of tight junction proteins, claudin-1, -4, -5, -7, and occludin, relative to cause-specific survival of squamous cell carcinoma of tongue patients, was analyzed here. The staining intensity of claudin-7 at the tumor's invasive front was strongly associated with disease-specific patient survival. It led to the conclusion that immunoreactive levels of claudin-7 could be used as markers when predicting the outcome of these patients.
1. REVIEW OF THE LITERATURE

Cancer

Cancer, or malignant neoplasm, is a common term for diseases in which the body has lost control over group of cells. These cells grow uncontrolled, invade to the surrounding tissues, and send metastases via blood or lymph vessels. Cancer is one of the leading causes of death worldwide: in the year 2008 about 7.6 million people died from cancer which was 13% of all deaths (Boyle et al. 2008). In the same year, cancer was the cause of death of 11,041 people in Finland (Finnish Cancer Registry 2008).

In general the nomenclature of cancer follows the cell type from which the tumour arises. For example, cancers from mesenchymal origin are called sarcomas, whilst leukemias are from hematopoietic cells, and lymphomas from lymphoid tissue, malignant neoplasms from epithelial origins are called carcinomas (Kumar et al. 2007).

1.2 Carcinoma of the oral cavity

Oral cancer is the 8th most common cancer among males and the 14th among females in the world (Da Silva et al. 2011). In year 2008, worldwide incidence of oral cancer in both sexes was 263,020 and the mortality was 127,654 (International Agency for Research on Cancer 2010). The occurrence of oral cancer is particularly high among men and it is more common in developing than developed countries (Petersen 2009). Typically, oral cancer patients are males over 60 years of age and only 0.4%-6% of cases are among young adults under 45 years. However, the incidence of oral cancer in this group has been increasing in both sexes recently (Hooper et al. 2009).

Over 90% of new oral cancer cases are squamous cell carcinomas (SCC) (Hooper et al. 2009). Clinically, oral squamous cell carcinoma (OSCC) includes lip and intraoral cancer (Scully et al. 2009). This classification varies depending on the
source, however. Intraoral cancer includes tongue (Union of International Cancer Control (ICD) C01-02), gum (ICD C03), floor of mouth (ICD C04), other unspecified mouth area (ICD C05-06), and salivary gland (ICD C07-08) (Petersen 2009). The tumour, node, and metastasis (TNM) system of cancer classification is principally used when oral squamous cell carcinoma is clinically described (table 1). Despite the use of new cancer treatments, about 50% of patients die within 5 years of diagnosis (Walker et al. 2003). At the time of carcinoma diagnosis, 8.5% of patients with well-differentiated tumours, and 48.5% with poorly differentiated tumours will have cervical metastasis (Kademani 2007).

Risk factors

**Tobacco** (smoked and smokeless) is the main risk factor for OSCC because it contains many carcinogens like nitrosamines and polycyclic aromatic hydrocarbons. Epidemiological studies have identified almost 80% of the oral cancer patients as smokers (Burket et al. 2008). Excessive **alcohol** (ethanol) consumption is considered to be the second most important risk factor for oral cancer (Reichart 2001). Ethanol is oxidized by alcohol dehydrogenases to acetaldehyde, which is a known carcinogen (Secretan et al. 2009). Tobacco and alcohol act synergistically, thus smokers and heavy alcohol drinkers carry an even higher risk for OSCC (Scully et al. 2009). Other risk factors for oral cancer include chewing **betel** and **areca nut** (Scully et al. 2009), **poor oral hygiene**, **social class**, **diet**, **human papilloma viruses**, **Candida albicans**, **immunosupression** after organ transplantation and patients in with HIV or AIDS, **inflammation** (Hoopers et al. 2009, Reichart 2001, Scully et al. 2009, Walker 2003), and premalignant lesions like **erythroplakia** and **leukoplakia** (Villa et al. 2011, Neville et al. 2002).
Table 1

<table>
<thead>
<tr>
<th>TNM Classification of carcinomas of the oral cavity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary tumour</strong></td>
</tr>
<tr>
<td>TX  Primary tumour cannot not be assessed</td>
</tr>
<tr>
<td>T0  No evidence of primary tumour</td>
</tr>
<tr>
<td>Tis Carcinoma in situ</td>
</tr>
<tr>
<td>T1  Tumour dimension ≤ 2 cm</td>
</tr>
<tr>
<td>T2  Tumour dimension &gt;2 cm ≤ 4 cm</td>
</tr>
<tr>
<td>T3  Tumour &gt;4 cm in greatest dimension</td>
</tr>
<tr>
<td>T4  Tumour invades to the surrounding tissues such as bone, deep muscle of tongue, and skin of the face</td>
</tr>
<tr>
<td><strong>Regional lymph nodes</strong></td>
</tr>
<tr>
<td>N0  No regional lymph node metastases</td>
</tr>
<tr>
<td>N1  Metastases in a single ipsilateral lymph node, dimension ≤ 3 cm</td>
</tr>
<tr>
<td>N2  Metastases in a single ipsilateral lymph node, dimension &gt;3 cm but ≤ 6 cm or multiple ipsilateral or contralateral lymphnodes, dimension ≤ 6 m</td>
</tr>
<tr>
<td>N3  Metastases in a lymph node &gt; 6 cm in greatest dimension</td>
</tr>
<tr>
<td><strong>Distant metastases</strong></td>
</tr>
<tr>
<td>M0  No distant metastases</td>
</tr>
<tr>
<td>M1  Distant metastases</td>
</tr>
<tr>
<td>MX  Distant metastases cannot not be assessed</td>
</tr>
<tr>
<td><strong>Stage grouping</strong></td>
</tr>
<tr>
<td>Stage 0  TisN0M0</td>
</tr>
<tr>
<td>Stage I  T1N0M0</td>
</tr>
<tr>
<td>Stage II  T2N0M0</td>
</tr>
<tr>
<td>Stage III  T3N0M0</td>
</tr>
<tr>
<td>T1-3N1M0</td>
</tr>
<tr>
<td>Stage IV  T4N0-1M0</td>
</tr>
<tr>
<td>T1-4N2-3M0</td>
</tr>
</tbody>
</table>

Modified from table 2. Joensuu et al. 2007
1.2.1 Tongue carcinoma

Tongue is the most common site for intraoral cancer and 25-40% of all squamous cell carcinoma cases of oropharyngeal cancers occur on the tongue. Base and lateral borders of the tongue are the most common site (Moore et al. 2000, Gorsky et al. 2004). Prognosis of the disease depends on the regional spread of carcinoma and its lymph nodes involvement. Tongue carcinoma has been reported to have the highest risk for neck metastases among intraoral cancers (Shah et al. 1995, Bello et al. 2010). About 50% of cases have metastases in lymph nodes and occult cervical metastases (<2-3mm diameter) are common (Gorsky et al. 2004, Jin et al. 2008), which are the main reasons for its poor prognosis.

The worldwide incidence of tongue cancer is difficult to determine precisely because it is frequently grouped together with other intraoral cancers and reliable statistics are difficult to obtain from developing countries. There is great variance in incidence rates between different geographic areas. In Europe, the incidence varies among countries from 1 to 8/100,000 people (Moore et al. 2000). In the year 2009 in Finland there were 159 new cases (Finnish Cancer Registry 2009).

1.3 Osteosarcoma

Osteosarcoma is a malignant tumour of mesenchymal origin, which produces bone. If myeloma and lymphoma are excluded, osteosarcoma is the most common primary malignant bone tumour. Osteosarcomas represent about 20% of all bone cancers diagnosed. They can occur at all ages, although 75% of patients are under 20 years of age. About 6-8% of cases are seen in the jaw (Burket et al. 2008).
1.4 Giant cell granuloma

Giant cell granuloma is also known as giant cell lesion. It is divided into two groups

1) Peripheral giant cell granuloma, soft tissue tumour
2) Central giant cell granuloma, bone tumour

Central giant cell granuloma is a benign tumour of young people under 30 years of age; it is more often in the mandible than the maxilla (Burket et al. 2008).

1.5 Giant cell tumour

Giant cell tumour, also known as osteoclastoma, are rare, typically benign, but locally aggressive bone lesions that represent 5% of all adult bone tumours (Mendenhall et al. 2006). They are typically diagnosed in 20-40 year old patients (Kumar et al. 2007). Giant cell tumours have been, very rarely, diagnosed as malignant, either primary or secondary (after radiotherapy or surgery). Malignancies are high-grade sarcomas with very poor prognosis (Bertoni et al. 2003). Even though giant cell tumours are histologically benign, about half of them recur after treatment and about 4% send metastases (Kumar et al. 2007).

1.6 Carcinoma growth, invasion, and metastases formation

Carcinogenesis is a multi-step transition process from a normal epithelial cell via a premalignant lesion to malignant carcinoma. It was first thought that a single mutation in the genome would result in carcinoma progression, but nowadays it is known that several mutations are required to initiate carcinogenesis (Lopez-Lazaro 2010). Although many different theories have been suggested regarding the carcinogenic process, and our knowledge of the process has increased enormously during recent decades, many critical parts remain unresolved.

Vineis et al. (2010) suggested that carcinogenesis can be summarized into two types of models: “(i) biological changes in the epithelium alone lead to malignancy and (ii) changes in stroma/extracellular matrix are necessary (along with changes in epithelium)”. In a normal epithelium, the epithelial cells are
under strict control and mutations in DNA are repaired immediately. During carcinogenesis, this mechanism does not function correctly, which leads to accumulations of mutations in two classes of genes. Oncogenes are responsible for the initiation and progression of cancer. They, among others, take part in the regulation of cell growth, differentiation, and signal transduction. Another important class of genes is the tumour suppressor genes that normally protect cells from cancer formation but in carcinogenesis their function is abnormal or reduced (Field et al. 1995, Sugerman et al. 1995, Hanahan et al. 2000 and 2011, Vineis et al. 2010). After these accumulating changes, cancer cells develop autonomy and no longer receive cues from the body for controlled cell growth. They increase their proliferation rate, develop limitless replication potential, and are at the same time resistant to apoptosis (programmed cell death) (Hanahan et al 2000, 2011). Changes in cell metabolism do also occur, including increase glycolysis, de novo fatty acid synthesis, and nucleotide biosynthesis, to satisfy the altered needs of carcinoma cells (Fritz et al. 2010).

During carcinogenesis alterations also take place in the surrounding stroma (De Wever et al. 2003). Tumour cells are not isolated from their surrounding microenvironment and they crosstalk with the stromal cells. As a matter of fact, tumours consist of carcinoma cells and tumour-associated host cells, which may include about half of the cell population of tumour tissue. Tumour-associated host cells include: blood and lymphatic endothelial cells, inflammatory cells, and fibroblasts. It is not know when host-cells become associated with tumour cells but it is thought to happen prior to tumour invasion (De Wever et al. 2008, Mareel et al. 2009, Pietras et al. 2010). Host cells are able to promote, for instance, angiogenesis, invasion, inflammation, and extracellular matrix (ECM) modeling through cell-cell contacts, producing growth factors, hormones, cytokines, and proteinases like matrix metalloproteinases (MMPs) (De Wever et al. 2008, Pietras et al. 2010).

Solid tumours are able to grow only 1–2 mm³ without vascularization; angiogenesis, the formation of new blood vessels is required, to bring nutrition and oxygen to cells and evacuate metabolic wastes in order to support larger tumour growth. This process is initiated by vascular endothelial growth factor
(VEGF) and its receptors, whose expression in cancer cells and tumour-associated cells, like macrophages, are upregulated due to hypoxic conditions. The architecture of these new vessels differs from normal blood vessels and their permeability is increased which eases intravasation, a tumour cell invasion into a blood vessel (Hanahan et al. 2000, Homer et al. 2000, and Roodink et al. 2010).

During malignant transformation, carcinoma cells can change their morphology, reduce intercellular and cell-matrix adhesions, and increase motility. This event is called epithelial-mesenchymal transition (EMT), which is typically seen in invasive fronts of primary tumours. EMT is a fast and reversible change in the cellular phenotype. Carcinoma cells that have gone through EMT can invade and metastasise, including intravasation, circulation in blood or lymphatic vessels, extravasation (escape of cells from circulation), and finally metastasize. Loss of junction proteins like E-cadherin, claudins, and zonula occludens-1 and increased expression of mesenchymal markers, like α-smooth muscle actin and fibroblast-specific protein-1, are typical events for EMT (Medici et al. 2006, Yanjia et al. 2006, Kalluri et al. 2009).

Cells have different kinds of strategies for migrations, which are divided based on cell morphology and migration pattern (single cell, multicellular).

**Individual cell migration:**

In amoeboid migration the cell is rounded or ellipsoid, leukocyte-type and it does not have mature focal adhesion. This is subdivided in to blebbly migration, where the cell is rounded and migrates by pushing without adhesion to substrates and pseudopodal, where the cell is slightly more elongated and makes weak adhesive interactions with substrates. In mesenchymal migration, in contrast, the cell has focal cell-matrix interactions, for example with integrins, which are glycoproteins that form receptors for ECM molecules (Wolf et al. 2006, Rathinam et al. 2010). Cell movement is produced by cytoskeletal contraction and cells have fibroblast-like spindle-shape morphology. Protease activity is observed.
**Multicellular migration:**

In chain migration, cells move in a strand-like fashion one after another. Collective migration is used, for example, in epithelial building, remodeling, and local carcinoma invasion. Cells can migrate as clusters, cohorts, multicellular strands or sheets. They maintain cell-cell junctions during the movement. Cells are able to change their migration strategy during invasion. They can shift from low adhesive to high adhesive migration, from collective to single cell migration, and from proteolytic to non-proteolytic migration. Roughly, an individual cell that uses protease-dependent migration shows a mesenchymal phenotype, whereas, an amoeboid phenotype is seen in non-protease migration. The use of matrix metalloproteinase inhibitors (MMPI’s) changes cell migration fashion from proteolytic to non-proteolytic amoeboid migration. The migration type for squamous cell carcinoma cells is often collective, but during dedifferentiation it may produce a disseminating single cell (Friedl *et al.* 2003, 2009, 2010, Mareel *et al.* 2009, Wolf *et al.* 2006).

Metastases are the main reason for death in cancer patients (Duffy *et al.* 2008). The ability to metastasize was first considered to come in the later stages of carcinogenesis because the primary tumour size has traditionally predicted the patient outcome. But this concept has been proven wrong. Even small tumours might be able to send metastases. This explains why small primary tumours might not be curable after surgery (Bernards *et al.* 2002).

Once carcinoma cells have invaded the surrounding stroma, the next step is intravasation. Cells in the circulation have to be able to resist hemodynamic forces and immune-mediated killing. After survival in circulation, they extravasate at distant sites and start to grow in a new microenvironment. This is actually the rate-limiting step in metastasis. If there is not a metastatic niche for survival, carcinoma cells fail to metastasize (Chambers *et al.* 1997, Gupta *et al.* 2006, Psaila *et al.* 2009, Hanahan *et al.* 2011).
1.7 Enteropeptidase in cancer

Enteropeptidase, also known as enterokinase, is a membrane bound serine protease. It consists of a 82-140 kDa heavy chain, which anchors the enzyme in the cell membrane, and a 35-62 kDa light chain, which is a catalytic center (Kitamoto et al. 1995). Enteropeptidase is produced in the proximal small intestine in the brush border of enterocytes and goblet, where it activates pancreatic-fluid derived trypsinogen to trypsin. This activation cascade leads to the activation of many other pancreatic enzymes and is very important in food digestion (Lu et al. 1999, Imamura et al. 2003). The expression pattern of enteropeptidase is very narrow, concentrated in the duodenum and, its expression in cancers has only been shown in duodenal cancer and tumour-derived epithelial cell lines of the prostate, colon and airway (Imamura et al. 2003, Cottrell et al. 2004).

1.8 Trypsins in cancer

Trypsins belong to the serine protease family. Trypsinogen, a latent form of trypsin from pancreatic juice, was among the first purified human enzymes. Figarella et al. found two isoforms of trypsinogens and named them trypsinogen-1 and trypsinogen-2, which are also referred to as cationic and anionic trypsinogens (Figarella et al. 1969, Rinderknecht et al. 1984). These two trypsinogens consist of 19% of all proteins present in the pancreatic juice (Guy et al. 1978). A third pancreatic trypsinogen isoform was later found and named trypsinogen-3. It is present at much lower level in the pancreatic juice (Rinderknecht et al. 1984). Interestingly, a fourth trypsinogen isoform was also found in the brain (Wiegand et al. 1993).

Trypsinogens are encoded by protease serine (PRSS) genes. PRSS1 and PRSS2, also as known T4 and T8, encode trypsinogen-1 and trypsinogen-2, whilst PRSS3, or T9, encodes trypsinogen-3 and trypsinogen-4, which are splice variants of this gene (Rowen et al. 2005, Wiegand et al. 1993). The nomenclature of these enzymes in the literature is confusing because Sheele et al. (1981) named
trypsinogens according to their isoelectric point, which gives an irregular numerical order to (See table 2).

Trypsinogens are secreted as latent zymogens and are activated by enteropeptidase (Imamura et al. 2003) which cleaves the acidic propeptide from trypsinogen leading to active trypsin (Kitamoto et al. 1995). Trypsins can also be autoactivated (Colomb et al. 1978) or activated by lysosomal cysteine protease cathepsin B (Szmola et al. 2003), but the role of this activation in cancer progression is unknown.

Table. 2 Different names of trypsinogens

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Synonym</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRSS1</td>
<td>Trypsinogen-1</td>
<td>Cationic trypsinogen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>trypsinogen-3 (Sheele et al. 1981)</td>
</tr>
<tr>
<td></td>
<td>TAT-1</td>
<td>Tumour-associated trypsin-1</td>
</tr>
<tr>
<td>PRSS2</td>
<td>Trypsinogen-2</td>
<td>Anionic trypsinogen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>trypsinogen-1 (Sheele et al. 1981)</td>
</tr>
<tr>
<td></td>
<td>TAT-2</td>
<td>Tumour-associated trypsin-2</td>
</tr>
<tr>
<td>PRSS3</td>
<td>Trypsinogen-3</td>
<td>Mesotrypsinogen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>trypsinogen-2 (Sheele et al. 1981)</td>
</tr>
<tr>
<td>PRSS3</td>
<td>Trypsinogen-4</td>
<td>Brain trypsinogen (Wiegand et al. 1993)</td>
</tr>
</tbody>
</table>

Trypsinogens were initially found to only be produced by pancreatic acinar cells, but Itkonen et al. (1996) demonstrated that low concentration of trypsinogen-1 and 2 are found in the serum of patients after pancreatectomy. Nowadays, the extrapancreatic expression of trypsins has been observed at least in epithelial cells of the stomach, small intestine, skin, adult and preterm lung, esophagus, kidney, liver, bile ducts, splenic and neuronal cells (Koshikawa et al. 1992 and 1998, Prikk et al. 2001, Ceredqvist et al. 2003), male genital tract and seminal fluid (Paju et al. 2000), brain (Wiegand et al. 1993), vascular endothelial cells (Koshikawa et al. 1997), Paneth cells of the gastrointestinal mucosa (Ghosh et al. 2002), colonic mucosa (Cottrell et al. 2004), synovial cells and synovial fluids (Stenman et al. 2005), and cerebrospinal fluids (Critchley et al. 2000).
The first trypsin-like serine protease in tumours was characterized from the membrane of Walker 256 carcino-sarcoma cells (LaBombardi et al. 1983). When Stenman et al. attempted to find target a protease for the tumour-associated trypsin inhibitor (TATI), they found two new trypsinogens from cyst fluid of mucinous ovarian tumours (Stenman et al. 1988). Further study showed that the amino-terminal amino acid sequence corresponded to pancreatic trypsinogen-1 and -2. They had also the same molecular weights, but different substrate specificity, enzyme stability, isoelectric point and responded to various protease inhibitors (Koivunen et al. 1989). The two trypsinogens were named tumour associated trypsinogen-1 and -2 (TAT-1 and TAT-2). The amino acid sequence of pancreatic trypsin-2 is almost identical to tumour-associated trypsin-2. There is only one base substitution (G 276 to A) (Sorsa et al. 1997).

Tumour-associated trypsin expression has since been detected, at least, in pancreatic cancer (Ohta et al. 1994), hepatocellular and cholangiocarcinomas (Terada et al. 1995), lung neoplasm (Kawano et al. 1997), colorectal cancers (Yamamoto et al. 2003), non-small cell lung cancer (Diederichs et al. 2004), and esophageal SCC (Yamamoto et al. 2001). Many cancer cell lines also produce trypsins, including colorectal, erythroleukemia, fibrosarcoma (Koivunen et al. 1991 (A), Williams et al. 2001), gastric carcinoma (Koshikawa et al. 1992), ovarian carcinoma (Miyagi et al. 1995), and tongue carcinoma cell lines (Nyberg et al. 2002).

Malignancy is strongly associated with the expression of trypsin in cancer. In fact, the level or trypsin-2 correlates with the metastatic potential of ovarian cancers (Koivunen et al. 1990); a highly invasive tongue carcinoma cell line (HSC-3) produced trypsin-2, whereas a less invasive OSCC cell line (SCC-25) did not (Nyberg et al. 2002). In colorectal carcinoma, trypsin-1 expression correlated with the aggressiveness of the cancer, whereas trypsin-2 was the dominant isoform in colon carcinoma tissue (Williams et al. 2001). In human esophageal squamous cell carcinoma, the levels of trypsin are associated with recurrence and poor prognosis (Yamamoto et al. 2001). Unlike trypsin-1 and trypsin-2, trypsin-4 appears to play a tumour suppressive role in esophageal squamous cell carcinomas and gastric adenocarcinomas (Yamashita et al. 2003).
Trypsins contribute to cancer progression by several mechanisms. They are able to degrade many ECM components like fibronectin, laminin, gelatins and type I, III, IV, and, V collagens (Koivunen et al. 1991 (B), Koshikawa et al. 1992, Moilanen et al. 2003). This process eases cancer cell migration and liberates ECM bound growth factors that facilitate carcinogenesis. Proteolytic activity is a very important feature for cancer. In addition to being able to degrade ECM on their own, trypsin can activate other proteases like proMMPs (Nyberg et al. 2006). Trypsin-2 can activate proMMP-9 in vitro at a very low molar ratio 1: 1000, and less efficiently proMMP-2 (Sorsa et al. 1997). In vivo high levels of trypsin-2 in ovarian tumour cyst fluids correlate with increased levels of MMP-9 but not MMP-2 (Paju et al. 2001). Other proMMPs that are activated by trypsin-2 in vitro include MMP-1, -3, -8 and -13 (Moilanen et al. 2003). Trypsins can also modulate cancer cell growth and adhesion via cell surface receptor activation (Miyata et al. 1998, 2000). Extrapancreatic trypsin-2 can activate proteinase-activated receptor-2 (PAR-2), which is a G protein coupled receptor that is activated by proteolysis (Alm et al. 2000, Soh et al. 2010). In gastric carcinoma cells, trypsin activates PAR-2, which then regulates the adhesion of integrin -α5β1 and -αvβ3 to fibronectin and vitronectin. This binding to ECM proteins activates focal adhesion kinases, which then interacts with cytoskeletal and intracellular signaling molecules (Miyata et al. 2000). In intrahepatic cholangiocarcinoma and colon carcinoma cells trypsins enhance cell proliferation by PAR-2 activation (Darmoul et al. 2004, Nakanuma et al. 2010).

1.9 Matrix metalloproteinases (MMPs)

MMPs are zinc-dependent endopeptidases that very efficiently degrade extracellular matrix and basement membrane components. In the year 1962, the first MMP, MMP-1, was found from the tail of a tadpole (Gross et al. 1962). To date, at least 24 distinct MMP genes have been found in humans, but only 23 different proteins have been discovered, because two identical forms of MMP-23 are coded by two different genes: mmp-23a and mmp-23b (Fanjul-Fernandez et al. 2010).
MMPs are the main processors of ECM and as such they participate tissue remodelling and repair processes in healthy tissues including such as ovulation, bone resorption, wound healing, angiogenesis, and embryogenesis. They also have important roles in various pathological tissue-destructive inflammatory and malignant conditions such as cancer, arthritis, periodontitis, autoimmune and cardiovascular diseases. Historically MMPs have been divided according to their substrate specificities to collagensases, gelatinases, stromelysins, and matrilysins. The list of substrates has grown and MMPs are now classified according to their structure (Figure 1. and Table 3).

The structure of all MMPs includes an N-terminal signal peptide, or pre domain that directs the protein to the secretory or plasma membrane insertion pathways, a prodomain that confers latency to MMPs, and a catalytic domain with the active site zinc atom. All MMPs except, MMP-7, -23, and -26, also include a C-terminal hemopexin (HPX)/vitronectin-like domain that is linked to the catalytic domain through a hinge region. It works primarily as a recognition sequence for the substrate (Hadler-Olsen et al. 2011). Instead, MMP-23 has unique cysteine-rich, proline-rich, and IL-1 type II receptor-like domains (Sternlicht et al. 2001, Pei et al. 2000).

MMPs are no longer considered to be simply ECM degrading enzymes. During ECM cleavage they can release proteins from cryptic sites. For example, MMP-2 cleaves laminin-5 γ2 subunit, which exposes a cryptic promigratory site that enhances cell migration (Gianelli et al. 1997). MMP-3, -7, -9, -13, and -20 can cleave type XVIII collagen. This leads to the release of endostatin, which is a potent inhibitor among others for angiogenesis and cell migration (Ferreras et al. 2000, Heljasvaara et al. 2005). MMPs can also release cytokines, chemokines, and growth factors from their membrane-anchored proforms (Nagase et al. 2006, Butler et al. 2009, Klein et al. 2011).
Figure 1. The protein structure of different MMPs. The following abbreviations are used in the Figure: Pre= signal sequence that directs MMPs into endoplasmic reticulum. Pro=prodomain with a zinc-interacting thiol (SH) group, Zn= zinc binding site, H= hinge region that connects catalytic domain to hemopexin domain, which mediates interactions with substrates. S-S= disulphide bond, Fi=collagen-binding fibronectin type II inserts, Fu= recognition motif for furin activation, Vn= vitronectin-like insert, TM= transmembrane domain, Cy= cytoplasmic tail, GPI= glycosylphosphatidylinositol domain, SA= N-terminal signal anchor, CA= cysteine array and Ig-like=immunoglobulin (Ig)-like domain. Modified from Egeblad et al. 2002 and Sternlicht et al. 2001.
Table 3. Nomenclature of MMPs

<table>
<thead>
<tr>
<th>Matrix metalloproteinase</th>
<th>Alternative names</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Collagenase-1, interstitial collagenase, fibroblast collagenase, tissue collagenase</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Gelatinase A, 72-kDa gelatinase, 72-kDa type IV collagenase, neutrophil gelatinase</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Stromelysin-1, transin-1, proteoglycanase, procollagenase-activating protein</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Matrilysin, matrin, PUMP1, small uterine metalloproteinase</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Collagenase-2, neutrophil collagenase, polymorphonuclear (PMN) collagenase, granulocyte collagenase</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Gelatinase B, 92-kDa gelatinase, 92-kDa type IV collagenase</td>
</tr>
<tr>
<td>MMP-10</td>
<td>Stromelysin-2, transin-2</td>
</tr>
<tr>
<td>MMP-11</td>
<td>Stromelysin-3</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Metalloelastase, macrophage elastase, macrophage metalloelastase</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Collagenase-3</td>
</tr>
<tr>
<td>MMP-14</td>
<td>MT1-MMP, MT-MMP1</td>
</tr>
<tr>
<td>MMP-15</td>
<td>MT2-MMP, MT-MMP2</td>
</tr>
<tr>
<td>MMP-16</td>
<td>MT3-MMP, MT-MMP3</td>
</tr>
<tr>
<td>MMP-17</td>
<td>MT4-MMP, MT-MMP4</td>
</tr>
<tr>
<td>MMP-18</td>
<td>Collagenase-4 (Xenopus; no human homologue known)</td>
</tr>
<tr>
<td>MMP-19</td>
<td>Rheumatoid arthritis synovium inflamed-1 (RAS-1) MMP-18</td>
</tr>
<tr>
<td>MMP-20</td>
<td>Enamelysin</td>
</tr>
<tr>
<td>MMP-21</td>
<td>Homologue of Xenopus XMMP</td>
</tr>
<tr>
<td>MMP-22</td>
<td>CMMP (chicken; no human homologue known)</td>
</tr>
<tr>
<td>MMP-23</td>
<td>Cysteine array MMP (CA-MMP), femalysin, Metaloprotease in the female reproductive trac (MIFR), MMP-21/MMP-22</td>
</tr>
<tr>
<td>MMP-24</td>
<td>MT5-MMP, MT-MMP5</td>
</tr>
<tr>
<td>MMP-25</td>
<td>MT6-MMP, MT-MMP6, leukolysin</td>
</tr>
<tr>
<td>MMP-26</td>
<td>Endometase, matrilysin-2</td>
</tr>
<tr>
<td>MMP-27</td>
<td>No name</td>
</tr>
<tr>
<td>MMP-28</td>
<td>Epilysin</td>
</tr>
</tbody>
</table>

Modified from Egeblad et al. 2002 and Klein et al. 2011
1.9.1 Regulation of MMPs

The quantitative levels and qualitative patterns of MMP expression vary between tissues, cell types, and also depend on diseases such as tumours and inflammatory conditions. MMPs are regulated at least at four different levels:

1) *Gene expression*: MMP gene expression is mainly regulated at the transcriptional level. It has been suggested that the expression of certain transcription factors is restricted in different cell types, which would explain why different MMPs are expressed in specific cell types or tissues, even though their promoters have very similar structures. Post-transcriptional mechanisms participate in the control of MMP expression like microRNA-222, which post-transcriptionally regulates the expression of MMP-1 (Sternlicht et al. 2001, Yan et al. 2007, Liu et al. 2009). Overall, MMP gene expression is regulated by various stimulatory and suppressive factors that interact with several signaling pathways.

2) *Compartmentalization*: Compartmentalization is the formation of cellular compartments. In this context, it means accumulation of enzymes like MMPs at different sites. This locating and concentrating of MMPs close to potential substrates, efficiently regulates MMP activity. In these compartments, MMPs bind to proteins like collagens, laminins, and fibronectin, and with differing binding interactions. MMPs can be localized, for example, in the nucleus, where they are associated with apoptosis (Aldonyte et al. 2009) or in exocytic vesicles. Mast cells, polymorphonuclear leukocytes, endothelial cells, and many cancer cells can store MMPs in these vesicles and release them to the extracellular environment (Handler-Olsen et al. 2011).

3) *Zymogen activation*: MMPs are mainly secreted in latent proforms, requiring activation either in extracellular milieu or on the cell surface to be catalytically active. MMP-11, -27, and MT-MMPs can be activated intracellularly by subtilisin-like serine proteinases because of their molecular structure (Sternlicht et al. 2001). ProMMPs are activated by the so-called cystein-switch model, where the pro-domain is removed from the catalytic site (Van Wart et al. 1990) (Figure 2). This can take place by different mechanisms, the most important of which is
direct cleavage of the pro-domain by other proteinases. Different proteolytic mechanisms include: 1) intracellular, 2) extracellular, and 3) cell surface-mediated mechanisms. The activation of most MMPs can be initiated by several serine proteinases or MMPs that are already activated. Furin, a subtilisin-like serine proteinase is located in the Golgi apparatus, where it activates intracellular MMPs that have a target sequence for furin, like all membrane bound MMPs (Thomas 2002, Ra et al. 2007). In an in vitro study, MMP-3 was activated extracellularly by plasma kallikrein (Nagase et al. 1990). Many other serine proteinases can also activate MMPs at least in vitro, such as plasmin, chymotrypsin, trypsin, and tumour-associated trypsin-2 (Nagase et al. 1990, Sorsa et al. 1997, Moilanen et al. 2003, Will et al. 1996). It should also be noted that the activated MMPs are able to activate proMMPs (Knäuper et al. 1996, Holopainen et al. 2003).

**Figure 2.** ProMMP activation. Pro=prodomain, Zn= zinc\(^{2+}\) ion at catalytic site, SH= free thiol of a conserved cysteine, ROS= reactive oxygen species, APMA=4-aminophenylmercuric acetate, MMPs=matrix metalloproteinases. Modified from Ra et al. 2007.
Activation of MMP-2 by MT1-MMP and tissue inhibitor of metalloproteinase 2 (TIMP-2) has been well characterized. MMP-2 is secreted as an inactive zymogen that is activated by MT1-MMP in a special mechanism where (TIMP-2) mediates proMMP-2 activation (Lehti et al. 2002). TIMP-2 binds to the active form of MT1-MMP in the plasma membrane. These two molecules work as a receptor for proMMP-2 and then TIMP-free MT1-MMP cleaves the Asn$^{37}$-Leu$^{38}$ peptide bond of proMMP-2 that causes an activated intermediate form. This is further processed to the fully active form by autoactivation (Zucker et al. 1998, Strongin et al. 1995). MMP activation can also happen by chemical agents like 4-aminophenylmercuric acetate (APMA), HgCl$_2$, N-ethylmaleimide, oxidized glutathione, sodium dodecyl sulfate (SDS), denaturating agents, heavy metal ions, disulfides, and reactive oxygen species (ROS). Heat treatment and low pH can also cause activation (Visse et al. 2003, Ra et al. 2007).

It is though that leukocytes and other cells can activate MMPs by producing oxidants but this is only on the grounds of in vitro studies. Fu et al. proposed that hypochlorous acid produced by neutrophil myeloperoxidase (MPO) can activate MMP-7 in vitro supporting a role for MMPs in inflammation (Fu et al. 2001). In vivo studies of ischemic mouse brains have demonstrated that proMMP-9 is activated by S-nitrosylation, which means that nitric oxide modulates the biological activity of MMP-9 by reacting with cysteine thiol to form an S-nitrosylated derivative (Gu et al. 2002).

Zymogens can also be activated by allostERIC activation. This is possible because the zymogen prodomain does not need to be removed to gain activity; only disruption of the zinc-thiol is needed. This can occur by post-translational modifications or interactions with non-substrate macromolecules as mentioned above. These cause conformational changes favoring conversion to an activated state. The cysteine-Zn$^{2+}$ allostERIC disruption leads to a transitional active state, enabling autolytic cleavage of the prodomain (Ra et al. 2007, Handler-Olsen et al. 2011). The proteolytic activation of MMPs is often stepwise. After removing the susceptible bait region in the middle of the propeptide, the rest of the propeptide
destabilizes, which allows intermolecular processing by partially activated MMP intermediates or other active MMPs (Nagase et al. 1997).

4) **Enzyme inactivation**: MMP activity in tissues is endogenously inhibited by general protease inhibitors like α-2-macroglobulin, which is a large serum protein that carries a bait region for enzymes. Cleavage of this region leads to conformational changes that trap the MMP in a cage-like structure (Nagase et al. 1994). Other known inhibitors include a membrane-bound MMP inhibitor called RECK (reversion inducing cyteine rich protein with Kazai motifs) (Oh et al. 2001), tissue factor pathway inhibitor-2 (TFPI-2) (Herman et al. 2001), and C-terminal fragment of the procollagen C-terminal proteinase (Mott et al. 2000).

Specific endogenous inhibitors for MMPs are tissue inhibitors of metalloproteinases (TIMPs) that bind to MMPs in a 1:1 stoichiometry. Four different TIMPs have been identified TIMP-1, TIMP-2, TIMP-3, and TIMP-4. Different TIMPs inhibit most MMPs, but those that have a deletion of the hemopexin domain, or if the domain is lacking naturally, bind less tightly to TIMPs than the full-length MMPs. There are always exceptions, such as MMP-14 on the cell surface, which is poorly inhibited by TIMP-1. TIMPs often block the autoactivation step of stepwise MMP activation, after the propeptide has been partially removed by proteinase. In this way, they inhibit MMP activation. TIMPs do not act as MMP inhibitors only, because TIMP-2 for example, takes part in MMP-2 activation (Woessner et al. 1999, Brew et al. 2000, Visse et al. 2003).

### 1.10. MMPs in carcinogenesis

Basement membrane (BM) is a thin, dense, sheet-like structure of specialized ECM beneath epithelial and endothelial cells. It consists of about 50 proteins and its main components are collagen IV and laminin (Kalluri 2003, Rowe et al. 2008). MMPs have long been associated with carcinoma invasion and metastasis formation because of their ability to degrade almost all ECM components, and in this way they facilitate the breakdown of physical barriers. This enables local invasion, intravasation, extravasation, and finally metastasis formation. However, MMPs have even more complex roles in carcinoma progression (Chambers et al.

1.10.1 Gelatinases MMP-2 and -9 in oral carcinoma

Two different soluble gelatinases have been found, MMP-2 and MMP-9. They are secreted as inactive zymogens and are activated later by other proteinases. Gelatinases can cleave denatured collagen, gelatin, for which they are named. MMP-2 and -9 have an important role in cancer progression because of their ability to degrade type IV collagen, one of the main components of basement membranes (Stetler-Stevenson et al. 1993). This is not the only mechanism by which they promote carcinogenesis. For example, MMP-2 cleaves laminin-5, a component of ECM. This leads to the exposure of a cryptic site, which leads to increased invasion (Giannelli et al. 1997). Lee et al. (2008) however, speculated that high concentrations of MMP-2 and MMP-9 could reduce natural killer cell-mediated cytotoxicity against an OSCC cell line, which might be one mechanism by which cancer cells escape the immune system.

Increased levels of MMP-2 and MMP-9 correlate with the aggressive nature of OSCC (Kurahara et al. 1999, Yoshizaki et al. 2001, Zhou et al. 2010). High expression of MMP-9 in patients OSCCs correlated with regional lymph node and/or distant metastasis and poor prognosis (Katayama et al. 2004), whereas high expression and gelatinolytic activity of MMP-2 and-9 in OSCC tumours was related to the invasiveness but not metastatic potential (Ikebe et al. 1999). Even though MMP-9 is mainly associated with the aggressive behavior of OSCCs, new studies have revealed that it might also have protective role (Luukkaa et al. 2010, Stokes et al. 2010).
MMPs that take part in cancer invasion are not always produced in carcinoma cells. MMP-2, in particular, is frequently expressed in stromal cells but carcinoma cells are able to utilize it (Thomas et al. 1999). ProMMP-2 is secreted from stromal cells and activated by MT1-MMP on the cell surface of HNSCC cells (Tokumaru et al. 2000). This same event is common in many other cancers. In invasive OSCC, MMP-2 mRNA was found from fibroblasts surrounding the tumour cells and lymph node metastases. Positive MMP-2 immunostaining was seen in the peripheral cell layer in neoplastic islands and in some fibroblast-like cells of tumoural stroma (Sutinen et al. 1998). Furthermore, the expression of MMP-9 has been found in OSCC cells and chronic inflammatory cells surrounding tumour islands (Impola et al. 2004).

1.10.2 MT1-MMP in oral carcinoma

MT1-MMP is a membrane bound MMP that has important roles in carcinogenesis. It is able to degrade ECM components like type I, II, and III collagens, laminins 1 and 5, fibronectin, and vitronectin (Itoh et al. 2006, Visse et al. 2003) but besides this it can enhance ECM degradation by activation of MMP-2 and MMP-13 (Knäuper et al. 1996). Sabeh et al. (2004) demonstrated that MT1-MMP is the major cell associated proteinase with collagenolytic activity during the invasion of cancer cells through the ECM.

Expression of MT1-MMP has been linked to the aggressive behavior of many cancers, including OSCC, and MT1-MMP is mainly secreted from OSCC cells (Myoung et al. 2002). Its expression correlates with invasiveness and lymph node metastasis (Polette et al. 1998, Myoung et al. 2002). De Vicente et al. (2007) also showed that OSCC tumour recurrence correlated with expression of MT1-MMP.

1.11 Synthetic peptide matrix metalloproteinase inhibitors (MMPI)

After MMPs significant role in cancer invasion and metastasis formation was proven, development of their synthetic inhibitors was commenced. In this field,
researchers did not take into account that MMPs take part into many other biological functions and ECM degradation in cancer progression is only one of them. In addition, some MMPs might even have protective roles in cancer (Korpi et al. 2008, Folgueras et al. 2004).

The first generation of peptidomimetic MMP inhibitors was low-molecular-weight-broad-spectrum inhibitors, which bound reversibly to the active site of MMP and in this way inhibited MMP activity. They were batimastat (BB94) and ilomastat (GM-6001), which mimic the cleavage sites of MMP substrates. Hydroxamic acid (-CONHOH) is their zinc-binding group, which then chelates the active site zinc ion (Brown et al. 1998, Overall et al. 2006). Batimastat was the first to be tested in human in phase I studies, where it was given intraperitoneally due to its poor solubility. Batimastat was locally quite toxic and no significant clinical responses were seen (Wojtowicz-Praga et al. 1996).

Marimastat (BB-2516) belongs to the second generation of MMP inhibitors. It has similar structure to batimastat, but good oral bioavailability. Marimastat showed very promising results in preclinical studies and was brought into phase I-III clinical trials, but results of the phase III trials have been disappointing (Wojtowicz-Praga et al. 1997, Zucker et al. 2000, Coussen et al. 2002). In a randomized phase III trial with metastatic breast cancer patients, marimastat did not have any effect on overall survival. In addition, many patients treated with marimastat suffered musculoskeletal toxicity (musculoskeletal syndrome), which includes joint and tendon pains, which can restrict the movements of limbs (Sparano et al. 2004). These symptoms are reversible and their severity seems to be dose-dependent (Brown et al. 1998).

One reason for failure of the broad-spectrum MMPI might be their ability to inhibit other zinc metalloproteinases, such as tumour necrosis factor-α-converting enzyme, a protease of the ADAMs (a disintegrin and metalloprotease) family (Yiotakis et al. 2008). This led to the development of more selective MMPIs. The first gelatinase specific MMPI was produced with the help of phage display peptide libraries. This hydrophobic peptide CTTHWGFTLC (CTT) inhibited the in vitro invasion of ovarian carcinoma, fibrosarcoma, Kaposi’s
sarcoma, breast carcinoma, and melanoma cell lines and also increased the survival of the human tumour xenografts (Koivunen et al. 1999). After that, the hydrophilic gelatinase specific MMP inhibitor GRENYHGCTTHWGFTLC (CTT2) peptide was developed. It inhibited MMP-9 activity and activation, and also inhibited in vivo and in vitro human tongue cancer cell invasion and intravasation (Heikkilä et al. 2006). The proMMP-9 specific peptide inhibitor named CRVYGPYLLC-peptide (CRV), which has only weak inhibitory effect on proMMP-2, and a weaker gelatinase inhibitor ADGACGYGRFSPCGAG-peptide (PPC), which inhibits both MMP-2 and MMP-9 activity, were also developed. CRV inhibited in vitro tumour cell migration and in vivo tumour xenograft growth (Björklund et al. 2004).

Novel MT1-MMP inhibitor GACFSIAHECGA-peptide or peptide G was discovered by Suojanen et al. 2009. It was able to inhibit MT1-MMP activity and carcinoma cell invasion in vitro. The growth of human tongue squamous cell carcinoma cell lines in vivo tumour xenografts was slower than control groups, which prolonged the survival of these peptide G treated mice (Suojanen et al. 2009).

It has been speculated that selective MMPs would be safer than the broad-spectrum ones. However, various MMPs are co-expressed in different types of cancers, which makes it very difficult to know which single MMP is the most critical to targeted.

1.12 Cell-cell junctions in carcinoma progression

Epithelial cells form highly organized tissues named epithelium, which is specialized to protect, secrete, absorb and transport materials. Epithelium lines both the outside and the inside cavities of the body. Stability of epithelium is maintained by cell junctions that are structures formed between two adjacent cells. They have been named tight junctions, adherens junctions, desmosomes and gap junctions (Giepmans et al. 2009). During carcinoma progression the rigidity of these junctions is changed. Most human cancers are of epithelial origin. One important moment of carcinoma progression is the loss of cell-cell contacts, which enables cancer cell invasion and finally metastasis formation. This process
also changes cell polarity and is a characteristic feature of EMT (Chidgey et al. 2007, Pinho et al. 2010, Singh et al. 2010).

1.13 Tight junctions

Tight junctions (TJ) or zonula occludens regulate cell-cell adhesions in epithelial and endothelial cell sheets. They are part of the epithelial junctional complex that also includes adherens junctions and desmosomes (Steed et al. 2010, Stukita et al. 2001) (Figure 3). TJs are the most apical of the junctional complex. They form spot-like intercellular contacts or "kissing points", where external leaflets of the lateral plasma membranes of adjacent cells appear to fuse (Gonzales-Mariscal et al. 2007) (Figure 3). At these points, the intercellular space is entirely obliterated, whereas in adherent junctions and desmosomes, opposite membranes are 15-20 nm apart (Stukita et al. 2001). TJs form semi-permeable barrier, which regulates movements of ions, solutions, and cells through the paracellular space (Steed et al. 2010). They separate the cells of apical and basolateral compartments, which have different lipids and integral membrane proteins, and in this way also maintain cellular polarity (Schneeberger et al. 1992).

In many physiological situations, it is necessary that different materials are selectively transported trough cell sheets. This can happen either by transcellular transport, which includes lipophilic diffusion and specific transport mechanisms for molecules like pumps, channels, and co-transporters, or paracellular transport which is size- and charge-selective and regulated by TJs (Berg et al. 2002, Gonzalez et al. 2007). TJs make strands that encircle the cells. The amount of strands differs among cell types and the stiffness of TJs is tissue dependent (Schneeberger et al. 1992). In the extracellular part of the TJ strands, there are aqueous pores that can be open or closed. They allow ion-selective diffusion along concentration gradients (Tsukita et al 2001, Tang et al. 2003). TJs are not as rigid structures as it was originally thought. They can be remodeled, for example, by caveolae and clathrin-mediated endocytosis (Findley et al. 2009, Steed et al. 2010).
Figure 3. Junctional complex and tight junctions. Junctional complex is encircled, TJ= tight junction, AJ= adherens junction, D= desmosome, kissing point= tight-junction strands on plasma membrane in the adjacent cells that form a paired tight-junction strand. Two headed arrows show transport routes through epithelial cell layer. Modified from Tsukita et al. 2001 and Gonzalez-Mariscal et al. 2007.

TJs consist of two types of proteins. Integral proteins (like occludin, claudins, tricellulin, junctional adhesion molecules (JAMs), a human homologue of Drosophila Crumbs and blood vessel/epicardial substance) are adhesion proteins that control the correct structure and function of TJs. Peripheral or plaque anchoring proteins (such as zonula occludens-1, -2 and -3 (ZO-1, ZO-2, ZO-3), membrane-associated guanylate kinase-1, -2 and -3 (MAGI-1, MAGI-2, MAGI-3) and multi-PDZ domain protein 1 (MUPP1)) are considered to be scaffold
proteins of the TJs, which also mediate protein-protein associations, cell signaling, and links to the actin cytoskeleton. Besides these, there are also many TJ-associated proteins (like cingulin, α–catenin, and ZO-1 associated nucleic acid-binding protein (ZONAB)) mediating cell proliferation, growth and differentiation (Gonzales-Mariscal et al. 2007, Martin et al. 2009, Balda et al. 2009).

1.13.1 Claudins and occludin

Claudins are a tight junction protein family that include 24 CLDN genes. Only 23 are found in humans because CLDN 13 is lacking. Their molecular weight varies from 20 to 34 kDa (Lal-Nag et al. 2009). Claudin originates from the Latin word “claudere” to close. They are integral proteins that are thought to form the backbone of tight junctions. Claudins consist of four transmembrane domains, two extracellular loops, and a carboxyl intracellular tail that contains a PDZ-binding motif (Figure 4). Through this motif, claudins are able to interact directly with the TJ’s PDZ containing proteins like ZO-1, ZO-2, and ZO-3 (Itoh et al. 1999 (A), Singh et al. 2010). The first and longer extracellular loop of claudins is thought to effect the paracellular ion selectivity (Colegio et al. 2002) and the second shorter one connects claudins to the claudins of the opposite cell membrane and functions as a receptor for a bacterial toxin: Clostridium perfringens enterotoxin (Fujita et al. 2000, Piontek et al. 2008).
Claudins are regulated at many different levels. Post-translational modification of claudins includes phosphorylation that leads to the decreased, or increased, paracellular permeability (Van Itallie et al. 2006) and palmitoylation that causes alterations in claudin localization (Van Itallie et al. 2005). In transcriptional regulation, the transcription factor Snail binds directly to the E-boxes of promoters of the claudin genes that lead to the complete repression of their promoter activity. A similar function for Snail has been described in EMT for E-cadherin (Martinez-Estrada et al. 2006). Claudin expression can also be regulated by several growth factors, such as epidermal growth factor (EGF) and transformin growth factor β (TGF-β), and inflammation associated cytokines like tumour-necrosis factor-α (TNF-α), interferon-γ, and interleukin-13, which downregulates claudins and causes paracellular permeability (Oliveira et al. 2007, Capaldo et al. 2009, Schulzke et al. 2009). Claudin expression is tissue specific and usually multiple claudins are expressed in the same tissue. They interact with nearby claudins in the same cell and in neighboring cells to form
homo- and hetero-dimers which create the tight junction strands (Van Itallie et al. 2006).

Mutations in claudin genes have been shown to cause diseases in human. In neonatal sclerosing cholangitis associated with ichthyosis (NISCH syndrome) the mutation in CLDN1 gene leads to progressive scaling of the skin and obstruction of bile ducts (Hadj-Rabia et al. 2004) whereas CLDN14 gene mutation results autosomal recessive deafness DFNB29 (Wilcox et al. 2001). Mutation of CLDN16 causes a rare Mg2+-wasting disease called familial hypomagnesaemia with hypercalciuria and nephrocalcinosis (FHHN) (Simon et al. 1999).

Occludin was the first identified transmembrane protein of TJ. Its molecular weight is 65 kDa (Furuse et al. 1993). Occludin consists of four transmembrane domains, two extracellular loops and it has a long carboxy-terminal and short amino-terminal cytoplasmic domain (Figure 5). Transmembrane domains and external loops are important for the paracellular permeability of TJs. Occludin might also cooperate with claudin-4, and probably other claudins, and in this way mediate selective paracellular permeability (Balda et al. 2000). Occludin has interactions with TJ proteins and TJ-associated proteins like ZO-1 and ZO-2 (Itoh et al. 1999, B).

Phosphorylation seems to be the most important mechanism of occludin regulation but there are other ways, like small GTPases that are GTP-binding proteins, proteases, cytokines, and transcription factor snail, which suppress the gene expression of occludin. Regulation of occludin affects mainly the TJ assembly and permeability (Feldman et al. 2005). Saitou et al. (1998) has shown the that expression level of occludin in epithelial cells correlates with the number of TJ strands, but a TJ strand can also be formed without it. Occludin expression has been found in many normal and abnormal tissues, and cell lines, and its expression is related to several diseases (Feldman et al. 2005, Förster 2008).
Figure 5. Occludin structure. TM= transmembrane domain, COOH= COOH terminus, (254 amino acids), NH₂=N-terminus (149 aminino acids) modified form Feldman et al 2005.

Several studies have shown that expression of TJ protein claudins are changed during tumourgenesis (Singh et al. 2010, Gonzales-Mariscal et al. 2007, Oliveira et al. 2007, Ouban et al. 2010). Their expression is downregulated in many cancers, which leads to the disruption of TJs and lost cohesion. Paradoxically, this is not always the case because researchers have observed that, in several carcinomas, claudin expression is in fact increased. The mechanism behind increased claudin expression and cancer progression is unclear, but it is believed that TJs are unable to work properly because of dislocation of claudins in the TJs. This leads to increased paracellular permeability, which allows free flow of nutrient and growth factors for the tumour. Changes in cell polarity, proliferation, and differentiation are also detected as well as mislocalization of claudins to the nucleus and cytoplasm (Matter et al. 2005, Dhawan et al. 2005). Even regulation of MMPs by claudins in carcinogenesis has been proposed (Oku et al. 2006).
1.13.2 Claudin-7 in cancer

Claudin-7 is a TJ protein which has a molecular weight of 22 kDa (Lal-Nag et al. 2009). It is downregulated in head and neck squamous cell carcinomas (HNSCC) compared to normal cells (Al moustafa et al. 2002). In prostatic adenocarcinomas, decreased claudin-7 expression is correlated with high tumour grade (Sheehan et al. 2007), whereas decreased expression of claudin-7 in squamous cell carcinoma of the oesophagus correlates with invasion and metastasis (Usami et al. 2006). The level of claudin-7 mRNA is already decreased as an early event in colorectal carcinogenesis (Bornholdt et al. 2011) and downregulation of claudin-7 in OSCC is associated with poor prognosis, advanced stages, and recurrence of disease (Lourenco et al. 2010 A and B). In oesophageal squamous cell carcinoma cells, claudin-7 was mislocalized during carcinogenesis, which resulted in increased invasion (Lioni et al. 2007). Claudin-7 is significantly overexpressed in human epithelial ovarian cancer (Tassi et al. 2008). Besides these examples, claudin-7 is also up- and downregulated in many other cancers (Singh et al. 2010, Oliveira et al. 2006, Martin et al. 2009 Ouban et al. 2010).
2. AIMS OF THE STUDY

At the time of this study’s was commencement, trypsin-2 was found in highly invasive tongue squamous cell carcinoma cells, whereas less invasive cell line did not express it (Nyberg et al. 2002). The mechanism behind trypsin-2 enhanced invasion was unclear. It was only known that trypsin-2 can activate MMP-9 and some other MMPs (Sorsa et al. 1997, Moilanen et al. 2003). The aim of this work was to analyze the expression and localization of trypsin-2 and MMP-9 in OSCC and mesenchymal tumours, and to reveal how trypsin-2 overexpression affects an OSCC cell line. High expression levels of MMP-9 were associated with the aggressive nature of OSCC (Kurahara et al. 1999, Yoshizaki et al. 2001, Zhou et al. 2010). How novel specific MMP peptide inhibitors would effect xenograft OSCC tumours in mice was investigated. Because trypsin-2 overexpressing tumours in mice had characteristics of EMT, and the expression of the tight junction protein claudin is known to change during this process (Singh et al. 2010), the expression of different claudins in the OSCC cell line and in tissue biopsies of OSCC patients and their correlation with the survival of the patients, was studied here.

The aims of the work were:

1. to reveal how trypsin-2 and MMP-9 are localized in OSCC cell line and in mesenchymal and epithelial tumours.

2. to analyze how overexpression of trypsin-2 affects the invasive properties of HSC-3 cells with in vivo and in vitro conditions.

3. to reveal how the novel specific MMP peptide inhibitors: antigelatinolytic CTT, PPC and proMMP-9 targeting CRV, effect tongue squamous cell growth, invasion, and metastasis formation.

4. to evaluate the capability of the antigelatinolytic CTT peptide fused with green fluorescent protein (GFP) in tumour imaging purposes.

5. to define the expression levels of different claudins in the tissue biopsies of OSCC patients and how they correlate with the survival of patients.
3. MATERIALS AND METHODS

3.1 Cell lines and cell cultures (I, II, II, IV)

The human tongue squamous cell carcinoma cell line, HSC-3, was purchased from the JCRB Cell Bank (JCRB 0623, Osaka National Institute of Health Sciences, Japan). Stable transfection of trypsin-2 and control vector to HSC-3 cells was made previously (Nyberg et al. 2002). Native HSC-3, HSC-3 + trypsin-2, and HSC-3 + control vector cells were cultured in a humid atmosphere of 5% CO₂ and 95% air at 37°C in 1:1 DMEM (Lonza,) and Ham’s Nutrient Mixture F-12 supplemented with 10% heat-inactivated fetal calf serum, 1 mM natrium pyruvate, 250 ng/ml fungizone (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 0.4 ng/ml hydrocortisone (Sigma-Aldrich). Selection of transfected cells was maintained by the addition of 500 μg/ml of geneticin to culture the medium (Invitrogen). Cell line authentication was done by LGC Standards and all the cell line profiles showed the same characteristics as the HSC-3 supplied reference.

3.2 Cell migration and proliferation in vitro (II)

To evaluate migration, 60 000 HSC-3 + trypsin-2 cells, and 60 000 HSC-3 + control vector cells were added to Transwell inserts (Costar) and cultured overnight. They were fixed for 15 min in 10% trichloroacetic acid, stained with crystal violet, and washed with H₂O. Unmigrated cells were removed and migrated cells on the underside of the membrane were counted under the microscope (50 × magnification, DMRB photo microscope, Leica Microsystems). For proliferation studies, 3000 cells were added to each well of a 96-well plate. Cells were first allowed to attach overnight and then cultured 24 hours in medium with or without serum. In the absence of serum, 0.5% lactalbumin was used. Proliferation was determined with the Cell Proliferation ELISA, BrdU kit from Roche, where BrdU incorporation to newly synthesized DNA
was measured in proliferating cells using an ELISA plate reader (Wallac) at 450 nm.

3.3 Apoptosis assays in vitro (II)

To evaluate the apoptosis level of HSC-3 + trypsin-2, and HSC-3 control vector cells, the cells were plated onto coverslips at a density of $1 \times 10^5$ cell per coverslip and cultured for 24 h in cell culture medium at 37°C in 5% CO₂. The Annexin-V-Fluos Staining Kit (Roche Diagnostics) was used to define apoptosis according to the manufacturer’s instructions with an Olympus BX61 microscope connected to an Olympus U-CMAB3 camera using AnalySIS-program. Apoptosis was measured from paraffin-embedded myoma tissue with the In Situ death detection Kit (Roche Diagnostics), according to manufacturers instructions.

3.4 Cell viability assay (III)

The effects of combined peptide therapy on HSC-3 cell viability were analyzed by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay (Sigma). Approximately $1 \times 10^5$ cells were plated in 24-well plates and allowed to attach overnight. A mixture of CTT/CTV/PPC peptides (1 mg/ml each) was added to the culture medium. After an incubation of 36 h, MTT reagent was added and the incubation was continued for 4 h. The viability was measured with the MTT reagent according to the instructions of the manufacturer (Sigma). The amount of viable cells was analyzed using microplate-reader (absorbance 570nm) (Labsystems).

3.5 Gelatin zymography (I)

In article I, total proteins from HSC-3 + trypsin-2 cells were extracted with lysis buffer (50 nM Tris, 10 nM CaCl₂, 2H₂O, 150 nM NaCl, 0.05% Brij-35, pH 7.5) and giant cell lesion tissue was homogenized in sample buffer (4 x loading buffer: 0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.0096% bromophenol blue). All
samples (10 μg/lane) were separated by 10% SDS-PAGE containing 1mg/ml fluorescently (2-methoxy-2.4-diphenyl-3 [² H] furanone; Fluka, Ronkonma NY) labeled gelatin (O’Grady et al 1984). After that, SDS was removed by 2.5% Triton-X and gelatinases were activated by incubation in 50mM Tris-HCl buffer (pH 7.8, 150 mM NaCl, 5 mM CaCl₂, 1 μM ZnCl₂) at 37°C overnight. The degradation of gelatin was visualized under long-wave UV light and gels were stained with 0.5% Coomassie brilliant blue R250. Gelatinolytic activity was measured by densitometry (QuantityOne, Bio-Rad Model GS-700 Imaging Densitometer, Bio-Rad).

3.6 Gelatin degradation assay (I)

A gelatin degradation assay was used to measure the activity of cleaved MMP-9. As a substrate, ¹²⁵I-labeled gelatin was used, which is degraded by the active MMP-9. Fifty ng of recombinant human proMMP-9 (Invitek) was treated with 0.15 IU human enterokinase (Prospec-Tany TechnoGene) for 90 min at 37°C, 25 mM ethylenediaminetetraacetic acid (EDTA) for 10 min at room temperature, or 2 mM APMA for 60 min at 37°C. MMP-9 or enterokinase alone were used as controls. To see if enterokinase cleavage makes MMP-9 more sensitive for further cleavage with APMA, proMMP-9 was first treated, as previously described, with enterokinase, and then incubated with APMA for either 20 or 60 min. All the samples were incubated in a total volume of 20 μl of buffer (50 mM Tris-HCl, pH 7.8, 200 mM NaCl, 1 mM CaCl₂) for 1 h at 37°C. Next, the samples were incubated with soluble ¹²⁵I-labeled gelatin (1.5μM) for 1 h at 37°C and 20% trichloroacetic acid was used to precipitate the undegraded gelatin. The supernatants radioactivity was counted with a γ-counter (Clinigamma, LKB Wallac).

3.7 Western blot (I, II, IV)

Total proteins were extracted from the HSC-3, HSC-3 + trypsin-2, and HSC-3 + control vector cells cultured in 250 mm² dishes by the Trizol® method (Invitrogen) or by lysing the cells in lysis buffer (50 mM Tris, 10 mM CaCl₂, 150
mM NaCl, 0.05% Brij. 35, pH 7.5). Approximately 7 -30 µg of protein samples were separated on 10% - 15% SDS-PAGE gels. Equal loading in the lanes was confirmed with Coomassie Brilliant Blue staining. The gels were then destained, and proteins were electrophoretically transferred to a nitrocellulose membrane (Bioscience). Nonspecific binding was blocked with 5% non-fat dry milk (Valio) at 37°C for 1 h. The membranes were incubated with primary antibodies overnight at RT. The primary antibodies used in the Western blot in this study are listed in Table 4. Membranes were washed and incubated with the secondary peroxidase-conjugated anti-mouse IgG antibody or peroxidase-conjugated anti-rabbit IgG antibody (Amersham Pharmacia Biotech) at RT for 1 h. An ECL Western blotting detection kit (Amersham Pharmacia Biotech) was used to visualize the proteins as described by the manufacturer instructions.

### Table 4. Antibodies and dilutions used in Western blotting

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin-2</td>
<td>mAb mouse</td>
<td>Itkonen et al. 1996</td>
</tr>
<tr>
<td>Trypsin-2</td>
<td>pAb rabbit</td>
<td>Koivunen et al. 1990 and 1991</td>
</tr>
<tr>
<td>Enterokinase</td>
<td>mAb mouse</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>MMP-13</td>
<td>mAb mouse</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>pAb rabbit</td>
<td>AbD Serotec</td>
</tr>
<tr>
<td>Laminin-332</td>
<td>pAb rabbit</td>
<td>Salo et al. 1999</td>
</tr>
<tr>
<td>Claudin-1</td>
<td>pAb rabbit</td>
<td>Zymed Laboratories</td>
</tr>
<tr>
<td>Claudin-7</td>
<td>pAb rabbit</td>
<td>Zymed Laboratories</td>
</tr>
<tr>
<td>Claudin-4</td>
<td>mAb mouse</td>
<td>Zymed Laboratories</td>
</tr>
<tr>
<td>Claudin-5</td>
<td>mAb mouse</td>
<td>Zymed Laboratories</td>
</tr>
<tr>
<td>Occludin</td>
<td>pAb rabbit</td>
<td>Zymed Laboratories</td>
</tr>
</tbody>
</table>

### 3.8 Silver staining (II)

Samples were run on 15% SDS-page with sample buffer, and the gel was stained according to PageSilver™ Silver Staining Kit instructions.
3.9 MMP and claudin cleavage in vitro (I, II)

Purified human recombinant proMMP-9 (1 μg) (Invitek) was treated with 3 IU of recombinant human enterokinase (Prospec-Tany TechnoGene Ltd) for 0 - 90 minutes at 37°C. Two μg of purified human recombinant prodomain – catalytic domain MT1-MMP (proMT1-MMP) (Invitek) was treated with purified autoactive human trypsin-2 (a kind gift from Prof. UH Stenman) or recombinant human trypsinogen-2, which was activated by 3 IU of recombinant human enterokinase (Prospec-Tany TechnoGene Ltd) for 1-120 minutes and overnight at 37°C. One μg of human recombinant claudin-1 or -7 with GST tags (Abnova) were treated with APMA activated human recombinant prodomain – catalytic domain MT1-MMP (1 μg) (Invitek) overnight at 37°C. All of the above-mentioned reactions were terminated by adding SDS-sample buffer and boiling the samples for 5 min. Proteins were separated by 11-15% SDS-PAGE and stained with Coomassie Brilliant Blue, PageSilver silver staining kit (Fermentas), or ECL Western blot was performed.

3.10 N-terminal sequencing (I)

Recombinant human MMP-9 was treated with enterokinase and the cleavage products were separated by 8% SDS-PAGE. After the gel was washed for 30 min in blotting buffer, proteins were electrophoretically transferred to a methanol soaked Problot membrane (Applied Biosystems) at 40 mA for 3 h. The membrane was then rinsed with distilled water and stained in 0.1% Coomassie Brilliant Blue for 5 min. The membrane was destained with 50% methanol, rinsed with distilled water, dried at RT and stored at -20°C. The N-terminal sequence from the cleavage product of 77-82kDa, which is known to be the active form of MMP-9, was analyzed with a Procise 492 protein sequencer (Applied Biosystems) using Edman chemistry at the Protein Sequencing Core Facility at the University of Oulu.
3.11 Immunohistochemistry (I, II, IV)

Human and myoma tissue sections (4-6 μm) were deparaffinised and rehydrated. The primary antibodies and specific information of staining protocol are listed in Table 5. Antigen retrieval was performed with 0.4% pepsin in 0.01 M HCl at 37°C for 1 hour or by microwaving the sections in citric acid, pH 6 or in Tris/EDTA (10 mM/L Tris, 1 mmol/L EDTA, pH 9) for 10 minutes or 8 minutes treatment with pronase (Tris-HCl, pH 7.6) at 37°C. Sections were washed twice with phosphate buffered saline (PBS) before endogenous peroxidase activity was blocked with Dako Real peroxidase-blocking solution or H2O2 in methanol for 30 minutes at room temperature. Sections were then washed twice with PBS and incubated with primary antibodies in a humidified chamber either at 37°C for 30 minutes - 1 hour, or at 4°C overnight. The sections were washed twice in PBS and incubated with secondary antibodies from Real Envision Detection System (DAKO), Vectastain ABC Elite kit (Vector Laboratories) or Histostain SP kit (Zymed Laboratories) according to manufacturer’s instructions. Color was developed by 3,3-diaminobenzidine tetrahydrochloride (DAB) or 3-amino-9-ethylcarbazole (AEC; Sigma). Sections were then counterstained with Mayer’s hematoxylin and mounted with Mountex (Histolab Products Ab), glycergel mounting medium (Dako) or Eukitt (Kindler). In study I, double immunohistochemical staining was also used. The first AEC method was used for trypsin-2 immunostaining, and after that, sections were washed, nonspecific binding was blocked, and sections were incubated with avidin-biotin-peroxidase complex and then stained with SG-color for visualization (Vector Labs). Negative controls were included (PBS, nonimmune rabbit or mouse serum instead of the primary antibody).
Table 5. Antibodies and staining protocols in immunohistochemistry

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Antigen retrieval</th>
<th>Incubation time and temperature</th>
<th>Visualization Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin-1 pAb rabbit, (Zymed Laboratories)</td>
<td>citrate b</td>
<td>1 h, RT</td>
<td>HS</td>
</tr>
<tr>
<td>Claudin-4 mAb mouse, (Zymed Laboratories)</td>
<td>citrate b</td>
<td>1 h, RT</td>
<td>HS</td>
</tr>
<tr>
<td>Claudin-5 mAb mouse, (Zymed Laboratories)</td>
<td>citrate b</td>
<td>1 h, RT</td>
<td>HS</td>
</tr>
<tr>
<td>Claudin-7 pAb rabbit, (Zymed Laboratories)</td>
<td>citrate b</td>
<td>1 h, RT</td>
<td>HS*</td>
</tr>
<tr>
<td>Claudin-7 pAb rabbit (Zymed Laboratories)</td>
<td>citrate b</td>
<td>1 h, RT</td>
<td>RE**</td>
</tr>
<tr>
<td>Occludin pAb rabbit, (Zymed Laboratories)</td>
<td>pronase</td>
<td>1 h, RT</td>
<td>HS</td>
</tr>
<tr>
<td>Trypsinogen pAb rabbit, Koivunen et al. 1990, 1991</td>
<td>pepsin</td>
<td>O/N, +4 C</td>
<td>VE</td>
</tr>
<tr>
<td>MMP-9 mAb mouse, Nikkari et al. 1996</td>
<td>pepsin</td>
<td>O/N, +4 C</td>
<td>VE</td>
</tr>
<tr>
<td>Lymphocyte marker, Leucocyte Common Antigen (LCA) mAb mouse, (Dako)</td>
<td>pepsin</td>
<td>O/N, +4 C</td>
<td>VE</td>
</tr>
<tr>
<td>Macrophage-marker CD68 mAb mouse, (Dako)</td>
<td>pepsin</td>
<td>O/N, +4 C</td>
<td>VE</td>
</tr>
<tr>
<td>Trypsin-2 mAb mouse, Itkonen et al. 1996</td>
<td>pepsin</td>
<td>O/N, +4 C</td>
<td>VE</td>
</tr>
<tr>
<td>MT1-MMP mAb mouse, (R&amp;D Systems)</td>
<td>Tris/EDTA</td>
<td>30 min, +37°C</td>
<td>VE</td>
</tr>
<tr>
<td>Pancytokeratin AE1/AE3 mAb mouse (Dako)</td>
<td>pepsin</td>
<td>1 h, +37°C</td>
<td>RE</td>
</tr>
</tbody>
</table>

RT=room temperature, O/N overnight, b= buffer,*IV,**III
HS= Histostain-SP kit, Zymed Laboratories
RE= Real EnVision Detection System K5007, Dako
VE= Vectastain ABC Elite kit, Vector Laboratories

3.12 Assessment of immunohistological staining (IV)

The intensity of immunohistochemical staining (claudin-1, -4, -5, - 7 and occludin) in tumour tissue was graded as + = weak (less immunostaining intensity compared with the histologically normal-appearing overlying epithelium), ++, =medium (same immunostaining intensity compared with the overlying epithelium) and +++ = strong (higher immunostaining intensity compared with the overlying epithelium). Quantitative immunostaining was assessed as follows: + = less than 25% of cells positive, ++ = 25-50% of cells positive, +++ = 50-75% of cells positive, ++++ = greater than 75% of cells positive. To ease the assessment ++ and +++ were considered as a medium immunostaining. The whole areas of the sections were screened. The assessments were carried out by two investigators and a final assessment was then performed jointly.
3.13 Fluorescent and laser scanning confocal microscopy (I, II)

Two different amounts of HSC-3 +trypsin-2 or HSC-3 control vector cells (1 × 10⁵ or 1 × 10³) were seeded onto coverslips and grown for 24 h in medium. In Study I protein secretion was blocked in cells with medium containing 1 μM Monensin for 1 h and 3 h before fixing. After that, the cells were washed with PBS, fixed in methanol at -20°C for 10 min, rinsed, and stored in PBS containing 0.02% sodium-azide at +4°C until analyzed. The coverslips were washed with PBS. Nonspecific binding was blocked with 5% normal goat serum (Dako) in PBS containing 3% bovine serum albumin (BSA) for 1 h at room temperature. Primary antibodies used in study I: monoclonal trypsin-2 (Itkonen et al. 1996), polyclonal MMP-9 (Calbiochem), and in study II: monoclonal fibronectin-1 (Sigma), polyclonal claudin-7, and Vilen et al. (unpublished results) claudin-1 (Zymed Laboratories) in PBS-3% BSA were incubated for 30-60 min at RT and rinsed in PBS. Secondary monoclonal and polyclonal antibody conjugates Alexa Fluor 488 and 568 (Molecular Probes Eugene) were diluted in PBS-0.1% BSA (1:400) and incubated for 30-60 min at RT. The coverslips were rinsed in PBS, incubated with TO-PRO-3 (1:1000)(Invitrogen)(I) for ten minutes, rinsed in PBS, then dH₂O and mounted with Vectashield H-1000 (Vector). In study I, 3-D co-localization analysis was performed as follows: image stacks were deconvolved and 3-D co-localization analysis of trypsin-2 and MMP-9 were performed by software Bitplane Imaris suite (Bitplane AG). In study II, sample evaluation was performed by using the AnalySIS-program with an Olympus BX61 microscope.

3.14 Tartrate-resistant acid phosphatase staining (I)

To identify osteoclasts from tumour sections, tartrate-resistant acid phosphatase staining was done according to Thompson 1966. In brief, slides were dewaxed, incubated first in 50 mM tartrate in acetate buffer (pH 5.2) at 37°C for 2 h and then in an acid phosphatase substrate buffer (25 % Michaelis acetate buffer, 0.16 % paraorsaniline, 0.16 % NaNO₂, 0.05 % naphthol AS-BI phosphate), pH 5.0 with 20 mM tartrate at 37 °C for 60 min. Finally, sections were washed and mounted.
3.15 Time-resolved immunofluorometric assay (II)

To evaluate the amount of secreted trypsin-2 in the cell culture medium of HSC-3 + trypsin-2 and HSC-3 + control cells, the medium was analyzed by time-resolved immunofluorometric assay as previously described (Itkonen et al. 1990).

3.16 Riboprobe synthesis and In situ hybridization (I)

Fluorescein-UTP riboprobe synthesis was used when in vitro transcriptions of sense and antisense trypsinogen probes were made using the RNA color kit (Amersham Pharmacia Biotech) as described previously (Paju et al. 2000). Tissue samples were fixed, paraffin embedded, dried for 2 h at 65°C, and mounted under RNAse free conditions. The in situ hybridization for trypsinogen was carried out as described (Paju et al. 2000) and the MMP-9 in situ hybridization in the bone tissues were performed as described in (Autio-Harmainen et al. 1992).

3.17 Reverse transcription polymerase chain reaction (RT-PCR) (I)

Purification of RNA from HSC-3 cells was done according to Trizol®kit (Invitrogen) instructions; 2 µg of total RNA with primers from SuperSriptIII-kit (Invitrogen) was used for the cDNA-reaction. Specific primers for amplifying enterokinase were 5’-TTG TTG TTC GTG GTG CCA TA 5’ and 5’-ACC AAC TTT GGT GCC AAC TC (outer-primers), and 5’-GAA AAT GGT CTG CCT TGC AT and 5’- TCT GGC TTT CTG TGT TTG GA (inner-primers). The nested RT-PCR reaction was performed with 2U Dynazyme polymerase (Finnzymes). The purified PCR-product was sequenced. For trypsin-4 RT-PCR primers were 5’-CTT CTG GGT GGA CGC ACT TGG (sense) and 5’-GGG GGC TTT AGC TGT TGG CA (antisense) and the PCR was performed with 0.6 U of AmpliTaq Gold (Applied Biosystems).
3.18 Microarray (II)

Approximately $1 \times 10^6$ HSC-3 + trypsin-2 and HSC-3 + control vector cells/flask were counted and cultured overnight. RNeasy mini kit (Qiagen) was used to isolate total cellular RNA from cells according to manufacturer's instructions at the Biomedicum Genomics core facility (Biomedicum Helsinki). Three replicates from each cell line were made. Experimental procedures for GeneChip® (Affymetrix) were performed according to the Affymetrix GeneChip® Expression Analysis Technical Manual. Microarray experiments were carried out using the Affymetrix Human Genome U133 Plus 2.0 chip, for analysis of over 47,000 transcripts.

3.19 Microarray data analysis (II)

To evaluate the changes in gene level between HSC-3 + trypsin-2 compared to HSC-3 control cells, raw data files (Affymetrix Cel-files) were imported to EzArray (www.ezarray.com) and chipster (http://chipster.csc.fi/) softwares and those were used according to the manufacturers’ instructions. These two programs were used to see the possible differences in the results of the two different algorithms. Data was first normalized by RMA (Robust Multi-Array) method in both softwares. Statistical testing in EzArray was performed by fold change. Genes with a 1.8 or higher fold change were listed, including both up and down regulated genes, whereas statistical testing in chipster was done as recommended in their tutorial (https://extras.csc.fi/biosciences/chipster-manual/tutorial1.html). Briefly, an empirical Bayes t-test was used to compare the groups and for multiple testing corrections Benjamini and Hochberg’s false discovery rate was used with a false discovery rate of 0.05.

3.20 Peptides (II and III)

Specific MT1-MMP inhibitor GACFSIAHECGA- peptide (peptide G), the corresponding scrambled control peptide CGAAPEACGIHS, antigelatinolytic CTTHWGF TLC (CTT) peptide, and its control peptide (GERGLETSC), were all custom made by Neosystem. ProMMP-9 targeting CRVYGPYLLC (CRV) and
CGYGRFSPPC (PPC) peptides were made as described by Björklund et al. (2004) and were a gift from PhD Erkki Koivunen (MD Andersson Cancer Center, Houston, TX, USA). In Study II, lyophilized peptides were diluted in microfiltered H2O, whereas in the animal experiments of study III the stock solution was made by diluting lyophilized peptides (20 mg/ml in dimethyl sulfoxide or 1 mg/ml in saline). Microfiltering of the solutions was performed before injections to the animal.

3.21 Tissue samples and patients (I and IV)

The ethical committee of Northern Ostrobothnia (Oulu University Hospital District, Finland) has approved the use of human samples in this study. Paraffin-embedded human mobile tongue squamous cell carcinoma (n=10), osteosarcoma (n=12), giant cell lesion from mandible (n=2) and giant cell tumour from vertebra (n=3) were obtained from the files of the Department of Pathology, Oulu University Hospital. Sections were deparaffinised, rehydrated, and subjected to immunohistochemistry and in situ hybridization (I). Gelatinases from patients’ giant cell lesions from the Oral and Maxillofacial Department of Oulu University Hospital were analyzed by zymography and trypsin-2 Western blotting (I). All cases of mobile tongue cancer (n=97) treated in the Oulu University Hospital between the years 1983 and 2005, that had enough histologic material, sufficient clinical data, and resection margins greater than 5 mm were included in study IV and immunohistochemical analysis was performed (IV). The tumours were histologically graded and staged using the current UICC and WHO-based classifications (Barnes et al. 2005, Sobin & Wittekind 2002) and invasive front grading was performed, which included five morphologic features that are scored from 1-4: degree of keratinization, nuclear polymorphism, number of mitoses, mode of invasion, and plasma lymphocytic infiltration (Bryne et al. 1991) (IV).
3.22 Clinicopathologic Characteristics of patients in study (IV)(Table 6.)

<table>
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<th>Sex</th>
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</tr>
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<td>Surgery</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Follow-up</td>
<td>Median time (Months)</td>
<td>Range (Months)</td>
</tr>
<tr>
<td>Unknown</td>
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</tr>
<tr>
<td>Invasive front grading</td>
<td>Low (5-10)</td>
<td>Death due to other cause</td>
</tr>
<tr>
<td></td>
<td>High (11-20)</td>
<td>Death due to unknown cause</td>
</tr>
<tr>
<td></td>
<td>Missing</td>
<td>4</td>
</tr>
</tbody>
</table>

3.23 Myoma Organotypic culture (II)

The study was approved by the Ethics Committee of the Oulu University Hospital. Myomas were prepared, stored, and the experiment was performed as described (Nurmenniemi et al. 2009) with a few modifications. The myoma discs were equilibrated with gentle agitation for 10 days in 4°C in medium which was changed every three days. The myoma disks were placed into Transwell inserts and 700 000 HSC-3 + trypsin-2 cells or HSC-3 + control vector cells in 50 µl medium were added on top of each myoma. The cells were cultured overnight and then myoma disks were transferred onto nylon disks on curved steel grids in 12-well plates with 1 ml medium. Medium was changed every three days and the collected medium were stored at -20°C. Myoma cultures were maintained for 10 days. The invasion inhibiting ability of peptide G was measured in the myoma model. Lyophilized peptides, peptide G, and scrambled control peptide were dissolved in H2O to make a 1 mM stock solution. The assay concentrations of 250 µM were prepared using culture medium. Broad spectrum MMP inhibitor Ilomastat (Millipore) was used as a control at a concentration of 50 µM. Myoma
disks were rinsed in culture medium for ten days and preincubated in peptide G, control peptide, or Ilomastat for 12 hours. The HSC-3 +/- trypsin-2 cells were also preincubated with peptide G, control peptide, or Ilomastat for 1 hour at 37°C before detaching the cells. Myoma disks with cells were maintained in culture medium containing 250μM peptide G and control peptide or 50 μM Ilomastat. Collagen I degradation products (Risteli et al. 1993) in the cell culture medium were measured using commercially available ICTP-RIA according to manufacturer's instructions (Orion Diagnostica Oy).

3.24 Quantification of Invasion (II)

As a marker for carcinoma cells pancytokeratin, immunostaining was performed as described previously (Nurmenniemi et al. 2009). Photos of myoma sections were captured at a magnification of 4 × with an Olympus BX61 microscope connected to an Olympus U-CMAB3 camera using the AnalySIS-program. Those areas where myoma tissue was less dense than normal, allowing artificial invasion, were excluded from the analysis. To calculate the maximal invasion (the distance of the deepest invading cell from the lower surface of the noninvasive cell layer), four deepest invading cells, or cell clusters, were measured per myoma section. The invasion index (1 - [non invading area/total area]) was measured as described (Gaggioli et al. 2007).

3.25 Animal experiments (II, III)

All of the animal experiments were approved by the Ethics Committee for Animal Experimentation in the State Provincial Office of Southern Finland, and also by the Ethics Committee for Animal Experiments at the University of Helsinki, in study III. The animals were maintained under standard conditions for temperature and humidity. Food and water was provided ad libitum and the well-being of the animals was inspected daily. In study II, tumour xenografts were initiated by inoculating subcutaneously 1.5 × 10⁶ detached HSC-3 +/- trypsin-2 and HSC-3 control vectors cells suspended in 200 μl serum-free culture medium were injected subcutaneously into both
flanks of 6- to 8-week old Harlan-Sprague Dawley athymic nude female mice (10/group) (Harlan Laboratories). The mice were weighed trice weekly, the tumour growth measured (width and length), and the survival observed. The criteria for euthanasia were weight loss more than 15 %, tumour size over 10 mm in diameter, or symptoms of suffering. When the first mouse reached the criteria for euthanasia, all were euthanized by cervical dislocation. The mice were dissected and macroscopic local invasion, as well as the number of enlarged lymph nodes was evaluated.

In study III, metastases formation after CTT treatment was measured. Mice (10/group) were treated with CTT (2 mg/ml) or saline. Sacrifice criteria and evaluation of metastases was identical to that described earlier. The combined peptide-therapy study was carried out by treating the mice (7/group) during days 4-8 daily with CTT (2 mg/ml) or CTT/CTV/PPC (all 1mg/ml) diluted in 0.9% NaCl/H2O. Tumour sizes were calculated with formula (Π/6) x A x B² (A= length, B=width of tumour). In the tumour targeting study CTT-Green fluorescent protein (CTT-GFP) and control peptides were synthesized as described (Reunanen et al. 2010). Tumours from HSC-3 cells (2 x 10⁶/inoculation) were made identically to those described in study II and tumours were allowed to grow 18 days. The mice (4/group) were injected either with saline, gelatinase targeting CTT-GFP, or non-functional His-GFP or Ala-CTT-GFP (all 1 mg/ml in 100 μl of PBS). All the mice were euthanized after 1 hour of injection and tumours were collected for fluorescence analysis, embedded in in Tissuetek-OCT, and frozen.

3.26 Tumour fluorescence analysis (III)

Frozen tumours from CTT-GFP treated mice were cut with microtome to 10 μm sections. To identify the tumour, tissue histological analysis was performed as described in Heikkilä et al. 2006. Fluorescence of the samples was detected with an Olympus microscope using filters for GFP (ex. 395±40 nm/em. 510±40 nm) and it was quantified from the three most intensive areas (magnification x 400) with the Quantity-One program (Bio-Rad).
3.27 Statistical analysis (I-IV)

In article I, results of MMP-9 activity are expressed as the mean ±SEM of experiments. Statistical significances were calculated by one-way variance (ANOVA) with Bonferroni's post-tests for multiple comparisons from co-compartmentalization data of MMP-9 and trypsin-2. Independent sample t-test (II) or Mann-Whitney (III) was performed to estimate the statistical significance between groups. Data are expressed as mean ± SD (III). Box plots are used in the description of invasion depth and invasion index. The line across the box indicates the median, the box contains the values between the 25th and 75th percentiles, and the whiskers show the highest and lowest values (II). The data of local tumour invasion into the fascia of xenograft tumours was analyzed by chi-square test (II). In article IV, the statistical association between patient survival and the variables studied was done using Kaplan Meier survival plots and Mantel-Cox log rank test. Univariate analysis with Cox's proportional hazards multiple regression model was then applied by adding known supplemental clinicopathologic prognostic variables that are believed to have an effect on prognosis. The proportional hazard assumption was verified by comparing estimated log (-log)(log-minus log [LML]) survival plots of the different categories used. Fisher’s exact test was used to test for association between groups. In all articles, results with P < 0.05 were considered statistically significant.
4. RESULTS

4.1 MMP-9 and trypsin-2 do not co-localize in bone tumours, whereas in OSCC they are co-compartmentalized (I)

Trypsin-2 is a very effective activator of MMP-9 in vitro (Sorsa et al. 1997). To evaluate the possible interactions of these two enzymes in vivo the expression and co-localization of MMP-9 and trypsin-2 in bone tumours was analyzed from osteosarcoma, giant cell lesion, and giant cell tumour. MMP-9 was expressed in osteosarcoma, whereas trypsin-2 was not detected in the tumour tissue at all. In giant cell lesion and tumours, MMP-9 expression was detected in mono- and multinucleated osteoclastic tumour cells. The active form of MMP-9 was observed in giant cell lesion tissue extracts by gelatin zymography and in giant cell tumours by immunohistochemistry with MMP-9 antibody that recognizes only active from of MMP-9. By Western blotting analysis of tissue extracts from giant cell lesions, 27-28 kDa trypsin-2 bands were detected. In immuhistochemistry, trypsin-2 was detected in lymphocyte-like and polymorphonuclear cells, whereas in giant cell lesions and tumours, mononuclear and osteoclastic tumour cells, and their macrophage precursors were negative. To further specify trypsin-2 containing cells, double staining for trypsin-2 and lymphocyte (LCA)/macrophage (CD68) was performed. This showed that trypsin-2 was expressed in LCA positive cells, whereas it was not found in CD68 positive cells. Double staining for MMP-9 and trypsin-2 in giant cell tumours showed that these enzymes are localized in different cell types. Although trypsin-2 and MMP-9 were not co-localized in tumours of mesenchymal origin, they might have different localization in carcinomas. Immunoreactivity of trypsin-2 was then analyzed from OSCC tissue samples. Positive staining for trypsin-2 was seen in the peripheral carcinoma cells and cancer islands in all tongue SCC samples. In addition, some fibroblasts and macrophages showed positive staining in surrounding tumour stroma. Trypsin-2 mRNA was detected in all carcinoma cells, stromal fibroblast, and macrophages, as well as in paraffin embedded cultured HSC-3 cells by in situ hybridization. A similar distribution of MMP-9 has been shown recently in OSCCs (Impola et al. 2004). Co-localization of trypsin-2 and MMP-9 in HSC-3 cells was
analyzed by confocal microscopy with or without 1 μM monensin treatment, which inhibits intracellular transport of proteins from the Golgi complex. In the absence of monensin, 58% (±2%) of total MMP-9 was co-compartmentalized with trypsin-2 and 36% (±4.8%) of all trypsin-2 was co-compartmentalized with MMP-9. Monensin treatment caused time-dependent redistribution of MMP-9 into separate vesicles. Gelatin zymography from HSC-3 cell extracts revealed both the pro- and active-form of MMP-9 in these cells whereas only the pro-form of MMP-2 was detected.

4.2 Cleavage of MMP-9 by enterokinase and expression of enterokinase in tongue squamous cell carcinoma cells (I)

The expression of enterokinase, a known activator of trypsin, in HSC-3 cells was demonstrated by nested RT-PCR and Western blot. To further investigate whether enterokinase was also able to activate proMMP-9, enterokinase and proMMP-9 were incubated together for up to 90 minutes, and the cleavage products analysed by SDS-PAGE. The results revealed that MMP-9 was converted to 77-82 kDa forms. N-terminal sequencing indicated that enterokinase cleaved recombinant proMMP-9 at Lys$^{65}$-Ser$^{66}$, and this cleaved MMP-9 was more susceptible to APMA activation than proMMP-9 without enterokinase treatment. Interestingly, gelatin degradation assay did not show any significant increase in MMP-9 activity after enterokinase treatment.

4.3 Trypsin-2 overexpression made tongue SCC cells more aggressive in vivo and in vitro (II)

The expression of trypsin-2 has previously been associated to the aggressive behavior of tongue SCC (Nyberg et al. 2002). Here, trypsin-2 overexpression in HSC-3 cells increased local invasion into subcutaneous HSC-3 + trypsin-2 xenograft tumours (P=0.002), as well as in the Transwell migration assay (P<0.001), and organotypic myoma model (P<0.001). During invasion into myoma substrate, HSC-3 + trypsin-2 cells degraded collagen I more efficiently
than the control cells. Collagen I is the main component of myoma tissue and its degradation products in culture medium represent collagenolytic activity of proteinases (Nurmenniemi et al. 2009). In addition, HSC-3 + trypsin-2 xenograft tumours in nude mice tended to be smaller than control tumours (P=0.102). In addition, HSC-3 + trypsin mice had more clinically enlarged lymph nodes (3.1 ±SD 1.6) than the control group (1.6 ±SD 1.2) (P=0.032). Histological analyses of tumours revealed that control cell tumours were mainly encapsulated and had necrotic areas, whereas HSC-3 + trypsin-2 cell tumours had invaded into the surrounding stroma and displayed characteristics of epithelial to mesenchymal transition. In in vitro adherent cultures, no statistically significant difference between proliferation of these cell lines was detected, but HSC-3 + trypsin-2 cells were slightly less apoptotic than the control cells.

4.4 Trypsin-2 increased carcinoma cell invasion by activating proMT1-MMP, increasing lam-332 degradation, and changing expression of metabolism related genes (II)

Recombinant proMT1-MMP (31 kDa) was cleaved after a 1 minute incubation by enterokinase activated recombinant trypsinogen-2 or human autoactive trypsin-2 into 23–25-kDa forms. Similar activation of proMT1-MMP by bovine trypsin has also been observed previously (Will et al. 1996). Western blot of cell extracts showed that MT1-MMP was differentially processed in HSC-3 + trypsin-2 cells compared to control cells suggesting that proMT1-MMP might also be processed by trypsin-2 in cells. Furthermore, the amount of 66 kDa degradation fragments of lam-332 was increased in HSC-3 + trypsin-2 cells. Increased amounts of laminin-332 have been found in invasive fronts of carcinomas (Katayama et al. 2004).

To understand how trypsin-2 overexpression affects gene expression, microarray data was analyzed by two different softwares (EzArray and Chipster). Trypsin-2 transfection changed gene expression in HSC-3 cells dramatically. Several of the up-regulated genes are involved in cell metabolism, such as UGT8, which encodes UDP-galactose ceramide galactosyltransferase and MAN1A1, which is involved in
glycoprotein biosynthesis. All the gene expression changes were not identical by EzArray and Chipster, however.

4.5 MMP inhibitors modulate tongue SCC cell behavior (II)

Non-selective, broad-spectrum peptide MMP inhibitors were promising new cancer drugs in two decades ago, but the clinical trials were disappointing. After that, new types of MMP inhibitors have been developed, which are more selective, like specific MT1-MMP inhibitor peptide G. It has been used in the current work to understand the role of MT1-MMP in the invasion process.

Overexpression of trypsin-2 made HSC-3 cells more invasive in the myoma model. When HSC-3 + trypsin cells were treated with the selective MT1-MMP inhibitor peptide G, their invasion ability decreased significantly (P=0.000), whereas peptide G treatment did not appreciably affect the invasion of control cells. The amount of collagen I degradation products was also increased. Unexpectedly, immunohistochemical staining demonstrated that blocking of MT1-MMP activity did not decrease the expression of MT1-MMP, but actually increased it. Broad-spectrum MMP inhibitor, Ilomastat, decreased invasion of all used cell lines.

4.6 CTT peptide treatment decreased OSCC tumour size in mice, whereas combined matrix metalloproteinase inhibitor therapy did not yield any extra benefit (III)

HSC-3 cells were injected subcutaneously into nude mice (n=10/group) and from day 4 to 8 postinjection they were treated with CTT peptide or saline. Carcinoma growth was measured three times a week and each experiment was terminated when the first mice reached the ending criteria. Antigelatinolytic CTT peptide treatment decreased the tumour size (P=0.006), but it did not have any remarkable effect on tumour spread to lymph nodes according to dissection results.
To evaluate the effects on combined antigelatinolytic and proMMP-9 inhibitor therapy to antigelatinolytic therapy, nude mice (n=7/group) with HSC-3 cell tumours were treated, similarly as earlier mentioned, with CTT or CTT/CRV/PPC combination. After 45 days of inoculation, no statistically significant difference in tumour size was evident between the treatment groups (P=0.17) (average size; CTT-group: 28.4 mm³ ± 62.5 and CTT/CRV/PPC-group: 162.3 mm³ ± 349). The experiment was ended because benefit was lacking in response to combinatory therapy.

4.7 CTT-Green fusion protein (CTT-GFP) targets tongue SCC cell tumour in mice (III)

HSC-3 xenograft tumours of nude mice (n=4/group) were initiated as described above. After 18 days, CTT-GFP or non-functional controls were injected into tail vein and allowed to circulate for 1 h; mice were then euthanized. Fluorescence of tumours in the CTT-GFP group was significantly higher than in the control group (P=0.025).

4.8 Expression of claudin -1, -4, -5, and -7 in HSC-3 cells, trypsin-2 overexpressing cells, and control vector cells (II and IV)

Claudin expression is known to change during carcinoma progression. Western blot analysis from the total extracts of HSC-3 cells showed several immunoreactive bands for claudin-1 (18 – 37 kDa), weak bands for claudin-4 (22 kDa) and -5 (23 kDa), and a strong band for claudin-7 (22 kDa). When comparing the expression of claudins between the more invasive trypsin-2 overexpressing HSC-3 cells and control cells, claudin-1 expression was increased 30% in HSC-3 + trypsin-2 cells, whereas claudin-7 expression was decreased 20%. There was no difference between claudin-4 and -5 expressions (Vilen et al. unpublished results). Similar results were seen in the myoma model with claudin-1 (Figure 6. Vilen et al. unpublished results) and -7.
Figure 6. Claudin-1 expression is increased in HSC-3 + trypsin-2 vs. HSC-3 + control vector cells in myoma invasion model.

4.9 MT1-MMP degrades claudin-1 and -7 in vitro (II)

Since claudin-1 and MT1-MMP co-localize on the cell-cell borders (Miyamori et al. 2001), and claudin-1 has been anticipated to participate in the MMP-dependent pathways, was assessed whether claudin-1 (unpublished data) and -7 could be processed by MT1-MMP. Both claudins were cleaved into smaller fragments by MT1-MMP within overnight incubation, *in vitro*.

4.10 Expression of claudin -1, -4, -5, -7, and occludin in superficial and invasive front of squamous cell carcinoma of tongue and their association with overall survival (IV)

In both the superficial parts and invasive fronts of tongue SCC samples, claudin-1 and -7 immunoreactivities were strong in intensity compared to the overlying normal-appearing epithelium. Claudin-4 was moderate and claudin-5 relatively weak, although there were some individual differences from this general trend. Immunoreactivity of occludin was generally very weak or negative.

There was no statistically significant association between patients’ cause-specific survival and the staining intensity or the staining quantity of claudins in superficial parts of samples by univariate analysis. In the invasive front of tongue
carcinoma, both low and high claudin-7 intensity, compared to normal epithelium, showed a statistically significant association with poor disease-specific survival (hazard rate: 3.42, 95% CI 1.16–10.10, P = 0.023 and hazard rate: 3.16, 95% CI 1.20–8.31, P = 0.02). In addition, low quantity of tumour cells in the invasive front stained by claudin-7 was associated with decreased patient survival (hazard rate 4.87, 95% CI 1.44–16.44, P = 0.01). Multivariate analysis that included patients’ age at diagnosis (<70 years versus >70), TNM stage, and gender as additional prognostic variables, was used to verify these associations. Being 70 years of age and over seemed to be the only independent variable that was associated with decreased patient survival; however TNM stage was also an independent factor for poor prognosis, but only in the claudin-7 quantity multivariate model. When additional factors were included in the model, the association between claudin-7 intensity and quantity, and disease-specific survival was reduced.

Claudin and occludin expression patterns did not show any statistically significant association between the histologic grading or the invasive front (Bryne’s) grading. Claudin-1 was the only claudin in superficial parts of tumours that had significant association between TNM staging and intensity of the staining, whereas the quantity of cells stained with the claudin-4 and -7 correlated with tumour TNM stage.
5. DISCUSSION

5.1 Trypsin-2-MMP cascade in oral carcinoma (I, II)

Trypsin-2 is an efficient activator of MMP-9 (Sorsa et al. 1997), and it may play an important role in the progression of tongue carcinomas, since trypsin-2-induced MMP-9 activation clearly increases the invasion of tongue oral squamous cell carcinoma (OSCC) cells in chorioallantoic membrane assay (Nyberg et al. 2002). In human ovarian tumour cyst fluids, trypsin-2 levels are associated with the activation of proMMP-9 (Paju et al. 2001). The activation level of MMP-9 in turn, may associate with shortened disease-free survival and high metastatic frequency OSCCs (Hong et al. 2000). Recent studies have shown, however, that MMP-9 might have a protective role in cancer. According to Bendrik et al. (2008), overexpression of MMP-9 in breast cancer leads to increased levels of endostatin, which causes decreased angiogenesis. While in salivary gland myoepithelial carcinoma, MMP-9 expression predicted better overall survival, and MMP-9 functioned as a tumour suppressor in a colitis-associated cancer (Garg et al. 2010, Luukkaa et al. 2010). In the tumours of regional metastases in head and neck SCC MMP9 expression was decreased (Stokes et al. 2010).

In this thesis work, trypsin-2 was present in OSCC tissue samples and co-expressed with MMP-9 in a tongue SCC cell line, whereas this same phenomenon was not seen in osteosarcoma, giant cell lesion and tumours. In an invasive tongue squamous cell carcinoma cell line (HSC-3) trypsin-2 was co-localized with MMP-9 in intracellular vesicles, which might be the mechanism of intracellular activation of MMP-9. In melanoma cells, MMP-9 is stored and transported in small cytoplasmic vesicles and active MMP-9 is found in ovarian cancer ascites-derived membrane vesicles (Graves et al. 2004, Schnaeker et al. 2004). Directed vesicular transport coupled with possible trypsin-2 mediated proMMP-9 activation inside cells may explain the aggressive behavior of tongue SCCs.

Similar to MMPs, trypsins are produced as inactive zymogens that require activation. Enterokinase, a known activator of trypsin-2, was also expressed in HSC-3 cells and it could also directly cleave MMP-9 in vitro (Imamura et al. 2003).
This did not yield an active form of MMP-9, but the finding does not rule out that this cleaved form of MMP-9 might not be more prone to other downstream activators. Unfortunately, the lack of proper enterokinase antibody for immunofluorescence staining prevented localization and co-localization studies.

MT1-MMP has been associated with increased invasiveness of OSCC and other cancers (Polette et al. 1998, Myoung et al. 2002). It is known that MT1-MMP has a central role in the initiation of ECM protein breakdown, one of the hallmarks of cancer. It also activates proMMP-2, which can degrade many components of BM (Thomas et al. 1999). In addition, it can directly degrade laminin and this way enhances the migration of cancer cells (Udayakumar et al. 2003). In the trypsin-2 overexpressing HSC-3 cells studied here, where MT1-MMP was processed in a divergent manner, increased amounts of laminin-332 were also seen. Production of laminin-332 has been detected in a number of tumour types, including HNSCC, and overexpression of laminin-332 has been associated with increased tumour invasiveness and more invasive tumour fronts of tongue SCC (Ziober et al. 2001, Ono et al. 1999). In addition, MT1-MMP can cleave laminin-332. The cleavage product of lam-332 γ2 chain then binds to epidermal growth factor receptor (EGFR) on cancer cell surfaces and promotes epithelial cancer cell migration (Koshikawa et al. 2000, Schenk et al. 2003). Trypsin-2 cleaved MT1-MMP and was most likely able to activate MT1-MMP in vitro. Because the N-terminus of MT1-MMP was blocked, however, most likely probably due to digestion circumstances, attempts to sequence the cleavage site failed. Similarly, the cleavage products could not by identified by mass spectrometry. Thus, the specific cleavage site of MT1-MMP by trypsin-2 remains unknown.

5. 2 Claudin-1 and -7 in oral carcinoma (II, IV)

Altered expression of different claudins correlates with cell invasion and carcinoma progression (Martin et al. 2009, Singh et al. 2010). In oral cancer, claudin-1 is over-expressed (Oku et al. 2006), whereas it is down-regulated in colorectal cancer (Martin et al. 2009). Claudin-1 was up-regulated here in invasive trypsin-2 overexpressing HSC-3 cells, which is in line with observations
where claudin-1 overexpression has been linked to increased invasion and the aggressive growth behavior of OSCC (Dos Reis et al. 2008). At the same time, claudin-7 expression was decreased in trypsin-2 overexpressing HSC-3 cells. Both the up-regulation and down-regulation of claudin-7 was associated with aggressive behavior and decreased survival of the OSCC patients, whereas claudin-1, although upregulated in cancer, did not have any association with prognosis.

Claudins can stimulate MT1-MMP-mediated proMMP-2 activation. Claudin-1 and MT1-MMP co-localize in cell-cell borders and cytoplasm (Miyamori et al. 2001). Claudin-1 provokes invasion of oral SCC cells by inducing the production of MT1-MMP and activation of MMP-2, which leads to increased cleavage of laminin-5 γ2 chains (Oku et al. 2006). In vitro MT1-MMP was able to process claudin-1 and -7. Because MT1-MMP and claudin-1 co-localize in vivo, it would be very tempting to conclude that MT1-MMP could break down TJs in some situations and thus disturb their function, which would lead to enhanced invasion.

![Figure 7. Effects of trypsin-2 on HSC-3 cell line](image_url)
5.3 Microarray results: a game of chance? (II)

Many microarray analyses have been counted out of OSCC samples, and there is considerable heterogeneity among the lists of affected genes (Choi et al. 2005, Erdem et al. 2008, Severino et al. 2008). In the microarray analysis performed here, many up-regulated genes in the trypsin-2 over-expressing cells were related to metabolism that is known to change during cancer progression (Fritz et al. 2010). To understand how much the analytical softwares would affect the results of the original data, the data was analyzed by two different data processing pipelines (EzArray and Chipster). The top 100 genes obtained by EzArray analysis consisted of 48 up- and 52 down-regulated genes. Of the 100 EzArray genes, 7 were not associated with a known function. Chipster analysis yielded 55 genes with significant changes (P<0.05): 25 up-regulated and 30 down-regulated. This indicates that the results of microarray data analysis can vary radically depending on the software used.

When we try to understand the significance of microarray results as a part of some biological systems, we need to understand that not all the genes that are changed are relevant. Some of the genes are secondarily altered by primary changes elsewhere (Miklos et al. 2004) and the same gene can be up-regulated in one cancer type or even cancer progression event and then down-regulated in another.

5.4 Effects of trypsin-2 and novel MMPI peptides on tongue SCC invasion (II, III)

Increased cell invasion, metastasis formation, and decreased apoptosis are typical features of aggressive cancer. The expression of trypsin-2 correlates with the metastatic potential and malignancy of tumours (Koivunen et al. 1990). Trypsin-2 overexpressing HSC-3 cells grew smaller tumours in nude mice than the control cells. However, they invaded more to surrounding fascia and, even mice that did not have clinically visible tumours had enlarged lymph nodes in trypsin-2 group. Unfortunately, real metastases could not be tracked from lymph nodes because of strict ending criteria of animal studies. Enlargement of the lymph nodes might be
a consequence of inflammation, as suggested by the presence of inflammatory cell reactions around invading cells or single or small clusters of carcinoma cells, termed “ occult metastases”, which are very common in tongue SCC patients. Furthermore, human subcutaneous tumours in mice metastasize very seldom (Cardiff 2010, Schuh 2004). Metastases are the main reason for cancer deaths. In the tongue SCC, the presence of occult metastases does not correlate with the tumour diameter, but will correlate with the tumour invasion depth (Jin et al. 2008). Results in mice support this data (study II results). Trypsin-2 transfection did not significantly change the proliferation rate of these cells and they were only slightly less apoptotic than control cells, but the cells invaded significantly better in the Transwell migration assay.

The invasion capability of carcinoma cells is traditionally analysed in basement membrane-like matrixes. One weakness of these models is that the matrix is not of human origin. Probably the most common in use is Matrigel, which is a protein matrix secreted by Engelbreth-Holm-Swarm mouse sarcoma cells (BD Biosciences). Its main components are laminin-111, collagen type IV, perlecan, and nidogen, but the structure and composition of in vivo basement membranes differ (Benton et al. 2010). Another commonly used invasion matrix is made of rat-tail type I collagen. In addition, human fibroblasts are often included in these matrices. The organotypic myoma model was used as an assay of carcinoma cell invasion. This model differs from those earlier mentioned because the animal-derived matrix is replaced by human uterine leiomyoma tissue obtained from routine surgical operations. This mimics very well the human tumour microenvironment because it contains ECM and BM components such as collagen I, III, IV, and laminins, in addition many cell types, such as smooth muscle cells, fibroblasts, lymphocytes, macrophages, and endothelial cells (Nurmenniemi et al. 2009). Trypsin-2 transfected cells invaded significantly better in this environment than control cells.

Since the most important role of MMPs in cancer progression was long thought to be exclusively destructive, the main goal of MMP research had long been to develop a working MMP inhibitor that would be effective and easy to dose.
Unfortunately, after numerous promising studies and unsuccessful clinical trials only one MMP inhibitor, Periostat (doxycycline hydrat), is in clinical use against periodontitis in the United States (Coussens et al. 2002). The main reasons for failure of the first MMPIs have been the lack of drug specificity, (because they also inhibited ADAMS family proteins), the use of late-stage cancer patients in clinical trials (whereas many preclinical studies demonstrated that MMIP should be used in early stage of cancer), and too low dosages in clinical trials (because of the side effects such as musculoskeletal pain and poor in vivo stability of some drugs) (Coussens et al. 2002, Sparano et al. 2004, Yiotakis et al. 2008).

Now that it is known that MMPs widely interact in biological functions, more targeted and specific MMP-inhibitors have been developed. An antigelatinalytic CTTHWGFTLC (CTT) peptide was developed and later its hydrophilic counterpart GRENYHGCTTHWGFTLC (CTT2) (Koivunen et al. 1999, Heikkilä et al. 2006). They inhibited cell invasion and the antigelatinalytic activity of tongue SCC (HSC-3 cells) in vitro and in vivo. CTT2 suppressed tumour growth and intravasation in mice xenograft tumours (Heikkilä et al. 2006). CTT targeted to the tumour microvasculature and also inhibited the tumour growth for breasts carcinoma, ovarian carcinoma, and Kaposi’s sarcoma in mice (Koivunen et al. 1999).

Increased gelatinalytic activity is associated with increased invasion but not metastasis formation in OSCC (Ikebe et al. 1999). OSCC xenograft tumours in mice showed similar results after antigelatinalytic CCT treatment. Primary tumours were smaller but CTT did not affect the tumour spread. When combination drug therapy, with mixture of antigelatinalytics and proMMP-9 inhibitor, was used the outcome was not improved compared to the CTT group. This raises the question that gelatinases might have a different role in cancer progression to what had been assumed and new studies have already been published that support this new concept (Bendrik et al. 2008, Garg. et al. 2010, Luukkaa et al. 2010, Stokes et al. 2010). Non-proteolytic invasion might also be more powerful than thought. More experiments are needed so that a satisfying conclusion can be drawn.
Even though the results from the earlier mentioned CTT studies did not yield the desired outcome, i.e. inhibition of metastasis, CTT might however have a role in the battle against cancer. CTT has been shown to target tumour microvasculature *in vivo* (Koivunen *et al.* 1999) and when fused with green fluorescent protein (GFP) it did not lose this character. So CTT-GFP fusion protein might be an interesting option in tumour imaging pre- and perioperatively in the future.

The expression of MT1-MMP has been connected with malignancy. In cancer cells, this membrane-bound protein is localized to the leading edge of migratory cells. Selective MT1-MMP inhibitor GACFSIAHECGA- peptide or peptide G has shown promising results. It inhibited the migration and invasion of cancer cells *in vitro* and *in vivo*, and decreased tumour size and increased the survival of mice with xenograft tumours. It also inhibited the MT1-MMP mediated activation of MMP-2 in HSC-3 OSCC cell line (Suojanen *et al.* 2009). In the tumour microenvironment mimicking myoma invasion model peptide G decreased invasion and collagen I fragmentation. Current results are promising but further studies are required to determine the real potential of this new potential drug candidate for cancer treatment.
1. CONCLUSIONS

1. The localization and compartmentalization of MMP-9 and its activator, trypsin-2, is different in OSCCs compared to bone tumours. All of these tumours produced both MMP-9 and trypsin-2, but MMP-9 and trypsin-2 were co-localized within the carcinoma cells and were co-compartmentalized within the same intracellular vesicles only in OSCCs. Furthermore, these results proved for the first time that enterokinase, the physiological activator of trypsin-2, was present in OSCC. Enterokinase also cleaved directly proMMP-9, but this processing did not result in MMP-9 activation. These results might explain the aggressive and invasive behaviour of OSCC.

2. Trypsin-2 overexpression makes OSCC cells more invasive and plastic in vitro and in vivo by activating MT1-MMP, cleaving laminin-332, changing gene expression profile, and cleaving claudin-7.

3. Antigelatinolytic therapy with CTT peptide was found to inhibit the growth of xenograft tumours but it had no effect on cancer spread; however, CTT fused with GFP could be a promising new tool in tumour imaging pre- and perioperatively.

4. Claudin-7 showed a statistically significant difference in immunohistochemical staining intensity and cause-specific patient survival in the invasive front of the tumours (mobile tongue SCC). High staining intensity of claudin 7 was also noted to be associated with decreased patient survival in the invasive fronts of the tumours when compared to same intensity staining. Claudins -1, -4, and, -5 and occludin showed no statistical association between staining intensity and cause-specific patient survival. The use of immunohistological analysis of claudin-7 expression levels of mobile tongue SCC might be useful for predicting the behavior of the tumour and patient outcome.
ACKNOWLEDGEMENTS

This study was carried out at the Department of Cell Biology and Oral Diseases, Biomedicum Helsinki, Institute of Dentistry, University of Helsinki and at the Department of Diagnostics and Oral Medicine, Institute of Dentistry, University of Oulu, during the years 2004-2012. Animal experiments were performed at the Viikki Laboratory Animal Center of the University of Helsinki. I thank the institute of Dentistry and Dean Jarkko Hietanen, for providing the facilities to my research.

I’m very thankful that I had an opportunity to have three amazing supervisors, Professor Timo Sorsa, Professor Tuula Salo, and Pia Nyberg, PhD. Without their help, this thesis would never have been possible. I want to thank Timo for his endless optimism, Tuula for her millions of ideas for new experiments and patience, and Pia for her tireless help with all the little details of experiments and the writing process.

I’m very grateful to Professor Jorma Keski-Oja and Professor Tuomo Karttunen for their careful revision of my thesis. I also want to thank my Thesis Committee members Professor Jorma Virtanen (in memoriam), Professor Jorma Keski-Oja, and Docent Petri Mattila for giving me advice and new ideas on my thesis project.

I wish to thank all the members of our research group especially: Docent Taina Tervahartiala, for the help of all areas of labwork, Juho Suojanen, PhD, for good advice on how to survive the PhD and Matti Laaksonen, PhD, for his easy-going attitude.

Special thanks go to my friends Heidi Holappa, DDS, Immi Kormi, DDS, Lotto Veistinen, DDS, and Eija Salminen, DDS, PhD. They have encouraged me and shared my scientific up- and downhills.

I acknowledge Ritva Keva, Jukka Inkeri, and Marjatta Kivekäs, as well as Maija-Leena Lehtonen, for their excellent technical assistance. I also want to thank
Docent Pirkko Pussinen for amazing statistical skills, Kirsti Kari, MSc, the head of the Scientific Laboratory, for organizing my basic working needs and also all of my colleagues and staff at the Institute of Dentistry.

I want to also thank all my collaborators: Docent Mika Hukkanen, Meeri Sutinen, PhD, Merja Ylipalosaari, PhD, Professor Anders Bjärtell, Annukka Paju, PhD, Virpi Haaparanta, Professor Ulf-Håkan Stenman, Fidel Salas, Professor Juha Risteli, Outi Itkonen, PhD, Docent Hannu Koistinen, Pia Heikkilä, PhD, Oula Penate-Medina, PhD, Per Ej Saris, Docent Jaana Hagström, Tanja-Maria Ranta, M.Sc., Justus Reunanen, PhD, Ibrahim Bello, PhD, Professor Ahti Niinimaa, Saara Kantola, PhD and Professor Ylermi Soini.

My warmest thanks go to Anna, and finally, I want to thank my brother Samuli and my parents Kaija and Seppo; especially my mum who warned me in the beginning that it is not going to be easy. She was right, but I managed.

This thesis study was supported by the Finnish Dental Society Apollonia, University of Helsinki Funds, HUCH- and OUCH-EVO grants.
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