FACTORS AFFECTING AGGRESSIVE ORAL TONGUE CANCER INVASION AND DEVELOPMENT OF IN VITRO MODELS FOR CHEMORADIOTherapy ASSay

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FACTORS AFFECTING AGGRESSIVE ORAL TONGUE CANCER INVASION AND DEVELOPMENT OF IN VITRO MODELS FOR CHEMORADIOThERAPY ASSAY

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**Abstract**

Tumor associated macrophages (TAMs) are linked to the invasion of oral tongue squamous cell carcinoma (OTSCC). We modified THP-1 leukemia cells to M1 (inflammatory), M2 (TAM-like) and R848 (imidazoquinoline-treated) type macrophages in order to examine their interactions with OTSCC-cells (HSC-3) by using different kinds of *in vitro* migration and invasion models. We observed that interaction of TAM-resembling M2-type macrophages with HSC-3 cells induced invasion and migration, whereas the influence of M1 macrophages reduced them.

Patient response to chemoradiotherapy is highly reliant on the characteristics such as the aggressiveness and stage of the cancer. Therefore, new methods for treatment testing are needed in order to design personalized therapies. We tested the applicability and consistency of human TME mimicking tissue methods for analyzing the effects of chemoradiation using commercial OTSCC cell lines. Based on our trials, both our human uterine leiomyoma tissue-based matrix models provide viable platforms for future *in vitro* chemoradiotherapy testing.

Conventionally pro-tumorigenic activities of matrix metalloproteinase (MMP)9 have been linked with oral squamous cell carcinoma, but recently its tumor-suppressor role has also been revealed. Our study provides strong evidence that MMP9 also has an anti-invasive effect in OTSCC and is a potential mediator of the protective effects of arresten in tongue cancer cells.

**Keywords:** chemoradiotherapy, matrix metalloproteinase, Myogel, myoma organotypic culture, squamous cell carcinoma, tongue cancer, tumor associated macrophage, tumor microenvironment
Väyrynen, Otto, Kielisyövän invaasioon vaikuttavista tekijöistä ja in vitro mallien kehittäminen kemosädehoidon testaamiseen.

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**Tiivistelmä**


**Asiasanat:** kasvaimen liittyvä makrofagi, kasvaimen mikroymympäristö, kemosädehoito, kielisyöppä, levyepiteelikarsinooma, matriksin metalloproteiniasi 9, Myogeeli, organotyyppinen myoomamalli
To people suffering from cancer
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Abbreviations

5-FU 5-fluorouracil
AJCC The American Joint Committee on Cancer
bFGF basic fibroblast growth factor
BrdU Bromodeoxyuridine / 5-bromo-2'-deoxyuridine
CCL chemokine (C-C motif) ligand
CD cluster of differentiation
CT computed tomography
CXCL chemokine (C-X-C) ligand
DNA deoxyribonucleic acid
EBRT external beam radiotherapy
ECM extracellular matrix
e.g. exempli gratia
EGF epidermal growth factor
EGFR epidermal growth factor receptor
EMT epithelial-mesenchymal transition
ENI elective nodal irradiation
EXTREME Erbitux in first-line treatment of recurrent or metastatic head and neck cancer
FGF fibroblast growth factor
Gy Gray
HNSCC head and neck squamous cell carcinoma
HPSE1 heparanase 1
HPV human papilloma virus
HS heparin sulfate
IGF insulin-like growth factor
IgG immunoglobulin G
IFN-ß interferon beta
IFN-γ interferon gamma
IL interleukin
IMRT intensity modulated radiotherapy
IR ionizing radiation
kDa kilodalton
KISS-1 KISS-1 gene for metastasis suppressor kispeptin
LPS lipopolysaccharide
M-CSF macrophage colony-stimulating factor
MHC  major histocompatibility complex
MIF  macrophage migration inhibitory factor
MMP  matrix metalloproteinase
MMPI matrix metalloproteinase inhibitor
mRNA messenger ribonucleic acid
MT1-MMP membrane type 1 matrix metalloproteinase
NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells
OSCC oral squamous cell carcinoma
OT oral tongue
OTSCC oral tongue squamous cell carcinoma
PBS phosphate-buffered saline
PCR polymerase chain reaction
PD-1 programmed cell death protein 1
PDGF-β platelet derived growth factor beta
SCC squamous cell carcinoma
SELEX systematic evolution of ligands by exponential enrichment
STAT signal transducer and activator of transcription
TAM tumor associated macrophage
TGF-β transforming growth factor beta
Th T helper cell
Treg T regulatory cell
TIMP tissue inhibitor of metalloproteinase
TLR toll-like receptor
TME tumor microenvironment
TNF-α tumor necrosis factor alpha
TNM tumor-node-metastases -classification of malignant tumors
UICC The Union for International Cancer Control
VEGF vascular endothelial growth factor
Original publications

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


* Equal contributions.
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1 Introduction

Oral squamous cell carcinoma (OSCC) is the most prevalent cancer of the head and neck area (Sarode, Sarode, Maniyar, Anand, & Patil, 2018). The prognosis of cancers in the oral cavity varies substantially depending on the location of the primary tumor. Tongue is the most perilous site due to its vulnerable anatomy with a dense lymphatic network, and because of this, oral tongue squamous cell carcinoma (OTSCC) has the potential for aggressive growth and rapid spread. The survival rate of OTSCC has not remarkably increased during the past few decades and treatment options remain limited, making it one of the deadliest cancer types (Sasahira & Kirita, 2018). Despite the advances in treatment approaches, the best way to improve prognosis lies in early detection of the disease. This highlights the importance of developing more accurate diagnostical methods and identifying reliable prognostic markers.

The tumor microenvironment (TME) involves the extracellular matrix (ECM) and the surrounding non-cancerous cells as well as a variety of soluble factors such as cytokines, chemokines and growth factors. In cancer, the normal interactions between cells and the ECM are disturbed, making the inner dynamics of the TME an interesting topic in the field of cancer research. Macrophages are a heterogeneous and plastic group of white blood cells that take part in the formation of TME and they are linked to tumor progression (Sica & Mantovani, 2012). Depending on the stimuli, macrophages can polarize into two main subtypes with divergent impacts on tumor cell behavior (Alves, Diel, & Lamers, 2018). This makes macrophages a prominent subject in the search for potential targets for therapeutic intervention.

Chemoradiotherapy is a firmly established approach in the treatment of head and neck cancers, including OTSCC. However, treatment is expensive and the practice is complicated to organize as well as it may cause severe side effects to patients. We have previously introduced human tissue-based matrix models that mimic the human TME (Nurmenniemi et al., 2009; Salo et al., 2015). This is the first time that human tissue based extracellular matrices were used as a chemoradiation test platform, as previous works have been mainly conducted using Matrigel – an EHS mouse sarcoma-based matrix model, or other xenobiotic substrates that lack the microenvironment of a human tumor.

Matrix metalloproteinases (MMPs) are a subfamily of proteases capable of degrading the components of ECM. MMP9 has been considered as a protumorigenic factor in oral cavity cancers, but evidence for the antitumorigenic roles of MMP9 has also been revealed (Vilen, Salo, Sorsa, & Nyberg, 2013). Moreover,
there are numerous *in vivo* studies implicating the protective function of MMP9 in various other types of cancers. On the other hand, *in vivo* data have remained highly controversial, suggesting that the action of MMP9 might fluctuate during different stages of the disease. To elucidate the role of MMP9, we used various models where production or activity of MMP9 was altered, including MMP9 silenced and arresten overexpressing HSC-3 cell lines with increased MMP9 expression, as well as using an MMP9 inhibitor.
2 Review of the literature

2.1 Oral tongue squamous cell carcinoma

Cancers of the head and neck (HNSCCs) are globally among the eighth most common malignancies (Bray et al., 2018), and squamous cell carcinoma (SCC) is the most common type of cancer in that area. SCCs account for approximately 90% of all oral cavity cancers as well (Hillbertz, Hirsch, Jalouli, Jalouli, & Sand, 2012; Rivera, 2015; Yan et al., 2010). The oral cavity is commonly defined as an area comprised of the lips, gingiva, retromolar trigone, hard palate, buccal mucosa, floor of the mouth and mobile tongue (Westra & Lewis Jr, 2017). Oral tongue squamous cell carcinoma (OTSCC) is the most common type of cancer of the oral cavity (Hillbertz et al., 2012; Marsh et al., 2011), covering the malignancies located in the oral tongue (OT) which comprises front two-thirds of the tongue muscle, e.g. the mobile tongue. Typical sites for OTSCC are the lateral borders and ventral surface of the OT, at the junction point where the tongue meets the floor of mouth (Piazza, Montalto, Paderno, Taglietti, & Nicolai, 2014; Rivera, 2015). The tongue is a vulnerable organ for malignancies due to its dense network of vascular and lymphatic vessels and close proximity to surrounding lymph nodes of the head and neck. Partly because of these anatomical disadvantages, OTSCC is frequently associated with lymph node metastases even at the early stages of the disease (Lim et al., 2006).

2.1.1 Epidemiology and risk factors for OTSCC

The recent release of the GLOBOCAN database (September 2018) includes an global estimate of 354,864 new cases of oral cancers (including lip cancer) for 2018 (Bray et al., 2018). SCCs of the mobile tongue cover the majority of all new cases, but the estimates about the percentage of OTSCCs should be evaluated critically since the cancer registries mostly represent data from developed countries, although the incidence of OTSCC is most prevalent in developing countries (Ng, Iyer, Tan, & Edgren, 2016). In Finland, a total of 128 new OTSCC cases (76 men, 52 women) were diagnosed in 2016 and the age-adjusted incidence rate has been steadily increasing during the past four decades from 0.46 new cases/100,000 inhabitants per annum in 1972-1976 to 1.34 in 2012-2016 (Finnish Cancer Registry, http://www.cancerregistry.fi). Cases of OTSCC occur primarily in patients over 45
years of age, with a median age of 62 at diagnosis (Appah et al., 2018; Majchrzak et al., 2014). Based on the most recent publication, the 5-year survival rate in Finland, used to estimate the prognosis of disease, is around 60% in OTSCC and it has improved only slightly over the past 20 years despite the advances in treatment (Mroueh et al., 2017). The incidence for OTSCC has been rising worldwide during the past few decades (van Monsjou, Schaapveld, van den Brekel, & Balm, 2015) and interestingly, there is an increasing trend in OTSCC cases among young adults, but the reason remains unclear (Ng et al., 2016). Notably, the increasing incidence is surprisingly high among white females who abstain from tobacco and alcohol (Farquhar et al., 2018; S. C. Patel et al., 2011). However, tobacco use and alcohol consumption are the well documented risk factors for OTSCC development, but recent studies indicate that there may be other emerging factors that remain yet unknown. The oncogenic human papilloma virus, and especially its subtype (HPV)16, has proven to contribute to the rising incidence of oropharyngeal cancers as well as cancers in the base of the tongue, but not in OTSCC (Liang, Lewis, Foote, Smith, & Kademani, 2008; Marur, D’Souza, Westra, & Forastiere, 2010; Ramqvist, Grün, & Dalianis, 2015). There are differences in risk factors and recurrence rate between older and younger patients suggesting that there may also be distinction in the genetic profiles of OTSCC between age groups (Farquhar et al., 2018).

2.1.2 Tumor microenvironment of OTSCC

Cancer cells along with the surrounding tissue comprised of extracellular matrix (ECM) and a heterogenous mixture of non-cancerous cells form an entity referred to as the tumor microenvironment (TME). The TME contains a variety of distinct cell types that promote tumor progression, such as fibroblasts, endothelial and inflammatory cells (Lorusso & Rüegg, 2008). Mutations that eventually initiate oral cancer occur in the epithelial cells, but the events that promote tumor progression involve the surrounding TME (Hanahan & Weinberg, 2000). In cancer, the normal interactions between tumor cells and ECM are altered and tumor cells tend to modulate their surroundings by releasing molecules, such as growth factors, that render the environment more suitable for tumor progression, for example via angiogenesis (Lorusso & Rüegg, 2008). Hence, TME is a topic of great significance in the field of cancer research.
2.2 Inflammation in OTSCC

Inflammation is an acknowledged predisposing factor for the development of several types of cancer, and the first links between tumors and chronic inflammation were discovered over a century ago (Mantovani et al., 2008). Inflammatory cells of the TME are known to participate in the development of cancer by releasing soluble factors such as enzymes, chemokines, cytokines and growth factors that are related to tumor growth and metastasis. In addition, non-steroidal anti-inflammatory drugs are known to reduce the risk of certain cancer types (Mantovani et al., 2008). However, it has been noticed that the quality of the inflammation matters and in some cases the exudate of the TME can restrain tumor development as a suppressive countermeasure by the host (Lisa M Coussens & Werb, 2002). In this context the dichotomy between “good” and “bad” inflammation raises questions of whether it could be possible to alter the patients immunity to be more unfavorable to tumor progression (Mantovani et al., 2008).

Certain key transcription factors, such as members of the transcription factor kappa B (NF-κB) protein family, partake in the regulation of inflammation in many cancer types, including oral squamous cell carcinomas (OSCCs) (Allen, Ricker, Chen, & Van Waes, 2007; Zheng, Yin, & Wu, 2011). NF-κBs act as tumor promoters in inflammation associated cancers, which cover approximately one fifth of all human cancers (Lisa M Coussens & Werb, 2002; Pikarsky et al., 2004). Members of NF-κB family proteins usually occur as dimers formed of subunits p65 (Rel A), Rel B, c-Rel, p105/p50 (NF-κB1), and p100/p52 (NF-κB2). The most abundant form of NF-κB dimers is the p50/p65 heterodimer, which is also the focus NF-κB form of this study. In the classic activation of the NF-κB signaling pathway, the p50/p65 subunits, which are stored in the cytosol of the cancer cell, are translocated into the nucleus when their inhibitor IκBα is phosphorylated and the inhibitor complex degrades. The p50/p65 subunits then activate the transcription of target genes upon binding to DNA (Oechinghaus, Hayden, & Ghosh, 2011). The activation of NF-κB in immune cells leads to induced production of cytokines that activates NF-κB in cancer cells and further results in induced chemokine production that attracts inflammatory cells into the tumor site. This NF-κB -mediated feed-forward loop functions in favor of tumor cells by promoting cell survival via induced expression of anti-apoptotic genes (Grivennikov, Greten, & Karin, 2010). In addition, NF-κB activation is crucial for the response of macrophages to inflammatory stimuli and this has proven to be a requirement for
tumor progression in various inflammation associated cancer types (Hagemann, Biswas, Lawrence, Sica, & Lewis, 2009).

2.2.1 Macrophages and their subtypes

Macrophages are inflammatory cells developed from monocytes and activated as they reach the inflamed site in the tissue. Macrophages are distributed throughout the body tissues and their main functions include the ingestion of dead cells, cell debris and foreign material as well as participation in inflammatory processes (Varol, Mildner, & Jung, 2015). Macrophages act as a part of the cell-to-cell immune defense system and they can be roughly divided into two subcategories (types M1 and M2) based on their phenotype, cytokine production profile and reactivity. The pro-inflammatory type M1 macrophages act as part of the antitumoral immunity by expressing cytokines such as IL-6, IL-12 and IL-23 as well as tumor necrosis factor alpha (TNF-α), which inhibits tumor progression. In addition, type M1 macrophages contribute to apoptosis by expressing major histocompatibility complex class I (MHC1) and class II (MHC2) that bind to tumor-specific antigens and further present them to T-cells. Cytokines secreted by M1 cells also promote type 1 T helper cells (Th1) and Th1 chemotraction. Th1 cells can be polarized from naïve Th cells into different types (e.g. Th1, Th2, Treg and Th17) with different cytokine expression profiles based on the stimulus they encounter. Th1 cells have anti-tumorigenic effects as they activate a cell-to-cell immunological response by further rendering macrophages cytotoxic towards cancer cells (Haabeth et al., 2011). Type M2 macrophages, on the other hand, have pro-tumorigenic, pro-angiogenic as well as anti-inflammatory functions as they express cytokines IL-4, IL-10 and IL-13, which promote the immunosuppressive regulatory T cell (Treg) and T helper 2 (Th2) cell response and attraction. Hence, type M2 macrophages closely resemble tumor associated macrophages (TAMs) (J. M. Brown, Recht, & Strober, 2017; Chanmee, Ontong, Konno, & Itano, 2014).

The mechanisms behind the polarization of macrophages into different phenotypes is based on the signaling derived from lymphocytes (T and B), tumor cells and stroma. M1 cells are commonly referred to as classically activated, while M2 cells are known as alternatively activated macrophages. In the canonical pathway, M1 cells are activated by interferon gamma (IFN-γ), lipopolysaccharide (LPS) and toll-like receptors (TLRs) via NF-κB and signal transducer and activator of transcription (STAT)1 regulation, whereas IL-4 and IL-13 stimulate the activation of M2 cells via STAT3 and STAT6 (Murray et al., 2014; Sica &
The M2 phenotype can be further subdivided into subtypes M2a, M2b and M2c depending on the quality of stimuli a macrophage receives (Hesketh, Sahin, West, & Murray, 2017). Also, a novel tumor associated macrophage (TAM)-like subset of M2 cells, referred to as M2d, has been identified. However, there is still ongoing debate concerning the categorization of macrophage subtypes and the dichotomy between M1 and M2 has remained as an established custom (Duluc et al., 2007; Takeya & Komohara, 2016).

### 2.2.2 Tumor associated macrophages

Tumor associated macrophages (TAMs) are essential components of the TME and they act as mediators between inflammation and cancer in almost all tumor types (Galdiero, Marone, & Mantovani, 2018). TAMs are associated with poor prognosis in oral cancers, especially high concentrations of CD68 – and CD163 -positive TAMs, which indicate the potential of TAMs as prognostic markers (Alves et al., 2018; He et al., 2014; C. Li, Shintani, Terakado, Nakashiro, & Hamakawa, 2002; Marcus et al., 2004). TAMs resemble type M2 macrophages and they act in a way which promotes the viability and progression of cancer cells by remodeling the TME into a immunosuppressive milieu for tumor growth (Hao et al., 2012). The origin of TAMs is still under discussion. For a long time, TAMs were believed to originate from the blood marrow (BM) and to be recruited as monocytic precursors to tumor sites by signals from the cells within the TME. However, recently this pattern of thought has gone through some updating, as there is new evidence suggesting that TAM precursors may originate as early as the embryonic state, rather than be simply derived from monocytes in the BM (Mantovani, Marchesi, Malesci, Laghi, & Allavena, 2017). The regulation of TAM functions is dependent on the signals in the TME, such as IL-4 and IL-13 derived from Th2 cells and cytokines and metabolites from tumor cells (Mantovani et al., 2017).

### 2.2.3 Cytokines and growth factors secreted by TAMs

TAMs affect tumor cells and the TME by suppressing adaptive immunity and by secreting tumor growth factors such as epidermal growth factor (EGF) and transforming growth factor beta (TGF-β) as well as various matrix metalloproteinases (MMPs) which can promote tumor growth and metastasis (Rubio, C., Munera-Maravilla, E., Lodewijk, 2018). TAMs show low expression of IL-12 and high expression of IL-10, which is also similar to the characteristics of
the M2 phenotype and TAMs are sometimes referred as M2-skewed (Chanmee et al., 2014; Sica & Mantovani, 2012). TAMs are often abundant in malignant tumors, where the expression of the main chemoattractant for monocytes, chemokine CCL2, is usually increased. In vitro studies show that TAMs express various pro-angiogenic and angiogenesis-modulating factors such as VEGF, FGF, TNF-α, interleukin 1β (IL-1β), platelet derived growth factor beta (PDGFβ) and various MMPs. The TME is also known to further stimulate the pro-angiogenic functions of TAMs, for example, via tumor hypoxia. Hypoxia can stimulate the release of pro-angiogenic factors from TAMs and TAMs are in fact shown to accumulate in hypoxic tumors (Murdoch, Muthana, Coffelt, & Lewis, 2008). The pro-tumorigenic functions of TAMs are also activated by direct cell-cell contact with tumor cells, yet the exact mechanisms behind this have not yet been uncovered (Komohara & Takeya, 2016).

2.3 Gelatinases (MMP2 and -9)

Matrix metalloproteinases (MMPs) are a subfamily of 24 enzymes in humans that are capable of degrading the structural proteins of the ECM. Previously, MMPs were grouped based on their specificity in degrading the proteins of the ECM, but this grouping became obsolete after the notion that MMPs can cleave also various substrates that are not present in the ECM. High levels of MMPs correlate with poor prognosis in numerous cancers and formerly MMPs were considered as merely pro-invasive factors because of their degrading action towards ECM. However, the association of MMPs in cancer progression has been revealed to be multifaceted and their potential, for example, as therapeutic targets is complicated (Jordan et al., 2004; Nissinen & Kähäri, 2014; Stamenkovic, 2003).

Two of the probably the most extensively studied MMPs in the field of cancer biology are MMP2 and -9 (gelatinase A and B, respectively) because of their association with tumor invasion and metastasis. They are enzymes capable of degrading components of ECM, but both enzymes are also able to cleave other ECM proteins as well, including denatured type I collagen, also known as gelatin. This ability of degrading gelatin is utilized in an established technique known as gelatin zymography, which is used to detect gelatinolytic activity in biological samples. MMP2 and -9 were originally referred to as type IV collagenases since they promote the hydrolysis of type IV collagen. Type IV collagen is a crucial part of the ECM and disturbances in the structure of this component play a significant role in the formation of malignancies (Toth & Fridman, 2001). Additionally, MMP2
and -9 are capable of shedding active proteins such as cytokines, growth factors and chemokines, from their membrane-anchored proforms (Klein & Bischoff, 2011). MMP2 is expressed by various cells such as endothelial cells, vascular smooth muscle cells, macrophages, neutrophils, T-lymphocytes and fibroblasts. MMP9 is expressed by a variety of normal and tumorigenic cells, including endothelial cells, vascular smooth muscle cells, neutrophils, macrophages, T-lymphocytes and fibroblasts. The expression of MMPs is regulated by growth factors and cytokines as well as by cell-cell contact. In addition, MMP expression may be regulated by interaction between cells and ECM components. Expression in tumor cells is mainly evoked by inflammatory stimuli (Arthur Z., Susanta K., Neuman, & Gregory I., 2013; Klein & Bischoff, 2011).

2.3.1 MMP2 and -9 molecular properties, activation and inhibition

MMP2 and -9 molecules consist of several functional parts including a pro-domain and the active enzyme domain followed by a zinc-binding domain, which form the catalytic region of the enzyme. Functions of the hemopexin domain concentrate mainly in substrate specificity and binding with tissue inhibitors of metalloproteinases (TIMPs). Finally, gelatinases have a fibronectin-like domain consisting of three repeats of fibronectin type II, which enables binding to gelatin, laminin and types I and IV collagen. The molecular structure of MMP9 is much more complex, but the distinct feature in MMP9 is the collagen V-like domain which MMP2 lacks (Opdenakker, Van den Steen, & Van Damme, 2001). The comparison of domain structures of MMP2 and -9 is illustrated in Fig 1. Similar to most MMPs, gelatinases are secreted as enzymatically inactive zymogens, proMMP2 and -9, which are then activated extracellularly when the pro-domain is cleaved. The activation is regulated by a so-called cysteine switch, which breaks the bond between cysteine in the pro-domain and the zinc ion (Zn$^{2+}$) in the active part (Vandenbroucke & Libert, 2014).

MMP9 (gelatinase B, 92-kDa type IV collagenase) was formerly considered merely as a pro-tumorigenic factor, because of its degrading action towards ECM and its role in releasing pro-angiogenic growth factors, such as VEGF. Later on, it has been revealed that MMP9 may also have anti-tumorigenic effects in various cancer types, such as prostate, breast, skin and colon cancers (Leifler et al., 2013; Martin & Matrisian, 2007; Pujada et al., 2017; Schäfer et al., 2012; Vilen et al., 2013; Walter et al., 2016).
There are different pathways for activation for MMP9, but the most studied mechanism is the enzyme proteolysis of the proMMP9 pro-domain. During activation, the 92-kDa pro-enzyme is cleaved to form the 83-kDa mature enzyme. Enzymatic activators include MMP2, -3, -7, -10, -13, -26 as well as the serine protease enterokinase and trypsin-2, of which MMP3 is regarded as the most potent activator (Vempati, Karagiannis, & Popel, 2007). Other known activation mechanisms for MMP9 are oxidation by reactive oxygen species, S-nitrosylation (covalent bonding of an -NO group to cysteine of the prodomain site) and allosteric activation (pro-domain is not removed but only formation of the cysteine- Zn\(^{2+}\) bond is disrupted) (Ra & Parks, 2007; Vilen et al., 2013).

MMP2 (gelatinase A, 72-kDa type IV collagenase) is expressed as a 72-kDa proform and it is activated into a 64-kDa mature enzyme by forming a complex with tissue inhibitor of metalloproteinase 2 (TIMP2). TIMP2 functions as a substrate for MMP14, which further proteolytically cleaves the pro-domain of proMMP2. In an alternative pathway, proMMP2 is activated by thrombin and activated protein C. As opposed to MMP9, pro-inflammatory stimuli do not increase the expression level of MMP2, although it also regulates inflammation via pro-inflammatory mediators, such as IL-1β (Klein & Bischoff, 2011).

MMPs are naturally regulated by α2-macroglobulin, a general protease inhibitor, and TIMPs, which function as endogenous inhibitors. TIMPs are proteins, consisting of a N- and C-terminal domains that each contain three disulfide bonds. The N-terminal domain binds to the active site of MMPs and their activity is inhibited reversibly. There are a total of four TIMPs identified in vertebrates and despite the overlap in TIMPs targeting, TIMP2, -3 and -4 are capable of inhibiting all MMPs, including gelatinases (Newby, 2012).

During the 1990’s and early 2000’s, a number of synthetic MMP inhibitors (MMPIs) were tested in clinical trials, but the programs were eventually terminated because of weak therapeutic efficacy or due to side effects (Cauwe, Van den Steen, & Opdenakker, 2007; Winer, Adams, & Mignatti, 2018). It was soon found, that prolonged treatment with MMPIs caused severe musculoskeletal pain and inflammation, which were not prognosticated in preclinical trials. More concerning, trials also showed that some MMPIs significantly decreased survival compared to patients receiving a placebo. Some studies also showed that elevated levels of MMPs resulted in decreased vascularization of transplanted tumors via increased conversion of plasminogen to angiostatin. In this regard, MMP inhibition may favor tumor vascularization (Lisa M Coussens, Fingleton, & Matrisian, 2002). The reasons for failure in MMPI trials were diverse, but perhaps the main problems
were insufficient knowledge of cancer biology and MMP complexity as well as the lack of inhibitor specificity. This was seen especially in the use of broad spectrum MMPIs which did not take into account possible anti-target MMPs, since later on it has been revealed that some MMPs may also have anti-tumorigenic effects (Vandenbroucke & Libert, 2014; Vilen et al., 2013). Recently, interest in MMPIs has surfaced again along with the increased knowledge of the complexity of MMPs (Vandenbroucke & Libert, 2014). Indeed, MMP inhibition still shows potential as a therapeutic approach in treatment of malignancies but there is a great challenge in determining the specific roles of particular MMPs in specific stages of tumor progression as well as generating limited-spectrum inhibitors.

![Fig. 1. Schematic structures of MMP2 and -9 proforms (modified from Visse & Nagase, 2003).](image)

### 2.3.2 MMP2 and -9 substrates

The substrate specificity of gelatinases is dictated by their active sites, which are distinct from other MMPs mainly by their fibronectin-type II domain (Hannocks et al., 2017). These substrates consist of a number of proteins including ECM components, other proteinases, proteinase inhibitors, growth factor binding proteins, cell surface receptors as well as intracellular molecules (Björklund & Koivunen, 2005). The substrates of MMP2 and -9 can be roughly divided into ECM and non-ECM originated substrates. The array of substrates for MMP2 and -9 is increasing as research progresses, which also widens the understanding of their pathological potential. Altogether, MMP2 and -9 are both capable of cleaving numerous proteins and the knowledge of their substrates and regulatory pathways...
is incomplete, making it challenging to unambiguously distinguish their pathophysiological roles. This study concentrates on the known substrates relevant from the point of view of tumor progression. The major identified and acknowledged substrates for MMP2 and -9 are listed in Tables 1 and 2 (Cui, Hu, & Khalil, 2017; Farina & Mackay, 2014; Hannocks et al., 2017; Rodríguez, Morrison, & Overall, 2010; Visse & Nagase, 2003).

Besides degradation of ECM structural components, the reported pro- and anti-tumorigenic effects of MMP2 and -9 include tumor promotion via release of cytokines, chemokines and growth factors as a result of substrate degradation. There is considerable overlap between MMP2 and -9 in substrates, mainly when it comes to degradation of ECM proteins that take part in the formation of the matrix scaffold such as collagen (see tables 1 and 2). Both MMP2 and -9 are involved in the activation of TNF-α, TGF-β and IL-1β, which promote tumor progression and inflammation, as well as enhancing cell invasion by generating metastin via cleavage of the tumor suppressor protein KISS-1 (Bauvois, 2012; Takino et al., 2003). There is also a variety of specific functions for both proteinases. For example, MMP9 is able to cleave plasminogen to form the active anti-angiogenic molecule angiotatin. MMP2 contributes to angiogenesis by cleaving laminin-5, which results in enhanced endothelial cell migration, and modulating the angiogenic activity of FGF via FGF-R1. MMP2 can also release insulin-like growth factors (IGFs) that promote tumor growth (Bauvois, 2012).

Increased MMP9 activity promotes the release of proangiogenic factors, such as VEGF and FGF, from the ECM making it a critical pro-angiogenic molecule and activator of the so-called angiogenic switch. On the other hand, MMP9 also releases anti-angiogenic factors such as endostatin and tumstatin, released by cleaving collagens XVIII and IV, as well as angiotatin which is formed after digestion of plasmin and plasminogen (Farina & Mackay, 2014). MMP9’s influence is also critical in cancer-related inflammation as it activates TGF-β, pro-inflammatory cytokines such as TNFα and IL-1β, as well as increases the activity of chemokines CXCL1, CXCL4, CXCL7 and CXCL8. As opposed to this, high MMP9 activity has been reported to promote the anti-tumorigenic activity via the innate immune response in breast cancer (Farina & Mackay, 2014; Leifler et al., 2013).
Table 1. Major substrates of MMP2 based on tissue origin. Common substrates between
MMP2 and -9 are marked with an asterisk (Cui et al., 2017; Farina & Mackay, 2014;
Hannocks et al., 2017; Rodriguez et al., 2010; Visse & Nagase, 2003).

<table>
<thead>
<tr>
<th>Structural proteins in ECM</th>
<th>Non-structural proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggrecan*</td>
<td>Active MMP9 and -13</td>
</tr>
<tr>
<td>BM-40/SPAR/Osteonectin*</td>
<td>Amyloid protein precursor</td>
</tr>
<tr>
<td>Brevican</td>
<td>Big endothelin-1</td>
</tr>
<tr>
<td>Collagen type I*, II, III*, IV*, V*, VII*, X* and XI*</td>
<td>Calcinon gene-related peptide (CGRP)</td>
</tr>
<tr>
<td>Elastin*</td>
<td>Complement protein C1q*</td>
</tr>
<tr>
<td>Entactin/Nidogen-1*</td>
<td>Eph B1 tyrosine kinase receptor</td>
</tr>
<tr>
<td>Fibrillin*</td>
<td>Fibroblast growth factor receptor (FGFR) -1</td>
</tr>
<tr>
<td>Fibrin*</td>
<td>Galectin-3*</td>
</tr>
<tr>
<td>Fibronecitin*</td>
<td>IGF-BP-3 and -5</td>
</tr>
<tr>
<td>Gelatin*</td>
<td>KiSS-1 protein/metaginin*</td>
</tr>
<tr>
<td>Laminin*</td>
<td>MMP-1 (trypsin-activated)</td>
</tr>
<tr>
<td>Neurocan</td>
<td>Monocyte chemoatractant protein MCP-3</td>
</tr>
<tr>
<td>Proteoglycan link protein*</td>
<td>Myelin basic protein*</td>
</tr>
<tr>
<td>Versican*</td>
<td>Myosin heavy chain*</td>
</tr>
<tr>
<td>Vitronectin*</td>
<td>Poly (ADP-ribose) polymerase (PARP)</td>
</tr>
<tr>
<td></td>
<td>Pregnancy zone protein*</td>
</tr>
<tr>
<td></td>
<td>Pro-IL-1β*</td>
</tr>
<tr>
<td></td>
<td>Pro-MMP-13*</td>
</tr>
<tr>
<td></td>
<td>Pro-MMP-2</td>
</tr>
<tr>
<td></td>
<td>Pro-MMP9*</td>
</tr>
<tr>
<td></td>
<td>Pro-TGF-β*</td>
</tr>
<tr>
<td></td>
<td>Pro-TNF-α*</td>
</tr>
<tr>
<td></td>
<td>Pro-uorokinase</td>
</tr>
<tr>
<td></td>
<td>Stromal cell derived factor (SDF)-1*</td>
</tr>
<tr>
<td></td>
<td>Troponin</td>
</tr>
<tr>
<td></td>
<td>Uorokinase receptor*</td>
</tr>
</tbody>
</table>
Table 2. Major substrates of MMP9 based on tissue origin. Common substrates between MMP2 and -9 are marked with an asterisk (Cui et al., 2017; Farina & Mackay, 2014; Hannocks et al., 2017; Rodríguez et al., 2010; Visse & Nagase, 2003).

<table>
<thead>
<tr>
<th>Structural proteins in ECM</th>
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<tbody>
<tr>
<td>Aggregan*</td>
<td>Big endothelin-1</td>
</tr>
<tr>
<td>BM-40/SPAR/Osteonectin*</td>
<td>Complement protein C1q*</td>
</tr>
<tr>
<td>Collagen type I*, III*, IV*, V*, VI*, X*, XI* and XVIII</td>
<td>Connective tissue-activating peptide-III (CTAP-III)</td>
</tr>
<tr>
<td>Elastin*</td>
<td>CXCL-10</td>
</tr>
<tr>
<td>Entactin/Nidogen-1*</td>
<td>CXCL-9</td>
</tr>
<tr>
<td>Fibrillin*</td>
<td>CXCL5</td>
</tr>
<tr>
<td>Fibrin*</td>
<td>CXCL6</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Galectin-3*</td>
</tr>
<tr>
<td>Fibronecclin*</td>
<td>Growth-regulated oncogene (GRO)-α</td>
</tr>
<tr>
<td>Gelatin*</td>
<td>IL2-Rα</td>
</tr>
<tr>
<td>Laminin*</td>
<td>Insulin</td>
</tr>
<tr>
<td>NG2 proteoglycan</td>
<td>Intercellular adhesion molecule (ICAM)-1</td>
</tr>
<tr>
<td>Proteoglycan link protein*</td>
<td>IFN-β</td>
</tr>
<tr>
<td>Versican*</td>
<td>KiSS-1 protein/metastin*</td>
</tr>
<tr>
<td>Vitronecclin*</td>
<td>Kit-ligand</td>
</tr>
<tr>
<td></td>
<td>Myelin basic protein*</td>
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<tr>
<td></td>
<td>Myosin heavy chain*</td>
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<tr>
<td></td>
<td>Plasminogen*</td>
</tr>
<tr>
<td></td>
<td>Platelet factor (PF)-4</td>
</tr>
<tr>
<td></td>
<td>Pregnancy zone protein*</td>
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<tr>
<td></td>
<td>Pro-IL-1β*</td>
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<td></td>
<td>Pro-IL-8</td>
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<tr>
<td></td>
<td>Pro-MMP13*</td>
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<tr>
<td></td>
<td>Pro-MMP9*</td>
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<tr>
<td></td>
<td>Pro-TGF-β*</td>
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<td></td>
<td>Pro-TNF-α*</td>
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<tr>
<td></td>
<td>Stromal cell derived factor (SDF)-1*</td>
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<tr>
<td></td>
<td>Substance P</td>
</tr>
<tr>
<td></td>
<td>Urokinase receptor*</td>
</tr>
<tr>
<td></td>
<td>α1-proteinase inhibitor</td>
</tr>
<tr>
<td></td>
<td>α2-macroglobulin*</td>
</tr>
<tr>
<td></td>
<td>αB-crystallin</td>
</tr>
</tbody>
</table>
2.3.3 Role of MMP2 and -9 in OTSCC

MMP2 is considered as a pro-tumorigenic factor in a variety of cancers because of its degrading effect on ECM components. MMP2 is consistently found overexpressed in HNSCCs and it is associated with poor prognosis, lymph node metastasis and higher metastatic capacity in OSCC (B. P. Patel, Shah, Shukla, Shah, & Patel, 2007; Rosenthal & Matrisian, 2006; Shrestha, Bajracharya, Byatnal, Kamath, & Radhakrishnan, 2017). MMP2 is also considered to predict poor prognosis in OTSCC along with its known activator MT1-MMP and inhibitor TIMP2 (Yoshizaki, Maruyama, Sato, & Furukawa, 2001) and strong MMP2 expression had a positive correlation with a higher risk of nodal recurrence (Gontarz et al., 2015). Recently, the Wnt5a-Ror2 signaling pathway was shown to enhance the migration, invasion and metastatic potential of OTSCC and to promote the expression of MMP2 (Sakamoto et al., 2017).

MMP9 has conventionally been considered as a pro-tumorigenic factor in HNSCCs and MMP9 overexpression is shown to predict poor prognosis in OTSCC patients (Ruokolainen, Pääkkö, & Turpeenniemi-Hujanen, 2005; Thangaraj et al., 2016). Nevertheless, the role of MMP9 seems not to be unambiguous and evidence for its anti-tumorigenic roles has also been revealed (Vilen et al., 2013). Previous studies have shown that although expression of MMP9 is associated with higher clinical stages and larger tumor size, there seems to be no correlation between higher MMP9 levels and positive lymph-node or distal metastases in OSCC patients. Also, decreased expression of MMP9 was detected in HNSCC primary tumors associated with regional metastases compared with non-metastatic tumors (C.-W. Lin et al., 2012; Stokes et al., 2010). Moreover, the mere presence of MMP9 is not necessarily a reliable prognostic measure while there is evidence showing a low ratio of activated form/proform of MMP9 in OSCC patients, which suggests that despite its expression MMP9 may not always be activated in OSCC (Kato, K., Hara, A., Kuno, 2005). The expression profiles of MMPs may also vary between HNSCC patients, possibly depending on metastatic stage, primary tumor site or host genetics, underlining the need for a more personalized approach in cancer treatment (Bates, Gomez Hernandez, Lanzel, Qian, & Brogden, 2018). There is also known to be functional variation as MMPs produced by different cell types may function in divergent ways (Kessenbrock, Plaks, & Werb, 2010). Selective inhibitors for MMP-2 and -9 have been shown to inhibit OTSCC growth and angiogenesis, but they did not prevent the spread of carcinoma cells in nude mice (Heikkilä et al., 2006; Suojanen et al., 2011). Instead, when inhibitors were
combined with proMMP9 targeting therapy, there was increased OTSCC tumor growth in mice (Suojanen et al., 2011).

### 2.4 Aptamers as potential anticancer agents

Aptamers are short (20-100 nucleotides) single-stranded DNA and RNA oligonucleotides generated using a modified SELEX (Systematic Evolution of Ligands by EXponential enrichment) procedure, selecting them from a large random sequence pool. The selection consists of several rounds where each round comprises three steps of binding, partition and amplification. The procedure begins with the synthesis of a very large oligonucleotide library ($10^{12-15}$ oligonucleotides) consisting of randomly generated sequences. These sequences in the library are exposed to the target ligand and those that do not bind the target are removed. The bound sequences are eluted and amplified by PCR and the tightest-binding sequences are identified. The SELEX procedure may normally take time as the rounds need repetition, but the number of rounds can be considerably reduced by using techniques such as high-throughput sequencing. The target ligands can be molecules of almost any kind, such as lipids, sugars or proteins. Aptamers can also be manufactured to carry molecules, for example drugs (Soldevilla, Villanueva, & Pastor, 2016; Tuerk & Gold, 1990). Aptamers bind to their targets with high affinity and specificity. Aptamers are smaller than antibodies (5-25 kDa), resulting in a high capability of penetrating tissues but also in faster metabolic clearance, which may affect bioavailability. Aptamers are relatively easy to synthesize, modify and store, and they have low immunogenicity and toxicity (Que-Gewirth & Sullenger, 2007). The first aptamer approved by the FDA (U.S. Food and Drug Administration) in 2004 was the VEGF-targeting Pegaptanib, developed for the treatment of age-related macular degeneration.

Aptamer-based immunotherapy is a promising field of cancer treatment based on the numerous successful clinical trials carried out with aptamer-constructs designed to modulate the immune response against cancer. A great benefit of aptamers in comparison to monoclonal antibodies is their plasticity; aptamers can be designed to work either as inhibiting/blocking antagonists or activating agonists for target molecules, such as cytokines or immune receptors. The very first DNA aptamer that reached clinical trials was AS1411– a nucleolin protein targeted aptamer trialed with renal cell carcinoma patients (Rosenberg et al., 2014; Soldevilla et al., 2016).
2.4.1 Heparanase in OTSCC and anti-heparanase aptamer

Heparanase is an endoglycosidase enzyme that cleaves heparin sulfate (HS) chains, which are important structural components of the ECM. Heparanase is normally secreted by various cell types, such as CD4+ T-cells, platelets, neutrophils as well as metastatic cells (Sanderson, Elkin, Rapraeger, Ilan, & Vlodavsky, 2017; Simmons et al., 2014). The degrading effect on the ECM is based on the ability of heparanases to hydrolyse the glycosidic bonds of HS chains attached to proteoglycans. This further allows tumor cells to penetrate endothelial cells of blood vessels and enables metastasis. In addition, HS is a reservoir of biological signaling molecules that are released to the environment by the action of heparanase. As HS degrades, it releases bound cytokines and growth-factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) into the ECM inducing the progression of cancer cells. On this regard, heparanase is recognized as an important factor in tumor progression as a pro-angiogenic mediator, but heparanase is also known to be involved in cell proliferation and enhancing the metastatic potential of tumor-derived cells (Nagler et al., 2007; Simmons et al., 2012; Vlodavsky et al., 2012). In addition, increased heparanase expression is associated with inflammation-related carcinogenesis via the activation of macrophages that secrete cancer-promoting cytokines, which further promote a tumorigenic microenvironment (Meirovitz et al., 2013; Vlodavsky et al., 2016).

Heparanase expression is associated with tumor progression in various cancer types, including cancers of the oral cavity (Ikuta, Podyma, Maruyama, Enomoto, & Yanagishita, 2001; Simmons et al., 2012). Heparanase expression was significantly increased in OSCC, including OTSCC, and high levels of heparanase correlated with Ki67 staining, indicating increased cell proliferation (Leiser et al., 2011). Elevated heparanase expression was found to positively correlate with tumor size, tumor stage and distant metastasis in OSCC, and heparanase levels were also found to have an inverse correlation with patient outcome in OTSCC (Nagatsuka et al., 2012; Nagler et al., 2007; Xia et al., 2014). Additionally, in OTSCC, heparanase expression correlated significantly with tumor stage and size in advanced-stage cases (TNM stages III-IV) but a similar correlation was not found in early-stages (TNM I-II) (Nagler et al., 2007).

In recent years, heparanase has aroused interest as a potential therapeutic target for cancer treatment. As described earlier, heparanase expression is increased in various malignancies and its association with tumor progression is well recognized.
However, in normal tissue heparanase expression is very low, making it easier to notice possible side-effects caused by heparanase inhibition. Also, there is only one enzymatically active form of heparanase found in humans (Sanderson et al., 2017; Vlodavsky et al., 2016). Currently, the most studied anti-heparanase agents are modified heparins and heparin mimetics. Heparin has a natural affinity towards heparanase due to its close resemblance to HS. These mimetic compounds are chemically modified in an attempt to suppress the natural anti-coagulant effect of heparin and at the same time to maintain its high affinity towards heparanase (Pisano, Vlodavsky, Ilan, & Zunino, 2014). Some heparin mimetics, such as Muparfostat, Roneparstat, Necuparanib and defibrotide, are being evaluated in clinical trials but there are also a number of other potential heparanase inhibitors currently under investigation in pre-clinical trials. These include heparanase neutralizing monoclonal antibodies as well as small molecules designed based on the recently deciphered protein structure of heparanase (Vlodavsky et al., 2016). Previously, it was shown that anti-heparanase aptamers 1.5 M Short and Long reduced the invasion of ovarian carcinoma and tongue carcinoma cell lines in both Matrigel and Myogel cell invasion assays. (Simmons et al., 2014, 2012). The action of these single strand DNA aptamers is based on their capacity to bind heparanase with high affinity and inhibit the enzymatic function. Anti-heparanase aptamers have proven to be stable in human serum in the initial studies and, importantly, they showed no signs of cytotoxicity. However, the 1.5 M Long aptamer species seems to have a tendency to form complexes with serum proteins with the cost of decreased affinity towards its target enzyme. Evaluation of these aptamers is currently under pre-clinical trials, but they show potential as therapeutic agents (Simmons et al., 2012).

2.5 Therapy in OTSCC

OTSCC is the most aggressive type of cancer manifested in the oral cavity even at early stages of the disease. In the past, cancers of the oral cavity, oropharynx and larynx were grouped together under the main heading of HNSCCs. However, this grouping is problematic especially when it comes to planning cancer treatment since SCCs occurring in the oral cavity tend to have worse overall prognosis than SCCs of the larynx and oropharynx (J. S. Brown, Shaw, Bekiroglu, & Rogers, 2012). The established treatment approaches for OTSCC include surgery, radiotherapy and chemotherapy, as well as various combinations of these treatments. Treatment selection depends on the pathological findings,
considerations of disease control and functional and cosmetic outcomes as well as availability of expertise (Huang & O’Sullivan, 2013). The Tumor-Node-Metastases (TNM) -classification is used for grading the range of spread of solid tumor cancers. OTSCC diagnosis is based on the histopathological findings and TNM staging on this basis is usually marked with the prefix p (pTNM). The TNM ranks are used to describe the size and invasion of the primary tumor (Tx, Tis, T0-T4), involvement of regional lymph nodes (Nx, N0-N3) and state of metastasis (Mx, M0-M1) (Edge, 2017). The prognostic staging groups (from I to IV) used for treatment planning are based on the TNM system. However, it is noteworthy that there are two parallel and slightly divergent staging systems currently in use; one from The Union for International Cancer Control (UICC) and one from The American Joint Committee on Cancer (AJCC).

OTSCC has a tendency for rapid spread to cervical lymph nodes at early stages via the lymphatic system of the tongue and the incidence of occult cervical metastases is relatively high. Examination protocols and treatment approaches for OTSCC may vary globally. In Finland the treatment is principally centralized in university hospitals and usually planned in multi-professional collaboration with specialists from the fields of otolaryngology, oral and maxillofacial diseases, oncology and plastic surgery. The cervical area is usually radiologically examined with a CT-scan and contrast enhanced MRI. Patients with advanced stage tumors may also receive a CT scan of the thoracic area to detect possible metastatic disease (H. Keski-Säntti, Atula, Törnwall, Koivunen, & Mäkitie, 2006).

The primary approach for localized small (stage I-II) tumors is surgical removal of the tumor with sufficient margins and on a case-by-case basis combined with localized radiotherapy. The widely accepted standard for an adequate resection margin distance is 5.0 mm from the tumor (Zanoni et al., 2017). In locally advanced (stage III-IV) tumors and in cases of nodal metastases, surgical resection is followed by chemoradiotherapy. Removal of larger tumors may be followed by autologous tissue transplants due to wide margins. In most advanced cases a partial or total glossectomy and possible neck dissection may be required. However, glossectomy is a highly disabling procedure as prognosis after total glossectomies remains poor (H. Keski-Säntti et al., 2018; H. T. Keski-Säntti, Markkola, Mäkitie, Bäck, & Atula, 2005; Mäkinen L.K, Hagström J, 2016).
2.5.1 Chemotherapy in OTSCC

Depending on the stage of the disease, surgical treatment of OTSCC is usually combined with adjuvant therapies. Patients with locally advanced disease and diagnosed with cervical lymph node metastases are treated with post-surgical combination therapies, consisting of radiotherapy and chemotherapeutic agents (Mäkinen L.K, Hagström J, 2016). A common obstacle in the use of chemotherapeutic agents is their toxicity even at therapeutic levels and limiting side effects can vary case by case. Also, postoperative and postradiotherapy fibrosis may hinder vascularity, making it difficult for the pharmacologic drug doses to reach the tumor site (da Silva, Hier, Mlynarek, Kowalski, & Alaoui-Jamali, 2012). The so-called EXTREME (Erbitux in first-line treatment of recurrent or metastatic head and neck cancer) regimen, comprised of cisplatin, 5-fluorouracil and cetuximab, is nowadays considered as a gold standard of care for the treatment of recurrent/metastatic HNSCC. However, the EXTREME regimen is not always eligible due to its toxicity and hence the combination of cetuximab and paclitaxel may be used as an alternative medication in selected cases (Guidi, Codecà, & Ferrari, 2018; Nakano et al., 2017; Szturz & Vermorken, 2017; J. B. Vermorken et al., 2008). There are also various alternative chemotherapeutic treatment protocols available for selected patients who may not be able to tolerate standard regimens. Some acceptable chemotherapeutic agents currently used in HNSCCs are briefly described below.

Cisplatin

Cisplatin was the first platinum-based agent approved for cancer treatment by the FDA in 1978 and led to the development of other metal-containing anti-cancer drugs (Dasari & Tchounwou, 2014). Cisplatin causes crosslinking of DNA inhibiting replication, leading to arrest in the G2 phase of the cell cycle and resulting to cell death (Hasegawa, Goto, Hanai, Ozawa, & Hirakawa, 2018). Side effects may include allergic reactions, myelosuppression and toxicities such as nephrotoxicity, which is the main dose-limiting side effect of cisplatin (Miller, Tadagavadi, Ramesh, & Reeves, 2010).
Carboplatin

Carboplatin is also a platinum-based chemotherapeutic drug with a close resemblance to cisplatin in terms of function and structure. It has a lower reactivity and binding kinetics towards DNA as well as reduced side effects compared to cisplatin. Despite the longer lasting excretion, carboplatin is usually better tolerated but less potent than cisplatin depending on the type of cancer (Dasari & Tchounwou, 2014).

5-fluorouracil

For decades 5-fluorouracil (5-FU) has been considered as the backbone of combination chemotherapies in various cancer types. 5-FU is an antimetabolite drug that blocks the synthesis of the pyrimidine thymidine via inhibition of thymidylate synthase, causing interference in DNA synthesis (J. J. Lee, Beumer, & Chu, 2016; Longley, Harkin, & Johnston, 2003).

Nivolumab

The checkpoint inhibitor Nivolumab is a human IgG4 monoclonal antibody targeting the programmed cell death protein 1 and its ligand (PD-1/PD-L1) pathway. The function of checkpoint inhibitor drugs is based on their ability to null the effect of certain proteins acting as checkpoints for immunological response, allowing the immune system to target cancer cells. The inhibition of PD-1/PD-L1 prevents downregulation of T cells, allowing them to recognize tumor cells and arbitrate their cell death. In 2016, Nivolumab gained FDA approval for the treatment of patients with recurrent metastatic HNSCCs with failed first-line platinum-based treatment and in 2017 it was also approved by the European Medicines Agency (Calles, Aguado, Sandoval, & Álvarez, 2019; Ferris et al., 2016; Forster & Devlin, 2018; Jelinek, Paiva, & Hajek, 2018; Saada-Bouzid, Peyrade, & Guigay, 2019).

Paclitaxel

Paclitaxel is an anti-mitotic compound originally extracted from the bark of the Taxus brevifolia tree. It disrupts the assembly of microtubules by stabilizing them, resulting in inhibition of cell division and inducing apoptosis or reversion to the G0 phase in the cell cycle. Paclitaxel was first indicated as a therapy for advanced
carcinoma of the ovary but is also used in the treatment of various other carcinomas as a single agent or in combination with a platinum-based agent (Kim et al., 2016; Nonaka, Ikeda, Fujisawa, Uehara, & Inokuchi, 2006).

**Docetaxel**

Similar to paclitaxel, docetaxel is a taxoid anti-mitotic agent that promotes microtubule stabilizing by preventing their depolymerization. Docetaxel is twice as potent and more soluble in water compared to paclitaxel. Stabilization of microtubules causes inhibition of the normal function of the microtubule network which is crucial for interphase of the cell cycle. This results in cell-cycle arrest and cell death. (Joerger, 2016; Kim et al., 2016).

**Methotrexate**

Methotrexate is an anti-cancer drugs that inhibits thymidylate synthase and the folic acid cycle. This leads to impaired nucleic acid synthesis and subsequent cell death. Methotrexate regimens are associated with severe side effects such as oral mucositis, myelosuppression and various toxicities (Campbell et al., 2016).

**Cetuximab**

Currently, the only targeted therapy in use for OTSCC treatment is the inhibition of the epidermal growth factor receptor (EGFR), which is overexpressed in approximately 30% of all epithelial tumors and 80-90% of HNSCCs (Ansell, Jedlinski, Johansson, & Roberg, 2015). EGFR is a transmembrane glycoprotein and its activation by ligand binding causes further activation of pathways that lead to transcription of genes responsible for cell growth, cell differentiation and apoptosis (Ang et al., 2002). Overexpression of EGFR is associated with reduced survival in HNSCCs patients (Kundu & Nestor, 2012). Cetuximab is a chimeric monoclonal IgG1 antibody targeting EGFR and it is commonly used in combination with radiotherapy and/or cisplatin. Cetuximab has a high affinity for the endogenous ligand-binding site of EGFR and prevents dimerization, internalization and autophosphorylation of the receptor. This affects tumor cells so that downstream signal transduction is blocked and thereby cell proliferation is reduced, cellular apoptosis increased and invasion prevented. Secondarily, EGFR inhibition promotes antibody dependent cell-mediated cytotoxicity via recruitment of
macrophages and natural killer cells (Kundu & Nestor, 2012; J. Vermorken & Specenier, 2013).

2.5.2 Radiotherapy in OTSCC

The main forms of radiotherapy are external beam radiotherapy (EBRT) and interstitial brachytherapy. EBRT is the most commonly used form of radiotherapy in OTSCC treatment. In brachytherapy a small bead-like source of short-range radiation is placed at the site of the tumor and it can be used also in combination with EBRT. Brachytherapy is best suited for treatment of early stage, superficial and well-defined primary tumors (Huang & O’Sullivan, 2013). EBRT is performed with a linear accelerator by using high energy photon radiation and the conventional radiotherapy consists of a 6-7 week treatment period with daily fractions of 2 Gy, given five days per week. The total radiation dosage per treatment period consists of 68-70 Gy for primary tumors and approximately 50 Gy for elective areas (Murthy et al., 2017; Saarilahti & Lindholm, 2011). Radiotherapy can cause severe side-effects in the form of fibrosis and ischemia of healthy tissues as well as reduced salivary functions and trismus. Additionally, radiotherapy may impair dental health and it also increases the risk of osteoradionecrosis (J. S. Brown et al., 2012). Careful fractioning is therefore necessary in order to minimize the radiation burden of healthy tissues. Recent advances in radiation oncology have introduced techniques such as intensity modulated radiotherapy (IMRT). Using IMRT, treatment can be 3D-optimized to conform the radiation beam to fit the exact area of the tumor site and hence a high dose of radiation can be delivered to the target with precision, while sparing surrounding healthy tissues (H. Keski-Säntti, Mäkitie, & Saarilahti, 2015). Due to the challenging anatomy of the head and neck area, IMRT is ideal for treatment of oral cavity cancers (N. Lee, Puri, Blanco, & Chao, 2005).

In cases of reliably resectable early stage tumors (T1-T2) with low risk of recurrence, radiotherapy is not considered necessary. Radiotherapy is usually issued post-operatively with or without chemotherapy in the treatment of high risk locally advanced disease (T3-T4) with positive margins and/or extranodal spread to enhance control of the primary tumor site, commonly referred to as loco-regional control (J. S. Brown et al., 2012). Some advanced stage tumors may require pre-operative radiotherapy approximately 4 weeks prior to surgery, which may delay wound healing and increase the risk of complications (Lindholm et al., 2006). Radiotherapy may also be employed as a primary treatment in the form of definitive
radiotherapy, which consists of EBRT as a single primary treatment and is usually issued at the primary tumors of early stage or sometimes in cases of inoperable tumors, refusal of surgery or as part of palliative care. In addition, radiotherapy may be used as a salvage treatment in cases of persistent or recurrent disease (Huang & O’Sullivan, 2013). Elective nodal irradiation (ENI) is used as a post-operative prophylactic measure in order to prevent possible micrometastases in risk regions, i.e., the cervical nodal area (Murthy et al., 2017).

2.5.3 Chemoradiotherapy in OTSCC

Chemoradiotherapy for OSCCs, including OTSCC, follows the principles of treatment based on the results of randomized trials for all HNSCCs. Post-operative chemoradiotherapy remains as an established approach as it is likely to reduce the risk of recurrence. (Fan et al., 2014). Some chemotherapeutic drugs and target agents can make tumor cells more sensitive to radiation, which is the principle of chemoradiotherapy in cancer treatment. Chemoradiotherapy is usually issued after surgery for high risk patients with unfavorable pathological features, such as positive resection margins and/or extracapsular nodal spread (Huang & O’Sullivan, 2013). The use of platinum-based drugs as radio-sensitizing agents improves the outcomes in patients with locoregionally advanced HNSCC, but with a cost of difficult toxicities to most patient (Mehra, Cohen, & Burtness, 2008).

Most HNSCCs express EGFR and inhibition of the receptor causes radiosensitization in tumor cells. Monotherapies with EGFR inhibitors have shown only limited benefit in HNSCCs. However, radiotherapy concurrent with EGFR-targeted therapy has proven to significantly increase survival and response rate in HNSCCs compared to radiotherapy alone. Cetuximab -based chemoradiotherapy also showed no association with excess toxic effects (Bonner et al., 2006; Feng et al., 2007). Cetuximab augments the radiation caused apoptosis and diminishes the capacity to repair radiation-induced damage in SCC cells (Harari & Huang, 2001).
3 Aims of the study

We are studying the fluctuating effects of MMP9 and the involvement of macrophages in OTSCC progression. Also, we have conducted in vitro testing of potential combination therapies suitable for the treatment of OTSCC using our novel 3D organotypic matrix models mimicking the TME of solid human tumors. This study elucidates the roles of matrix metalloproteinases as well as inflammatory cells in the progression of OTSCC. Furthermore, this study is part of a project in which we aim to develop a fast and reliable in vitro method for analyzing patient cancer cell samples in order to recognize those patients who will benefit from chemoradiotherapy and to whom therapy might cause recurrences and metastases. The goal of these analyses is to provide more personalized treatment for cancer patients.

The primary research questions of this study are:

1. What is the significance of macrophages (M1 and M2) in the invasion process of OTSCC (I)?
2. How does chemoradiation affect the invasion of OTSCC analyzed in our novel 3D organotypic models (myogel and myoma discs)? Can these models be harnessed as a method for chemoradiation testing in the future (II)?
3. What is the role and mechanism of action of MMP9 in OTSCC in vitro invasion (III)?
4 Materials and methods

The materials and methods used in the original articles of this study are listed in Table 1 and the most important methods used in this thesis are further described below.

Table 3. Methods used in the original publications.

<table>
<thead>
<tr>
<th>Level</th>
<th>Method Used in</th>
<th>Used in</th>
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<tr>
<td>Human</td>
<td>Myoma organotypic culture</td>
<td>I, II, III</td>
</tr>
<tr>
<td></td>
<td>Myogel invasion assay</td>
<td>II, III</td>
</tr>
<tr>
<td>Animal</td>
<td>Mouse xenocraft model</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Matrigel invasion assay</td>
<td>I-III</td>
</tr>
<tr>
<td>Cells</td>
<td>Cell culture</td>
<td>I-III</td>
</tr>
<tr>
<td></td>
<td>Establishment of type M1, M2 and R848 macrophages</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Establishment of transfected HSC-3 arresten cells</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Establishment of MMP9 silenced HSC-3 cells</td>
<td>III</td>
</tr>
<tr>
<td>Cell experiments</td>
<td>Efferocytosis</td>
<td>III</td>
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<tr>
<td></td>
<td>Migration analyses</td>
<td>I-III</td>
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<tr>
<td></td>
<td>Horizontal migration (Scratch wound healing assay)</td>
<td>I, III</td>
</tr>
<tr>
<td></td>
<td>Vertical migration (Transwell migration assay)</td>
<td>II, III</td>
</tr>
<tr>
<td></td>
<td>Chemotactic migration</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Irradiation assay</td>
<td>II</td>
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<td></td>
<td>Proliferation assay (ELISA BrdU)</td>
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<td></td>
<td>Clonogenic assay</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Adhesion assay</td>
<td>III</td>
</tr>
<tr>
<td>RNA</td>
<td>Microarray</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>RNA extraction and PCR</td>
<td>III</td>
</tr>
<tr>
<td>Protein analyses</td>
<td>Antibody array</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Immunohistochemistry</td>
<td>I-III</td>
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<tr>
<td></td>
<td>Immunofluorescence</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Zymography</td>
<td>I-III</td>
</tr>
<tr>
<td></td>
<td>Gelatinase assay (EnzChek)</td>
<td>II</td>
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<tr>
<td></td>
<td>Immunoblot analysis (Western blot)</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Others</td>
<td>Statistical analyses</td>
<td>I-III</td>
</tr>
<tr>
<td></td>
<td>Quantification methods</td>
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<td></td>
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<tr>
<td></td>
<td>EMT</td>
<td>II</td>
</tr>
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</table>
4.1 Cell experiments

4.1.1 Native cell lines

Human tongue squamous cell carcinoma cell lines HSC-3, SAS, SCC-25 and human malignant melanoma G361 cells as well as breast adenocarcinoma MDAMB-231 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F-12, 1:1 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 50 μg/ml ascorbic acid, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B and 0.4 μg/ml hydrocortisone (all from Sigma-Aldrich). Human embryonic kidney HEK-293 cells, human gingival fibroblasts (GF) and human adult low-calcium high-temperature HaCaT cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, 250 ng/ml amphotericin B and 1 mM sodium pyruvate (all from Sigma-Aldrich). GFs used in this study were obtained from biopsies of healthy gingiva as described earlier (Kylmäniemi, Oikarinen, Oikarinen, & Salo, 1996). HPV16 immortalized human oral epithelial cells (IHGK) were cultured in Keratinocyte SFM (Gibco) supplemented with 5 ng/ml human recombinant epidermal growth factor, 50 μg/ml bovine pituitary extract (both from Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B and 100 μM CaCl2 (all from Sigma Aldrich). THP-1 human leukemia cells were cultured in advanced RPMI supplied with 10% FBS (both from Life Technologies) supplemented with 2 mM L-Glutamine (Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml fungizone (all from Sigma-Aldrich). The complete list of cell lines used in this study and their corresponding distributors/references is shown in table 2.
Table 4. Cell lines used in the original publications.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Distributor/References</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP-1</td>
<td>Human leukemia cells</td>
<td>ATCC TIB-202</td>
<td>I</td>
</tr>
<tr>
<td>(M1 macrophage)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(M2 macrophage)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(R848 macrophage)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSC-3</td>
<td>Epithelial human tongue squamous cell carcinoma</td>
<td>JCRB-0623</td>
<td>I-III</td>
</tr>
<tr>
<td>(shMMP9 HSC-3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(arrHSC-3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAS</td>
<td>Epithelial human tongue squamous cell carcinoma</td>
<td>JCRB-0260</td>
<td>II, III</td>
</tr>
<tr>
<td>SCC-25</td>
<td>Epithelial human tongue squamous cell carcinoma</td>
<td>ATCC CRL-1628</td>
<td>II, III</td>
</tr>
<tr>
<td>G361</td>
<td>Human malignant melanoma</td>
<td>ATCC CRL-1424</td>
<td>III</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Human caucasian breast adenocarcinoma</td>
<td>ATCC HTB-26</td>
<td>III</td>
</tr>
<tr>
<td>HEK-293</td>
<td>Human embryonic kidney cell</td>
<td>ATCC CRL-1573</td>
<td>III</td>
</tr>
<tr>
<td>HGF</td>
<td>Human gingival fibroblasts</td>
<td>(Kylmäniemi et al., 1996)</td>
<td>III</td>
</tr>
<tr>
<td>IHGK</td>
<td>HPV16 immortalized human oral epithelial cells</td>
<td>(Oda, Bigler, Mao, &amp; Disteche, 1996)</td>
<td>III</td>
</tr>
<tr>
<td>HaCaT</td>
<td>Human adult low-calcium high-temperature cells</td>
<td>(Boukamp, 1988)</td>
<td>III</td>
</tr>
</tbody>
</table>

4.1.2 Polarized, transduced and transfected cell lines

To study the interactions between OTSCC cells and macrophages (Mf), THP-1 human leukemia cells were polarized into type M1 and M2 Mfs and R848 Mfs as previously described (Tjiu et al., 2009). In co-culture experiments HSC-3 cells were labeled with Vybrant CM-Dil (red) and Mfs with Vybrant DiO (green) (both from Life Technologies).

When the effect of MMP9 -silencing was studied, HSC-3 cells were stably transduced with three different commercial human GIPZ MMP9 lentiviral shRNAmir particles (Thermo Fischer Open Biosystems) according to the manufacturer’s instructions. The transduced cells were selected with puromycin antibiotic (Sigma-Aldrich). HSC-3 cells transduced with the non-silencing scrambled GIPZ lentiviral shRNAmir particles (Thermo Fischer Open Biosystems)
were used as a control (control HSC-3 cells). MMP9 silencing was further confirmed by semi-quantitative PCR and zymography.

To study the effect of arresten, HSC-3 cells were stably transfected with arresten plasmids to overexpress human arresten (arrHSC-3) with Geneticin G418 selection as previously described (Aikio et al., 2012).

4.2 Invasion experiments

4.2.1 Myoma organotypic culture

The uterine leiomyoma tissues used in myoma organotypic cultures and Myogel assays were obtained during routine surgeries after the informed consent of the donors. The use of myoma tissue was approved by the Regional Ethics Committee of the Northern Ostrobothnia Hospital District (statement number 35/2014). The myoma samples were cut in discs with an 8 mm biopsy punch and the discs were then placed inside Transwell® inserts (diameter 6.5 mm; Corning). 3 × 10^5 or 7 × 10^5 HSC-3 cells in 50 µl medium were added on the top of the myoma disc and incubated in 37 °C overnight. After cell attachment, the discs were shifted onto nylon membranes (Prinsal Oy) resting on steel grids placed in 12-well plates with 1 ml of culture medium. Cells were cultured on the top of the myoma disc for 10–14 days in a humidified atmosphere of 5% CO² at 37 °C. The culture media was changed every 3-4 days.

4.2.2 Myogel invasion assay

The uterine leiomyoma -based Myogel extracellular matrix (2.4 mg/ml of protein, 0.2% agarose in serum free culture medium) was likewise developed in our laboratory for the invasion assay. For the Myogel invasion assay the filter of a 24-plate Transwell® insert (pore size 8 μm) was coated with 50 µl of Myogel extracellular matrix (Salo et al., 2018, 2015) and incubated at room temperature for 30 min. Next, 500 µl of normal medium with 10% FBS was added in the bottom chambers and 50000–70000 cells in 200 µl medium, in which FBS was replaced with 0.5% lactalbumin, were seeded into the upper chambers. The cells were allowed to invade for 72 h and then fixed with 10 % trichloroacetic acid (TCA) and stained with Toluidine Blue or alternatively with 0.1 % Crystal Violet. The cells and excess dye were removed from the upper side of the membrane and the dye of
migrated cells was eluted with 1% SDS. Absorbance was measured at 650 nm using a Victor2 Microplate Reader (Perkin Elmer Wallac). Alternatively, the membranes were detached from the inserts, attached to glass slides and photographed with a microscope using Leica Application Suite (LAS) software (Leica Microsystems). The area of cells in relation to total membrane area was measured and calculated using QWin V3 software (Leica Microsystems).

4.2.3 Matrigel invasion assay

In the Matrigel invasion assay, 10 000 unlabeled Mfs or HSC-3 cells were cultured at the bottom of the lower chambers of the BioCoat Tumor Invasion system (BD Biosciences) according to the manufacturer’s instructions. Vybrant DiO-labeled HSC-3 cells were cultured in the upper insert in serum-free medium containing 0.1% BSA. Cells in the lower chambers were cultured with or without serum. Cells were cultured for up to 96 hours and the invasion was determined by measuring the absorbances at 24, 48 and 96 hours at 490/535nm Ex/Em. A second set of experiments were then done with an uncoated reference plate in order to measure the cell migration.

4.3 Migration analyses

In cell migration experiments we used different types of assays for horizontal and vertical as well as for chemotactical migration analyses.

4.3.1 Horizontal migration

Horizontal cell migration was tested using the Oris Pro cell migration assay (Platypus technologies), scratch-wound assay and Ibidi inserts (Ibidi, Planegg/Martinsried). In the Oris Pro migration assay, Vybrant CM-Dil -labeled (red) HSC-3 and Vybrant DiO -labeled (green) THP-1 cells (5000 each) were seeded on Oris Pro cell migration 96-well plates and allowed to attach with inserts overnight. Next, the inserts were removed, the cells washed with PBS and medium with 1% FBS was added. Migration was monitored for 48 hours by photographing the wells with an Evos FL Cell Imaging System (Life Technologies) and the area of migrated, labeled cells was analyzed with QWin3 Software (Leica Microsystems). Each experimental setting was performed in quadruplicate.
In Ibidi experiments, 20 000 HSC-3 cells were applied in Ibidi inserts on 24-well plates and allowed to grow to confluence with the inserts attached in the middle of the wells. Inserts were then removed and cell migration over the empty area was monitored with an Evos FL Cell Imaging System (Life Technologies). Finally, the empty area was measured with Fiji software (Schindelin et al., 2012). Each experimental setting was performed in triplicate.

In scratch-wound assays, 2.5 x 10^5 shMMP9 or control HSC-3 cells were applied in 24-well plates and allowed to attach overnight. The next day, the cell cultures were wounded with a 1 ml pipet tip, and the wells were rinsed with serum-free medium before adding medium containing 1 % FBS. The cells were photographed as above and the open area in between the two cell edges was measured with QWin V3 or Fiji software. The results were calculated as a percentage from the original empty area (0 h). In some cases, 24-well plates were coated with rat tail type I collagen, fibronectin or Matrigel® (detailed description in original publication II) before the cells were applied and re-coated after cell attachment.

4.3.2 Vertical migration

The vertical cell migration was studied with a Transwell® migration assay (Corning) with 8 µm pores. The membrane inserts were equilibrated with 600 µl normal medium for 1 h before adding the cells. 70 000 arresten overexpressing, shMMP9 and corresponding control HSC-3 cells as well as SAS, SCC-25 or MDA-MB-231 cells were seeded into the upper chamber of Transwell® inserts in medium containing 1 % FBS while the medium in the lower chamber contained 10 % FBS. In some cases, CTT2 was added to the upper chamber in serum free medium. Cells were allowed to migrate for 24 or 48 h and were then fixed with 10 % trichloroacetic acid (TCA) and washed with dH2O. The cells were allowed to dry and were then stained with 0.1 % Crystal Violet. The upper side of the insert membranes were polished with a cotton swab to remove the non-migrated cells. The membranes were detached and photographed with a microscope and the area of migrated cells was measured visually and the percentage of cell area was determined using QWin V3 software (Leica Microsystems). Alternatively, the cells were stained with Toluidine blue, the dye of the migrated cells was eluted in SDS and the absorbance was measured at 650 nm using a Victor2 Microplate Reader (Perkin Elmer Wallac).
4.3.3 Chemotactic migration

When chemotactic migration was studied, a similar setting was assembled in which Transwell® membrane inserts were first coated with fibronectin. Then HSC-3 cells were seeded in the upper chambers and HSC-3-cells, M1- or M2- or R848-Mfs were seeded into the lower chamber or some lower chambers were left without cells. Chemotactic cell migration was also tested with the xCELLigence system (Roche Diagnostics) where HSC-3 cells were seeded to the upper inserts containing electrodes in migration plates. Macrophage -conditioned media (Mf-CM) were then added to the lower chambers and the cells were allowed to migrate for 48 hours.

4.4 Chemoirradiation assays

The effects of irradiation and test drugs on OTSCC cells were examined in different environments, using both 2D and 3D *in vitro* methods. Invasion, proliferation and clonogenic viability were examined in this study. The chemoirradiation assay methods are described briefly below.

4.4.1 Invasion assays

Invasion assays (myoma organotypic culture or Myogel invasion assay) were established as described above and the cell cultures in different experiments were irradiated at doses of 2, 4 and 8 Gy with a Varian Clinac iX linear accelerator (15 MV photon beam) at a dose rate of 4Gy/min and a field size of 30cm*30cm in the Radiotherapy Unit of Oulu University Hospital. An unirradiated control group (0 Gy) was included in each experiment. Cells were treated with an antibody against heparanase (Anti-HPSE1), 1.5 M short protected heparanase aptamer (inverted 3 ′-base dT5) and Erbitux (Merck Serono).

When the joint effect of irradiation and cell invasion was examined, the irradiated and invaded HSC-3 cells were collected from the bottom wells of a 24-well Transwell invasion experiment described earlier. The collected cells were then cultured for up to 4–10 passages after irradiation, and in each experiment 0, 2, 4 and 8 Gy -irradiated clones had equal passage numbers.
4.4.2 Proliferation assay

HSC-3 control cells and shMMP9 or arrHSC-3 cells as well as irradiated and invaded HSC-3 cells were seeded in normal media on 96-well culture plates. The cell proliferation was determined after 24 and 48 h and in some cases after 72 h using the Cell Proliferation ELISA BrdU kit (Roche) according to the manufacturer's protocol. Absorbance was measured at 450 nm using a Victor2 Microplate Reader (Perkin Elmer Wallac). Wells containing only media were measured as a blank control.

4.4.3 Clonogenic assay

Irradiated and invaded HSC-3 were seeded on a 6-well plate in triplicate and on a 24-well plate in quadruplicate. For drug treatments, HSC-3 cells were seeded in normal media on a 24-well culture plate in quadruplicate and allowed to attach 6 h and the drugs were added at the indicated concentrations. The next morning, the cells were irradiated as described above. Drugs and medium were changed after 3 days. Cells were cultured a total of 7 days, then fixed and stained with crystal violet. The clonogenic viability of the cells was calculated as previously described (Franken, Rodermond, Stap, Haveman, & van Bree, 2006). The results represent the average of three separate experiments.
Fig. 2. Chemoirradiation assay methods. A. BrdU assay. BrdU (Bromodeoxyuridine / 5-bromo-2'-deoxyuridine) is incorporated into synthesized DNA of dividing cells. The incorporation of BrdU is determined using anti-BrdU and by measuring the intensity of absorbance, which reflects the cell proliferation. B. Clonogenic assay. Cells are seeded on plates at low densities. The effect of irradiation and drugs on cell survival is determined based on the cell’s ability to form colonies. C. Myoma organotypic culture. Cells are cultured on top of a myoma disc and they invade into the tissue. Invasion rate is determined by analyzing the invasion depth and area from myoma samples. D. Myogel invasion assay. Cells are cultured on top of a Myogel layer where they invade into the gel and through the membrane underneath. Excess cells and dye are removed from the top side, invaded cells are fixed and stained and invasion is determined by measuring the absorbance. Created using Servier Medical Art (Servier 2019), licence CC BY 3.0.

4.5 Zymography and gelatinase assay

Subconfluent cell cultures were cultured for 24 h and media were collected for zymography. In some cases, conditioned media (CM) from HSC-3 cells, polarized Mfs or their co-cultures were collected. HaCaT, HGF, IHGK, SCC-25 and HSC-3 (control and shMMP9) and MDA-MB-231 cells were cultured in Opti-MEM (Gibco) and control and arrHSC-3 in 1% lactalbumin (Sigma-Aldrich) medium. Conditioned medium was then concentrated with a speed-vac device, and analyzed with gelatin zymography using the same media protein amount or sample volume.
The gelatinase activity of the conditioned medium was analyzed with an EnzChek® Gelatinase Assay Kit (Molecular Probes) according to the manufacturer's protocol. The fluorescence was measured after 3 h incubation at 485/535 nm using a Victor2 Microplate Reader (Perkin Elmer Wallac) and the intensities were normalized to the cellular soluble protein concentration.

4.6 Ethical considerations

The mouse study was approved by the Animal Care and Use Committee at the University of Oulu and the National Animal Care and Use Committee of Finland (OLH-2006-02521/Ym-23, OLH-2006–01987/Ym-22, ESLH-2008-09631-Ym-23, ESLH-2008-03956-Ym-23).

Oral cancer tissue samples were obtained from patients who had signed an informed consent form. The patient data inquiry has been approved by the National Supervisory Authority for Welfare and Health (VALVIRA), #6865/05.01.00.06/2010 (5.10.2010), and the Ethics Committee of the Northern Ostrobothnia Hospital District, statement 49/2010 (16.8.2010).

Uterine leiomyoma tissue was obtained during routine surgeries after the informed consent of the donors. The study was approved by the Regional Ethics Committee of the Northern Ostrobothnia Hospital District. Use of patient material (myomas) for this study was approved by the Northern Ostrobothnia Hospital District Ethics Committee (statement #8/2006, amendment 19/10/2006 and statement #35/2014, 28.4.2014).
5 Results

5.1 Macrophages are actively involved in OTSCC progression

5.1.1 Macrophages merge with OTSCC cells in co-cultures partially due to efferocytosis

We investigated the effect of macrophages on a highly aggressive OTSCC cell line, HSC-3. To study the cell-cell interactions between macrophages and tumor cells we polarized THP-1 cells into inflammatory M1, pro-tumorigenic (TAM-like) M2 and imidazoquinoline-treated R848 subtypes and co-cultured them with HSC-3 tongue carcinoma cells. Macrophages were first primed with phorbol 12-myristate 13-acetate (PMA). Polarization towards M1 and M2 types was done by incubating THP-1 cells in the presence of IFN-γ and LPS or in the presence of IL-4 and IL-13, respectively. Additionally, we used the imidazoquinoline-compound R848, a TLR7/TLR8-agonist, for the polarization of THP-1 cells into R848 macrophages. We also used PMA-type macrophages, which are THP-1 cells that have been polarized with PMA alone without additional stimuli and resemble TAM-like macrophages. HSC-3 cells were labeled with Vybrant CM-Dil (red) and macrophages with Vybrant DiO (green). In further analyses with confocal microscopy, co-localization and the fusion of HSC-3 cells with macrophages could be seen (Fig. 1B, E, H and K-M in I). The cells were treated with amiloride (AMI), an efferocytosis inhibitor, in order to evaluate whether the incorporation of HSC-3 cells and macrophages was due to phagocytosis. Fusion of cells was clearly observed in co-cultures treated with dimethylsulfoxide (DMSO, used as a vehicle) and BAY 11-7082, a NF-κB inhibitor (Fig. 2 in I). In cultures treated with AMI fusion was nearly absent, suggesting that the witnessed merging of HSC-3 cells and macrophages was at least partially a result of efferocytosis (Fig. 2 in I).

5.1.2 Co-cultures of macrophages and OTSCC cells altered the cytokine expression profile in vitro

Serum free media from HSC-3 cells, the three macrophage subtypes as well as from the three co-cultures of macrophages with HSC-3 cells were collected and analyzed for cytokine profiles with a RayBio Human Cytokine Antibody Array. The only four cytokines that were expressed in higher levels in HSC-3 cells compared to
macrophages were PDGF-BB, angiogenin, Macrophage migration Inhibitory Factor (MIF) and TIMP2 (Fig. 3D in I). The fold change of these four cytokines also increased in co-cultures of HSC-3 with macrophages compared to mono cultures of macrophages. Levels of cytokines associated with OSCC and increased invasion, such as EGF, macrophage colony-stimulating factor (M-CSF), TGF-β, TIMPs, PDGF-BB and MIF were increased in co-cultures with HSC-3 and M2 macrophages (Fig. 3 B-C in I). Also, the very same cytokines were down-regulated in co-cultures with HSC-3 and M1 macrophages, suggesting that these cytokines may be potential targets in further studies of OSCC.

5.1.3 M2 macrophages induced the migration and invasion, whereas M1 macrophages reduced the invasion of OTSCC cells

The effect of macrophage-conditioned medium (Mf-CM) on the horizontal migration of HSC-3 cells was tested with Ibidi cell culture inserts. HSC-3 cells treated with Mf-CM migrated slower than cells treated with DMSO (Fig. 4D, E in I, only M2 Mf-CM shown). BAY-11-7082 did not have any effect on HSC-3 migration, but on the other hand it had a slight reversing effect on the Mf-CM inhibition of HSC-3 cell migration (Fig. 4 E in I), suggesting that the factor or factors in Mf-CM causing the inhibitory effect towards HSC-3 migration is/are at least partially mediated by NF-κB.

To further study the effect of macrophages on HSC-3 cell invasion through a 3D barrier, we used our myoma-based organotypic model. HSC-3 cells and macrophages were co-cultured on top of myoma discs. M2- and PMA-type macrophages induced invasion of HSC-3 cells in myoma (p = 0.02 and 0.002 respectively) while M1 macrophages reduced invasion (p = 0.034) compared to monoculture of HSC-3 cells (Fig. 5C, D in I), indicating the pro-invasive effect of M2 macrophages and invasion inhibiting effect of M1 macrophages. In another myoma experiment the HSC-3 cells were cultured on top of myoma discs and the discs were incubated in Mf-CM. This setting on the other hand showed no induction in HSC-3 cell invasion (Fig. S2 in I), suggesting that although Mf-CM contains pro-migratory signals it does not necessarily induce invasion. Instead, the close proximity of macrophages is needed for both induction and inhibition of HSC-3 cell invasion. Also, when medium from this experiment performed with Mf-CM was analyzed by gelatin zymography, we found that all macrophage types produced substantially more MMP9 than HSC-3 cells. Interestingly, MMP9 production in pro-inflammatory M1 macrophages was stronger than in pro-tumorigenic M2 type
cells. The difference stayed the same in co-cultures with HSC-3 cells in which most of the MMP9 is likely produced by macrophages (Fig 5E, F in I).

5.1.4 M2 macrophages express a surplus level of NF-κB p50 homodimers, which can be crucial for OTSCC progression

Since we found that Mf-CM had an inhibitory effect on HSC-3 cell migration likely due to NF-κB regulated molecules, we further explored the expression and activity of NF-κB subunits p50 and p65 in HSC-3 and macrophage co-cultures. The p65/p50 heterodimer activates NF-κB inducing genes, whereas the p50/p50 homodimer is associated with induction of tumor-progressive genes.

We conducted immunofluorescence analysis of HSC-3 and macrophage cells by confocal microscopy. Both NF-κB p50 and NF-κB p65 subunits were present in the cytoplasm of HSC-3 cells (Fig. 6A and B in I). In M2 macrophages, the p50 subunit was present in the nuclei (Fig. 6A in I) but the p65 subunit, on the other hand, was present mostly in the cytoplasm of M2 macrophages, indicating that there may be an excessive amount of p50 in the nuclei of M2 macrophages. This may cause a dysregulation in NF-κB activation and in this way contributes to the induction of tumor-progressive genes.

Also, in HSC-3/M2 macrophage co-cultures the close proximity of M2 macrophages seemed to induce NF-κB dysregulation in HSC-3 cells, represented as a lack of translocation of p65 and indicating the presence of the p50 homodimer (Fig. 7 and 9 in I). In HSC-3/M1 macrophage co-cultures, the close vicinity of HSC-3 seemed to alter the NF-κB activity in M1 macrophages, but did not cause changes in HSC-3 cells (Fig 8 A-B and 9 in I).

5.1.5 HSC-3 cells and the TME of myoma tissue attract macrophages

To study the role of TME in vivo, collagen gel and myoma tissue containing human gingival fibroblasts with or without HSC-3 cells were transplanted into nude mice. The transplanted myoma tissue accompanied with HSC-3 cells was found to be histologically more necrotic and cancer-like than myoma tissue transplants without HSC-3 cells. Further analyses showed that the myoma transplants containing HSC-3 cells were more abundant in macrophages than transplants without cells (Fig. 10 J in I). This indicates that TME alone has a chemoattractive effect towards macrophages, but the presence of tumor cells amplifies this effect. In addition, when OTSCC tissue sections obtained from cancer patients were double-stained...
with antibodies for TAMs (CD163) and NF-κB subunits p50 and p65, we found that the samples were redundant in macrophages within direct contact with carcinoma cells (Fig. 11 G, H in I).

5.2 Human extracellular matrices in chemoradiation testing

We further investigated the role of TME by using our human uterine leiomyoma-based ECM models and also studied the potential and consistency of these novel models for in vitro chemoradiation studies. Our hypothesis was that the combination of anti-heparanase aptamers and ionizing radiation (IR) may enhance the anti-invasive effect in the myoma model. Aptamers were tested along with cetuximab (Erbitux) as adjuncts to chemoradiation testing. We have previously introduced two different uterine leiomyoma-based matrix models, the 3D myoma disc and Myogel models, and we tested them in parallel to find out whether they offer consistent and comparable environments for chemoradiation testing with OTSCC cells. Both 3D myoma disc and Myogel models were harnessed for settings, in which the effects of IR and the combined effect of IR and heparanase 1 (HPSE1) inhibitors were analyzed.

5.2.1 Inhibition of HPSE1 combined with ionizing radiation may induce cell invasion

First, we analyzed the combination of IR, anti-heparanase 1 (HPSE1) antibody and anti-heparanase aptamers on the invasion of HSC-3 cells in myoma discs. HSC-3 cells were cultured with HPSE1 and anti-heparanase aptamer 1.5 M short protected (inverted 3'-base dT5) for 12 days, then irradiated with doses of 0, 2, 4 and 8 Gy. After irradiation the myomas were cultured for another 6 days, after which they were prepared for pancytokeratin AE1/AE3 antibody-stained histological sections and the invasion area and invasion depth were measured. We found that irradiation alone was able to reduce the invasion area with a dose of 4 Gy (Fig. 1A in II) and invasion depth with 2 and 4 Gy (Fig. 1B in II). Anti-HPSE1 and 1.5 M short protected aptamer decreased the invasion area and depth compared to controls. Irradiation combined with anti-HPSE1 or 1.5 short protected aptamer however had surprisingly an increasing effect on invasion area and depth with doses of 4 and 8 Gy, suggesting that inhibition of HPSE1 in combination with IR may actually induce cell invasion.
5.2.2 The combination of IR and HPSE1 inhibition have no synergic effect on invasion or clonogenic viability

We continued using the Myogel model and evaluating its feasibility with the chemoradiation setting described above. We used OTSCC cell lines SCC-25, SAS and HSC-3 in order to model a variety of cell lines from less invasive to aggressive, respectively. In this setting we also included an EGFR-directed monoclonal antibody, Erbitux (cetuximab), as a control in addition to anti-HPSE1 antibody and 1.5 M short aptamer. With the HSC-3 cell line, IR alone had only a slight reducing effect on the invasion in the Myogel Transwell invasion assay. The drug treatments alone also seemed to reduce invasion compared to untreated, non-irradiated cells. However, the increase in IR seemed to dose dependently induce invasion compared to the corresponding non-irradiated cells, but this effect was only witnessed in the highly aggressive HSC-3 cell line but not with other two cell lines examined (Fig. 2A in II). In the SCC-25 cell line IR alone decreased the invasion with doses of 4 and 8 Gy, but the drug treatments alone did not have any significant effect (Fig 2B in II). In a moderately invasive SAS cell line, IR alone significantly decreased the invasion dose-dependently (Fig. 2C in II). Also, an IR dose of 4 Gy combined with anti-HPSE1 or Erbitux treatments significantly decreased invasion of SAS cells. Thus, our data indicate that IR and drug treatments seemed to have no clear synergic effects on invasion with the OTSCC cell lines used.

To assess the effect of anti-HPSE1, 1.5M short protected heparanase aptamer and Erbitux in combination with IR on the cell viability of HSC-3 cells we performed a clonogenic assay. Anti-HPSE1 and the 1.5M short protected heparanase aptamer both had only a minimal effect. Instead, Erbitux alone showed a diminishing, yet not statistically significant effect on the clonogenic viability (Fig. 8 in II). IR alone showed a dose dependent reducing effect on cell survival. Our data suggest that these treatment combinations have no synergistic effects on cell viability and therefore show no potential as a therapy approach.

5.2.3 IR has a durable effect on the induction of matrix metalloproteinases (MMP)2 and -9

To examine the role of matrix metalloproteinases (MMPs) in the behavior of aggressive OTSCC cells we evaluated the effects of IR on the expression of MMP-2 and -9 as well as various other markers linked to cell invasion in HSC-3 cells by immunohistochemistry. With an IR dose of 8 Gy we observed an increased number
of HSC-3 cells that were positive for MMP-2 and -9 localized in the invasion area of myoma samples (Fig. 3A and B in II). In addition, we analyzed the levels of Ki-67 in 2 Gy irradiated myoma samples but found no changes in the immunoreactivity compared to non-irradiated control samples, indicating that IR did not result in changes in proliferation of HSC-3 cells (Fig. 4A and B in II). Furthermore, the level of HPSE1 was increased in myomas irradiated with 2 Gy (Fig. 4G-J in II). We also analyzed common markers for the epithelial-mesenchymal transition (EMT) and found that the level of E-cadherin was significantly decreased whereas the level of vimentin was increased when myomas were irradiated with a dose of 2 Gy (Fig. 4D and F in II). These results suggest that low doses of IR may increase the expression of MMPs and HPSE1 and also initiate EMT in OTSCC cells cultured in the myoma model.

We next examined the possible long-term effects of IR on the progression of HSC-3 cells by establishing clones from previously irradiated HSC-3 cells after harvesting the cells from the bottom of Myogel Transwell assays wells. After harvesting, two cell clones were cultured over 4-10 passages and cells in each experiment shared the same passage number. These subcultures of previously irradiated and invaded cells were then analyzed. We observed these clones had proliferation and clonogenic viability similar to native HSC-3 cells (Fig. 5A and B in II). Also, in the Myogel Transwell invasion assay the clones showed no difference in invasion capability compared to controls (Fig. 5C in II). Immunoblots showed no significant changes in the phosphorylation of EGFR, Akt and Erk1/2 for these clones, yet there was a trend to lower phosphorylation of Akt and Erk1/2 with IR doses of 4 and 8 Gy compared to control cells (Fig. 5D in II). We then used zymography and EnzChek in order to determine the MMP and gelatinase activities within these cell clones. In zymography we witnessed dose dependently increased levels of proMMP2 and -9, active MMP2 as well as the total level of these enzymes compared to non-irradiated control cells (Fig 6A-C in II). Furthermore, the overall gelatinase activity, assayed by EnzChek, showed no dose dependent changes, suggesting specific induction for MMP2 and –9 (Fig. 6D in II). Next, we analyzed E-cadherin and vimentin by immunoblots but found no changes (Fig. 7A-B in II) and also the level of HPSE1 remained unchanged (Fig. 7A and C in II). These results suggest that no long-term effects on invasion, proliferation, clonogenic viability nor activation of signaling pathway proteins or EMT occur in post-irradiated HSC-3 cells, but there seems to be a specific induction in the expression of MMP2 and -9.
5.3 MMP9 has a fluctuating role in OTSCC progression

MMP9 has been considered merely as a pro-tumorigenic factor in OSCC, but there is also a body of evidence stating otherwise. We hypothesized that MMP9 may also have a anti-tumorigenic effect in the progression of OSCC and to further elucidate the role of MMP9, we used OTSCC cell lines along with various models where production or activity of MMP9 was altered. These models included MMP9 silenced and arresten overexpressing HSC-3 cell lines with increased MMP9 expression, as well as using an MMP9 inhibitor.

5.3.1 Gelatinase inhibitor CTT2 increases invasion of OTSCC cells in myoma model

We first examined the effect of a gelatinase inhibitor on the motility of HSC-3, SCC-25 and SAS tongue carcinoma cell lines and MDA-MB-231 breast carcinoma cell line by using CTT2, an inhibitor for both MMP2 and -9. CTT2 inhibited the migration of MDA-MB-231 cells (48 h p<0.05, Fig. S1C) and SAS (24 h and 48 h p<0.05, Fig. S1D in III) cells, but had no effect on SCC-25 cells (Fig. S1E in III). Interestingly, CTT2 slightly increased the migration of HSC-3 cells (p = 0.057; Fig. 1B in III). We also found that when HSC-3 cells were treated with the gelatinase inhibitor CTT2 at the time of seeding, they invaded significantly deeper in the myoma organotypic culture compared to control cells (Fig. 1C-D in III). Also, the invasion index was higher after CTT2 treatment (Fig 1E in III).

5.3.2 Silencing of MMP9 reduced proliferation but increased migration and invasion of OTSCC cells

In the second experimental setting we used HSC-3 cells that were silenced for MMP9 (shMMP9). To confirm the silencing of MMP9 in these transduced cells, we measured the mRNA and relative protein levels by PCR and zymography, respectively (Fig. 2A and B in III). Similar to previous inhibition with CTT2, shMMP9 cells migrated significantly faster compared to control cells in the scratch wound assay and in the Transwell® migration assay (Fig. 2C and D in III). Also, in myoma organotypic culture the invasion area of shMMP9 cells was significantly increased, but there was no difference in the invasion depth between shMMP9 cells and control cells (Fig. 3A-C in III).
5.3.3 Tumor-suppressive effects of arresten may mediate through expression of MMP9

We have previously shown that HSC-3 cells, transfected to overexpress arresten (arrHSC-3), had reduced migration but increased expression of MMP9 (and slightly reduced MMP2 expression) (Fig. 5 A and C in III). We used arrHSC-3 cells as a third model to examine the effect of altered MMP9 activity on the cell motility. We first tested how CTT2 treatment affected the cell migration of arrHSC-3 with elevated MMP9 expression and found that after 24 h CTT2 was again able to slightly increase the migration of control cells. However, after 48 h CTT2 increased the migration of arrHSC-3 to the same level with the control cells (Fig. 5B in III) and subsequently the MMP9 levels were increased in these cells due to migration and longer incubation time (Fig. 5C in III). Finally, we confirmed the tumor-suppressive effect of arresten by utilizing our human myoma-based organotypic model. In myoma organotypic culture the cell invasion of normal HSC-3 cells was almost completely inhibited by 500 nM exogenous arresten (Fig. S5A-D in III). Endogenous arresten also significantly increased the proliferation of HSC-3 cells (Fig. S2C in III) – opposite to what was seen with shMMP9 cells (Fig. S2B in III). The decrease in cell invasion was also observed with arrHSC-3 cells in myoma organotypic culture (Fig S6A-D in III).
6 Discussion

6.1 The significance of macrophages in the invasion process of OTSCC

There is still a lack of reliable biomarkers for diagnostics in OTSCC. Tools to predict the progress of the disease are of high importance for successful treatment. The cornerstone of personalized cancer medicine is to recognize the patient’s genetics as well as the distinctive characteristics of the tumor and its microenvironment. OTSCC progression is dependent on the tumor microenvironment (TME), which includes tumor associated macrophages (TAMs). As the normal interactions between tumor cells and ECM are disturbed, tumor cells tend to modulate their environment so that it is more suitable for themselves. The presence of TAMs is linked with poor prognosis, but the mechanisms are yet unknown. TAMs are crucial components of the TME as they participate in regulation of tumor growth, immune suppression, angiogenesis and metastasis (Chanmee et al., 2014; Evrard et al., 2019). In our study (I) we chemically modified THP-1 leukemia cells to M1 (inflammatory), M2 (TAM-like) and R848-type macrophages and examined their interactions with HSC-3 cells. We showed that co-culturing with macrophages affected the migration and invasion potential of HSC-3 tongue cancer cells. Moreover, it altered the phenotype of both cells and substantially affected the cytokine secretion profile. High levels of cytokines such as EGF, TGF-β1 and M-CSF were found in HSC-3 cells when the cells were cultured with M2 macrophages, suggesting that interaction with TAM-like M2 cells favors tumor progression by altering the cytokine expression profile of the carcinoma cells. TGF-β1 has a dual role in cancer progression: in early stages it inhibits proliferation and induces apoptosis, whereas in later stages it switches to a cancer promoting direction and induces EMT in tumor cells (D. Li, Xu, Lu, Dong, & Wang, 2015; Z. Lin et al., 2014; Wang et al., 2011). EGF is strongly associated with HNSCCs, including OSCC (Koontongkaew, 2013) and overexpression of EGFR is associated with reduced survival (Kundu & Nestor, 2012). M-CSF takes part in the differentiation of hematopoietic stem cells into macrophages and it is associated with macrophage migration and survival. (Hume & MacDonald, 2012; Ries, Hoves, Cannarile, & Rüttinger, 2015). The activation of the M-CSF/CSF-1R (receptor) pathway is linked with growth and metastasis in various cancer types and blocking it can inhibit cancer progression. M-CSF takes
also part in the polarization of macrophages into the M2 subtype via a synergistic effect of transcription factors c-Jun and NF-κB (Yujiao Yang et al., 2014). Hence, the cytokines at issue may be potential therapeutic targets for further studies in OTSCC.

The presence of M2 macrophages induced the invasion of HSC-3 cells in the myoma model, whereas the influence of M1 macrophages reduced it. Our results also suggest that close proximity with macrophages may be essential for both pro-invasive and inhibitory effects in HSC-3 cells. The pro-tumorigenic functions of TAMs have been reported to be activated by direct cell-cell contact with tumor cells, although the mechanism behind this is not yet clear (Komohara & Takeya, 2016). However, macrophage-conditioned media seemed to have an inhibitory effect on the horizontal migration of HSC-3, likely due to NF-κB regulated molecules. In addition, we observed that the myoma tissue alone acted as a chemoattractant to macrophages, but this effect was even stronger when HSC-3 cells were present in the myoma tissue. This supports our observations made with previous experiments that myoma tissue mimics the human TME in several ways (Dayan et al., 2012; Nurmemmniemi et al., 2009; Teppo et al., 2013; Åström, Heljasvaara, Nyberg, Al-Samadi, & Salo, 2018).

We found M2 macrophages to have an excess amount of p50 in the cytoplasm, that may be causing a dysregulation in NF-κB activation and in this way contributes to the induction of tumor-progressive genes and OTSCC progression. The p50 presence was manifested as a lack of translocation of the p65 homodimer. Cell-cell contact with M2 macrophages and HSC-3 cells seemed to induce NF-κB dysregulation in HSC-3 cells. High expression of NF-κB is associated with increased migration and invasion in OSCCs (Tang et al., 2017) and selective inhibition of this NF-κB was able to reduce the metastatic potential of cancer cells both \textit{in vitro} and \textit{in vivo} (Tanaka et al., 2011). The results suggest that TAMs are actively involved in the progression of tongue cancer, and thus TAMs should be considered as possible therapeutic targets in the future for OTSCC treatment. Currently, there are a number of therapeutic strategies focusing on macrophage targeting that have showed promising results in clinical trials, for example, immune checkpoint inhibition therapies (Cassetta & Kitamura, 2018; Poh & Ernst, 2018). Therefore, a better understanding of the interactions between tumor cells, TME and TAMs is required.
6.2 HPSE1 inhibition combined with IR may induce invasion in OTSCC

For decades, research on improving radiotherapy focused almost entirely on the cancer tumor itself while biological interactions between stroma and tumor were ignored.

We have introduced a human 3D myoma disc organotypic model (Nurmenniemi et al., 2009) and a gelatinous leiomyoma matrix, MyoGel (Salo et al., 2018, 2015) for \textit{in vitro} human cell invasion experiments. The hypoxic myoma discs mimic the TME of solid human tumors, and they contain a variety of non-vital cells and soluble invasion related factors, such as lysyl oxidase (Teppo et al., 2013). MyoGel, on the other hand, provides a soluble human TME matrix for cancer studies.

Compared to the commercial mouse tumor derived matrix, Matrigel®, MyoGel contains, \textit{e.g.}, latent and active MMP-2, tenascin-C, and collagen types XII and XIV, which are not present in Matrigel® (Salo et al., 2015). A metastatic niche is required for the survival and outgrowth of metastasizing cancer cells (Chin & Wang, 2016; Kaplan et al., 2005; McAllister & Weinberg, 2014; Sceneay, Smyth, & Möller, 2013). Formation of this niche is associated with the deposition and remodeling of ECM, the presence of hypoxia, growth factors, cytokines and chemokines (Eltzschig & Carmeliet, 2011; Psaila & Lyden, 2009; Sleeman, 2012). Interestingly, since the composition of myoma tissue shares the characteristics of the metastatic niche, myoma models enable an approach for preclinical drug and chemoradiation testing.

We tested the feasibility of both of these models for chemoradiation experiments on OTSCC cells (II). We have previously shown that anti-heparanase aptamers inhibited the invasion of HSC-3 cells in the myoma model (Simmons et al., 2014). Here, we tested the combined effects of aptamers and ionizing radiation (IR) on the invasion and proliferation on HSC-3 cells. Similar to our previous results (Simmons et al., 2014), the HPSE1 antibody and 1.5M Short aptamer inhibited the invasion of HSC-3 cells in the myoma model and Myogel assay, confirming the consistency of both invasion models. In addition, we detected increased expression of HPSE1, MMP2 and MMP9 in HSC-3 cells after irradiation in the myoma model. Surprisingly, anti-heparanase aptamers induced the invasion of HSC-3 cells after irradiation with doses of 4 and 8 Gy, suggesting that there may be alternative invasion regulating pathways activated upon HPSE1 inhibition and IR. However, with this experimental setting the invasion was not induced with two other OTSCC cell lines, both low in heparanase expression. Knockdown of
heparanase has been shown to upregulate the expression of MMP2 and -9 (Y. Yang et al., 2015; Zcharia et al., 2009). With respect to this, increased MMP activity may explain the induced invasion of HSC-3 cells in our experiments.

6.3 IR induces the expression of MMP2 and -9

The benefit of IR in cancer treatment has been questioned in the light of evidence concerning IRs possible pro-tumorigenic effects and activation of pathways associated with tumor progression and radioresistance. Undoubtedly, radiotherapy is beneficial and prolongs survival, but the negative effects are also well acknowledged (Moncharmont et al., 2014; Perri et al., 2015; Sundahl, Duprez, Ost, De Neve, & Mareel, 2018).

We found that levels of MMP2 and -9 were induced in HSC-3 cells due to IR in the myoma model. Similar induction of MMP2 and -9 has been witnessed in previous studies with irradiated cancer cells (Kargiotis, Geka, Rao, & Kyritsis, 2010; Moncharmont et al., 2014; Sundahl et al., 2018). However, we did not detect any long-term enhancement in the invasive potential of HSC-3 cells nor did the IR have any effect on the activation of ERK and PI3K/Akt pathways that are known to contribute to tumor pathogenesis. When we examined clones of previously irradiated and invaded HSC-3 cells, we found that the levels of proMMP2 and -9 as well as active MMP2 were increased dose-dependently with the IR exposure to the parental cells. Nonetheless, this did not have any effect on the activation of ERK or PI3K/Akt signaling. We surmise that IR may induce the expression of HPSE1 which then induces the expression of MMP2 and -9 in the HSC-3 cells that already contain high levels of HPSE1.

As we have previously shown, the myoma model initiates the expression of vimentin in HSC-3 cells, which is characteristic for the epithelial-mesenchymal transition (EMT) (Nurmenniemi et al., 2009). The EMT is a process where epithelial cells gain pro-invasive characteristics as they lose their polarization and attachment to basement membrane and to neighboring cells (Lamouille, Xu, & Derynck, 2014; Radisky & LaBarge, 2008). We detected loss of E-cadherin and subsequent gain of vimentin in the irradiated myoma model but not on the test plate. This indicates that although IR induces EMT in the myoma model, the environment is crucial for the initiation of the EMT-process. Hence, the myoma model provides a more realistic 3D test environment for in vitro studies for cancer cells compared to plastic.
6.4 Anti-tumorigenic roles of MMP9

Elevated MMP9 expression generally seems to associate with poor prognosis in OTSCC patients. However, some MMPs, including MMP9, are considered as both tumor suppressors and tumor promoters. In vivo data have remained highly controversial, suggesting that the action of MMP9 might be fluctuating, depending on, for example, the tumor stage, aggressiveness and genetic mutations of the tumor cells. We showed in our study (III) that inhibition and silencing of MMP9 resulted in increased migration and invasion of the highly aggressive OTSCC cell line HSC-3. The silencing of MMP9 also led to decreased proliferation. This is in line with the increased overall motility observed since tumors that are large in volume due to a high proliferation rate are not necessarily the most aggressive ones. We also witnessed elevated MMP9 expression in arresten overexpressing HSC-3 (arrHSC-3) cells, that showed reduced motility. Furthermore, inhibiting MMP9 with CTT2 (inhibiting both MMP9 and MMP2) restored the migration of arrHSC-3 cells. Recently, we have detected increased MMP9 expression in a less aggressive HSC-3 clone that was transduced to overexpress MMP8 (Åström et al., 2017). In addition, in the first study (I) HSC-3 cells invading in myoma tissue in the presence of M1 macrophages showed a higher level of MMP9 compared to HSC-3 cells in the presence of M2 macrophages, which is the phenotype displayed by most TAMs.

Observations made in studies with different types of cancers showing MMP9 as a protective factor, conflict with the tumor promoting role it is often associated with. For example, increased levels of MMP9 in primary tumors seem to protect from lymph node metastases in HNSCC (Stokes et al., 2010). Also, the general idea of dichotomous roles of MMPs in cancer progression needs updating as there is evidence indicating that MMPs can also have alternation and fluctuation in their effects on tumor progression, as seen with MMP9 as well as other MMPs (Vilen et al., 2013; Winer et al., 2018). A selection of studies where MMP9 is associated with anti-tumorigenic effects and improved outcome are listed in Table 5.

We state that MMP9 indeed has a fluctuating role in the aggressive oral carcinoma microenvironment and in some cases MMP9 can potentially act as a tumor suppressive agent. This is not the first study revealing evidence of the multifaceted role of MMP9 in cancer, which indicates that the prevalent opinion about MMP9s influence should be viewed critically and the subject needs further investigation. For a long time MMP9 has been considered merely as a pro-tumorigenic factor due to its degrading effect towards the ECM (Jordan et al., 2004), yet recently its protective role has also been reported in numerous studies as
described above. Although, in the literature, MMP9's pro-tumorigenic functions are described more frequently, the data suggesting otherwise cannot be neglected and some protective mechanisms have already been acknowledged and generally approved. One of the identified mechanisms is MMP9's ability to inhibit angiogenesis via production of angiostatin. Rightfully, this raises a question of whether the role of MMP9 in tumor progression should be re-evaluated, especially in the light of results gained from failed clinical trials with numerous MMPIs (Cauwe et al., 2007; Vandenbroucke & Libert, 2014; Winer et al., 2018).

Table 5. Studies portraying the protective effects of MMP9 in tumor progression.

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<thead>
<tr>
<th>Cancer</th>
<th>Main relevant finding</th>
<th>Reference</th>
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<tr>
<td>Lung</td>
<td>High stromal MMP9 expression was an independent positive prognostic factor for disease-specific survival.</td>
<td>(Stenvold et al., 2012)</td>
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<tr>
<td>HNSCC</td>
<td>MMP9 mRNA was significantly decreased in primary tumors of HNSCCs with metastasis compared with non-metastatic tumors.</td>
<td>(Stokes et al., 2010)</td>
</tr>
<tr>
<td>Prostate</td>
<td>Vaccinia virus-mediated gene transfer of MMP9 resulted in regression prostate cancer growth.</td>
<td>(Schäfer et al., 2012)</td>
</tr>
<tr>
<td>Breast</td>
<td>MMP9 gene transfer by adenovirus decreased tumor growth dose dependently in breast cancer.</td>
<td>(Leifler et al., 2013)</td>
</tr>
<tr>
<td>Skin</td>
<td>Elevated levels of MMP9 caused reduction in subcutaneous tumor angiogenesis via increased angiostatin expression in integrin α1-null mice.</td>
<td>(Pozzi et al., 2000)</td>
</tr>
<tr>
<td>Skin</td>
<td>Reduction in MMP9 plasma levels resulted in decrease of angiostatin leading to increased tumor angiogenesis and growth.</td>
<td>(Pozzi et al., 2002)</td>
</tr>
<tr>
<td>Skin</td>
<td>Percentage of higher malignant grade carcinomas increased in MMP9-deficient mice compared to wild-type mice.</td>
<td>(Coussens et al., 2000)</td>
</tr>
<tr>
<td>Colon</td>
<td>MMP9 had tumor suppressing effect in colitis associated carcinoma by sustaining epithelial mucosal integrity via activation of the EGFR-Sp1 pathway.</td>
<td>(Pujada et al., 2017)</td>
</tr>
<tr>
<td>Oral</td>
<td>Epithelial-derived MMP9 acted as a tumor suppressor colitis associated carcinoma via the “MMP9-Notch1-ARF-p53 axis” pathway, regulating apoptosis, cell-cycle arrest and DNA damage.</td>
<td>(Walter et al., 2016)</td>
</tr>
<tr>
<td>Breast</td>
<td>MMP9 have may anti- or pro-tumorigenic effect depending on the stage, grade and location of the tumor of the oral cavity.</td>
<td>(Vilen et al., 2013)</td>
</tr>
<tr>
<td>Breast</td>
<td>Expression of MMP9 in stromal cells correlates with poor survival, whereas MMP9 expression in tumor cells predicts better survival in breast carcinoma.</td>
<td>(Pellikainen et al., 2004)</td>
</tr>
<tr>
<td>Breast</td>
<td>Overexpression of MMP9 caused increased generation of antiangiogenic factors and induced tumor regression.</td>
<td>(Bendrik et al., 2008)</td>
</tr>
</tbody>
</table>
7 Summary and conclusions

In cancer, the relations between tumor cells and ECM are disturbed which makes the TME an interesting topic in the field of cancer research. As shown in study I, especially type M2 macrophages actively take part in the invasion of OTSCC cells in the myoma model by altering the cytokine expression of the tumor cells. This is also the first time that OTSCC cells are shown to fuse with macrophages, which may in fact portray a mechanism of how pro-tumorigenic factors are spread to the surrounding stroma. In addition, tongue carcinoma cells are attracted by macrophages and the TME of myoma tissue. This further highlights the importance of both macrophages and the TME in cancer progression.

The general viability of myoma organotypic culture and Myogel models has been proven in numerous peer reviewed studies. However, in study II the feasibility of human tissue-based matrix models was tested for chemoradiation trials. Our analysis demonstrates the reliability and consistency of both of our ECM models and their applicability for further chemoradiation studies. Moreover, we studied the combined effect of HPSE1 inhibition and IR and found that this combination may induce the invasion of OTSCC cells. Also, IR seems to have a sustained inducing effect on the expression levels of MMP2 and -9.

Based on the fact that there are numerous studies implicating both anti- and pro-tumorigenic functions of MMP9 in various cancer types, we studied MMP9 in OTSCC using different approaches, such as gene silencing and specific MMP inhibitors as well as OTSCC cell lines overexpressing MMP9. In study III we revealed new evidence of the multifaceted role of MMP9 in OTSCC as we found that MMP9 has an inhibiting effect on the motility of the highly aggressive OTSCC cell line HSC-3. Our results suggest that MMP9 may also have anti-migratory effects that still remain unknown.
Lähdeluettelo


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Osajulkaisut


* Equal contributions.

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