Pirjo Åström

REGULATORY MECHANISMS MEDIATING MATRIX METALLOPROTEINASE-8 EFFECTS IN ORAL TISSUE REPAIR AND TONGUE CANCER
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Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium F101 of the Department of Physiology (Aapistie 7), on 14 November 2014, at 12 noon
Abstract

Tissue repair and cancer progression involve similar mechanisms, including degradation of extracellular matrix in which matrix metalloproteinases (MMPs) play essential roles. The action of MMPs is important in normal physiological processes but MMPs also contribute to various pathological conditions.

MMP-8 belongs to a family of collagenases with a diverse set of substrates. MMP-8 action is involved in skin wound healing and in various human cancers. The function of MMP-8 in cancer appears to be highly complex and varies depending on the cancer type and location. Little is known about the involvement of MMP-8 in oral physiology and pathology. The aim of this study was to clarify the role of MMP-8 in oral tissue repair and oral tongue squamous cell carcinoma (OTSCC).

Studies with MMP-8 deficient mice revealed that the function of MMP-8 in tissue repair is highly dependent on the spatial aspects. In alveolar bone, MMP-8 increased inflammation and affected collagen metabolism. In tongue wounds, MMP-8 impaired early healing and reduced transforming growth factor (TGF-β1) levels. This study also revealed the protective role of MMP-8 in OTSCC patients, in agreement with previous studies indicating positive features of MMP-8 in cancer. Low MMP-8 level and high vascular endothelial growth factor (VEGF)-C levels in tumors correlated with worse prognosis in these patients.

In mouse tongue fibroblast cell cultures, MMP-8 reduced TGF-β1 signaling molecule phosphorylated Smad2 levels and impaired the collagen contraction ability. TGF-β1, apoptosis factor Fas-ligand (Fas-L) and estrogen receptors (ERs) were identified as novel MMP-8 substrates. In OTSCC cell cultures, MMP-8 impaired cell migration and invasion. Diminished TGF-β1 levels were involved in the defective migration of MMP-8 overexpressing cells. Moreover, MMP-8 affected the expression of MMP-9, MMP-1, cathepsin-K, VEGF-C and TGF-β1. In mouse models of OTSCC, MMP-8 protected against tumor development but was not able to prevent metastasis formation.

The main findings of this study were that 1) MMP-8 action in tissue repair depends on the site of the injury and 2) in OTSCC, MMP-8 has tumor suppressive effects, but in mouse, MMP-8 does not inhibit metastasis formation. In addition, 3) four novel MMP-8 substrates (TGF-β1, Fas-L, ER-α and -β) were identified that may explain the spatial and diverse roles of MMP-8.

Keywords: fibroblasts, matrix metalloproteinases, squamous cell carcinoma, tongue neoplasms, wound healing
Åström, Pirjo, Matriksin metalloproteinaasi-8:n vaikutuksia välittävät säätemekanismit suun kudosvaurioiden paranemisessa sekä kielisyövässä.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Hammaslääketieteien laitos, Diagnostiikka ja suun limakalvosairaudet; Medical Research Center Oulu; Finnish Doctoral Program in Oral Sciences

*Acta Univ. Oul. D 1263, 2014*

Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

**Tiivistelmä**

Kudosvaurioiden paranemiseen ja syövän etenemiseen liittyvät useita samankaltaisia mekanismeja. Molemmissa toimivat soluväliitamin muokkaamiseen osallistuvat proteasit, joista matriksin metalloproteinaaseilla (MMP) on tärkeä merkitys; ne osallistuvat lukuisiin elimistön keskeisiin fysiopatologisiin prosesseihin.

Kollagenaasi MMP-8 muokkaa monentyyppisiä molekyylejä. Se on mukana ihohaavan paranemisessa ja useissa syövissä. MMP-8:n toiminta syöpätiloissa on hyvin moninainen riippuen syöpätyyppistä ja sijainnista. Väittöstudiumissa selvityttiin MMP-8:n merkitystä suun kova- ja pehmytkudosvaurioprosesseissa sekä kielisyövässä, joissa se on ollut tuntematon.

MMP-8 poistogeeneissä hiirillä tehdyissä pehmyt- ja kovakudoshaavoissa MMP-8:n vaikutusmekanismit riippuvat kohdekudoksesta. Alveoliluun paranemisen yhteydessä MMP-8 lisäsi tulehdusta ja osallistui kollageenin muokkaamiseen. Akuutin kielihaavan paranemisessa MMP-8 hidasti haavan umpumusta ja vähensi transformoivan kasvutekijä-β1:n (TGF-β1) määrää. Kuten useissa muissakin syövissä, myös kielisyövässä todettiin MMP-8:lla olevan suojavaiva vaikutus. Potilaan ennuste huononi, jos kasvainsolukon matala MMP-8-taso yhdistyi korkeaan verisuonten kasvutekijä-C:n (VEGF-C) määrään.


Tässä väittökirjatutkimuksessa on kolme päälöydöstä: 1) MMP-8:n vaikutus kudoksen paranemisprosessen riippuu vauriokohdasta, 2) MMP-8 on kielisyövän kehittymisessä puolustusmekanismi- line molekyli, mutta sen lisääntynyt tuotto ei hiirikokeissa estänyt etäpesäkkeiden muodostusta, 3) MMP-8:illä löydettiin neljä uutta kohdemolekyyliä (TGF-β1, Fas-L, ER-α ja -β), joiden muokkaus saattaa osin selittää MMP-8:n monipuoliset kudos- ja prosessiesitteet vaikutukset.

**Asiasanat:** fibroblastit, haavan paraneminen, kielien kasvaimet, levyepiteelikarsinooma, matriksin metalloproteinaasit
Acknowledgements

This study was carried out at the Department of Diagnostics and Oral Medicine, Institute of Dentistry, University of Oulu. I thank the head of the Institute, Prof. Pertti Pirttiniemi, DDS, PhD, for providing the research facilities and an opportunity to conduct this study.

I am deeply thankful for my supervisor, Prof. Tuula Salo, DDS, PhD, for her support and patience during these years. Her vast knowledge combined with brilliant ideas and enthusiasm for science is admirable and inspiring. Her encouragement and trust gave me the strength to overcome the moments of disbelief. She was always available when I needed help. I am thankful for all the cheerful and genuine moments that I have shared with her!

I am grateful for Emma Pirilä, MSc, PhD, and Meeri Sutinen, MSc, PhD, for providing valuable guidance during the years. I wish to express my thanks to Emma for being the key person to introduce me into the world of research with her inspiring attitude in science. I deeply thank Meeri for her valuable support and expert assistance in cell experiments with her friendly and calm touch. I owe my thanks to Prof. Timo Sorsa, DDS, PhD, for his assistance in planning the experiments and critical review of the original studies. I also thank Prof. Solve Hörkkö, MD, PhD, for her warm-hearted support and guidance. I am grateful for the thesis follow-up group members Prof. Ylermi Soini, MD, PhD, and Docent Ritva Heljäsvaara, MSc, PhD, for their valuable ideas and guidance on the way.

I thank Prof. Veli-Matti Kähäri, MD, PhD, and Docent Marko Hyytiäinen, MSc, PhD, for critical revision of my thesis and valuable comments. Pertti Väyrynen, MA, PhD, and Deborah Kaska, MSc, PhD, are acknowledged for the careful language revision and Saana Piltonen for Finnish language check.

All the other co-authors are acknowledged for their valuable contribution to this study: Carolina Bitu, Soili Kallio-Pulkkinen, Saara Kantola, Joonas Kauppila, Virva Kervinen, Jarkko Korpi, Maija Lahtinen, Niko Lehtonen, Riitta Lithovius, Esa Lääärä, Helinä Mäklin, Pia Nyberg, Ari Ristimäki, Sirpa Salo, Marja Siponen, Sini Skarp, Leo Tjäderhane, Otto Väyrynen, Anu Väänänen and Merja Ylipalosaari in Oulu, Heidi Heikkola and Suvi-Tuuli Vilen in Helsinki, Nilva Cervigne, Ricardo Coletta and Marcela Hernández in Brazil, Elin Hadler-Olsen and Gunbjorg Svineng in Norway, Gareth Thomas in U.K. and Carlos López-Otín in Spain. I owe special thanks to Prof. Coletta, DDS, PhD, for providing me an opportunity to visit his lab in Piracicaba, Brazil and Michelle Agostini, DDS, PhD, for introducing me a valuable new method there. I thank Hannu
Vähänikkilä, MSc, and Paula Pesonen, MSc, for their assistance in statistical analyses and Seija Leskelä for her expertise in figure preparations. I also thank the personnel at the Laboratory Animal Center for their kind assistance with the animal experiments.

I owe my warm thanks to the rest of the team in Oulu, current and previous, Ilkka Alahuhta, Ehsanul Hogue Apu, Ibrahim Bello, Priscila Campioni, Hanna-Leena Jämsä, Johanna Korvala, Arja Kullaa, Sini Nurmenniemi, Virve Pääkkönen, Maija Risteli, Aleksi Rytönen, Elias Sundqvist and the younger generation of the team for creating the friendly working atmosphere. I especially thank Sini and Carolina for their friendship and for the nice moments and supportive conversations at the office. I am grateful for the skillful technicians Maritta Harjapää, Sanna Juntunen, Eeva-Maija Kiljander, Tanja Kuusisto, Maija-Leena Lehtonen, Henna-Riikka Niskala and Merja Tyynismaa for their expert and kind assistance. Mallu and Emuli are especially acknowledged for their significant technical assistance in this study and committed attitude for the work.

It has been my pleasure and honor to be part of this great group!

I owe my thanks to Anna, Anni and Johanna for sharing the first trip into biochemistry and being supportive friends since then. All my friends outside the research world are thanked for sharing valuable non-scientific time. Annukka and Mellu deserves special thanks for offering cheerful moments that have been a guaranteed time out of this project.

My warmest thanks to my parents Arja and Veikko and my siblings Heidi, Heikki, Piia and Tiina for their care and support. Heidi deserves extra acknowledgement for her artistic skills and assistance with the figures. I thank my parents-in-law Anna-Greta and Paavo for their warm-hearted support and the rest of the family-in-law for their support in many ways. My special thanks for all the family members for taking care of the children. You have been of priceless help!

I express my deepest appreciation and loving thanks to Mikko for his love, support and endless understanding. Finally, all my gratitude for my beloved children, the wisest supervisors of my life, Lassi, Taneli, Eevi and Santeri, for sharing the happy and exciting everyday life.

This study was financially supported by Finnish Doctoral Program in Oral Sciences, Finnish Dental Society Apollonia, Cancer Foundation of Northern Finland and Ida Montin Foundation, all which are gratefully acknowledged.

Oulu, September 2014

Pirjo Åström
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>C1</td>
<td>human oral squamous cell carcinoma cell line</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>CTT</td>
<td>CTTHWGFTLC peptide</td>
</tr>
<tr>
<td>CXC</td>
<td>chemokine C-X-C motif</td>
</tr>
<tr>
<td>CXCL</td>
<td>chemokine C-X-C motif ligand</td>
</tr>
<tr>
<td>ECIS</td>
<td>electric cell-substrate impedance sensing</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td><em>e.g.</em></td>
<td><em>exempli gratia</em></td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>Fas-L</td>
<td>fas-ligand</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FN</td>
<td>fibronectin</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HNSCC</td>
<td>head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HPV</td>
<td>human papilloma virus</td>
</tr>
<tr>
<td>HSC-3</td>
<td>human squamous cell carcinoma cell line</td>
</tr>
<tr>
<td>HSPG</td>
<td>heparan-sulfate proteoglycan</td>
</tr>
<tr>
<td>ICTP</td>
<td>carboxyterminal telopeptide of type I collagen</td>
</tr>
<tr>
<td>IIINTP</td>
<td>aminoterminal telopeptide of type III collagen</td>
</tr>
<tr>
<td>IFMA</td>
<td>immunofluorometric assay</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP-10</td>
<td>interferon inducible protein-10</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KGF</td>
<td>keratinocyte growth factor</td>
</tr>
</tbody>
</table>
LIX lipopolysaccharide-induced CXC chemokine
LTBP latent TGF-β binding protein
MCP monocyte chemotactic protein
MIG monokine induced by interferon-gamma
MIP macrophage inflammatory protein
MiR micro RNA
MMP matrix metalloproteinase
Mmp8−/− matrix metalloproteinase-8 deficient
MMPI MMP inhibitor
MPO myeloperoxidase
mRNA messenger RNA
MT-MMP membrane-type matrix metalloproteinase
NO nitric oxide
OSCC oral squamous cell carcinoma
OTSCC oral tongue squamous cell carcinoma
PG proteoglycan
PMN polymorphonuclear neutrophil
PSMAD phosphorylated Smad
rh recombinant human
RT-PCR reverse transcriptase polymerase chain reaction
SCC squamous cell carcinoma
SDS sodium dodecyl sulfate
shRNA small hairpin RNA
SLC small latent complex
TATA box cis-regulatory element
TGF transforming growth factor
TIMP tissue inhibitor of metalloproteinase
TMA tissue microarray
TME tumor microenvironment
TNF tumor necrosis factor
TNM tumor, node, metastasis
TUNEL terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF vascular endothelial growth factor
wt wild type
Original publications

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


*Equal contribution
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1 Introduction

Tissue repair and cancer progression comprise largely the same cellular processes, actions of various bioactive molecules and degradation of extracellular matrix (ECM). In all these events, matrix metalloproteinases (MMPs) play essential roles. The controlled action of MMPs is important in completion of normal physiological processes, but disturbed proteinase balance is involved in various pathological conditions. Traditionally MMPs have been considered devastating factors in the progress of pathological conditions. Our current understanding of the mechanisms by which MMPs participate in these processes includes much more than the degradation of ECM structural macromolecules. MMPs are known to regulate a wide range of specific substrates, ranging from growth factors to cell adhesion molecules, and thereby affect basically all cellular processes. Because of the versatility of MMP substrates and specific features of various cells and tissues, the mechanisms of MMP actions are even more complicated and seem to vary spatially and temporally.

Matrix metalloproteinase-8 (MMP-8) belongs to a group of collagenases that degrade fibrillar collagens with high efficiency. In addition, a number of other structural proteins as well as bioactive factors have been identified as MMP-8 substrates. MMP-8 is strongly connected to inflammatory conditions and it was originally identified from polymorphonuclear neutrophils (PMN). Thereafter, MMP-8 production was also detected in other cells, e.g. in fibroblasts and oral squamous cell carcinoma (SCC) cells.

The role of MMP-8 in skin wound healing involves regulation of the inflammatory response, and in the absence of MMP-8 activity, mouse wound healing is delayed. However, MMP-8 seems to be involved also in the regulation of inflammatory-independent cellular processes and was among the first MMPs found to exhibit protective effects in cancer. Although a growing number of studies indicate that MMP-8 mediates beneficial effects in various human cancers, some studies indicate the opposite. Almost nothing is known about the role of MMP-8 in oral wound healing and oral tongue squamous cell carcinoma (OTSCC). The incidence of OTSCC is constantly increasing, and unfortunately, it is still characterized by poor prognosis. The early diagnostics and recognition of the most aggressive carcinomas is of high priority in order to improve the treatment planning. Resolving the role of MMP-8 in normal oral tissue repair could also lead to an understanding of its roles in OTSCC. Defining the molecular basis of disease progression provides essential knowledge needed for the
development of novel clinical tools for diagnostics as well as the treatment of cancer.

The role of MMP-8 in normal oral tissue repair, including healing of hard and soft tissue wounds, and in OTSCC was of particular interest in this study. The work was conducted by carrying out three experimental oral tissue repair models with MMP-8 deficient (\textit{Mmp8-/-}) mice. Human OTSCC samples were examined to evaluate the potential association of MMP-8 with disease related variables. To evaluate the role of MMP-8 in OTSCC progression, two different models of mouse OTSCC were used. In addition, to verify the effects of MMP-8 on the behavior of cells from the oral cavity \textit{in vitro}, mouse tongue derived fibroblasts and human OTSCC cell cultures were examined using diverse methods. The study was expected to provide novel knowledge about the role of MMP-8 in the oral environment and in the behavior of oral derived cells, and hence increase our overall understanding of the molecular mechanisms of MMP-8 activity in oral tissue repair and OTSCC.
2 Review of the literature

2.1 Wound healing

Successful healing of a wound proceeds through a highly controlled interplay of cells of distinct tissue patterns and involves a complex signaling network comprising various growth factors and cytokines. Efficient wound healing first protects the host from pathogens and blood loss and then step-wise regenerates the injured tissue. The process also requires the strictly controlled action of tissue degrading proteases and their natural inhibitors. (Broughton et al. 2006, Steffensen et al. 2001).

Traditionally, the wound healing process is divided into three interdependent and temporally overlapping phases that are presented in Figure 1: 1) hemostasis and inflammatory phase, 2) proliferative phase (re-epithelialization and granulation tissue formation), and 3) remodeling phase (Broughton et al. 2006). The original wound size substantially affects the length of each phase and the overall healing time.

Skin wound healing has been studied extensively and the literature is based mainly on the knowledge of dermal wounds. However, other tissues undergo the same basic cell biological events in the healing process, although some tissue-specific variations do exist. (Häkkinen et al. 2000).
Fig. 1. The main events of the three overlapping phases of wound healing: hemostasis and inflammatory phase (I), proliferative phase (II) and remodeling phase (III). The duration of each step depends on various factors including wound location and size. The overall healing time in wounds of the oral mucosa is substantially shorter than those of skin wounds. The remodeling of hard tissue (bone) however may last for years.

**Hemostasis and inflammatory phase**

Immediately after wounding, tissue injury activates the hemostasis cascade that is aided by vasoconstriction (= narrowing of the blood vessels) (Stadelman et al. 1998). Aggregated platelets form a clot that consists of matrix proteins, including, for example, collagen, thrombin, fibrin and fibronectin (FN). This non-structured matrix prevents further bleeding and serves as an initial scaffold for the cells that invade the wounded area. The injury triggers a release of inflammatory factors from platelets that play an essential role in the initial step of healing and
participate in the formation of a provisional matrix. The early inflammatory signals also result in vasodilation that facilitates cellular traffic into the site of injury. (Broughton et al. 2006, Midwood et al. 2004).

The inflammatory response begins in the very early moments of healing. It ensures the protection against pathogens but also results in the activation of other cellular events mediated by the release of important cell signaling molecules. (Broughton et al. 2006). The factors influencing the initiation of inflammation by the influx and activation of leukocytes include, e.g. extracellular matrix (ECM) fragments, antimicrobial peptides, cytokines, lipid mediators and receptors (Parks et al. 2004). Acute inflammation in response to tissue injury is an essential physiological event to prevent infection. However, previous studies suggest that no single inflammatory cell type is essential for healing (Dovi et al. 2003, Egozi et al. 2003, Szpaderska et al. 2003), but instead, they may actually interfere with the wound healing rate and matrix deposition (Dovi et al. 2003, Martin et al. 2003). However, the importance of inflammation, in general, is emphasized by the fact that people with genetic deficiencies in crucial components of the inflammatory response are vulnerable to infections, and without treatment, infection is life threatening (Bunting et al. 2002). These deficiencies may also result in delayed wound healing, indicating the importance of inflammation in tissue repair (Etzioni et al. 2009, Peters et al. 2005, Wada et al. 2011).

The recruitment of the first inflammatory cells, polymorphonuclear neutrophils (PMN), starts within minutes after injury. They are drawn by the response to various cytokines and bacterial products. (Brinkmann et al. 2004, Jones 2004). Neutrophils are primarily responsible for phagocytosis of the bacteria and cellular debris. They release proteases, such as matrix metalloproteinases (MMPs), that digest the ruptured tissue and bacteria. (Martin & Leibovich 2005, Steffensen et al. 2001). In addition, reactive oxygen free radicals generated by neutrophils assist in cleansing and sterilizing the injury site (Yager & Nwomeh 1999). Once the neutrophils have accomplished their task in the most acute phase of inflammation, monocytes arrive into the wounded area and transform into macrophages. They continue removing the pathogens by phagocytosis and synthesizing nitric oxide (NO) that forms toxic compounds to kill bacteria. (Martin & Leibovich 2005, Witte & Barbul 2002). Moreover, activated macrophages participate in the initiation of the proliferative phase by producing various growth factors and cytokines [such as transforming growth factors (TGF) -β and -α, vascular endothelial growth factor (VEGF), interleukin
(IL)-1 and tumor necrosis factor (TNF)-α that mediate angiogenesis and fibroplasia (Leibovich & Ross 1976, Rappolee et al. 1988).

**Proliferative phase**

The next phase of the healing process involves re-epithelialization by epidermal keratinocytes and begins concurrent with the inflammatory phase. During re-epithelialization a new extracellular matrix called granulation tissue is formed. Keratinocytes arise from the basal layers of the wound edges and when exposed to the new provisional matrix, change from a stationary to a migratory phenotype. (Odland & Ross 1968). Sweat glands and hair follicles are also sources of keratinocytes in skin, but not in mucosa (Häkkinen et al. 2000). Epidermal growth factor (EGF) and TGF-α, for example, stimulate the epithelial cells to proliferate and further construct the protective barrier. Angiogenesis also starts at this stage as endothelial cells are stimulated to proliferate and form new capillaries in response to VEGF that is produced by various cells such as keratinocytes. (Bao et al. 2009, Barrientos et al. 2008).

A granulation tissue consisting of newly formed blood vessels, inflammatory cells (such as macrophages) and proliferating fibroblasts begins to form (Singer & Clark 1999). The fibroblasts are thought to invade into the injured area from the undamaged surrounding tissue (Hinz et al. 2007). However, there are also other sources of fibroblasts such as pericytes that are residents of vascular endothelium of capillaries and bone marrow mesenchymal progenitor cells (Häkkinen et al. 2000, Opalenik & Davidson 2005). Although the functions of fibroblasts from different sources may vary depending on their origin, one of the most important tasks of these cells is the production of matrix components, such as collagens, which serve to contract the wound matrix in granulation tissue to ensure rapid wound closure (Gabbiani et al. 1971, Häkkinen et al. 2000, Ruszczak 2003). During the wound repair process, fibroblasts undergo a phenotypic change from a quiescent state to become proliferative and migratory, and finally, as the healing proceeds further into a matrix producing, stationary but wound contracting phenotype (Darby & Hewitson 2007). Moreover, fibroblasts produce keratinocyte growth factor (KGF)-1, KGF-2 and IL-6 that drive keratinocyte migration, proliferation and differentiation in the epidermis (Broughton et al. 2006).
**Remodeling phase**

In the final stage of healing, the wound is fully re-epithelialized and the most time consuming phase involving matrix reorganization begins. The injured area starts to increase the elasticity and strength of the tissue. During the matrix deposition, glycosaminoglycans (GAGs), proteoglycans (PGs) and FN produced by fibroblasts are replaced with type I collagen. In granulation tissue, type III collagen predominates (Namazi et al. 2011), but eventually type I collagen becomes the major collagen type in the healed area. (Broughton et al. 2006). A secretion of TGF-β1 by, e.g. wound macrophages stimulates the transformation of fibroblasts into α-smooth muscle actin (α-SMA) producing wound contracting myofibroblasts (Hinz et al. 2007, Singer & Clark 1999). The final phase of healing also includes scar formation, which is a particular feature of skin wounds. A continued low rate of synthesis and catabolism of collagen is needed for the transition from granulation tissue to scar in a process that requires the action of MMPs. (Broughton et al. 2006, Singer & Clark 1999). Scar formation is characterized by the accumulation of ECM that is rich in collagen and FN. Additionally, the arrangement of collagen fiber bundles and elastic fibers in the scar differs from that of normal healthy tissue. (Larjava et al. 2011). Eventually, the scar strength will comprise only approximately 70–80% of the normal skin strength. (Broughton et al. 2006, Singer & Clark 1999). The mechanism of scar-free healing of oral mucosa and fetal skin wounds has been extensively studied. Identifying the key features leading to scarless repair could assist in developing methods for skin scar prevention or treatment of chronic wounds.

### 2.1.1 Special characteristics of mucosal wound healing

Although wound healing proceeds through the same basic mechanisms in various tissue compartments, some differences do exist. For example, scar formation is typical in dermal wounds, whereas oral mucosal wounds are clinically distinguished by faster and basically scar-free wound healing. In addition, the composition and organization of the ECM deposited differ in mucosal wounds and skin wounds. (Larjava et al. 2011).

Murine tongue wounds heal rapidly and with slight inflammation (Chen et al. 2010, Schrementi et al. 2008, Szpaderska et al. 2003). Likewise, wounded human fetal skin xenografted into mouse is characterized by decreased inflammation with diminished levels of pro-inflammatory cytokines, compared to adult skin.
(Liechty et al. 1998, Liechty et al. 2000). Fetal wound healing also progresses rapidly with minimal scar formation (Whitby & Ferguson 1991). Interestingly, fetal fibroblasts show similarities to oral mucosal fibroblasts (Larjava et al. 2011, Schor et al. 1996). One of the most studied factors mediating the scar formation related events is TGF-β1. The levels of TGF-β1 are low both in oral mucosal and fetal wounds compared to adult skin wounds. (Lin et al. 1995, Szpaderska et al. 2003). TGF-β1 has also been shown to upregulate type III collagen expression in non-scarring fetal fibroblasts compared to post-natal fibroblasts (Rolfe et al. 2007). A wound healing study with mouse fetuses suggested that IL-10, which activates macrophages, plays a critical role in reducing both inflammation and scarring in fetal repair (Liechty et al. 2000). However, the mechanisms of mucosal wound healing are not completely established and even mucosal wounds of different origins in the oral cavity have their own special characteristics (Larjava et al. 2011).

### 2.1.2 Tooth extraction socket wound healing

Tissue repair after tooth removal follows the basic wound healing steps described above. However, some unique features also exist since the process involves both hard and soft tissue healing. (Cardaropoli et al. 2003, Hollinger & Wong 1996, Lin et al. 1994). The formation and maturation of a blood clot starts in the extraction socket immediately after tooth removal (Amler 1969, Lin et al. 1994). As in the case of skin injury, this first matrix serves as a protective barrier against foreign bodies and pathogens in the oral cavity, but also as a mediator of cell signaling molecules, as well as a base for cell movement (Lin et al. 1994, Witte & Barbul 1997). The infiltrating fibroblasts produce matrix molecules and replace the coagulation. The extraction socket filled with a connective tissue and inflammatory cells is covered by epithelium. As the healing proceeds, the socket walls will be resorbed by osteoclasts and new trabecular bone begins to form. (Araujo & Lindhe 2005, Cardaropoli et al. 2003). The final recovery of the extraction socket is time consuming as the newly formed tissue in the extraction socket is first filled with woven bone and eventually replaced by lamellar bone and marrow (Kingsmill 1999).
2.1.3 Tooth apical periodontitis

Tooth apical periodontitis is an inflammatory state with destruction of periradicular tissues. It rises from persistent inflammation or infection of dental pulp called pulpitis. Pulpal tissue is exposed to pulpitis by several outside threats such as microbes, and chemical or mechanical irritants. The inflammation in pulpal tissue usually proceeds slowly. The inflamed granulation tissue is rich in various inflammatory cells (neutrophils, monocytes/macrophages, lymphocytes, eosinophils) and the cellular damage initiates the release of inflammatory mediators. The inflammatory cells recruit other cells to the injured area, including osteoclasts that cause degradation of root apex area bone. Inflammatory mediators stimulate the release and action of proteolytic enzymes, such as MMPs and serine proteases, that participate in the destruction of the periodontal ligament. (Graunaite et al. 2012).

2.2 Carcinomas

Carcinoma is a common nomenclature for the cancer arising from squamous epithelial cells. It is the most common type of human cancer, other types being, e.g. adenocarcinomas (arising from non-squamous epithelia), sarcomas (mesenchymal origin), leukemias (hematopoietic cells) and lymphomas (lymphoid tissue). Cancer, in general, is considered as a pathological condition in which a group of cells in the body is no longer under normal control. Cancer results from genetic alterations in tumor cells that are caused by external mutagens and inheritable factors, for example. However, development into a malignant tumor is a highly complex and progressive process and requires various other aspects, including cellular and environmental factors. (Hanahan & Weinberg 2011). As the carcinoma progresses, the cells grow in an uncontrolled manner and finally invade across the epithelial basement membrane (BM) into surrounding tissue. Metastases from the primary tumor through lymphatic or blood veins eventually cause most of the carcinoma related deaths. (Kalluri 2003).

It has long been recognized that carcinomas and their surrounding stroma include cellular and molecular events that closely resemble processes of wound healing (Schäfer & Werner 2008). Harold Dvorak noted that tumors are “wounds that do not heal” (Dvorak 1986). Several clinical observations support the assumption that previous injuries are preconditions for tumorigenesis (Schäfer & Werner 2008). In addition, molecular evidence supports this assumption. The
gene expression profiles of fibroblasts exposed to serum show similarities to those of tumor cells exposed to their stroma (Chang et al. 2004). The tissue repair models and tongue carcinoma examined in mouse in this study are illustrated in Figure 2.

**Fig. 2. The oral phenomena and locations of interest in this study.**

### 2.2.1 Tumor microenvironment

The tumor microenvironment (TME) consists of various cells, including proliferating tumor cells, fibroblasts, endothelial cells, leukocytes, macrophages and adipocytes as well as ECM and blood vessels (Whiteside et al. 2008). The composition of tumor-derived ECM is biochemically distinct from normal ECM (Bissell & Radisky 2001, Butcher et al. 2009). The interaction between the cancer cell and its surroundings partly occurs through transmembrane receptors. Hence, the tumor itself influences its surroundings (and *vice versa*), and the TME cells accelerate the tumor-promoting processes, such as angiogenesis, inflammation,
ECM remodeling and invasion by producing growth factors, cytokines and proteinases. (Pietras & Ostman 2010).

2.2.2 Oral tongue squamous cell carcinoma (OTSCC)

The majority of the cancers in the head and neck region are located in the oral cavity (Scully et al. 2009). Cancer of the oral cavity (including pharynx) is estimated to be the tenth most common malignancy in the U.S. (Siegel et al. 2013). Oral cancers comprise the neoplastic disorders of the oral cavity, including lip, buccal mucosa, floor of the mouth, lower and upper alveolar ridges, oropharynx, hard palate, retromolar gingiva and the mobile tongue (Hillertz et al. 2012). Oral squamous cell carcinoma (OSCC) originates from the mucosal epithelial lining and covers over 90% of all cancers in the oral cavity (Neville & Day 2002). The incidence of oral cancer is higher in men than in women and in persons over 60 years old. Geographically viewed, the highest incidence of OSCC occurs in Pakistan, Brazil, India and France. (de Camargo Cancela et al. 2010). Regardless of increased knowledge about the molecular basis of OSCC and improvements in treatment and diagnostics, oral cancer is still characterized by poor prognosis (Salo et al. 2014).

Oral tongue squamous cell carcinoma (OTSCC) comprises 25–40% of all oral carcinomas and covers the SCCs located in the anterior two thirds of the tongue. In Finland, the incidence of OTSCC has increased by ~3-fold in men and ~2-fold in women during the last four decades (Finnish Cancer Registry). Lymph node metastases are associated with tongue cancer more frequently than in other types, presumably because of the histological features of the tongue. The muscularized structure and dense lymphatic network offers favorable conditions for cancer invasion and metastasis. The clinical course of OTSCC is also hard to predict since the occurrence of metastasis is not related to the size of the primary tumor. (Bello et al. 2010). Despite the improvements in surgery and adjuvant radiotherapy, which are the most important treatment methods for tongue malignant tumors, the 5-year survival rate of OTSCC patients has remained around 50% (Majchrzak et al. 2014). The prognosis of OTSCC patients is significantly worse than that of those patients with similar lesion in some other oral cavity subsites such as oropharynx, larynx and hypopharynx (Rusthoven et al. 2008, Sano & Myers 2007) and approximately 20% of patients with early stage OTSCC die of the disease (Ganly et al. 2012).
Risk factors of oral cancer

The risk factors for OTSCC include basically the same lifestyle related, and environmental factors as OSCC in general, namely tobacco exposure and alcohol consumption (Scully & Bagan 2009). Compared to non-smokers, the risk for OSCC is 4–7 times greater in cigarette smokers. Smoking and alcohol consumption are independent risk factors, but they also show synergistic effects in combination. (Lubin et al. 2009, Talamini et al. 1990). Ethanol enhances the action of various carcinogens (Seitz & Stickel 2007) and increases the permeability of mucosa. Smoking in turn further increases the effects of acetaldehyde burden after alcohol exposure. (da Silva et al. 2011). Mouthwashes containing high concentrations of ethanol are also reported to increase the OSCC risk (McCullough & Farah 2008). Chewing of betel quid/areca nuts is regarded as an important risk factor for OSCC (Chen et al. 2008). Other lifestyle related risk factors include, e.g. poor oral hygiene (Moreno-Lopez et al. 2000, Rosenquist 2005, Zheng et al. 1990) and insufficient consumption of fruits and vegetables (Lucenteforte et al. 2009).

Besides lifestyle related factors, genetic predisposition has been widely explored and seems to play some role in susceptibility to head and neck cancer, especially in younger patients (Copper et al. 1995, Foulkes et al. 1996, Garavello et al. 2008). Moreover, some oral diseases characterized by chronic inflammation, such as lichen planus (García-García et al. 2012), and relatively rare heritable disorders, such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) syndrome (Rautemaa et al. 2007), are connected to a higher risk for developing oral cancer.

Viral infections, such as human papilloma virus (HPV), exhibit oncogenic capability in various human cancers, including some cancers of the oral pharynx (Hillbertz et al. 2012, Syrjänen et al. 2011). Although it has been proposed that HPV infection may play a role also in tongue cancer (Dahlgren et al. 2004, Mork et al. 2001), the study by Liang et al. (2008) questioned this assumption in the case of mobile tongue SCC. The authors showed that HPV infection is not a significant factor contributing to the etiology, pathogenesis or clinical outcome of OTSCC. Moreover, HPV infection is not a potential factor explaining the increased prevalence of OTSCC in younger populations. However, some unknown virus infections may well account for the increase in OTSCC prevalence, especially in young, non-smoking populations, since in these patients, the typical TP53 mutations detected in smokers and HPV positive cancers are not
present. (Li et al. 2014). However, efforts to identify such viruses or other etiological factors explaining the global increase in OTSCC incidence have not been successful thus far.

**Diagnosis and prognostic factors of tongue cancer**

Of all cancers in the oral cavity, OTSCC is the most commonly diagnosed malignancy. Carcinoma in the tongue is highly invasive and metastases to draining lymph nodes are detected more often than in other oral cancer types. The unpredictable clinical course of OTSCC is partly due to the high rates of occult metastases found in patients with a very small primary tumor. (Bello et al. 2010).

The prognosis and treatment planning of tongue cancer relies largely on defining the tumor size, lymph node involvement and metastasis occurrence (TNM-stage = tumor, node, metastasis) (Sobin & Wittekind 2002). TNM-stage is still regarded as the most important prognostic method and other clinical prognostic factors, including patient age, are of weaker prognostic value (Bello et al. 2010, Kantola et al. 2000). There are also various histopathological prognostic factors and histological grading systems available, but none of them is currently in clinicopathological use (Bello et al. 2010). Bryne’s malignancy score, for example, is based on various morphological features (degree of keratinization, nuclear polymorphism, number of mitoses, pattern of invasion and lymphoplasmacytic infiltration) and has been reported as a significant prognostic indicator in oral cancer (Bryne et al. 1989, Bryne et al. 1992, Gluckman et al. 1997, Piffko et al. 1997). Other methods used to histopathologically evaluate the tumor invasive front include Anneroth’s malignancy grading system (Anneroth et al. 1987), the Brandwein-Gensler method (Brandwein-Gensler et al. 2005, Brandwein-Gensler et al. 2010) and the evaluation of tumor budding (Almangush et al. 2014, Wang et al. 2011a). Specific molecular prognostic indicators for OTSCC are still lacking. Much effort has been made to find markers that could be used to determine disease course and identify the most aggressive malignancies. Identifying such markers would in turn provide tools for clinicians to plan more personalized treatment for OTSCC patients. (Soland & Brusevold 2013).

**2.3 The extracellular matrix (ECM)**

An individual organ of a multicellular organism is comprised of different tissues. Tissues are composed of mixed populations of cells embedded within a complex
meshwork of ECM. Previously, ECM was considered simply as a scaffold that provided a structural support for the cells, tissues and organs. However, after extensive ECM research during the past decades, ECM is now recognized to be far more than that. It mediates intercellular communication and regulates various aspects of cell behavior, including cell proliferation, migration, changes in cell shape, differentiation and survival. Thereby, ECM also determines largely the tissue functionality. The ECM microenvironment constitutes of an interlocking mesh of various structural and functional proteins produced particularly by fibroblasts. The arrangement and sequence of ECM proteins are highly conserved and typically comprise multiple domains that are independently folded. The protein domains can bind to cell adhesion receptors, such as integrins, to form cell-ECM connections enabling the transmission of cell signaling molecules across the cell membranes. Moreover, various regulatory roles are carried out by growth factors and cytokines engaged within the ECM. (Daley et al. 2008, Fan et al. 2014, Frantz et al. 2010, Hynes 2009, Kim et al. 2011).

Rather than being just an inert entity of proteins, ECM is a dynamic and physiologically active assembly that is constantly remodeled by various cell actions. The synthesis and degradation of ECM occur particularly during physiological processes, such as development, growth, differentiation and wound healing. The remodeling of ECM is regulated by various factors, including complex proteolytic cascades under strict control. Disturbance in the protease activity toward ECM components may contribute to various disease states. (Daley et al. 2008, Lukashev & Werb 1998). Thereby, comprehension of ECM structure and function has contributed significantly to the understanding of such conditions, including chronic wounds, inflammatory diseases, as well as cancer invasion and metastasis.

2.3.1 Composition of the ECM

Fibrous proteins and PGs are the main classes of molecules composing the ECM. PGs consist of a protein core covalently linked to GAG polysaccharide chains. The majority of the extracellular interstitial space of tissue is filled by hydrated gel-forming PGs. The family of PGs includes small leucine-rich PGs, modular PGs and cell-surface PGs that perform a variety of functions in, e.g. regulating cell behavior, inflammatory response, binding of growth factors, angiogenesis and co-receptor functions. Collagens represent the major group of fibrous proteins of the ECM. (Frantz et al. 2010). They are also the most abundant proteins in
mammals, comprising approximately 30% of the total protein mass of a multicellular animal (Myllyharju & Kivirikko 2001). The majority of collagen molecules form a triple-stranded helix, which then assembles into fibrils or networks depending on the collagen type. In vertebrates, twenty-eight collagen types have been identified, and they can be subdivided based on their supramolecular assemblies. (Gordon & Hahn 2010, Ricard-Blum 2011). Other main fibrous components of ECM are laminins, elastins and FNs. FNs bind to collagens and PGs providing an important platform for cell migration and cell-matrix adhesions (Stoffels et al. 2013). Elastins provide elastic features that allow the tissue to stretch when needed and then recover to its initial form (Muiznieks et al. 2010). Laminins form sheet-like structures and initiate the assembly of BMs (Li et al. 2003).

BM are specialized, dense structures of ECM that directly interact with epithelia and endothelia and provide a functional barrier separating differential compartments of tissues. BM is an active transmitter of signals and hence contributes to the regulation of various cell actions. (Daley & Yamada 2013, Kalluri 2003). BMs in different tissue compartments show tissue-specific features and approximately 50 different proteins are identified in BMs. However, BMs in general are mainly composed of collagens (accounting for 50% of all proteins, the major type being type IV collagen), laminins, nidogens, heparan-sulfate proteoglycans (HSPGs) and perlecans. (Kalluri 2003). In tumor progression, BMs play crucial roles in regulating angiogenesis. The endothelial cell migration during new capillary formation requires proteolytic activity to degrade BMs, for example by MMPs, and both angiogenic- and antiangiogenic factors are released. (Campbell et al. 2010).

ECM also serves as a reservoir for various bioactive molecules, such as cytokines and growth factors, which can be rapidly released and activated by proteolytic enzymes. These bioactive factors contribute to ECM function, e.g. by regulating the synthesis and turnover of ECM proteins and controlling cell actions. (Hynes 2009, Schönherr & Hausser 2000). Growth factors and cytokines are usually localized to BMs and bind to PGs containing GAGs, typically heparan or heparan-sulfate. Fibroblast growth factor (FGF), VEGFs and TGFs -α and -β, for example, all play various essential roles in both physiological and pathological conditions and HSPGs mediate the movement of these growth factors and protects them from the action of proteolytic enzymes. (Fan et al. 2014, Hynes 2009, Schönherr & Hausser 2000, Vaday & Lider 2000).
2.3.2 ECM degradation

The controlled degradation of the ECM is a crucial process in various physiological events, including differentiation, morphogenesis, growth and wound healing. It enables essential functions such as removal of unnecessary components of the matrix and assembly and remodeling of ECM structures. (Basbaum & Werb 1996). However, aberrant ECM degradation can promote pathological conditions, such as tumor progression, arthritis, periodontal diseases, fibrosis, and chronic wound healing (Nagase & Woessner 1999).

There are several proteinase families, such as serine proteinases, cysteine proteinases and MMPs that influence ECM dynamics. They degrade the structural components of ECM and thereby create smaller fragments that can act as bioactive signaling molecules. Proteolytic degradation of ECM releases bound bioactive molecules, which can be then further regulated by proteinases leading to activation or inhibition of their function. MMPs are also able to regulate the function of cell membrane receptors and thereby influence the progression of signaling cascades. (Basbaum & Werb 1996, Toriseva & Kähäri 2009). Proteinase activity is required for various cell actions, most importantly cell migration and invasion. MMPs are a well-characterized group of proteinases and their roles, e.g. in wound healing and various human cancers, have been under investigation for decades. Nevertheless, their mechanisms of actions are not completely understood and the evidence remains partly contradictory. (Noël et al. 2008, Page-McCaw et al. 2007).

2.4 Matrix metalloproteinases (MMPs)

MMPs, also designated matrixins, are endopeptidases belonging to a metzincin superfamily of enzymes. Metzincins are characterized by the presence of a highly conserved motif containing three histidine residues capable of zinc ion binding and a glutamate residue. (Gomis-Ruth 2003). Since the first collagen degrading MMP (later identified as MMP-1) was reported in 1962 by Gross & Lapiere during tadpole tail metamorphosis (Gross & Lapiere 1962), a total of 24 vertebrate MMPs have been identified, 23 of which have been found in humans (Visse & Nagase 2003). MMPs target a highly diverse set of substrates and they are collectively able to degrade all the ECM components. In addition, their specific capabilities to cleave growth factors, cytokines, receptors, cell adhesion molecules, as well as other proteinases and their inhibitors largely determines
their roles in regulating cell behavior. (Chakraborti et al. 2003, Kessenbrock et al. 2010, Page-McCaw et al. 2007). By these pivotal actions, MMPs are involved in various physiological processes, such as embryogenesis, inflammatory cell action, wound healing and bone remodeling, but also in pathological conditions, such as cancer, chronic wound development and arthritis (Nagase & Woessner 1999). The possible MMP related functional mechanisms are illustrated in Figure 3.

Although the MMP family members are genetically distinct, they nevertheless share several common structural features (Figure 4). All MMPs are produced as prepro-enzymes and most of them are secreted into the ECM and their N-terminal predomain is cleaved off. MMP-23 is an exception in that its N-terminal structure contains a membrane targeting signal anchor and the single cleaved domain regulates both activation and secretion (Pei et al. 2000). The propeptide domain contains the cysteine switch domain (PRCGXPD) that ligates the catalytic zinc and maintains the proMMP latency. Some MMP prodomains carry a furin cleavage site and are subjected to activation inside the cell by, e.g. furin (Pei & Weiss 1995). The catalytic domain of MMPs includes the zinc binding domain (HEXXHXXGXXH) and Ca$^{2+}$ or Zn$^{2+}$ dependent activation requires a neutral pH. Other MMP functional domains include a hinge region (composition varies among MMPs), type II fibronectin inserts (in MMP-2 and MMP-9), a hemopexin domain (lacking in MMP-7, -23 and -26) and a transmembrane domain (in MT-MMPs). (Knäuper et al. 1996a, Nagase & Woessner 1999, Visse & Nagase 2003).
Fig. 3. The proposed MMP mediated functions. Some identified MMP-8 targets are indicated in green in the figure (Page-McCaw et al. 2007, Pellinen et al. 2012, Soria-Valles et al. 2014). (P-G-P: Pro-Gly-Pro, LI X: lipopolysaccharide-induced CXC chemokine). Modified and reprinted by permission from Macmillan Publisher Ltd: Nature Reviews Molecular Cell Biology (Page-McCaw et al. 2007), copyright (2007).
2.4.1 Classification of MMPs

MMPs can be subdivided into six groups based on their sequence similarity, substrate specificity and domain organization (presented in Figure 4) as follows:

1) Collagenases comprise MMPs -1, -8 and -13. MMP-18 is the fourth identified collagenase found in *Xenopus*. The common functional feature of this enzyme group is their ability to process type I, II and III collagens. The cleavage occurs at a specific site and yields products $\frac{1}{4}$ and $\frac{3}{4}$ the length of the initial molecule. The resulting fragments are then rapidly denatured to gelatins and may be further processed by other proteinases. 2) Gelatinases MMP-2 and -9 effectively cleave gelatin. Their structure includes type II fibronectin inserts, which enables binding to ECM components, such as collagens and laminins. MMP-9 also cleaves type I-III collagens. 3) Stromelysins MMP-3 and -10 share similar substrate specificity. 4) Matrilysins MMP-7 and -26 lack the hemopexin-like domain, which is included in the structure of almost all the other MMPs. They cleave various ECM components, but especially for MMP-7, numerous non-ECM substrates such as Fas-ligand (Fas-L) have been identified (Mitsiades *et al.* 2001, Powell *et al.* 1999). 5) Membrane-type MMPs (MT-MMPs) include MMP-14, -15, -16, -17, -24 and -25 that are also named MT1-MMP – MT6-MMP correspondingly. These proteinases are either membrane associated (MT1-MMP, MT2-MMP, MT3-MMP and MT-5-MMP) or anchored to the membrane via glycosylphosphatidylinositol (GPI) (MT4-MMP, MT6-MMP). 6) Other MMPs include those matrixins that do not fall into any of the above mentioned groups. These comprise MMP-11 [termed stromelysin-3, but differs from stromelysin-1 (MMP-3) in sequence and substrate specificity], -12, -19, -20, -21, -23, -27 and -28. (Sternlich & Werb 2001, Visse & Nagase 2003).
Fig. 4. The domain structures of the MMPs. Pre: signal sequence, Pro: propeptide with a free zinc-ligating thiol (SH) group, Catalytic: catalytic domain with zinc-binding site (Zn), H: hinge region, Hemopexin: hemopexin/vitronectin domain, S: disulfide bond, II: collagen-binding fibronectin type II inserts, F: furin-susceptible site, TM: transmembrane domain, C: cytoplasmic tail, GPI: glycosylphosphatidylinositol-anchoring domain, Vn: vitronectin insert, C/P: cysteine/proline-rich domain. Modified from Sternlich & Werb (2001) and reprinted with the permission from Annual Reviews.

2.4.2 Regulation of MMPs

The well-coordinated regulation of MMPs is implemented at various levels, including modulation of gene expression and activation by their physiological activating agents, as well as inhibition by protein inhibitors. Furthermore, their activity is controlled by substrate availability and affinity. (Ra & Parks 2007) MMP substrates show considerable overlap, especially among ECM components (at least in vitro). In order to control biochemical redundancy, substrate selectivity is further determined by enzyme affinity and compartmentalization. Compartmentalization also ensures that the cells do not release proteases in an
indiscriminate manner and are able to respond to outside signals effectively. (Parks et al. 2004, Sternlicht & Werb 2001).

**Transcriptional and post-transcriptional regulation of MMPs**

The expression levels of matrix metalloproteinases are low under normal conditions in healthy tissues. Since substrate specificities of MMPs are largely overlapping, but also individual MMPs may have unique targets, the importance of transcriptional control is inevitable. **MMP** promoters are composed of several **cis**-elements that are regulated by a diverse group of **trans**-activators. Simply, MMP promoters can be divided into three groups, based on the basic promoter conformation, containing 1) TATA box and activator protein (AP)-1 sites (**MMP1**, 3, 7, 9, 10, 12, 13, 19 and 26), 2) TATA box, but no AP-1 site (**MMP8**, 11 and 21) or 3) neither TATA box nor proximal AP-1 (**MMP2**, 14 and 28). The similarities in **MMP** promoters are not necessarily related to their other structural similarities. For example, structurally and functionally very similar collagenases MMP-1 and -8 and also gelatinases MMP-2 and -9 differ in their gene promoter structure. (Yan & Boyd 2007). Moreover, **MMP2** is exceptional among **MMPs**, since it is usually transcribed constitutively and mostly regulated at the level of activation (Strongin et al. 1995). Most of the **MMP** genes are induced or suppressed by various factors, including chemical agents, growth factors and physical stress that affect multiple signaling pathways (Nagase & Woessner 1999, Sternlicht & Werb 2001). Many of the cytokines and growth factors, including, EGF, KGF, VEGF, basic FGF, nerve growth factor (NGF), TNF-α, platelet derived growth factor (PDGF), TGF-β, ILs, interferons (IFNs) and extracellular matrix metalloproteinase inducer (EMMPRIN) have shown regulatory effects on MMP expressions. These factors can induce the expression of c-fos and c-jun proto-oncogenic products that bind to the common MMP promoter region **AP-1**. However, not all MMPs contain this **cis**-element; MMP-8, for example, is the only collagenase that does not contain the AP-1 binding site. (Birkedal-Hansen et al. 1993, Borden & Heller 1997, Sternlicht and Werb 2001).

Although the regulation of gene expression is generally accomplished at the transcriptional level, post-transcriptional regulation of MMPs in the cytoplasm also occurs (Yan & Boyd 2007). For example, TGF-β participates in maintaining the mRNA levels of MMP-2 (Overall et al. 1991) and MMP-11 (Delany & Canalis 2001). Other examples include α3β1 integrin that stabilizes MMP-9.
mRNA (Iyer et al. 2005), whereas doxycycline reduces the stability of MMP-2 mRNA (Liu et al. 2003).

Activation of MMPs

Most MMPs are secreted as inactive proenzymes (also called zymogens) that are activated by targeting the N-terminal prodomain (Visse & Nagase 2003). The propeptide domain and catalytic domain are the two functional domains common to all MMPs. The amino acid sequences in the regions involved in the activation mechanisms are highly conserved. The propeptide domain contains the unpaired cysteine sulfhydryl group that binds to the catalytic zinc-ion. To enable activation of MMPs, the thiol-Zn2+ bond needs to be disrupted and the cysteine-thiol group is replaced by a water molecule. This mechanism of activation is referred to as a “cysteine switch”. (Van Wart & Birkedal-Hansen 1990). The thiol-Zn2+ interaction can be broken by three mechanisms: 1) stepwise direct cleavage of the pro-domain (proteinases), 2) reduction of the free thiol (oxidants, non-physiological reagents) or 3) allosteric perturbation (e.g. sodium dodecyl sulfate; SDS). Both thiol reduction and allosteric control lead to autolytic cleavage of the prodomain. (Ra & Parks 2007). In addition to proteinases and non-physiological reagents, activation of MMPs may occur due to low pH (Tjäderhane et al. 1998) or heat treatment (Van Wart & Birkedal-Hansen 1990).

The activation of proenzymes can take place in the extracellular milieu, by intracellular activation or on cell surfaces. The in vivo activation is accomplished mostly by proteinases in the extracellular milieu in a stepwise manner. (Visse & Nagase 2003). However, chemical activation has also been reported to take place in vivo; Gu et al. (2002b) showed the activation of MMP-9 by NO during cerebral ischemia. In addition, reactive oxygen species have been recognized as important in vivo activators of MMPs especially in inflammatory conditions (Sorsa et al. 1998, Weiss et al. 1985). Some MMPs, including all membrane bound MMPs, contain a target sequence for proprotein convertases or furin (Visse & Nagase 2003). The furin cleavage was first reported by Pei & Weiss (1995) demonstrating that proMMP-11 is intracellularly activated by furin. MMPs containing the furin cleavage site are activated inside the cells, before their secretion. The extracellular activation of MMPs is thought to be accomplished by other MMPs and serine proteases, such as plasmin (Carmeliet et al. 1997, Mazzieri et al. 1997) and tumor associated trypsin-2 (Nyberg et al. 2002, Sorsa et al. 1997). The MMP activation cascades by other MMPs are very complicated and several issues need to be
resolved to ensure the in vivo relevance of all the reported activation mechanisms. These include at least whether the suggested activating MMPs are co-expressed and co-localized at sufficient levels with their target MMPs. (Ra & Parks 2007). Some mechanisms, however, are well described and generally accepted, such as MMP-2 activation on the cell membrane. The activation of MMP-2 proceeds through mechanisms requiring active MMP-14 and co-operation of tissue inhibitor of metalloproteinase (TIMP) -2. (Hernandez-Barrantes et al. 2000, Strongin et al. 1995).

The non-proteolytic activation by chemical agents such as p-aminophenylmercuric acetate (APMA), SDS, HgCl₂ and free oxygen radicals (Van Wart & Birkedal-Hansen 1990, Visse & Nagase 2003) proceed in a stepwise manner and produce several intermediates. Allosteric activation of MMPs includes disruption of the thiol-Zn²⁺ interaction without actual removal of the prodomain. This type of activation is utilized by substrate zymography using SDS as an activator for zymogens. The disruption of thiol-Zn²⁺ interaction leads to formation of a transitional active state that is further processed by autolytic cleavage. (Ra & Parks 2007).

**Inhibition of MMPs**

TIMPs are specific endogenous inhibitors of MMP activity in vivo. They are 21–29 kDa homologous molecules that bind MMPs in a 1:1 stoichiometric fashion. In vertebrates, four TIMPs (TIMP-1, -2, -3 and 4) have been identified. Like MMPs, their expressions are also regulated during various physiological conditions requiring tissue remodeling. (Visse & Nagase 2003). In pathological conditions, their levels are altered and they are known to affect various events, such as in vitro cell invasion, tumorigenesis and angiogenesis in which MMPs are known to be involved (Gomez et al. 1997, Johansson et al. 2000). TIMPs also have biological functions independent of their MMP inhibitory activities, including the growth-promoting activity of TIMP-1 and TIMP-2 (Chesler et al. 1995, Hayakawa et al. 1992, Hayakawa et al. 1994). Other in vivo MMP inhibiting molecules comprise α-macroglobulin (Barrett 1981), tissue factor pathway inhibitor-2 (Herman et al. 2001), membrane bound β-amyloid precursor protein (Miyazaki et al. 1993), C-terminal fragment of the procollagen C-terminal proteinase enhancer (Mott et al. 2000), and RECK (GPI-anchored glycoprotein) (Oh et al. 2001, Takahashi et al. 1998). In plasma, α-macroglobulin is abundant and thereby it is the major MMP inhibitor in tissue fluids, whereas TIMPs most
likely act when local inhibition is needed. Moreover, α-macroglobulin inhibition is irreversible unlike TIMPs that can inhibit MMPs in a reversible manner. (Sternlicht & Werb 2001).

MMPs and regulations of their actions in pathological conditions, such as cancer, have been studied for decades. Although synthetic MMP inhibitors (MMPIs) have offered tools for both research and clinical trials targeting MMP related diseases, the lack of specificity limits the range of their usefulness. The peptidomimetic inhibitors such as GM6001 inhibit various MMPs by binding to the active site. There are also inhibitors with more specific target MMPs, including the CTT peptide that selectively inhibits MMP-2 and -9, but not MMP-8, -13 or -14 (Koivunen et al. 1999). Wide spectrum synthetic inhibitors in clinical use include, e.g. tetracyclins (Golub et al. 1994) and bisphosphonates (Woo et al. 2006). (Overall & López-Otín 2002).

### 2.4.3 MMPs in wound healing

Wound healing involves highly coordinated functions of various cell types from distinct tissue compartments. To be completed successfully, the signaling molecules, their receptors and numerous ECM molecules should be expressed in a temporally limited and spatially confined manner. (Toriseva & Kähäri 2009).

MMPs were first thought to mainly participate in the resolution phase of wound healing because of their collagen remodeling ability. Surprisingly, it has turned out to be challenging to prove this action in vivo. Some evidence, however, supports the roles of MMP-2, -3, and -9 in this process. (Mulholland et al. 2005). The contraction of the wound is also an important effector of the closure. At least MMP-3 is known to participate in this process (Bullard et al. 1999). It plays a role in the formation of organized actin bundles, but does not affect the levels of wound contracting myofibroblasts. Knowledge about the diverse functions of MMPs in regulating various bioactive factors has increased, and they are known to participate in controlling all the aspects of wound healing, from the very start of the inflammatory response to the late maturation phase of the ECM (Gill & Parks 2008). Regardless of the multiple beneficial and sometimes dual roles of MMPs in wound healing, their actions need to be kept under strict control throughout the healing cascade. Aberrant MMP levels have been reported in chronic wounds and their actions are connected to disturbed growth factor activity. (Trengove et al. 1999, Yager & Nwomeh 1999). In addition, various viral
and bacterial infectious diseases are characterized by excess MMP activity (Elkington et al. 2005).

The MMP genes are highly inducible by various agents when tissue damage occurs. MMPs are produced by multiple cells operating in wound healing, such as inflammatory cells, fibroblasts and epithelial cells. (Gill & Parks 2008, Parks et al. 2004). MMP-1 (Ashcroft et al. 1997b, Inoue et al. 1995, Saarialho-Kere 1998, Vaalamo et al. 1997), MMP-3, MMP-10 (Madlener et al. 1998, Saarialho-Kere 1998), MMP-9 (Lund et al. 1999, Madlener et al. 1998, Salo et al. 1994) and MMP-13 (Lund et al. 1999) are expressed by wound keratinocytes. Stromal cells are known to express at least MMP-1, MMP-3 (Saarialho-Kere 1998), MMP-13 (Ravanti et al. 1999a, Ravanti et al. 1999b, Ravanti et al. 2001, Vaalamo et al. 1997), MMP-8 (Pirilä et al. 2007) and MMP-9 (Okada et al. 1997). MMP-13 is produced by osteoprogenitor cells at the sites of new bone formation during the tooth extraction socket healing process (Devlin 2000). MMP-13 also participates in the formation of granulation tissue in skin; mice lacking MMP-13 have reduced experimental granulation tissue growth and a delay in myofibroblast arrangement (Toriseva et al. 2012). MMPs -2 and -9 are produced by osteogenic and connective tissue cells during alveolar bone regeneration (Accorsi-Mendonca et al. 2008). MMP-2 and -9 are also localized in the tooth extraction socket granulation tissue where they are produced by various cell types (Silva et al. 2001, Zecchin et al. 2005). In addition, MMP-2 is expressed by fibroblasts and is detected in the leading epidermal edge of the wound (Oikarinen et al. 1993, Salo et al. 1994). Inflammatory cells release and express, for example, MMP-8 (Tschesche et al. 1991) and MMP-9 (Kjeldsen et al. 1992) by neutrophils and MMP-1 (Gu et al. 2002a), MMP-8 (Prikk et al. 2001), and MMP-12 by macrophages (Kerkelä et al. 2000, Madlener et al. 1998). Many of the actions of MMPs in wound healing are related to the regulation of inflammation and re-epithelialization, and consistently several of their specific matrix and non-matrix substrates are involved in these processes.

**MMPs in inflammatory phase**

Inflammation is the key step in initiating various cellular responses to injury. In any disease that involves inflammation, MMPs are present and usually show misregulated or increased levels. The activation mechanism of MMPs by reactive metabolites, often produced by leukocytes, is relevant, especially in inflammatory conditions and indicates that MMPs play crucial roles during inflammation. (Fu et
al. 2001, Peppin & Weiss 1986, Weiss et al. 1985). On the other hand, oxidative mechanisms also participate in restraining proteolytic activity (Fu et al. 2003). The above mentioned mechanisms may well play considerable roles in regulating MMP actions in inflammation, but their exact in vivo relevance still remains to be elucidated.

The matrix proteolysis itself serves as a hallmark of the inflammatory process and has a variety of effects on the course of the inflammation and tissue repair (Parks et al. 2004). As an example, MMP-1 has been indicated as the key proteolytic enzyme to initiate the bone resorptive phase in apical periodontitis (Hong et al. 2004, Lin et al. 2002). In addition, MMP-7 and gelatinases MMP-2 and -9 participate in the progression of periapical inflammation (Belmar et al. 2008, Buzoglu et al. 2009, Gusman et al. 2002, Letra et al. 2013, Panagakos et al. 1996, Shin et al. 2002, Ueda & Matsushima 2001). In addition, collagenases MMP-8 (Wahlgren et al. 2002) and -13 contribute to various events of apical periodontitis (Leonardi et al. 2005, Uitto et al. 1998) and their actions are also driven by their surroundings; in inflamed chronic human periodontitis and in vitro oral mucosa tissue cultures, MMP-13 is expressed by epithelial cells, when exposed to a collagenous matrix or cytokines. This suggests the importance of MMP-13 in collagenolysis and local cell invasion in inflamed oral tissue (Uitto et al. 1998).

In addition to inflammation driven ECM degradation, MMPs are able to process many of the factors participating in the initiation of the inflammatory phase (cytokines, growth factors, and receptors) and thereby activate or inhibit their functions. They also release bioactive factors from the surrounding tissue, as well as regulate receptor function. In addition, MMPs participate in creating chemokine gradients that guide cell migration, proliferation and differentiation and aid in extravasation of leukocytes from the bloodstream to the site of injury. (Gill & Parks 2008).

The chemokines play a central role in controlling inflammation and repair by attracting and recruiting the immune effector cell population (Barrientos et al. 2008, Nathan 2002). MMPs have numerous tools to control chemokines and the direct cleavage of the molecule can enhance or inhibit the activity or lead to antagonism. CC group of chemokines participate in monocyte chemotaxis and the actions of MMPs mostly result in reduced activity of these factors. The cleavage may, for example, result in a fragment that acts as a receptor antagonist inhibiting downstream signaling. (McQuibban et al. 2000, McQuibban et al. 2002, Van Den Steen et al. 2003b). Neutrophil chemotaxis is regulated by C-X-C motif (CXC)
chemokines that show a more fluctuating response to MMP processing (McQuibban et al. 2001, Tester et al. 2007, Van Den Steen et al. 2003b). Processing of the chemokine C-X-C motif ligand (CXCL) -12 [stromal cell derived factor (SDF) -1] by MMPs may even result in the formation of neurotoxic proteins (Zhang et al. 2003). In addition, MMPs can regulate chemokines indirectly by acting on some other substrate which then mediates the regulatory roles, e.g. by binding or retaining the chemokines or formation of chemotactic gradients (Corry et al. 2002, Li et al. 2002, Pruijt et al. 1999). After wounding, the epithelial cells start to express, e.g. CXCL8 (CXCL1 in mouse) that assembles in syndecan-1 heparan sulfate chains. The simultaneous expression of MMP-7 is responsible for creating the chemokine gradient by shedding the syndecan-1 ectodomain. The upcoming gradient controls the neutrophil influx and activation. (Li et al. 2002). Another interesting example of chemokine participation in inflammation involves the lipopolysaccharide induced chemokine (LIX; CXCL5). LIX is processed by MMP-2 (Song et al. 2013), MMP-8 (Balbín et al. 2003, Tester et al. 2007, Van Den Steen et al. 2003b) and MMP-9 (Song et al. 2013, Van Den Steen et al. 2003b), and all these MMP actions on LIX have also some in vivo evidence. MMP-8 processing of LIX leads to enhanced activity and subsequent recruitment of inflammatory cells (Balbín et al. 2003, Tester et al. 2007).

MMPs regulate also other cytokines and growth factors that carry out numerous functions in the repair process and exhibit both pro- and anti-inflammatory features. The growth factors or growth factor receptors reported as MMP targets include fibroblast growth factor receptor (FGFR) -1 (Levi et al. 1996), VEGF (Bergers et al. 2000), heparin-binding epidermal growth factor-like growth factor (HB-EGF) (Suzuki et al. 1997) and TGF-β1 (Mu et al. 2002, Yu & Stamenkovic 2000). Moreover, MMPs play a dual role in regulating IL-1β, a potent pro-inflammatory factor. At least MMPs-2, -3 and -9 are able to activate the IL-1β precursor (Schönbeck et al. 1998), but on the other hand, there is evidence that the same MMPs are responsible for the inactivation of the biologically active IL-1β (Ito et al. 1996). In addition, IFN-β (Nelissen et al. 2003) and the crucial pro-inflammatory factor TNF-α (Gearing et al. 1994, Moss et al. 1997) are prone to MMP action.

Inflammation also serves as a defense against pathogens and affects apoptosis. MMP-7 seems to participate in these processes by activating pro-α-defensins that participate in the battle against pathogens (Wilson et al. 1999), as
well as by shedding membrane bound Fas-L, and thereby releasing a pro-
apoptotic soluble Fas-L (Powell et al. 1999).

**MMPs in re-epithelialization**

Simultaneously with inflammation, re-epithelialization occurs to cover the exposed surface. This requires alteration of cell-cell and cell-matrix interactions at the wound margins and subsequent migration of epithelial cells. (Singer & Clark 1999). Several MMPs take part in epithelial cell migration with multiple mechanisms. MMP-1 participates in epithelialization by at least increasing keratinocyte migration due to collagen denaturation (Pilcher et al. 1997). The actions of MMP-7 to facilitate cell migration include targeting the adherens junctions by cleavage of E-cadherin (McGuire et al. 2003). Interestingly, the ability of MMP-7 to shed syndecan-1 and affect inflammation is also related to its ability to regulate epithelial cell migration through a mechanism controlling α2β1 integrin conformation (Chen et al. 2009). Other MMPs that have been reported to take part in epithelial cell migration include MMP-9 (Bove et al. 2007, McCawley et al. 1998), and MMP-10 (Krampert et al. 2004, Madlener et al. 1996), but the exact mechanisms of their function are largely unknown.

Behind the migratory front, the proliferation of epithelial cells comprises another part of re-epithelialization. At least MMP-2, MMP-9 (Sigurdson et al. 2003), MMP-14 (Atkinson et al. 2007) and MMP-28 (Saarialho-Kere et al. 2002) take part in regulating this process. MMPs participate also in cell differentiation in airway epithelia in which MMP-7 and -9 expression and activity appear to be high during the later state of wound healing as the epithelial cells differentiate (Coraux et al. 2005). Although MMPs in various cellular processes of wound healing and inflammation have been studied intensively for decades, many of their functions still need to be resolved and confirmed in vivo.

**2.4.4 MMPs in cancer**

The classical view of MMPs in cancer as solely connective tissue degrading enzymes is declining as novel substrates for individual MMPs are found. The initial MMP1 used in the clinical trials broadly inhibited various proteinases and the first studies were conducted at a time when only a few of the MMPs were even identified. Since then, many additional MMPs have been identified, but also other enzyme family groups have emerged. (Overall & López-Otín 2002). In
addition to cancer cells themselves, also stromal cells in TME produce MMPs. Some of the MMPs may be produced to offer protection for the host, whereas some MMPs might be stimulated by tumor cells to benefit tumor growth and to resist host defense. During tumor growth and metastasis, the tumor cells need to interact with the ECM, embedded cytokines and growth factors as well as numerous different cell types. Keeping in mind the complexity of tumor progression and diverse source and roles of MMPs in this process, it is not surprising that the role of these proteinases is such a tricky task to resolve. A failure of clinical trials using MMPIs has become more understandable as the complexity of the proteinases in the TME has emerged. (Bissell & Radisky 2001, Deryugina & Quigley 2006, Kessenbrock et al. 2010).

Tumor progression involves alterations in tumor stroma, including changes in turnover of fibrillar collagens. During the early tumorigenesis MMPs undoubtedly play an essential role in ECM and BM degradation and thereby facilitate the formation of a tumor favoring microenvironment. (Erler & Weaver 2009, Noël et al. 2008). In addition to ECM modulation, MMPs are known to contribute to all stages of cancer progression. These include both early stage tumorigenic events such as malignant transformation, angiogenesis and primary tumor growth and later stage processes such as invasion and metastasis. (Hua et al. 2011, Johansson et al. 2000, Overall & López-Otín 2002).

Genetic alterations in numerous MMP genes result in altered levels and are known to affect the disease course in various human cancers (Ye 2000). The literature describes highly diversified proposals for the roles and prognostic values for individual MMPs in cancer progression. The variety in type of the cancer examined, methods used and the number of samples undoubtedly has an influence on the end result and conclusions of each study. For some MMPs, such as MMP-14 (MT1-MMP) and collagenase MMP-1, the findings from different in vivo and in vitro studies are quite uniform and they are generally accepted to act as tumor promoters and indicate worse prognosis. (Hadler-Olsen et al. 2013, Poincloux et al. 2009, Vihinen & Kähäri 2002). Collagenase MMP-13 is also acknowledged as a tumor promoter in various cancers (Ala-alho and Kähäri 2005, Vihinen & Kähäri 2002), including head and neck cancer (Luukkaa et al. 2006). Gelatinase MMP-2, first described as BM collagenase, was initially considered to be responsible for BM degradation indicating its tumor promoting role (Liotta et al. 1980). Another gelatinase, MMP-9 was long thought to be only a negative actor in cancer (Vandooren et al. 2013, Vilen et al. 2013). However, for MMP-9 as well as for other MMPs, also protective features have emerged, although they
were previously considered as devastating proteinases in cancer (Martin & Matrisian 2007, Noël et al. 2008, Vilen et al. 2013). In HNSCC, the tumors with regional metastases show lower MMP-9 levels (Stokes et al. 2010). The known antitumorigenic mechanisms of MMP-9 are related to its anti-angiogenic functions (Hamano et al. 2003). Beneficial roles of MMP-3 in cancer progress are verified in mammary tumor progression (Witty et al. 1995) and in mouse experimental skin SCC (McCawley et al. 2004). The origin of some MMPs, such as MMP-12 in TME, seems also to define their dual roles (Kerkelä et al. 2002). In addition, at least MMPs -8, -11, -12, 19, -26 and -27 have been reported to have positive effects in specific circumstances. However, the molecular mechanisms by which the MMPs exhibit their pro- and antitumorigenic actions remain largely to be elucidated. It is likely that the role of individual MMP in cancer progression depends also on its temporal and spatial expression, as well as the circumstances in the surrounding tumor stroma. (López-Otín & Matrisian 2007, López-Otín et al. 2009, Noël et al. 2008).

**MMPs in tongue cancer**

The mechanistic roles of MMPs in HNSCC and OSCC are still mainly unknown, and for OTSCC, specifically, no individual MMP has been identified consistently as responsible for disease progression or aggressiveness.

Gelatinases are perhaps the most widely studied proteinases in OTSCC, most likely partly because of the simple methodology (gelatin zymography) required to investigate them. Numerous studies have used specific gelatinase inhibitors or anticancer agents to examine their effects on tongue cancer cell behavior in vitro and in vivo. In the xenograft mouse model, the gelatinolytic activity was localized in the invasive front of the tongue tumor. Interestingly, gelatinolytic activity was not present in non-invasive skin tumors established from the same cell lines (Hadler-Olsen et al. 2011). Specific gelatinase inhibitors CTT-1 and CTT-2 inhibit HSC-3 tongue carcinoma cell growth when xenografted in mouse subcutaneously (Heikkilä et al. 2006, Suojanen et al. 2011). CTT-1 failed to reduce the spreading of carcinoma cells (Suojanen et al. 2011), but CTT-2 in turn decreased the blood vessel density and improved the survival of mice with xenografts (Heikkilä et al. 2006). Various tongue carcinoma cell lines in vitro show reduced gelatinase expression and decreased migration and invasion abilities in response to agents with anticancer effects, including berberine (in SCC-4 cells) (Ho et al. 2009) and the cyclooxygenase-2 (COX-2) specific
inhibitor celecoxib (in a cell line established from human OSCC of the tongue) (Kwak et al. 2007). In addition, gypenosides inhibit SCC-4 cell invasion and migration as well as downregulate MMP-9 (Lu et al. 2008). In HSC-3 tongue carcinoma cells, trypsin-2 co-localizes with MMP-9 in intracellular vesicles. The authors suggested that this joint appearance is a unique feature for epithelial derived cancers, since the co-localization was never detected in tissues from bone tumors. (Vilen et al. 2008).

The studies evaluating the potential prognostic value of gelatinases in OTSCC have been partly contradictory. MMP-2 and -9 are expressed more often in primary tumors with lymph node metastasis than in primary tumors with no detectable metastasis (Zhou et al. 2010). Likewise, another study reported that high gelatinase expression in tumor and tumor stroma associated with the appearance of lymph node metastasis in human OTSCC samples. Moreover, gelatinases were linked to type IV collagen degradation and poorer overall survival of the patients (Fan et al. 2012). However, a study exploring OTSCC primary tumor samples using a relatively low sample number (n = 23) failed to connect MMP-9 expression to disease aggressiveness (Guttmann et al. 2004). Another study reported that MMP-9 (or VEGF) does not associate with the presence of metastasis in OTSCC. Nevertheless, a slight but non-significant increase in MMP-9 and VEGF levels in metastatic tumors compared to non-metastatic tumors was observed. (Henriques et al. 2012). Moreover, a study examining early tongue cancer samples found no disease predicting value for MMP-2 or -9, but identified VEGF as a prognostic factor predicting tumor invasion (Kim et al. 2006).

The role of collagenases in OTSCC is not very well established. A study examining T1N0M0 and T2N0M0 patient primary tumors indicated that nuclear MMP-13 predicted OTSCC patient survival. The expression of MMP-13 was also associated with invasion depth and tumor size. However, in that study, cytoplasmic expression of MMP-2, -8 and -9 was not linked to either tumor growth or invasion depth and thereby was not considered to have prognostic value in OTSCC. (Mäkinen et al. 2012). Another trial examining OTSCC samples found no association of MMP-13 levels with histological grading, cancer stage or patient survival (Ylipalosaari et al. 2005). Instead, the authors found that integrin αvβ6 expression, which was previously linked to enhanced migration of malignant oral keratinocytes by enhancing MMP-9 levels (Thomas et al. 2001), downregulated MMP-13 expression (Ylipalosaari et al. 2005). MMP-1 is downregulated by microRNA (miR) -222 in the cell line established from an
OTSCC patient sample. Silencing of MMP-1 with small interfering RNA (siRNA) reduced the invasion of the aggressive cells and the authors suggested that miR-222 regulates the invasion of OTSCC through MMP-1 regulation. (Liu et al. 2009). In addition, the immunohistochemistry (IHC) analysis of patient OTSCC samples revealed that the expression levels of MMP-1 (and MMP-2) are significantly higher in tumors compared to the levels in control tissue adjacent to the tumor (Zhang et al. 2011b). Evidence for the role of MMP-8 in OTSCC has remained very limited, although MMP-8 is expressed by various tongue carcinoma derived cell lines (Moilanen et al. 2002).

In addition to gelatinases and collagenases, also other MMPs, such as MT1-MMP (MMP-14), are reported to contribute to OTSCC progression. A study using a selective MT1-MMP inhibitor provided in vitro evidence that the inhibition of this particular proteinase significantly reduces migration and invasion ability of tongue cancer cells (Suojanen et al. 2009). Another study examining tongue cancer patient samples identified MMP-2 and its activator MT1-MMP, as well as TIMP-2 expressions to be associated with tumor metastatic recurrence and worse prognosis of the patient (Yoshizaki et al. 2001). The tumor promoting role of MT1-MMP in OTSCC is further supported by the study exploring trypsin-2 using both in vitro and in vivo methods. The authors showed that this serine proteinase enhanced tongue carcinoma cell invasion, e.g. through activating MT1-MMP. (Vilen et al. 2012). Moreover, activation of MT1-MMP, (and also MMP-2) by claudin-2 seems to enhance tongue cancer cell invasion by increased cleavage of laminin-5 γ2 chains (Oku et al. 2006). The invasive front in human OTSCC is characterized by the increased expression of matrilysins MMP-7 and -26 compared to inner parts of the tumor. Although the finding could indicate their role in tongue cancer cell invasion, their expression showed no predictive value for the metastatic potential of OTSCC, (de Amorim et al. 2010). A study using tissue microarray (TMA) and IHC to explore MMP-7 and -25 in OTSCC revealed that high MMP-7 expression associates with the presence of occult cervical metastases, increased invasion depth and higher tumor grade. Moreover, MMP-7 was found to predict a poor outcome. (Mäkinen et al. 2013).

2.5 Matrix metalloproteinase-8 (MMP-8)

A proteinase from a granule fraction from a preparation containing human polymorphonuclear leucocytes was reported already in the 1960s (Lazarus et al. 1968). Later, MMP-8 was isolated and characterized (Macartney & Tschesche
1983) and MMP8 was cloned and sequenced from a peripheral leucocyte mRNA extract of a chronic granulocytic leukemia patient (Hasty et al. 1990). MMP-8 belongs to the collagenase group of MMPs and it is also referred to as neutrophil collagenase and collagenase-2 (Van Lint & Libert 2006, Visse & Nagase 2003). The neutrophil secreted form of MMP-8 is mainly synthesized in the maturing leukocytes in bone marrow and stored in specific granules of PMN neutrophils prior their exit to the blood stream (Hasty et al. 1986, Murphy et al. 1980). As a response to extracellular stimuli, MMP-8 is released from the activated neutrophils (Hasty et al. 1986, Tschesche et al. 1991).

The collagenases are able to cleave native fibrillar collagens with varying efficiencies. MMP-8 prefers type I collagen over type III, whereas MMP-1 processes type III collagen most effectively, and MMP-13 is the most efficient in degrading type II collagen (Hasty et al. 1987, Knäuper et al. 1996a, Mallya et al. 1990). The primary cleavage of type I collagen occurs at a specific site within the triple helical collagen and produces typical ¼ and ¾ length α chain fragments that can be further processed by other proteinases such as gelatinases. In addition to type I collagen, MMP-8 processes various ECM structural macromolecules, including type II, III, VII and X collagens, aggregan, tenascin and gelatin. (Fosang et al. 1994, Van Lint & Libert 2006).

The MMP8 gene is located in the MMP gene cluster in chromosome 11q22.3 (long arm) (Pendas et al. 1996). MMP-8 was initially found in molecular sizes of 55 kDa and 75 kDa (Hasty et al. 1986, Hasty et al. 1987). PMN leukocyte derived proMMP-8 is ~80 kDa in size and is further processed into the active 75 kDa form. The non-PMN type MMP-8 (molecular forms of 40-60 kDa) is produced, e.g. by gingival (Abe et al. 2001) and rheumatoid synovial fibroblasts (Hanemaaijer et al. 1997), odontoblasts (Palosaari et al. 2000), oral mucosal (Hanemaaijer et al. 1997) and skin keratinocytes (Bachmeier et al. 2000), chondrocytes (Chubinskaya et al. 1996, Cole et al. 1996), melanoma cells (Giambernardi et al. 2001), Jurkat T leukemia cells (Kim et al. 2001) and oral SCC cells (Moilanen et al. 2002, Moilanen et al. 2003) in vitro. (Dejonckheere et al. 2011, Van Lint & Libert 2006). In vivo evidence has indicated that MMP-8 is located in various cells, including human bronchial monocytes/macrophages (Prikk et al. 2001), plasma cells (Wahlgren et al. 2001), rheumatoid synovial fibroblasts (Hanemaaijer et al. 1997), rat wound fibroblasts (Pirilä et al. 2001), human articular cartilage chondrocytes (Chubinskaya et al. 1999), epithelial cell (Pirilä et al. 2001, Prikk et al. 2001, Tervahartiala et al. 2000), oral SCC cells (Moilanen et al. 2002) and breast cancer cells (Agarwal et al. 2003). MMP-8
from secretory granules of neutrophils can be rapidly released upon outside stimulus and is therefore not dependent on transcriptional regulation. MMP-8 expression is downregulated in vitro by TGF-β1 in human odontoblasts and pulpal cells (Palosaari et al. 2000), as well as in SCC cells (Moilanen et al. 2002). In addition, MMP-8 expression is increased in vitro by II-1β in ovarian cancer cells (Stadlmann et al. 2003) and in human gingival fibroblasts (Abe et al. 2001). Moreover, TNF-α upregulates, whereas doxycycline downregulates MMP-8 expression in rheumatoid synovial fibroblasts and endothelial cells (Hanemaaijer et al. 1997). The function of MMP-8 gene is also regulated by alternative splicing (Hu et al. 1999). MMP-8 is activated by various factors, including tumor associated trypsinogen-2 (Moilanen et al. 2003), reactive oxygen species (Saari et al. 1990) and bacteria derived proteases (Sorsa et al. 1992) and other MMPs, such as MMP-7 (Dozier et al. 2006), MT1-MMP (Holopainen et al. 2003), MMP-3 (Knäuper et al. 1993) and MMP-10 (Knäuper et al. 1996b).

MMP-8 contributes to the progress of a number of a human diseases listed in Figure 5 (Dejonckheere et al. 2011, Sulkala et al. 2007, Van Lint & Libert 2006), wherein the mechanisms of its action seem to depend on its temporal and spatial expression, as well as its origin (Dejonckheere et al. 2011).
2.5.1 MMP-8 in inflammation

Both anti- and pro-inflammatory properties have been reported for MMP-8 in various disease states. MMP-8 processes LIX (CXCL5) on both ends and the cleavage creates a neutrophil chemotactic form (Balbin et al. 2003, Tester et al. 2007, Van Den Steen et al. 2003b). Neutrophil migration through corneal stroma is mediated by ECM degradation by MMP-8 and production of a chemotactic collagenous matrix derived peptide Pro-Gly-Pro (PGP) (Lin et al. 2008). In addition, MMP-8 processes other inflammatory cytokines, including IL-8 (CXCL8) and human and mouse granulocyte chemotactic protein-2 (GCP-2, CXCL6) (Van Den Steen et al. 2003b), as well as monokine induced by IFN-γ.
(MIG), IFN-γ-inducible protein (IP) -10 (Van den Steen et al. 2003a) and monocyte chemotactic protein (MCP) -1 (McQuibban et al. 2002). The pro-inflammatory effects of MMP-8 are demonstrated in various inflammatory conditions. MMP-8 deficiency protects mice from TNF-α-induced hepatitis (Van Lint et al. 2005). The pro-inflammatory properties of MMP-8 were also discovered by Solan et al. (2012), who showed that increased circulating MMP-8 activity and mRNA levels in patients with septic shock correlated with decreased survival of the patient. Moreover, the authors reported that both genetic and pharmacological MMP-8 inhibition in the mouse model of sepsis reduced the inflammation and improved survival. The in vitro ability of MMP-8 to activate a pro-inflammatory transcription factor nuclear factor κB was also described. (Solan et al. 2012).

Although MMP-8 recruits neutrophils into the sites of inflammation, it also reduces the neutrophil count in mouse acute lung injury (Owen et al. 2004). In lung injury repair, MMP-8 has variable effects depending on the experimental model used. In a study exploring ventilator-induced lung injury, lack of MMP-8 in mice diminished pro-inflammatory cytokine levels, whereas it increased anti-inflammatory cytokine levels (Albaiceta et al. 2010). Quintero et al. (2010) illustrated macrophage inflammatory protein (MIP)-1α inactivation by MMP-8 and increased acute lung inflammation in MMP-8 deficient (Mmp8/-) mice. The anti-inflammatory action of MMP-8 in granulocytic allergen induced inflammation of airways is linked to its ability to regulate inflammatory cell apoptosis (Gueders et al. 2005). Likewise, in a study exploring lung inflammation during endotoxemia, an absence of MMP-8 increased S100A8 and S100A9 levels in mouse, resulting in increased inflammation in Mmp8/- mice, since these two proteins are able to trigger a pro-inflammatory reaction (González-López et al. 2012). A bleomycin induced lung injury model also provided further evidence for the anti-inflammatory effects of MMP-8 (Craig et al. 2013). In mouse inflammatory arthritis, MMP-8 likewise exhibits anti-inflammatory actions by regulating neutrophil apoptosis (Cox et al. 2010, García et al. 2010). An inflammatory-related role of MMP-8 is also reported in the mouse skin wound healing model, in which MMP-8 was connected to resolution of inflammation (Gutiérrez-Fernández et al. 2007).

The variability of the evidence related to the nature of MMP-8 in inflammation indicates that the mechanism of its action in various conditions most likely depends on multiple factors, including temporal and spatial aspects.
2.5.2 MMP-8 in wound healing

Little is known about the role of MMP-8 in wound healing and the evidence available is partly contradictory. The most well-established function of MMP-8 is related to its role in regulating inflammation by participating in neutrophil recruitment and clearance through various mechanisms (Dejonckheere et al. 2011). MMP-8 is the most abundant collagenase in acute skin wounds (Nwomeh et al. 1999). However, it is also present — even with higher levels — in chronic non-healing ulcers (Amato et al. 2013, Nwomeh et al. 1999, Pirilä et al. 2007). The essential role of MMP-8 in the skin wound healing process is evident in the mouse model. Mmp8-/- mice skin wounds show impaired healing with increased inflammation and altered neutrophil appearance (Gutiérrez-Fernández et al. 2007). Another study demonstrated that excess MMP-8 is not beneficial, but instead impairs normal tissue repair in rat skin wounds (Danielsen et al. 2011). Moreover, although neutrophils may interfere with the wound healing rate in some circumstances (Dovi et al. 2003), PMN neutrophils with MMP-8 expression play a beneficial role in rat liver repair after reversible biliary obstruction by maintaining essential collagenase activity (Harty et al. 2010). In lung injury repair, most of the studies indicate that the main role of MMP-8 is in the regulation of inflammation. In addition, in bleomycin mediated lung injury, MMP-8 exhibits profibrotic actions by IL-10 cleavage (García-Prieto et al. 2010) and also by reducing the levels of IP-10 and MIP-1α (Craig et al. 2013).

2.5.3 MMP-8 in cancer

Although MMPs have long been considered tumor promoters due to their ability to degrade extracellular matrix, there is increasing evidence for a protective role of MMP-8 in various human cancers (López-Otin & Matrisian 2007, López-Otin et al. 2009). Human and animal studies have shown a tumor suppressive role of MMP-8 in various cancers, including skin (Balbin et al. 2003) and breast cancers (Agarwal et al. 2003, Decock et al. 2008). In contrast, MMP-8 expression levels in ovarian cancer correlates with poor prognosis and more advanced stage of the disease (Stadlmann et al. 2003). Moreover, Waldenström’s macroglobulinemia, a cancer affecting B cells, is characterized by higher MMP-8 transcription levels in peripheral blood B cells (Zhou et al. 2011). In OTSCC patient samples, no correlation was found between cytoplasmic MMP-8 expression and invasion depth or tumor size (Mäkinen et al. 2012).
In colorectal cancer, serum MMP-8 levels were increased and indicate disease course. MMP-8 may participate in the immune response that targets the primary tumor, since serum MMP-8 levels also correlated with blood neutrophil count and inflammatory properties of the primary tumor (Väyrynen et al. 2012). In breast cancer, plasma MMP-8 levels did not predict disease occurrence, but were assumed to have a protective role against distant lymph node metastases (Decock et al. 2008). In HNSCC, plasma levels of MMP-8, however, showed no disease course indicative value, but associated with tumor stage (Nurmenniemi et al. 2012, Pradhan-Palikhe et al. 2010). Instead, high levels of the MMP-8 inhibitor TIMP-1 predicted poor outcome in HNSCC (Pradhan-Palikhe et al. 2010).

The MMP8 gene promoter polymorphism +17C/G has been connected to decreased risk for developing lung cancer, which further supports the protective role for MMP-8 in tumor progression (González-Arriaga et al. 2008). However, this polymorphism did not have an effect on overall survival of the patients. A study identifying possible MMP polymorphisms in hepatocellular carcinoma, did not find an association of MMP-8 polymorphisms with disease risk (Qiu et al. 2008). In contrast, in malignant melanomas, the higher promoter activity due to gene variation is related to a higher risk of developing malignant melanomas (Dębniak et al. 2011) and serum MMP-8 may increase melanoma spreading (Vihinen et al. 2008).

In vitro studies have provided some clue to the mechanisms by which MMP-8 mediates the protective effects in cancer. The comparison of two breast cancer cell lines with divergent metastatic potentials revealed that MMP-8 is expressed in higher levels in the non-metastatic cell line (Montel et al. 2004). A genetic manipulation of these cells lines confirmed that by inverting the expression levels of MMP-8 the metastasis ability likewise changed and suggested a tumor suppressive role of MMP-8. In breast cancer cells, MMP-8 induces the expression of IL-6 and IL-8 and regulates the course of cancer related inflammation (Thirkettle et al. 2013). Moreover, MMP-8 can inactivate β1 integrin activity in breast cancer cells, which may influence cell invasion ability (Pellinen et al. 2012). In addition, MMP-8 increased melanoma and lung carcinoma cell adhesion and thereby hindered cell invasion through Matrigel® (Gutiérrez-Fernández et al. 2008). The study also showed that tumor cell MMP-8 expression in human breast cancers associated with lower appearance of lymph node metastasis. Moreover, the antimetastatic mechanism of MMP-8 action is evidenced in mouse and
includes inhibition of TGF-β1 action by MMP-8 cleaved decorin in breast cancer cells (Soria-Valles et al. 2014).

### 2.6 Transfoming growth factor-β1 (TGF-β1)

TGF-β1 is a multifunctional cytokine that is produced by virtually all cells (Blöbe et al. 2000). TGF-β1 belongs to the TGF-β superfamily together with the structurally related bone morphogenetic proteins (BMPs), activins, inhibins and growth differentiation factors (GDFs) (Kingsley 1994). In mammals, TGF-β exists in three isoforms (TGF-β1-3) that have similar effects through the same receptor signaling system (Massague 1990).

TGF-β1 is synthesized as a preproprotein that first undergoes N-terminal processing in the endoplasmic reticulum. Next, a homodimer is formed through disulfide bridges to yield the proenzyme molecule containing an N-terminal propeptide. The propeptide is cleaved off by furin convertase (Dubois et al. 1995) and the resulting fragments form a small latent complex (SLC) in which the dimer TGF-β1 is non-covalently bound to the latency associated peptide (LAP). Finally, a large latent TGF-β1 complex (LLC) is formed when latent TGF-β binding protein (LTBP) covalently attaches to the SLC. LTBPs enable proper folding and secretion of TGF-β1 (Miyazono et al. 1991). Once secreted, LTBP attaches the complex to the ECM via its N-terminus. (Saharinen et al. 1999, ten Dijke & Arthur 2007). LTBP can be released from ECM by proteolytic cleavage by for example MMP-14 (MT1-MMP) (Tatti et al. 2008).

As presented in Figure 6 below, the active dimer TGF-β1 needs to be released from the latent complex to enable binding to its cell surface heterodimer receptor and the resulting activation of a signaling pathway through, e.g. Smads. This activation can be accomplished by various chemical, physical and biological mechanisms. (Brown et al. 1990, Lawrence et al. 1985). Proteinases, such as MMP-9 (Yu & Stamenkovic 2000) and MMP-14 (Karsdal et al. 2002, Mu et al. 2002) can act as TGF-β1 activators, but also other MMPs (Morris et al. 2003) and plasmin (Lyons et al. 1990) may contribute to this activation. In contrast, some proteinases, such as neutrophil elastase (Chen et al. 1997) and cathepsin-K (Zhang et al. 2011a) degrade TGF-β1 and thereby may participate in its inactivation. The receptor family is comprised of structurally and functionally similar transmembrane serine/threonine kinases referred to as type I and type II receptor subfamilies. TGF-β1 ligand binding to receptor I recruits constitutively phosphorylated receptor II and coupling of these receptors leads to the formation
of a heterotetramer complex. The ensuing phosphorylation of the receptor I kinase domain by receptor II triggers a signaling cascade mediated by Smad dependent or some of the Smad independent pathways. The Smad signaling pathway proceeds through a series of phosphorylations initiated by the phosphorylation of receptor-regulated Smad (R-Smad; e.g. Smad2 and Smad3), which allows their association with common mediator Smads (co-Smad; e.g. Smad4). (Massague & Chen 2000, Shi & Massague 2003, ten Dijke & Hill 2004).

TGF-β1 plays pivotal roles in various cellular processes, including proliferation, differentiation, apoptosis and migration (Akhurst & Derynck 2001, Blobe et al. 2000), as well as participates in angiogenesis (Pardali & ten Dijke 2009) and regulation of ECM synthesis and fibrosis (Leask & Abraham 2004). TGF-β1 contributes to the regulation of inflammation (Han et al. 2012) and myofibroblast differentiation and hence exhibits crucial roles in wound healing (Pakyari et al. 2013, Sarrazy et al. 2011). In tumor cells, TGF-β1 is the most frequently upregulated TGF-β isoform. It has a number of roles in cancer progression, e.g. induction of the epithelial to mesenchymal transition (EMT), and mediates both positive and negative effects on tumor progression. (Derynck et al. 2001).
3 Outlines of the study

MMPs play versatile roles in tissue repair and carcinoma progress and their expressions and actions seem to depend on temporal and spatial factors. The function of MMP-8 in tissue repair is not completely understood and partly contradictory evidence has been presented. Fibroblasts contribute critically to the tissue repair process by contracting the wound surroundings and synthesizing ECM macromolecules. However, the role of MMP-8 in oral tissue repair and tongue fibroblast action is poorly understood. Accumulating evidence suggests that MMP-8 has positive features in various human cancers through a number of mechanisms, but its role in OTSCC is unknown. Since OTSCC shows highly aggressive features and is associated with poor prognosis, the prediction of the disease course is of high importance in order to improve patient treatment planning. Understanding the molecular mechanisms involved in OTSCC cell behavior is essential for the development of novel diagnostic and treatment strategies. MMP-8 driven antitumorigenic mechanisms have been studied in various human cancer cells in vitro, but not in OTSCC cell lines. The overall aim of the work was to investigate the role of MMP-8 in tissue repair and tongue carcinoma processes. More specifically, we aimed:

1. To identify the role of MMP-8 in alveolar bone and tongue tissue repair processes. The study was accomplished by using a MMP-8 deficient mouse model.
2. To explore the effects of MMP-8 on fibroblasts and more specifically the contribution of MMP-8 in the regulation of TGF-β1 signaling. TGF-β1 plays essential roles in stimulating fibroblasts and plays a number of roles in wound healing, including scar formation in skin.
3. To examine the role of MMP-8 in human and mouse OTSCC and evaluate its potential in predicting patient survival.
4. To identify the protective mechanisms of MMP-8 action in OTSCC by studying tongue carcinoma cell behavior in vitro and the effect of MMP-8 on the metastatic potential of these cells in orthotopic mouse model of OTSCC in vivo.
4 Materials and methods

The materials and methods used in the original articles are summarized in Table I and the most unique methods of the present thesis are briefly described.

Table 1. Methods used in the original publications.

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4.1 Human samples

Human carcinoma resection samples were collected for histopathological analyses from the patients with diagnosed OTSCC during the years 1981–2001 at the Oulu University hospital. The samples were analyzed for Bryne’s malignancy score, microvascular density (factor VIII, CD31), and thickness as well as various cancer related factors [COX-2, αvβ6 integrin, laminin-5 γ2-chain (laminin-332), estrogen receptors-α and -β, VEGF-C and MMPs -2, -7, -8, -9, -20 and -28]. The patient clinical data are described in detailed in the original articles III and IV. The tumor-related factors were collected from the hospital files (tumor grade, TNM stage) and the patients’ present status from the death statistics of Finland.

4.2 Animal experiments

4.2.1 Animals

*Mmp8-/-* mice in a C57BL/6J/129/SvJ background were a kind gift from Prof. Carloz Lopez-Otín, University of Oviedo, Spain. The mice bred and developed normally after disabling the *Mmp8* gene (Balbin *et al.* 2003). C57BL/6J (Harlan, Holland) mice were used as controls. *Mmp8-/-* mice were used in tissue repair models and carcinogen induced OTSCC. In addition, blood was collected to analyze serum TGF-β1 with an enzyme-linked immunosorbent assay (ELISA). BALB/c nude mice (Charles River, Germany) were used in an orthotopic model of OTSCC. The number of mice as well as the anesthesia and analgescs used are described in more detail in the original articles I–IV.

4.2.2 Mouse tissue repair models

*Tooth extraction procedures*

The second maxillary molars of *Mmp8-/-* and wild type (wt) mice (four weeks old) were removed with a dental probe under anesthesia and given analgesics after the operation. At day four or seven following the tooth extraction, the mice were sacrificed and the skulls fixed and processed into histological sections to determine the total inflammatory cell count and for IHC analyses of newly synthesized type III collagen.
The right side mandibular molars of \textit{Mmp8}\textsuperscript{-/-} and wt mice (four weeks old) were extracted and after four days, the mice were sacrificed and jaw bones and alveolar mucosa from the healing area were removed and frozen in liquid nitrogen. The extracted proteins were used to determine cytokine levels with the RayBio mouse inflammatory array (RayBio) and myeloperoxidase (MPO) levels with ELISA (Hycult Biotechnology). Degradation product levels of type I (carboxyterminal telopeptide ICTP) and type III (aminoterminal telopeptide IIINTP) collagens were analyzed with Western blotting, MMP-9 with gelatin zymography and TIMP-1 levels with reverse zymography.

\textit{Periapical lesion induction}

Pulpal exposure was performed on mandibular first molar of \textit{Mmp8}\textsuperscript{-/-} and wt mice (four weeks old) under anesthesia by using dental burs (Komet ISO 806 104; Komet Group). Microbial infection was ensured by leaving the pulp chambers open. After three weeks, the mice were sacrificed and the skulls were processed for histological analyses or scanning with a peripheral quantitative computerized tomographic (pQCT) (Stratec XCT 960A, Norland Stratec Medizintechnik) device to examine the periapical lesion area.

\textit{Experimental tongue wounds}

Wounds were created to the dorsal right side of \textit{Mmp8}\textsuperscript{-/-} and wt mice (three to four months old) tongues with a 1 mm biopsy-punch. The animals were sacrificed at selected time points (6 h, 24 h, 48 h, 4 d, 11 d) and the tongues collected for histological analyses to measure wound widths and IHC analyses to examine TGF-\(\beta\)1, LIX and \(\alpha\)-SMA levels or frozen in liquid nitrogen and processed into protein extraction samples to examine TGF-\(\beta\)1 and phosphorylated (P) Smad2 with Western blotting.

\textbf{4.2.3 Mouse models of OTSCC}

\textit{Carcinogen induction of mouse OTSCC}

Tongue SCC was induced in 13–16 weeks old \textit{Mmp8}\textsuperscript{-/-} and wt mice by smearing the left dorsal half of their tongues with carcinogenic 4-Nitroquinoline-N-Oxide
(4NQO, Sigma) (10 mg/ml in propylene glycol). The treatment was performed under slight anesthesia and repeated three times per week for 12 weeks. The mice were sacrificed at 55 weeks of age and the tongues prepared for histological analyses and IHC to analyze ERs.

Orthotopic mouse model of OTSCC

The HSC-3 cells with and without MMP-8 overexpression (2x10^4 cells; described in the next section) were injected into the lateral part of the tongues of BALB/c nude mice. The mice were weighed daily and the tongues and lymph nodes were collected for histological analyses after three weeks (or less if the mouse had to be sacrificed due to weight loss or health problems).

4.3 Cell experiments

4.3.1 Cell lines and cell culture sample analyses

Mmp8-/- and wt mouse tongues were used to establish fibroblast primary cell cultures. The tongues were cut, washed and sectioned onto petri dishes. The tissue pieces were covered with fibroblast culture medium and the growing fibroblasts transferred by trypsinization to cell culture flasks. MMP-8 expression and localization was studied with immunofluorescent staining in human OSCC C1 cells (Ylipalosaaari et al. 2005) and with RT-PCR in HSC-3 cells with and without 10 nM estrogen (Steraloids, Inc) treatment. Human OTSCC cell lines HSC-3 (ATCC, USA), SCC-15 and SCC-25 (Japan Health Science Resources, JRCB 0623, Japan) were used to establish stably MMP-8 overexpressing (MMP-8+) cell lines with a lentiviral vector containing a MMP8 or null control sequence (Amsbio). The cells were selected with puromycin and MMP-8 overexpression confirmed by PCR and Western blot. Short hairpin (sh) RNAs in lentiviral particles were used to silence the expression of MMP-9 (shMMP-9) (Thermo Fischer Open Biosystems) and cathepsin-K (Santa Cruz Biotechnology) in the HSC-3 cell line. OSCC cells were used to study various cell processes, including apoptosis (TUNEL), proliferation (BrdU) and adhesion (ECIS). The invasion (Matrigel® and myoma), migration (horizontal and vertical) and collagen contraction assays are briefly described in the next sections.
Fibroblasts or their conditioned media were examined for TGF-β1 and PSmad2 protein levels with Western blotting. In some cases, MMP-8-treated TGF-β1, intact TGF-β1 (10 ng/ml R&D Systems) or exogenous rhMMP-8 (0 ng, 5 ng/ml, 10 ng/ml and 50 ng/ml; Protealmmun) was added to the media and PSmad2 levels were examined. Newly synthesized type III collagen and α-SMA levels were examined by IHC. Mmp8 mRNA levels were detected with RT-PCR.

OTSCC cells or their conditioned media were examined for VEGF-C, TGF-β1, MMP-1 and EMT markers (E-cadherin, slug, N-cadherin, vimentin) with Western blotting, MMP-9 with gelatin zymography, TGF-β1 with ELISA and MMP8 mRNA levels with RT-PCR or qRT-PCR. In addition, stationary and migrating MMP-8+ and control HSC-3 cells were subjected to gene expression microarray with Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix).

4.3.2 Myoma invasion assay

The invasion of OTSCC cells was examined by using 3D organotypic myoma cultures (Nurmenniemi et al. 2009). Human uterine leiomyoma samples were obtained during routine surgeries at the Oulu University hospital. All the donors provided their written consent for the tissue use in research. The myoma samples were punch biopsied to obtain equal sized disks and placed into Transwell® inserts (Corning Inc.). 7x10^5 MMP-8+ and 3x10^5 shMMP-9 and corresponding control cells were seeded on top of the myoma disks in 10% FBS culture medium. The medium was added into the wells to obtain an air-liquid surface. The next day, the myomas were transferred onto nylon disks lying on steel grids in 12-well plate with culture medium. The cells invaded for 10 or 14 days and the myomas were processed into histological samples to examine the area of invaded cells and maximum invasion depth. IHC was used to examine the levels of EMT markers (E-cadherin, vimentin) in MMP-8+ and control cells invading into the myoma disks.

4.3.3 Migration assays

Migration of fibroblasts and OTSCC cells was studied with a scratch assay. Confluent cell layers were wounded with a pipette tip and the migration was followed by photographing the cultures at regular intervals. In the case of shMMP-9 cells, scratch migration was studied also using matrix components [bovine serum albumin (BSA), type I collagen, FN, Matrigel®] as a migration
base. Vertical migration was studied in Transwell® inserts. In case of MMP-8+ and control HSC-3 cells, vertical migration was studied also with exogenous rhTGF-β1 (R&D System), rhVEGF-C (ProspecBio) and CTT-2. In addition, ibidi inserts were used to create open areas to MMP-8+ and control HSC-3 cell cultures. The cells were monitored to obtain phase-contrast time-lapse images and cell movement was analyzed by using the maximally stable extremal regions (MSER) approach (Matas et al. 2004).

4.3.4 Collagen contraction assay

*Mmp8−/−* and wt mouse tongue fibroblasts (7x10⁵) were embedded into collagen gels containing eight volumes of type I collagen (BD BioScience), one volume of 10 x Dulbecco’s modified eagle medium (DMEM) and one volume of cells in fetal bovine serum (FBS) or in 5% lactalbumin (0.5% in gel) media with and without TGF-β1 (R&D Systems; 1 ng/ml in gel). The gels (1 ml) were added into 24-well plates and the contraction was followed by photographing the wells at regular intervals. When TGF-β1 was included in the gels, the serum free culture media also contained 1 ng/ml TGF-β1.

4.4 *In vitro* cleavage assay

Recombinant human (rh) TGF-β1 (R&D Systems), Fas-L (Calbiochem) and ERs -α and -β (Invitrogen) were incubated with and without activated rhMMP-8 (ProtealImmun or Chemicon) in incubation buffer with and without MMP inhibitors at 37°C o/n. The samples were analyzed with Western blotting.

4.5 Ethical considerations

The human studies were approved by the Ethical Committee of the Faculty of Medicine, University of Oulu, Finland (III/IV: 18/2003, IV: 8/2006 and 26/2006). The animal experiments were approved by the Animal Care and Use Committee at the University of Oulu, Finland or the National Animal Care and Use Committee of Finland (I: 113/05 and 046/06, II: OLH-2006-02543/Ym-23, ESLH-2007-07454/Ym-23 and ESLH-2008-09648/Ym-23, III: OLH-2006-02544/Ym-23 and ESLH-2007-06458/Ym-23) or Animal Welfare Committee at the University of Tromsø, Norway (IV: 6374).
5 Results

5.1 MMP-8 plays divergent roles in oral wound healing

5.1.1 Tissue repair in MMP-8 deficient mice results in varying outcomes depending on the location

Knowledge of the role of MMP-8 in wound healing is restricted mainly to skin wound healing studies with partly contradictory results (Danielsen et al. 2011, Gutiérrez-Fernández et al. 2007). The previously established finding (Gutiérrez-Fernández et al. 2007) that Mmp8-/− mouse skin wound healing is delayed, was confirmed with a small group of mice (Suppl Fig 1 in II). The role of MMP-8 in healing of soft and hard tissue in the oral environment was studied using Mmp8-/− mice. New trabecular bone formation was examined in wt and Mmp8-/− mice extraction sockets after removing the second maxillary molars. Based on the histological analyses, the trabecular bone areas 4 d and 7 d after tooth removal were similar in wt and Mmp8-/− mice (Fig 1A–D and I in I). To examine periapical inflammation, mice were subjected to pulpal exposure in first mandibular molars. The analyses of periapical lesions were conducted using histological and radiological methods (Fig 2 in I) to investigate the surface areas of ligament spaces and the granulation tissue lesion size in both healthy and infected roots. MMP-8 deficiency had no effect on these parameters.

Using the same Mmp8-/− mouse model, MMP-8 action was further studied in tongue wound healing. Interestingly, unlike in skin, tongue wound healing revealed a dramatic increase in wound early contraction in these mice (Fig 1A–B in II).

5.1.2 MMP-8 affects collagen metabolism

Further analyses revealed significantly increased levels of newly synthesized type III collagen in Mmp8-/− mice bone granulation tissue after 4 d of healing of a tooth extraction socket (Fig 1E–H and J in I). The difference was not detected, however, in the samples collected after 7 d of healing. Interestingly, the increase in type III collagen was also observed in cultured Mmp8-/− tongue fibroblasts (Suppl Fig 5 in II). Moreover, the effect of MMP-8 on collagen metabolism was evidenced by determining the degradation product levels of type I (ICTP) and
type III (IIINTP) collagens by Western blot. The protein extraction samples of Mmp8-/− mice jaw bone and mucosa after teeth removal revealed higher ICTP and IIINTP levels in samples (Figure 4A in I).

5.1.3 MMP-8 affects inflammation and MMP-9 levels in healing extraction sockets, but not in tongue wounds

The analysis of jaw bone 4 d after the removal of all right side mandibular molars, revealed a diminished total inflammatory cell count in Mmp8-/− mice compared to wt mice. Determination of MPO levels reflecting myeloperoxidase positive cells (mainly neutrophils) confirmed the reduction in inflammatory response (Fig 3C–D in I). In contrast, no difference was observed in the MPO expressing inflammatory cells during the mouse tongue wound healing (Suppl Fig 2A–B in II). The jawbone and alveolar mucosa cytokine levels also showed various changes in Mmp8-/− mice (Fig 3A–B in I). In alveolar mucosa, the most dramatic decrease (≥40%) was observed in IL-6, MIP-1γ and -1α and a corresponding increase (≥40%) in II-1β and Fas-L. In contrast, Fas-L was the most decreased factor in jaw bone. Other considerably (≥40%) diminished cytokines in Mmp8-/− jaw bones were IL-6, Fractalkine, IL-3 and granulocyte colony stimulating factor (GCSF), whereas the highest increase (≥40%) was observed in IFN-γ, granulocyte macrophage colony stimulating factor (GM-CSF), and keratinocyte derived cytokine (KC). The results of the cytokine array represent the average of four samples per genotype. Hence, the observations from this analysis are only directional and statistical differences could not be established. Among the most affected cytokines in Mmp8-/− mice tooth extraction wounds, Fas-L, an apoptosis factor, showed a difference between the two tissue types examined. Further in vitro experiments with recombinant human proteins revealed Fas-L to be an MMP-8 substrate (Fig 3E in I).

MMP-9 and TIMP-1 levels were determined by zymography and reverse zymography, respectively. Overall, the levels of MMP-9 were higher and TIMP-1 levels lower in jaw bone compared to that of alveolar mucosa. MMP-8 deficiency decreased MMP-9 levels, but had no effect on TIMP levels (Fig 4C–D in I). In tongue wounds, however, MMP-8 deficiency had no effect on MMP-9 levels (Suppl Fig 2C in II).
5.1.4 MMP-8 regulates TGF-β1 levels in mouse tongue wounds and fibroblasts in vitro

The unchanged inflammatory response and MMP-9 levels suggested that in tongue wounds, MMP-8 may affect factors other than those observed in skin wounds (Gutiérrez-Fernández et al. 2007) and tooth extraction sockets healing in this study (I). TGF-β1 contributes to a variety of cellular processes during wound healing, including wound contraction. IHC analyses revealed significantly increased TGF-β1 levels in Mmp8-/− mouse tongue wounds at 24 h (Fig 1C–D in II). By this method, the location of the factor under interest can be determined and the analyses performed from the chosen sites. In addition, the protein extraction samples from wounded mouse tongues were subjected to TGF-β1 Western blot. The analysis confirmed the alteration of the dimer form of TGF-β1 (Fig 1E in II). The result, however, now represented a larger area around the wound, and perhaps therefore, the peak time point of the levels was detected in 6-h wounds, in contrast to the peak in the levels in 24-h wounds in IHC staining analyses.

Fibroblast primary cell cultures were established from tongues of male wt and Mmp8-/− mice to investigate the possible role of MMP-8 in the behavior of these cells and in TGF-β1 action. The morphology confirmed the fibroblastic phenotype in all cell lines and no changes were observed in proliferation between the genotypes (Suppl Fig 6B in II). The ability of wt fibroblasts to express Mmp8 was first confirmed by RT-PCR (Fig 2A in II). Next, the fibroblasts were confirmed to express TGF-β1 detected as a molecular size corresponding to that of the dimer form. In cell extracts of wt fibroblast only, also a fragment smaller than the TGF-β1 dimer was detected. Moreover, TGF-β1 could be detected only in Mmp8-/− fibroblast culture media (Fig 2B in II).

The preliminary finding from the cell culture samples prompted an investigation of the ability of rhMMP-8 to process rhTGF-β1. Western blot analysis of the reaction products clearly demonstrated that the dimer, active TGF-β1 is a substrate of MMP-8 in vitro (Fig 2C in II). In the reduced gels (stained with Coomassie) the same was observed as diminished levels of the monomer TGF-β1.
5.1.5 MMP-8 decreases TGF-β1 signaling and collagen gel contraction in mouse tongue fibroblasts in vitro

TGF-β1 activates the cellular signaling cascade detected as phosphorylation of Smads. The observed increase in TGF-β1 was confirmed to also increase PSmad2 levels in Mmp8-/− fibroblasts (Fig 2D in II). Further cell experiments confirmed that exogenously added MMP-8 in Mmp8-/− fibroblast cultures can reduce the elevated PSmad2 levels to the lower level. The effect was directly related to the amount of MMP-8 added to the cultures (Fig 2E in II). In addition, MMP-8 reduced α-SMA expression of Mmp8-/− fibroblasts (Suppl Fig 7 in II). Moreover, exogenously added TGF-β1 triggered the PSmad2 dependent signaling cascade in Mmp8-/− fibroblasts, but when TGF-β1 was first treated with MMP-8, the detected PSmad2 levels were substantially lower (Fig 2F in II).

The absence of MMP-8 in mouse tongue wounds allowed the wounds to contract faster compared to those of wt mice. When fibroblasts from wt and Mmp8-/− mouse tongues were embedded in collagen gel lattices, the gels with Mmp8-/− fibroblasts reached substantially smaller sizes than the gels with wt fibroblasts (Fig 3A–B in II). Since the Mmp8-/− fibroblasts showed changes in PSmad2 levels, the effect of TGF-β1 alone was examined in serum free conditions. The exogenously added TGF-β1 did not have an effect on the wt fibroblasts and hardly any reduction in gel size was observed. However, Mmp8-/− cells reduced the size of collagen gels under similar culture conditions (Fig 3C in II).

5.2 MMP-8 has a multitude of tumor suppressive effects on OTSCC behavior in vivo and in vitro

5.2.1 Low MMP-8 and high vascular endothelial growth factor-C levels in OTSCC patient samples predict worse outcome of the disease

OTSCC is a highly aggressive cancer characterized with poor prognosis (Bello et al. 2010). To identify potential new molecular prognostic factors for OTSCC, 90 human mobile tongue SCC surgical resection samples of all stages were examined by using Bryne’s malignancy score, microvascular density and thickness. In addition, various factors potentially involved in OTSCC progress, were examined by IHC staining analyses. Tumor thickness did not predict patient
survival (Table I in III). Of the twelve factors studied [COX-2, αβ6 integrin, laminin-5 γ2-chain (laminin-332), estrogen receptors-α and -β, VEGF-C and MMPs -2, -7, -8, -9, -20 and -28], only MMP-8 and VEGF-C showed potential prognostic value (Table III in III and Fig 3 in IV). The relative mortality rate of the patients was significantly higher (with a hazards ratio of 3.7 by the Cox proportional hazards model) when the carcinoma cells were negative for MMP-8 compared with those with positive MMP-8 immunostaining. The Kaplan-Meier method was used to illustrate that MMP-8 expression in the SCC tumor cells correlated with better survival of the OTSCC patients. The association of MMP-8 with patient survival seemed to be stronger in women, but the difference did not reach statistical significance (Fig 1 in III). Furthermore, high VEGF-C levels with low MMP-8 levels predicted patient survival even better than the levels of MMP-8 or VEGF-C alone (Fig 3 and Suppl Fig 6 in IV).

5.2.2 MMP-8 protects mice from developing OTSCC but does not prevent metastasis

The association of MMP-8 with patient survival raised the question of whether MMP-8 could protect from OTSCC in the early development of the carcinoma. To examine potential protective features of the host MMP-8, wt and Mmp8-/- mice tongues were exposed to carcinogenic 4-Nitroquinoline-N-Oxide to induce experimental OTSCC. The efficiency of this agent to induce OTSCC was not very high, since from the group of 24 wt mice, only one mouse eventually developed a carcinoma. Dysplasia was observed in one third of the mice. Interestingly, the study revealed a higher incidence of dysplastic changes and carcinomas in Mmp8-/- mice (Table IV and Fig 2 in III). The difference was especially prominent in females; the number of dysplasia or carcinoma cases was 67 percent points higher when the mice lacked MMP-8 (83% vs. 17%). The same was detected also in males and the corresponding difference between Mmp8-/- and wt mice was 20 percent points (45% vs. 25%). However, when the metastatic potential of MMP-8 overexpressing HSC-3 cell was studied in the orthotopic murine model of OTSCC, no reduction in formation of lymph node metastases was found (Suppl Fig 1B–C in IV). The results of the experiment, however, suggested that MMP-8 retards OTSCC disease progress, since the mice with tumors from MMP-8 overexpressing cells survived distinctly longer than the control group mice (Suppl Fig 1A in IV).
5.2.3 MMP-8 inhibits OTSCC cell invasion and migration in vitro

To reveal the mechanism behind the positive effects of MMP-8 in OTSCC, in vitro studies were conducted with various oral SCC cell lines. The expression of MMP-8 in SCC C1 cells was located by immune fluorescent counterstaining with actin to cell membranes and in granules in subcellular sites (Fig 3A in III). Three MMP-8 overexpressing OTSCC cell lines (HSC-3, SCC-25 and SCC-15) were created by lentiviral transduction of the MMP8 gene (Fig 1A in IV). MMP-8 overexpression was detected by RT-PCR and Western blot. The anti-invasive effects of MMP-8 in vitro were demonstrated by using myoma tissue as a base for cells to invade. Various cellular processes, such as proliferation, apoptosis and migration as well as EMT, take part in carcinoma growth and cell spreading. The main cellular process that MMP-8 targeted was migration (Fig 2A–C in IV), whereas proliferation (not shown) or apoptosis showed no changes in the HSC-3 cell line (Suppl Fig 2A in IV). The differences in migration of the most aggressive cell line, HSC-3, were further examined using various approaches, including horizontal migration and cell movement analyses after wounding with ibidi cell culture inserts and vertical migration in Transwell® chambers. The experiments confirmed the inhibitory effect of MMP-8 on the migratory ability of these cells (Fig 2A–C and 7C in IV).

5.2.4 MMP-8 changes the proteinase and growth factor expression pattern in OTSCC cells in vitro

The gene expression patterns of control and MMP-8 overexpressing HSC-3 cells in the stationary and migrating phenotypes were determined by mRNA microarray analyses (Suppl Fig 3 and Suppl Fig 4 in IV). The expression levels obtained from the analysis represented the average of three repeats for both cell lines. Those genes showing more than 1.5 fold change in their expression between the control and MMP-8 overexpressing cell lines were subjected to further considerations. The genes with the highest differences were evaluated for their previously established importance in cancer. Eventually, the most interesting down regulated factors were considered to be VEGFC and MMP1. The decreases in VEGF-C and MMP-1 levels by MMP-8 overexpression in HSC-3 and SCC-25 cell lines were confirmed also at the protein level by Western blotting (Fig 3A and Fig 6A in IV). Interestingly, in addition, microarray analyses implied that MMP-8 expression resulted in increased MMP9 gene expression levels in migrating HSC-
The difference was confirmed in stationary cell cultures also by analyzing MMP-9 protein levels in all three MMP-8 overexpressing cell lines with gelatin zymography (Fig 4A in IV).

No changes were detected in TGFBI mRNA expression levels by microarray analysis. However, the established connection between MMP-8 and TGF-β1 in tongue wounds (Fig 1C–D in II) prompted the investigation of TGF-β1 protein levels in OTSCC cells as well. First, the decreased TGF-β1 level was observed with Western blot analysis in MMP-8 overexpressing HSC-3 cells. ELISA analysis further confirmed that the level of the active form of TGF-β1 was diminished, especially when compared to the amount of total TGF-β1 (Fig 7A–B). Furthermore, collagenases cathepsin-K and MMP-8 were found to co-regulate each other’s expressions in HSC-3 cells (Fig 6B in IV).

5.2.5 Interaction between MMP-8 and estrogen actions

The trend of the differential effect of MMP-8 in the genders detected in patient sample analyses and in the mouse study prompted further studies on the female sex related factor estrogen and ERs. MMP-8 overexpression in HSC-3 cells did not change the expression of estrogen or ERs based on microarray analyses (not shown, IV). However, estrogen was found to increase MMP-8 expression in HSC-3 cells (Fig 3B–C in III). When the cells were exposed to estrogen treatment, higher levels of MMP-8 protein were localized, particularly in cell membrane extracts. Moreover, the potential participation of MMP-8 in regulating estrogen action was evidenced in vitro by the finding that rhMMP-8 cleaves rhERs -α and -β (Fig 5A in III). By IHC detection, the expression of ERs -α and -β were also confirmed to be present in mouse and human tongue SCCs. A slight correlation between ER-β expression with better prognosis was found (Fig 4 and Table III in III).

5.2.6 The ability of MMP-8 to reduce OTSCC cell migration involves regulation of TGF-β1

Although VEGF-C and MMP-9 levels showed changes due to MMP-8 overexpression in HSC-3 cells (Fig 3A and Fig 6A in IV), these factors did not seem to be mediators of the reduced migratory ability in these cells. Despite the finding that silencing of MMP-9 with lentiviral shRNA particles in HSC-3 cells significantly increased the cell invasion and migration ability (Fig 4 and Fig 5 in
IV), gelatinase inhibition by CTT-2 in MMP-8 overexpressing cells did not improve their vertical migratory ability to the levels of control cells. Exogenous addition of VEGF-C also did not restore the ability of the cells to migrate (not shown, IV).

MMP-8 overexpressing cells showed no changes in TGF-β1 mRNA level (not shown, IV), but the protein level of TGF-β1 was reduced (Fig 7A–B in IV). A clear difference was observed in the vertical migration of wt and MMP-8 overexpressing cells through Transwell® chambers. Exogenously added active TGF-β1 increased HSC-3 migration first (24 h, not shown) in both control and MMP-8 overexpressing cells. Interestingly, TGF-β1 restored the difference in the migratory ability of the MMP-8 overexpressing cells to the levels of control cells. After 48 h, the difference in the cell migration was no longer statistically significant, although still some reduction could be detected in the vertical migration of MMP-8 overexpressing cells. Finally, after 72 hours, the migration levels were equivalent between the cell lines (Fig 7C in IV).
6 Discussion

MMP-8 contributes to several physiological and pathological conditions, including inflammation, wound healing and cancer progression (see Figure 5 above). The studies of the present thesis were conducted to resolve the role of MMP-8 in repair processes of hard and soft tissue in the oral cavity and in human and mouse oral tongue cancer. Subsequently, the effect of MMP-8 was analyzed using mouse tongue MMP-8 deficient fibroblasts and MMP-8 overexpressing OTSCC cell lines in vitro. The main novel findings of MMP-8 action in the studies I–IV are summarized in Table 2 and discussed in the next sections in more detail.

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<th>Table 2. The main findings of the MMP-8 role in the present study.</th>
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6.1 MMP-8 in hard and soft tissue repair processes

The mechanisms of MMP-8 actions in acute wound repair processes have been resolved previously only in the skin of mouse (Gutiérrez-Fernández et al. 2007) and rat (Danielsen et al. 2011). Healing of an alveolar bone socket after tooth extraction, and wounds in Mmp8/−/− mouse tongues were excellent models to study MMP-8 action in both soft and hard tissue repair processes.

Based on our tooth extraction healing studies, MMP-8 did not affect the overall healing rate of the new trabecular bone or periapical lesions (I). However, MMP-8 expression was related to the regulation of collagen metabolism and inflammatory response leading to altered levels of MMP-9. The participation of other MMPs, such as MMP-2 and -9 in granulation tissue (Accorsi-Mendonca et al. 2008, Silva et al. 2001), and MMP-13 (Devlin 2000) in the sites of new bone formation, has previously been demonstrated in rat tooth extraction socket
healing. The knowledge about MMP-8 in hard tissue repair is very restricted. MMP-8 is expressed by collagen expressing osteocytes, osteoblasts and chondrocytes in rat mandible and hind limb growth plate, which suggests its role in collagen remodeling in bone and cartilage development (Sasano et al. 2002). In addition, the highest MMP-8 expression can be detected in the early moments of bone healing in experimental rat bone defect, before the highest collagen I expression (Itagaki et al. 2008).

Mmp8-/- mouse skin wounds are characterized by increased MMP-9 levels and increased inflammation with reduced healing rate (Gutiérrez-Fernández et al. 2007). Unlike in skin, wounds in Mmp8-/- mouse tongues showed increased early contraction, but no effect on MMP-9 levels (II). These results suggest that mechanisms of MMP-8 action that take place in keratinized masticatory mucosa of the tongue differ from those in skin. In contrast to mouse experiments, excess MMP-8 in rat acute skin wounds inhibited the repair process (Danielsen et al. 2011).

In mouse tongue wounds, MMP-8 deficiency increased the levels of active TGF-β1. We showed that MMP-8 was able to cleave TGF-β1 and regulate TGF-β1 signaling in fibroblasts (II). This suggests that MMP-8 may also regulate the action of fibroblasts during wound contraction. However, the skin wounds of Mmp8-/- mice showed increased PSmad2 levels, but a reduced healing rate (Gutiérrez-Fernández et al. 2007). In our study, the increase in PSmad2 levels in Mmp8-/- tongue fibroblasts could explain the accelerated collagen gel contraction, since MMP-8 seemed to diminish α-SMA expression in these cells.Interestingly, TGF-β1 overexpression in mouse inhibited full thickness skin wound healing, but accelerated partial thickness ear wound healing, suggesting that TGF-β1 signaling has variable effects depending on the depth and location of the wound (Tredget et al. 2005). This highlights the importance of the variations in ECM composition and the characteristics of the cells originating from different locations. Moreover, fibroblasts from human skin and oral buccal mucosa showed differences in the TGF-β1 induced myofibroblast differentiation (Meran et al. 2007, Meran et al. 2008), and the level of myofibroblasts in pig skin was lower than in the oral mucosa (Mak et al. 2009). In pig oral mucosa, the amount of TGF-β1 and PSmad3 immunopositive cells was also low compared with skin wounds. Interestingly, although the number of myofibroblasts was significantly elevated in oral wounds, they showed significantly less contraction than the maturing wounds in the skin. (Mak et al. 2009). In our early stage tongue wounds, the amount of α-SMA positive myofibroblasts was altogether low without any clear differences
Mmp8-/- and control wounds. Interestingly, in TGF-β1 stimulated human dermal fibroblasts, IL-10 up-regulated MMP-8 mRNA expression and reduced the amount of myofibroblasts. IL-10 also inhibited scar formation when injected into mouse dermal wounds. (Shi et al. 2013). However, since the level of extracellular TGF-β1 in various tissues is different, it may, at least to some extent, explain the variable outcomes of the tissue repair processes in bone, skin and oral mucosa. Our results demonstrating that TGF-β1 could be cleaved by MMP-8, may thus to some extent explain the discrepancy in wound healing rates of skin and oral mucosa in the MMP-8 deficient mouse model.

### 6.2 MMP-8 as a prognostic tool in head and neck cancers

A major clinical challenge in oncology is the prediction of the disease course and identification of the most detrimental cases. The studies identifying the association of MMP-8 with disease progress or patient survival are somewhat controversial depending on the cancer and the analytical methods used to measure the expression and level of MMP-8. There are studies using the evaluations of plasma or serum levels of MMP-8, tumor and inflammatory cell MMP-8 levels, and the variations in MMP8 gene expression. A number of studies in cancers of tissues other than the head and neck suggest that the presence of MMP-8 in cancer is a positive sign for the disease progression, such as in melanoma (Palavalli et al. 2009), breast cancer (Decock et al. 2008) and lung cancer (González-Arriaga et al. 2008). However, in the case of colorectal cancer (Väyrynen et al. 2012), ovarian cancer (Stadlmann et al. 2003) and also melanoma (Dębiak et al. 2011, Vihinen et al. 2008) high levels of MMP-8 predicts worse disease prognosis.

In this study, MMP-8 positivity in OTSCC cells was found to predict better survival of the patients and the phenomenon was even stronger in women (III). MMP-8 staining in inflammatory cells did not show association with survival of the patients, which suggests that in OTSCC, the protective role of MMP-8 is not related to the mechanisms mediated by neutrophil derived MMP-8. However, in another study of OTSCC samples, the cytoplasmic MMP-8 level did not associate with the invasion depth or the size of the tumor (Mäkinen et al. 2012). There are only a few studies measuring serum or plasma levels of MMP-8 as an indicator of the HNSCC disease occurrence or state. In those studies, the HNSCC disease course could not be predicted based on plasma MMP-8 levels (Nurmenniemi et al. 2012, Pradhan-Palikhe et al. 2010), but the levels associated with tumor stage
The finding that high levels of MMP-8 inhibitor TIMP-1 in HNSCC predict poor outcome (Pradhan-Palikhe et al. 2010) supports the protective action of MMP-8 or some other TIMP-1 target proteinase.

Using several prognostic factors that associate with each other could provide more accurate prediction of the disease status and future course. In colorectal cancer, for example, serum MMP-8 levels correlate with blood neutrophil count and MMP-8 associates with the inflammatory properties of the primary tumor (Väyrynen et al. 2012). In HNSCC, MMP-8 association with TIMP-1, ICTP and IIINTP has been examined but no connection was found (Nurmenniemi et al. 2012). The association can also be useful when evaluating the potential mechanisms behind the action of a specific proteinase. In this study, low MMP-8 expression strongly correlated with high VEGF-C expression. Furthermore, this combination was associated with poor cancer specific survival of OTSCC patients (III). Since we found decreased VEGF-C expression in MMP-8 overexpressing SCC cells (IV), it is possible that MMP-8 regulates VEGF-C also in vivo and some of the protective features are mediated through this action. The co-expression of MMP-9 with MMP-8, similar to our findings in HSC-3 cells overexpressing MMP-8, has been also found in breast carcinomas (Decock et al. 2007). This combination might also be valuable in prediction of OTSCC disease course, since MMP-9 alone was slightly connected to better survival of the patient, but without statistical significance (III). In addition, the revealed connection between MMP-8 and ERs in vitro should be further validated, especially in cancers that show gender related differences in incidence or disease progress. Sex hormones may also be involved in the progress of OTSCC, since ER-α expression levels in oral SCC (Colella et al. 2011) and ER-β levels in HNSCC (Shatalova et al. 2011) are increased compared with healthy tissue.

6.3 Mechanisms of MMP-8 action in cancer

Knowledge of the molecular mechanisms involved in various stages of the disease can lead to the development of novel methods for diagnostics and treatment of cancers. With the present knowledge about the complex roles of MMPs, it is not surprising that the first trials with MMPIs showed unfavorable clinical outcomes (Overall & López-Otin 2002). The abundance of research techniques available today has enabled studies identifying the specific mechanisms of individual MMPs in cancer. Next, the wide range of methods used to reveal MMP-8 mediated mechanisms in human cancers are discussed.
6.3.1 Methodological aspect of the studies resolving the role of MMP-8 in cancer

There are several ways of analyzing the impact of various molecules in the carcinogenesis and progression of tumors. In the following sections, the current knowledge and the outcome related to the importance of MMP-8 in cancer progression using human clinical samples, animal models or in vitro methods are briefly discussed.

The assessment of circulating MMP-8 levels in cancer patients’ prognosis could provide an easy diagnostic tool to predict disease course. However, using serum or plasma samples, very limited information of MMP-8 mediated mechanisms in carcinogenesis is obtained. Currently, the results related to the relevance of circulating MMP-8 levels as a cancer progression prognosticator vary, and clinically relevant recommendations for its use in any cancer, including HNSCC, cannot be given (Decock et al. 2008, Nurmenniemi et al. 2012, Pradhan-Palikhe et al. 2010, Väyrynen et al. 2012). Partially, in addition to differences in cancers, the variations may result from differences in samples and their preparation. Both serum and plasma have been analyzed by using either immunofluorometric (IFMA) (Mäntylä et al. 2003) or commercial ELISA assays to measure MMP-8 levels.

The studies using IHC analyses to detect MMP-8 in patient samples vary in terms of sample collection, processing, IHC methods and data interpretation. These variations may account, at least in part, for the differential results obtained for MMP-8 in various human cancers. First, the antibodies used can display varying results depending on, e.g. their specificity, sensitivity, retrieval methods and the controls used (Soland & Brusevold 2013). The starting material has also varied: MMP-8 has been stained in formalin fixed biopsy, tumor resection or TMA block sections. The wider resection samples allow an overview of cancer and TME compartments unlike small biopsy or TMA samples. In the present study (III), MMP-8 was evaluated separately from the inflammatory cells and tumor cells of OTSCC resection samples, whereas in the OTSCC TMA samples, only cancer cells were analyzed (Mäkinen et al. 2012). A protective role of MMP-8, similar to our findings in OTSCC, was revealed for breast tumor specimens using archived resection material (Gutiérrez-Fernández et al. 2008). In that study, MMP-8 levels in cancer cells correlated with the lower incidence of metastasis and with better overall prognosis. Similarly, in our study, the protective effect of MMP-8 in OTSCC was related to expression in cancer cells and significantly
associated with the patient survival (III). In addition, we revealed the association of low MMP-8 and high VEGF-C in OTSCC patient samples which associated with poor prognosis (IV). Interestingly, MMP-1 and VEGF-C co-expressions have been reported in some studies as a combination predicting poor prognosis in cancer (Tao et al. 2012, Xu et al. 2013). The study evaluating MMP-8 as a disease prognostic tool used OTSCC TMA samples, but there MMP-8 was not associated with tumor size or invasion depth (Mäkinen et al. 2012). TMA material was also used in the study of ovarian cancer progression, where, in contrast to the results in breast cancer (Gutiérrez-Fernández et al. 2008) and in our OTSCC studies (III, IV), a connection between high MMP-8 level and poor prognosis was found (Stadlmann et al. 2003).

Cell culture experiments provide numerous approaches to study the molecular basis of MMP-8 function in vitro. MMP-8 expression in cultured SCC cells was first detected over ten years ago using a sandwich enzyme immunoassay system (Shimada et al. 2000) and PCR methods (Moilanen et al. 2002). Here, we could localize MMP-8 in the cell membrane and cellular granules of OTSCC cells (III), similar to localization in activated PMN leukocytes (Owen et al. 2004). In leukocytes, where MMP-8 has a crucial role during the inflammation process, membrane bound MMP-8 was shown to be highly resistant to TIMP inhibition.

Cell functional studies of proliferation, apoptosis or adhesion can easily be determined with commercially available kits. Those detected changes may potentially reflect on cell migration, invasion and metastasis ability also in vivo. Cell migration is usually studied by horizontal migration of scratch/wound healing assays (ibidi cell culture inserts or scratching with a pipette tip), ECIS, and vertical migration with Transwell® chambers. Here, the effect of MMP-8 on cell migration was studied using both horizontal (scratch assay and ibidi inserts) and vertical (Transwell®) migration that revealed that MMP-8 reduced the migration of HSC-3 cells in vitro. Interestingly, MMP-8 overexpressing cells also expressed increased levels of MMP-9 and silencing of MMP-9 in HSC-3 cells improved the cell migration (IV). Since the migration was clearly reduced by MMP-8 overexpression, we compared the gene expression profile of these cells to the control cells of both the stationary and migratory phenotypes. In the migratory phenotype, the expression of MMP9 showed a clear difference in mRNA level, but for example, the difference in VEGFC levels in stationary cells was not detected in migrating cells (IV).

Invasion in vitro is determined by cells able to penetrate tissue barriers, particularly BM structures (Kramer et al. 2013). The most widely used model to
study cell invasion is commercially available Matrigel® (BD Bioscience) as a base for cell invasion through porous membranes in Boyden chambers (Boyden 1962) such as commercially available Transwell® chambers (Corning®). However, it is questionable if the method accurately simulates the ECM of human cancer, since Matrigel® is derived from mouse sarcoma tumor composed of BM matrix components. The effect of MMP-8 on breast cancer invasion was demonstrated by increased ability of MMP-8 downregulated cells to pass through Matrigel® in Transwell® chambers (Agarwal et al. 2003). The myoma invasion assay is an excellent model to study human cancer cell invasion (Nurmenniemi et al. 2009). Obviously, the method cannot completely replace experimental animal studies, but it has some advantages compared to the current in vitro invasion assays; myoma is solely human tissue and mimics the microenvironment of the tumor. Of the three MMP-8 overexpressing cell lines used in the myoma invasion assay, HSC-3 cells showed the highest invasive potential, whereas SCC-15 cells invaded least, corresponding to the previous understanding about the difference in the aggressiveness of these cells (Ramos et al. 1997, Scott et al. 1988). Here, we were able to show that MMP-8 also affected cell invasion in the myoma model (IV). A cell spot microarray technique using various human cancer cell lines revealed that MMP-8 regulates β1 integrin activity and this action may contribute to cell invasion ability (Pellinen et al. 2012). In breast cancer cells, instead, the regulation of IL-7 and -8 levels by MMP-8 may play an important role in controlling inflammation, but the effect of this action on cell invasion remains unknown (Thirkettle et al. 2013).

The experimental in vivo evidence of the molecular mechanisms of MMP-8 action in cancer has come from the studies using Mmp8-/- mouse models. The first study indicated the protective role of host MMP-8 in skin tumor progression (Balbín et al. 2003). Thereafter, the same mouse model was used to show the protective effect of MMP-8 in the formation of melanoma metastases (Gutiérrez-Fernández et al. 2008). Using the same model, we found that the host MMP-8 protected from OTSCC development after exogenous application of carcinogen (III). However, with this method, the mice did not develop metastases. In these transgenic animals, the normal immune system contributes to cancer progression, unlike in the classical mouse back subcutaneous tumor xenograft model. Moreover, the antitumor effect of MMP-8 in breast cancer cells was demonstrated by injecting the cancer cells into the mouse mammary fat pad (Soria-Valles et al. 2014). It should be kept in mind that in these immune deficient nude mouse studies, including various orthotopic mouse models analyzing human cancer cells,
some interactive mechanisms related to normal immune system may left unrevealed. In addition, it should be noted that when human cancers are studied in mouse, species specific differences may confound the results. When the function of human cells with substantial overexpression of proteinase, such as MMP-8, is evaluated in vivo, several regulatory mechanisms (such as TIMPs) most likely also affect proteinase actions.

The next step in cancer progression followed by invasion is the formation of metastases. Depending on the cancer, the cells do metastasize usually either through lymphatic or blood vessels. However, although the overexpression of MMP-8 in HSC-3 OTSCC cells reduced the migration and invasion in vitro and the size of primary tumor in mouse tongue, there was no difference in the metastatic potential of the cells in the orthotopic mouse model (IV). The abnormal immune response of the nude mice, and possible inhibitory factors involved in the process, may explain at least partly the result from this model. Instead, genetic manipulation of MMP-8 in two breast cancer cell lines, originating from the same parental cell line, but with opposite MMP-8 expressions and metastatic phenotypes, also strongly indicated the antimetastatic actions of MMP-8 (Agarwal et al. 2003, Montel et al. 2004). In addition, MMP-8 overexpression in murine melanoma cells, increased their adhesion to type I collagen and laminin-1, and suppressed the metastatic ability of these cells (Gutiérrez-Fernández et al. 2008). The antimetastatic action of MMP-8 in vivo was further demonstrated in mouse by injecting breast cancer cells into the tail vein. This study showed that MMP-8 also regulated the MiR-21 level that is able to inhibit various tumor-suppressor genes. (Soria-Valles et al. 2014).

The present study revealed that MMP-8 overexpression in tongue SCC cells substantially affects the proteinase and growth factor pattern (e.g. MMP-9, MMP-1, cathepsin-K, VEGF-C and TGF-β1) of the cells (IV). These changes most likely cause positive changes in the behavior of the SCC cells in vitro, but the contribution of these factors to the cancer progression in vivo remains to be elucidated in future studies. MMP-1, for example, is generally viewed as a devastating proteinase in tumor progression and has prognostic value in various human cancers (Ala-aho & Kähäri 2005). Recent studies further confirm this proposal and reveal some putative mechanisms behind the MMP-1 action (Liao et al. 2012, Tahara et al. 2010, Tao et al. 2012, Xu et al. 2013, Yang et al. 2013).

In summary, evidence accumulating from studies with diverse approaches will be required to eventually uncover the multifunctional complex roles of MMP-8 in various cancers, including OTSCC. In general, consistency in sample
handling and methodology seems to be crucial when patient samples (blood, carcinoma tissue) are analyzed for MMP-8 levels. However, in experimental cell studies, the effect of MMP-8 seems to be quite uniform and the methodology does not seem to play an important role. The possibility to repeat the analyses and use diverse methodology provides extra reliability for the in vitro observations.

6.4 Interaction between MMP-8 and growth factors and cytokines – role in modulating TGF-β1

The number of in vitro and in vivo substrates identified for MMPs is constantly increasing. The group of identified MMP-8 non-structural mouse or human substrates includes cytokines and growth factors, such as LIX (Balbín et al. 2003, Tester et al. 2007, Van Den Steen et al. 2003b), MIG, IP-10 (Van den Steen et al. 2003a) and MCP-1 (McQuibban et al. 2002), and also regulators of cytokine/growth factor actions, such as decorin (Soria-Valles et al. 2014). In this study, the in vivo relevance of the identified novel in vitro substrates Fas-L (I), ER-α and -β (III) and TGF-β1 (II) was only partly established.

Fas-L is an apoptotic factor transmitting its effects by stimulating the cell membrane receptor (Fas) and altered expression of Fas/Fas-L is connected to cancer (Ashkenazi & Dixit 1999). In addition to MMP-8 action on Fas-L as shown in this study, MMP-7 can also cleave Fas-L (Mitsiades et al. 2001, Powell et al. 1999). In this study, the altered levels of Fas-L in alveolar bone and mucosa of Mmp8-/− mice suggest an in vivo link between Fas-L and MMP-8 (I). However, we are still lacking direct evidence of the in vivo cleavage by MMP-8.

Estrogen and ERs are widely recognized in cancer progression (Ortona et al. 2014). The link between MMP-26 and estrogen in cancer has been reported in various studies showing also that estrogen increases MMP-26 levels (Li et al. 2004, Nishi et al. 2013, Pilka et al. 2004). Moreover, a mutual regulation between MMP-26 and estrogen signaling seems to exist, since MMP-26 is able to proteolytically target ER-β (but not ER-α) and thereby its expression in breast cancer appears to provide positive effects (Savinov et al. 2006). Here, we found that MMP-8 was able to cleave both ERs and also MMP-8 expression was stimulated by estrogen in HSC-3 cells (III). If this mutual regulation occurs in vivo, it could partially explain the stronger association of MMP-8 expression and disease specific survival in female patients, as well as the differences between male and female mice in the carcinogen exposed OTSCC progression (III). Interestingly, in addition to MMP-8 induction in cancer cells, estrogen increases
TGF-β1 secretion by dermal fibroblasts (Ashcroft et al. 1997a, Stevenson et al. 2008).

TGF-β1 is activated by various MMPs suggesting that MMPs can positively regulate TGF-β1 mediated actions (Karsdal et al. 2002, Mu et al. 2002, Yu & Stamenkovic 2000). The possible degradation of TGF-β1 has been shown to occur by neutrophil elastase (Chen et al. 1997) and cathepsin-K (Zhang et al. 2011a). TGF-β1 instead has been shown to upregulate MMP-13 expression in head and neck carcinoma cells leading to enhanced invasion (Leivonen et al. 2006) although it downregulates MMP-8 expression in human tongue SCC cells (Moilanen et al. 2002). The mutual regulatory system between MMP-8 and TGF-β1 was confirmed by various experiments in this study. At least some of the results from the animal and cell experiments could be explained by the ability of MMP-8 to decrease the levels of active TGF-β1, most likely by direct proteolytic degradation. MMP-8 is also able to inhibit TGF-β1 signaling by an indirect mechanism that involves proteolytic action against decorin (Soria-Valles et al. 2014). The role of MMP-8 in regulating TGF-β1 signaling in fibroblasts could explain the changes in Mmp8/- mouse wound healing and increased contraction of collagen gels by Mmp8/- fibroblasts (II). The levels of PSmad2 and α-SMA positive myofibroblasts in the tongue wound healing experiment remained unchanged in this study. However, a recent study found increased MMP-8 expression simultaneously with a decrease in the myofibroblasts in TGF-β1 stimulated fibroblasts in response to IL-10 (Shi et al. 2013).

MMP-8 expression in OTSCC cells in vitro results in defects in cell invasion and migration and HSC-3 cells showed decreased levels of active TGF-β1 without changes in the TGFB1 gene expression levels (IV). TGF-β1 induces the migration and Matrigel® invasion of HSC-3 cells (Takayama et al. 2009), similar to what we found here (IV). Moreover, a recent study showed that TGF-β1 production by stromal cells increases podoplanin (PDPN) expression, leading to enhanced invasion of oral SCC cells in vitro (Hwang et al. 2014). Downregulation of TGF-β1 levels by MMP-8 might at least partly explain the defects in the migratory ability of MMP-8 overexpressing OTSCC cells, since exogenously added rhTGF-β1 restored the ability of the cells to migrate (IV). TGF-β1 regulates EMT in oral cancer cells via Snail transcription factor and increases cell invasion (Qiao et al. 2010, Quan et al. 2013, Sun et al. 2008). Interestingly, TGF-β1 inhibits the proliferation of normal epithelial cells but in turn, enhances growth, invasiveness and metastasis of cancer cell (Leivonen and Kähäri 2007). This may at least partly result in variations in the TGF-β1 signaling.
cascades. The expression of Smad4 declined in OTSCC cells and the overexpression of this transcription factor mediates tumor suppressive effects by inhibiting OTSCC cell proliferation and metastasis ability as well as increasing apoptosis (Wang et al. 2011b). In human OSCC (stages III and IV), TGF-β1 expression is associated with disease recurrence, shorter survival and higher risk of lymph node involvement (Chen et al. 2012).

Finally, to resolve whether MMP-8 action towards TGF-β1, or other substrates identified in vitro, plays a relevant role in particular physiological or pathological conditions would require additional highly specific in vivo models.
7 Conclusions

The present study provided evidence suggesting various mechanisms that mediate the effects of MMP-8 in oral tissue repair and OTSCC cell behavior in vivo and in vitro. The main conclusions of the present study were:

1. MMP-8 plays diverse roles in hard and soft tissue repair in mouse. MMP-8 regulates the inflammation and collagen metabolism in tooth extraction sockets. MMP-8 deficiency 1) does not change the tissue repair of the alveolar bone tooth extraction socket, 2) increases tongue wound contraction rate, 3) but delays skin wound repair.

2. MMP-8 action results in diminished TGF-β1 levels in mouse tongue wounds and reduces TGF-β1 signaling in mouse tongue fibroblasts in vitro. MMP-8 cleaves and inactivates active TGF-β1, as shown by recombinant human proteins in vitro.

3. MMP-8 expression in human OTSCC predicts better survival of OTSCC patients, and protects from OTSCC development in mouse. Low levels of MMP-8 associate with high VEGF-C levels in human OTSCC, and this combination predicts a worse outcome of patients even more accurately than these markers separately.

4. In vitro experiments indicate that the tumor suppressive role of MMP-8 in OTSCC cells could be related to its connection with the concomitant changes in the expression levels of other cancer associated molecules, such as MMP-1, MMP-9, cathepsin-K, VEGF-C and TGF-β1.
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REGULATORY MECHANISMS MEDIATING MATRIX METALLOPROTEINASE-8 EFFECTS IN ORAL TISSUE REPAIR AND TONGUE CANCER