THE REGULATION OF TONGUE CANCER INVASION BY PROTEASES AND TUMOR MICROENVIRONMENT

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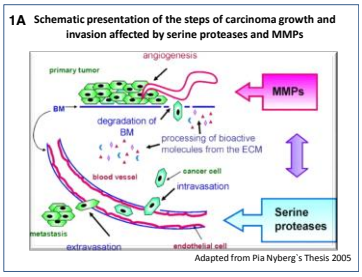
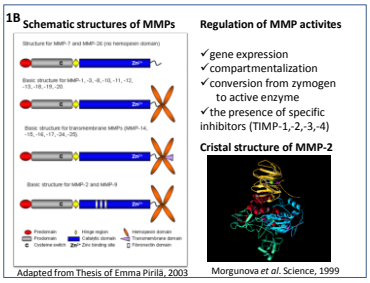
Introduction

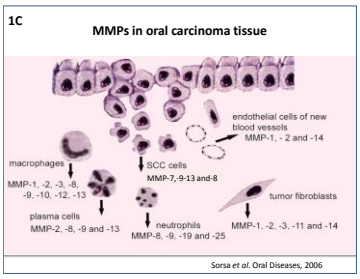
Oral squamous cell carcinoma (OSCC) is the most common type of cancer of the oral cavity constituting more than 90% of all cases (Muir and Weiland, 1995). In Finland, there are about 250 new cases per year and the number, especially of tongue carcinomas, has been increasing (Finnish Cancer registry, Cancer statistics at www.cancerregistry.fi). The World Health Organization (WHO) expects a worldwide increase in incidence in the next few decades (Massano *et al*., 2006). In the past three decades, despite advances in investigation and treatment, the overall prognosis has remained largely unchanged. The most common site currently affected is the tongue and at this site OSCC is particularly aggressive with almost half of the patients dying of the disease. (Annertz *et al.,* 2002)

Part 1. Metallo- and Serine Proteinases in Oral Tongue Squamous Cell Carcinoma (OTSCC)

Increasing evidence supports the view that extracellular proteinases, such as the matrix metalloproteinases (MMPs) and serine proteases, mediate many of the changes in the microenvironment during tumor progression (Fig 1 A). MMPs are a family of 23 human zinc-dependent endopeptidases, first described almost half a century ago (Gross and Lapiere, 1962). They play a crucial role in various physiological processes and in diseases, such as cancer. The general structure of MMPs shows three domains that are common to almost all MMPs, the pro-peptide, the catalytic domain, and the hemopexin-like C-terminal domain that is linked to the catalytic domain via a flexible hinge region (Fig 1B). MMPs are initially expressed in an enzymatically inactive state and they require proteolytic cleavage by convertases, which occurs intracellularly by furin or extracellularly by other MMPs or serine proteinases (Sternlicht and Werb, 2001). Proteolytic activity of MMPs can be regulated at different levels: gene expression, compartmentalization, conversion from zymogen to active enzyme, and, the presence of specific inhibitors. TIMPs (tissue inhibitors of metalloproteinases) are a group of four homologous specific inhibitors that by binding tightly and non-covalently to MMPs inhibit their catalytic extracellular activity (Overall and Lopez-Otin, 2002). However, TIMPs may also be part of the activation process where MMP-2 requires TIMP-2 that is bound to one molecule of MMP-14 via its catalytic domain and also is bound to pro-MMP-2 via its hemopexin domain. A second molecule of MMP-14 then catalytically activates MMP-2. Compared with carcinomas originating from other tissues, the amount and activity levels of MMPs are generally increased in OSCCs (Strongin, 2006). Cells in the microenvironment of many cancers express most of the proteinases that are released into the extracellular space and influence multiple events in tumor progression (Fig 1C).

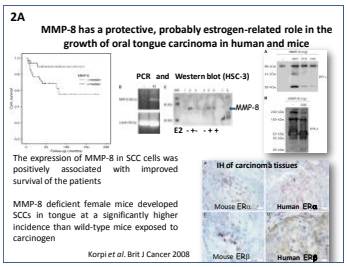


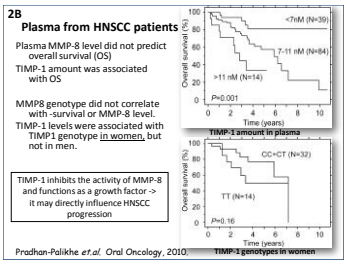


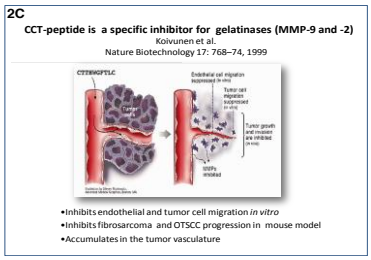


Interstitial collagenases, MMP-1, -8 and -13, fragment fibrillar collagen types I-III and V as well as proteoglycans. Collagen fragments are further degraded by gelatinases (MMP-2 and -9) that also modulate basement membrane type IV collagen, anchoring filament type VII collagen and hemidesmosomal component, laminin-5 (currently called lam-322). Deregulation of gelatinases (MMP-2 and -9), collagenases-1 and -3 (MMP-1 and -13) and MMP-14 are associated with tumor progression in head and neck cancers (Sutinen *et al.,* 1998, Thomas *et al*., 1999, Ruokolainen *et al*., 2004). However, MMP-8 (collagenase-2), mainly synthesized in bone marrow and secreted by neutrophil leukocytes during inflammation, has a protective role in cancers. Balbin *et al*. (2003) were the first to show that absence of this protease strongly increases the incidence of skin tumors in mice. We demonstrated that OSCC cells express MMP-8 (Moilanen *et al*., 2002), and showed that MMP-8 expression is associated with improved survival of the tongue cancer patients, especially in females (Korpi *et al.,* 2008, Fig 2A). We were also able to demonstrate demonstrate the protective role of MMP-8 in a mouse model: MMP-8 deficient females developed tongue SCCs at a significantly higher incidence than wild-type mice when exposed to carcinogen. Consistently, estrogen was able to induce the expression level of MMP-8 in cultured oral carcinoma cells. Thus, in contrast to most MMPs, MMP-8 seems to have a protective, gender related role in the growth of tongue SCCs (Korpi *et al.,* 2008, Fig 2A). However,

based on our recent data MMP-8 plasma level or genotype of head and neck cancer patients did not correlate with the outcome of the patients. Instead, TIMP-1 (inhibitor for MMP-8) correlated with the poor survival (Fig 2B). Plasma TIMP-1 levels were associated with *TIMP1* genotype in women but not in men, and the *TIMP1* genotype in women predicted survival suggesting that TIMP-1 may have a direct role in the progression of SCC (Pradhan-Palikhe *et al*., 2010). *The tumor destructive and suppressive mechanisms of proteinases are currently still only partially known and further analyses are required for the validation of the functional and clinical significance of MMPs and TIMPs in tongue cancer.*





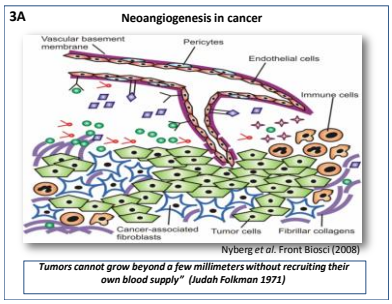


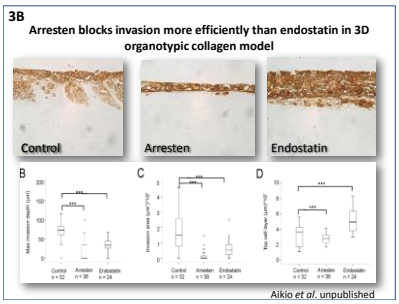
The notion that MMP-mediated ECM degradation leads to cancer cell invasion and metastasis has been a guiding theory in MMP research over thirty years (Liotta *et al*., 1980). The discovery that inhibition of MMPs suppresses the invasive potential of tumors in animal studies was implemented into clinical trials. Yet, these broad spectrum MMP inhibitor studies failed to increase survival rate of the patients (Coussens *et al*., 2002). Together with Dr. Erkki Koivunen´s research group we discovered a cyclic CTT1-peptide, which is a selective inhibitor for MMP-2 and MMP-9, but not for other MMPs (Fig 2C). CTT1 inhibits growth of various tumors improving survival of mice. We also showed that CTT1 specifically target blood vessels *in vivo* (Fig 2C). We demonstrated that oral tongue HSC-3 cells, transfected with trypsin-2 gene, are more invasive in the chicken CAM-model, and this induced intravasation is most likely derived from the increased MMP-9 activity since pre-treating the cells with CTT1-peptide abolished the intravasation (Nyberg *et al.,* 2002). Similar to CTT1 the hydrophilic derivative, CTT2-peptide, was effective in inhibiting the gelatinolytic activity *in vitro*, the growth and vessel formation of tongue HSC-3 carcinoma xenografts increasing significantly the survival of the mice (Heikkilä *et al*., 2006). Recently, we have discovered peptide G, a selective MMP-14 inhibitor. The peptide effectively inhibited the migration and invasion of cancer cells *in vitro*. Furthermore, *in vivo* the peptide reduced the growth of tongue carcinoma xenografts and prolonged the survival of mice (Suojanen *et al.,* 2009).

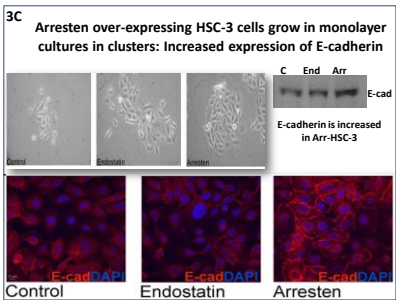
TATI (tumor associated trypsin inhibitor) and two isoforms of trypsins, trypsin-1 and trypsin-2 (tumor associated trypsinogen-1 and -2) were first isolated and purified from ovarion tumors (see review Nyberg *et al*., 2006). Cancer associated trypsins differ from their pancreas counterparts based on their enzymatic and chemical properties. We have shown that trypsin-2 efficiently activates MMPs, especially gelatinase MMP-9, at a remarkable low concentration *in vitro* (Sorsa *et al.,* 1997). Interestingly, we also demonstrated that trypsin-2 efficiently fragments type I collagen (Moilanen *et al*., 2003). After transfecting trypsin-2 into HSC-3 cells, MMP-9 was secreted in an active form. Using the chicken chorionallantoic membrane intravasation assay (CAM) (Kim *et al*., 1998), we demonstrated that trypsin-2 transfected HSC-3 cells were 60 % more invasive than the control cells and the induced invasive capacity was inhibited by TATI (Nyberg *et al*., 2002). In trypsin-2 transfected HSC-3 cultures the production of "protective" MMP-8 was noticeably decreased as compared to normal HSC-3 cells (Moilanen *et al*., 2003). We also revealed that tongue SCC tissue sections do sporadically express trypsin-2 and enterokinase in carcinoma cells and macrophages. In addition, MMP-9 was co-located with trypsin-2 in intracellular vesicles of HSC-3 cells (Vilen *et al.,* 2008). Recently we found that trypsin-2 also processed MMP-14, and the processed form was secreted from try-2 transfected HSC-3 cells (Vilen *et al*., unpublished data). Transfected cells also cleaved lam322 chain and regulated the amount of tight junction proteins, claudin 1 and 7 (Vilen *et al*., unpublished data). We have previously shown that the altered levels of claudin 7 (either strong or low immunoreactivities) is associated with decreased survival of oral tongue SCC patients (Bello *et al*., 2008). Claudins have previously been shown to take part in carcinoma growth and invasion processes at least to some extent through MMP-dependent pathways (Miyamori *et al*., 2001). Oku *et al*. (2006) demonstrated that claudin 1 provokes invasion of oral SCC cells via inducing the production and activation of MMP-2 and MMP-14. These MMPs have previously been shown to promote epithelial cancer cell migration by producing the cleavage product of lam322 gamma2 chain which binds on epidermal growth factor receptor (EGFR) on cancer cell surfaces (Schenk *et al.,* 2003). We propose that trypsin-2 - MMP-9 and trypsin-2 - MMP-14 cascades are important in directional modulation of the extracellular matrix in tongue carcinoma invasion.

Part 2. Anti-angiogenic Peptides in OTSCC

The degradation of ECM components and other extracellular molecules may generate fragments with new bioactivities that inhibit angiogenesis (reviewed by Nyberg *et al.* 2005, Fig 3A). For example, biologically active endostatin is generated via cleavage of type XVIII collagen by MMP-3, -7, -9, -13, and -20 (Heljasvaara et al., 2005). Recombinant endostatin efficiently blocks angiogenesis and suppresses primary tumor and metastasis growth in experimental animal models without any side effects, toxicity or drug resistance (Nyberg *et al.* 2005). Endostatin inhibits tumor growth not only by acting via endothelial cells but also by directly affecting the behavior of carcinoma cells. In fact, endostatin significantly reduces invasion of endothelial as well as tumor cells by inhibiting the activity of distinct MMPs, like MMP-9 (Kim *et al.,* 2000, Nyberg *et al.,* 2003). In addition to endostatin, there are several other anti-angiogenic and cryptic fragments derived from different BM molecules. The non collagenous domains of certain collagens are particularly a good source of these inhibitors. Type IV collagen derived arresten is such a molecule. Arresten was shown to inhibit angiogenesis and tumor growth *in vivo* (Sudhakar *et al.,* 2005). We have recently stable transfected endostatin and arresten cDNA fragments to HSC-3 cells (Aiko *et al*., manuscript) which after transfection secreted those antiangiogenic peptides to culture media. Surprisingly, endostain transfection increased the size of tumors in nude mice over control whereas arresten transfection reduced tumor growth. Arresten tumors were less vascularized and less invasive than endostatin- and control-HSC-3 xenografts. Arresten inhibits tongue carcinoma cell (HSC-3) migration and wound healing (Fig 3B). Arresten blocks invasion more efficiently than endostatin in 3D organotypic collagen model (Fig 3B). Arresten increased the amount of E-cadherin. It also induced apoptosis by increasing the amount of Bax, an inductor of apoptosis, and decreases Bcl-xL, an inhibitor of apoptosis, in the mitochondrial Bcl-family signaling pathways (Fig 3C). We conclude that, although both endostatin and arresten are collagen-derived angiogenesis inhibitor arresten is also a potent inhibitor of tongue SCC invasion and inductor of apoptosis. Since loss of E-cadherin is a marker for epithelial to mesenchymal transition (EMT) we hypothesize that arresten peptide is able to reverse EMT in tongue carcinoma cells (Aiko *et al*., manuscript in preparation).



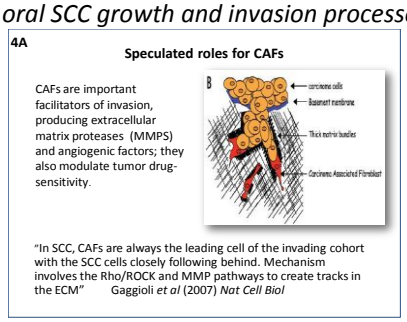


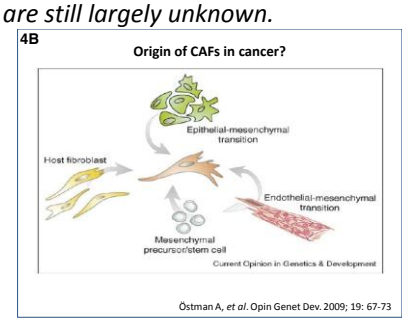


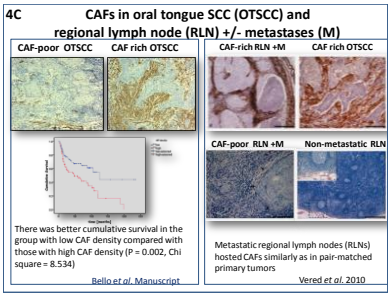
Part 3. Carcinoma Associated Fibroblasts in OTSCC

Tumor microenvironment is now considered to be an active participant in solid epithelial tumor progression and not merely a structural pathway for tumor invasion (Baglole *et al*., 2006, Kessenbrock et al., 2010). Stromal cells, extracellular molecules and hypoxia all play an important but a complex role in tumor progression. Microenvironment also influences the response to anti-tumor therapies (Ruan *et al*., 2009). Stromal myofibroblasts (MFs) also called as carcinoma associated fibroblasts (CAFs) have effect on tumor cell proliferation, survival, angiogenesis, invasion and metastasis by producing a wide range of growth factors, cytokines and proteases, including MMPs (Kalluri and Zeisberg, 2006; Kessenbrock *et al.*, 2010). A recent study has also shown that an additional mechanism involves the "hitching a ride" model where carcinoma cells invade on tracks that have already been created by CAFs with the latter cells always serving as the leading cell invading cohort and the carcinoma cells closely following behind (Gaggioli *et al*., 2007; Fig 4A). Interestingly, the origin and the mechanism of activation of CAFs are possibly distinct in different tissues, and may include various combinations of cell types and signalling (Li *et al.,* 2007). Abundance of CAFs or increased expression of CAF associated proteins has been linked with bad prognosis in many epithelial malignancies including the oral cavity (Kellermann *et al*., 2007). During oral carcinogenesis CAFs are identified mainly after the development of carcinomas, not in pre-malignant lesions (Vered *et al.,* 2007, 2009). *In vitro* oral SCC cells can induce a myofibroblastic phenotype of primary fibroblasts and this trans-differentiation is dependent on TGF-β1 (Lewis *et al*., 2004; Fig 4B). Emerging evidence suggests that a major source of tumor-associated myofibroblasts is carcinoma cells through epithelial-mesenchymal transition (EMT) (reviewed by Tse & Kalluri, 2007). However, there is also data supporting that CAFs originate from circulating bone marrow derived mesenchymal stem cells (BMMSC) (reviewed by McAnulty, 2007). Recent studies suggest that MMPs may also stimulate cancer progression by inducing epithelial cells to trans-differentiate into activated myofibroblasts (Radisky *et al*., 2007). It is also possible that CAFs are at the beginning bystanders who during carcinoma progression turn into

key players of the cancer process (Ostman and Augsten, 2009). We have recently shown that, indeed, increasing CAF density is strongly associated with elevated mortality in oral tongue SCC (Bello *et al*., submitted; Fig 4C) and that CAFs are common to both primary and metastatic SCC (Vered *et al.,* 2010; Fig 4C). Based on our demonstration of CAFs within metastatic regional lymph nodes in OTSCC we have hypothesized that CAFs can also facilitate metastases either by co-metastasizing and/or being recruited to lymph nodes (Vered *et al.,* 2010). *It will be a challenging task to confirm this hypothesis. In addition, the origin of CAFs in oral cancers and the mechanism of how they participate in oral SCC growth and invasion processes are still largely unknown.*

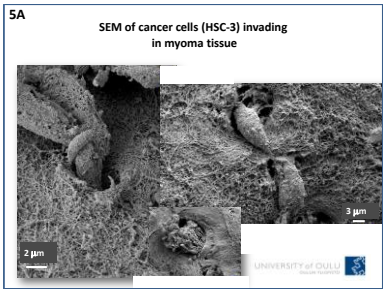


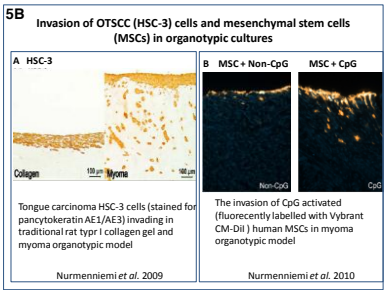


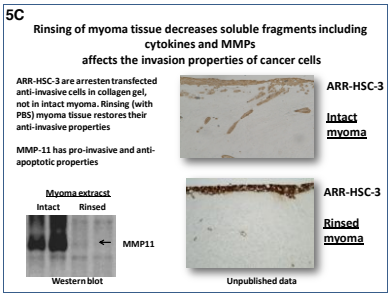


Part 4. Organotypic Myoma Model for OTSCC Invasion Studies

Finally, invasion and metastasis are challenging phenomena to study. We have recently developed an organotypic invasion model where carcinoma cells invade in leiomyoma tissue obtained from routine surgical operations after informed consent of the donors (Fig 5A, B). It fulfills the repertoire of organotypic assays by providing the first simple and easy-to-manipulate, fully human assay for studying invasion and metastasis (Nurmenniemi *et al.,* 2009, 2010). It has also potential to be developed as the first large scale invasion assay for the screening of anti-invasion or anti-metastasis compounds. We have further developed the model by rinsing off the soluble factors (Fig 5C), and testing the invasion of other cell lines including activated bone marrow derived mesenchymal stem cells (Fig 5B). In the future it should still be modified by adding e.g. living fibroblasts, endothelial cells and macrophages, and thus expand the potential applications of myoma organotypic model in studies of the highly complex cascade of OTSCC invasion.







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References

Annertz *et al.* Int J Cancer, 2002. **Baglole** *et al.* Immunol Invest 2006. **Balbin** *et al.* Nat Genet, 2003. **Bello** *et al.* HumanPathol, 2008. **Coussens** *et al*. Science, 2002. **Gaggioli** *et al.* Nat Cell Biol, 2007. **Gross** and **Lapiere** Proc. Natl. Acad. Sci. USA, 1962. **Heikkilä** *et al.* Int J Cancer, 2006. **Heljasvaara** *et al.* Exp Cell Res 2005. **Kalluri** & **Zeisberg** *Nat Rev Cancer*, 2006. **Karnoub** *et al.* Nature, 2007. **Khanna** *et al.* J Natl Cancer Inst, 2009. **Kellermann** *et al.* Histopathol, 2007. **Kessenbrock** *et al.* Cell 2010. **Koivunen** *et al*. Nat Biotechnology, 1999. **Korpi** *et al.* Brit J Cancer, 2008. Lewis *et al*. *Br J Cancer*, 2004. **Li** *et al.* J Cell Biochem, 2007. **Liotta** *et al.* Nature, 1980. **Massano** *et al.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2006. **Miyamori** *et al*. J Biol Chem, 2007. **McAnulty** Int J Biochem Cell Biol, 2007. **Moilanen** *et al*. J Pathol 2002. **Moilanen** *et al*. Biochemistry, 2003. **Muir** and **Weiland**, Cancer, 1995. **Mäkelä** *et al*. Exp Cell Res, 1999. **Nurmenniemi** *et al*. Am J Path 2009, **Nurmenniemi** *et al*. Exp Cell Res, 2010, **Nyberg** *et al, Front Biosci, 2008.* **Nyberg** *et al*. J Dent Res, 2002. **Nyström** *et al*, J Pathol, 2005. **Oku** *et al*. Cancer Res **2006**. **O'Reilly** *et al*. Cell 1997. **Overall** & **Kleifeld** *Nat Rev Cancer*, 2006. **Overall** & **Lopez-Otin** Nat Rev Cancer, 2002. **Ostman** &**Augsten** *Curr Opin Genet Dev* 2009. **Pradhan-Palikhe** *et al.* Oral Oncol, 2010. **Radisky** *et al.* J Cell Biochem 2007. **Raman** *et al.* Cancer Lett, 2007. **Ruan** *et al.* J Cell Biochem, 2009. **Ruokolainen** *et al. Clin Cancer Res*. **Schenk** *et al*. J Cell Biol, 2003. **Siller-Lopez** *et al.* Gastroenterology 2004. **Sorsa** *et al*. Oral Diseases, 2004. **Sorsa** *et al.* J Biol. Chem, 1997. **Sudhakar** *et al.* J Clin Invest, 2005. **Suojanen** *et al*. Cancer Biol Ther, 2009. **Strongin** *Cancer Metast Rev* 2006. **Sutinen** *et al*. Brit J Cancer 1998. **Thomas** *et al.* Oral Oncol, 1999. **Tse** & **Kalluri** *J Cell Biochem* 2007. **Van Lint & Liber**, *Cytokine & Growth Factor Rev* 2006. **Vered** *et al*. Oral Oncol, 2007. **Vered** *et al*. In J Cancer, 2010.