

# **Effects of soluble factors released by oral squamous cell carcinoma on osteoclasts**

*A thesis submitted to McGill University in partial fulfillment of the  
requirements of the degree of Master's of Science*

M.Sc. Thesis

Mohammed Alkindi, B.D.S., SSC-OMFS

Supervisor:

Dr. Svetlana Komarova

*Graduate and Postdoctoral Studies*

*Faculty of Dentistry*

*McGill University*

*Montreal, (Quebec), Canada*

February, 2011

**Copyright © 2011 Mohammed Alkindi**

## Table of Contents

1	INTRODUCTION AND BACKGROUND .....	1
1.1	General Introduction .....	1
1.2	Environmental Risk Factors .....	2
1.2.1	Tobacco .....	2
1.2.2	Alcohol.....	3
1.2.3	Viruses .....	3
1.3	Genetic Risk Factors .....	4
1.3.1	Oncogenes and Tumor Suppressor Genes .....	4
1.3.2	Epidermal Growth Factor Receptor (EGFR) .....	5
1.3.3	Nuclear Factor-Kappa B .....	5
1.4	Bone invasion by OSCC .....	6
1.5	Bone Microenvironment .....	9
1.5.1	Osteoclasts .....	9
1.5.2	OPG/RANK/RANKL and their Roles in Bone Homeostasis .....	10
1.6	Interactions of Cancer Cells with Bone Microenvironment.....	13
2	MATERIALS AND METHODS.....	17
2.1	Materials.....	17
2.2	OSCC human cell-lines .....	17
2.3	BHY and HN cultures .....	18
2.4	Osteoclast culture .....	18
2.5	Osteoclast identification and quantification.....	19
2.6	RNA isolation and real time PCR .....	19
2.7	Statistics .....	20
3	RESULTS .....	21
3.1	Soluble factors produced by squamous cancer cells stimulate osteoclast formation from RANKL-primed precursors .....	21
3.2	Soluble Factors Released by BHY and HN cell lines Increase Osteoclasts Survival.....	23
3.3	Mechanism of Osteoclastogenic Effect of OSCC-Derived Factors .....	24
4	DISCUSSION AND CONCLUSION.....	28
4.1	Osteoclastogenic effects of bone-invasive OSCC.....	28
4.2	Mechanism of osteoclastogenic action of OSCC .....	29
4.3	Conclusion and Recommendation.....	32
5	REFERENCES .....	33

## LIST OF FIGURES

Figure1 : Osteoblast role in osteoclast activation .....	12
Figure2 : Effect of OSCC-derived factors on osteoclast formation.....	22
Figure3 : Effect of OSCC-derived factors on osteoclast survival.....	23
Figure4 : Role of MAPK pathways on osteoclastogenic effects on OSCC-derived factors.....	25
Figure5 : Role of PI3K/AKT/mTOR pathway on osteoclastogenic effects on OSCC-derived factors .....	27
Figure6 : Suggested signaling pathways involved in mediating osteoclastogenic effects of OSCC .....	30

## ABBREVIATION

1 $\alpha$ ,25(OH) <sub>2</sub> D <sub>3</sub>	25-dihydroxy vitamin D <sub>3</sub>
ATP	Adenosine triphosphate
BHY-CM	BHY-condition medium
BMCs	Bone marrow cells
CTR	Calcitonin receptor
DMEM	Dulbecco's modified eagle medium
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
HSV	Herpes simplex virus
IL-6	Interlukin 6
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony stimulating factor
MMP-1	Matrix metalloproteinase 1
NFAT	Nuclear factor for activating T-cells
NF- $\kappa$ B	Nuclear factor kappa B
OAF	Osteoclast activating factor
OC	Osteoclast
OCIF	Osteoclastogenesis inhibitory factor
ODF	Osteoclast differentiation factor
OSCC	Oral squamous cell carcinoma
OPG	Osteoprotegerin
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase-C
pOB	Primary osteoblast
PTHrP	Parathyroid hormone related protein
RANKL	Receptor activator of NF- $\kappa$ B ligand
TGF- $\beta$	Tumor growth factor $\beta$

TNF	Tumor necrosis factor
TRAP	Tartrate-resistant acid phosphatase
uPK	Urokinase type plasminogen activator
VFFBG	Vascularised free fibula bone graft

## ABSTRACT

**Objective:** Bone invasion represent significant problem in managing head and neck cancers, however the mechanisms of interactions between oral squamous cell carcinoma (OSCC) and bone cells are poorly understood. We hypothesized that tumor cells can directly stimulate osteoclastogenesis.

**Methods:** OSCC cell lines, bone-invasive BHY and metastatic but not bone-invasive HN were cultured and conditioned medium (CM) was collected. Osteoclast formation from RAW 264.7 mouse monocytic cell-line was assessed.

**Results:** When RAW 264.7 were primed with receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) and then treated with BHY-CM, marked 2-6 fold induction of osteoclastogenesis was observed. In contrast, HN-CM did not significantly affect osteoclastogenesis. In addition, BHY-CM, but not HN-CM promoted survival of mature osteoclasts. Using pharmacological inhibitors, we found that Protein kinase C (PKC)/Extracellular signal-regulated kinase (ERK)1/2/Mitogen activated protein kinase (MAPK) p38 as well as Phosphatidyl-inositol 3-kinases (PI3K)/Serine/threonine protein kinase Akt/Mammalian target of rapamycin (mTOR) pathways mediate BHY-CM induced osteoclastogenesis.

**Conclusion:** OSCC-cells produce soluble factors that stimulate osteoclastogenesis from RANKL-primed precursors. Tumor-derived factors act by stimulating ERK1/2 and p38 MAPK pathways in osteoclast precursors.

## RESUME

**Objectif:** L'invasion du tissu osseux est un problème majeur dans le traitement des cancers de la tête et du cou, cependant les mécanismes d'interactions entre le carcinome de cellules de squamous oral (OSCC) et les cellules du tissu osseux sont mal compris. Nous avons posé comme hypothèse que les cellules tumorales peuvent stimuler directement le phénomène d'ostéoclastogenèse.

**Méthodes:** Deux différentes populations cellulaires de la lignée OSCC furent utilisées: les cellules BHY ayant un potentiel de colonisation du tissu osseux et les cellules HN ayant un potentiel métastatique mais non colonisant. Ces deux lignées cellulaires ont été cultivées et le milieu de culture conditionné (CM) a été collecté. La formation de cellules ostéoclastiques à partir de cellules de la lignée monocyttaire de souris RAW 264.7 a été évaluée.

**Résultats:** Une augmentation significative du phénomène d'ostéoclastogenèse d'un facteur 2 à 6 fut observée lors d'une activation des cellules RAW 264.7 avec RANKL suivit d'un traitement avec BHY-CM. De plus, la survie des cellules ostéoclastiques matures était favorisée en présence de BHY-CM uniquement. L'utilisation d'inhibiteurs pharmacologiques nous a permis de mettre en évidence que la stimulation du phénomène d'ostéoclastogenèse induite par BHY-CM est médiée par les voies de signalisations PKC/ERK/p38 et PI3K/AKT/mTOR.

**Conclusion :** Les cellules OSCC produisent des facteurs solubles stimulant la formation d'ostéoclastes à partir de précurseurs activées par RANKL. Les facteurs dérivés de tumeurs agissent en stimulant les voies de signalisation ERK1/2 et p38 dans les précurseurs ostéoclastiques.

## ACKNOWLEDGEMENTS

I am deeply grateful to my supervisor, **Dr. S.V. Komarova** who was very helpful in guiding me throughout my research experiments and editing my thesis.

I would like also to thank **Dr. Michel El-Hakim**, oral and maxillofacial surgery program director for his support throughout my research period, and the **Oral and Maxillofacial Surgery Division** at McGill University for funding this research project and covering my travel expenses to present this work at the American Head and Neck Society 2010 Annual Meeting.

I would like to thank **Dr. Osama Hussein**, PhD student at Dr. Komarova's lab, who was always available to help, encourage, demonstrate and share his ideas throughout all stages of the experimental procedures.

I am also thankful to *all lab members* **Dr. Gulzhakhan Sadvakassova, Dr. Kerstin Tiedemann, Dr. Damien Le Nihouannen, Jenna Fong, Shahrzad Rafiei and Osama Maria** who were kindly available for assistance and guidance throughout the research project, **Dr. Le Nihouannen** also translated the abstract into French language.

Last but not least, I am very grateful to my mother, father and wife **Karima, Ghazi and Amani** for all of their great support and patience.

## **1 INTRODUCTION AND BACKGROUND**

### **1.1 General Introduction**

Oral squamous cell carcinoma (OSCC) is the eleventh most common cancer worldwide, accounting for approximately 90% of all oral cancers (Fonseca 2009). In Canada, it is estimated that 3,400 new cases of oral cancer will be diagnosed in 2010, with an estimated mortality of about 33%. Oral cancers are about twice as common in men as in women, and are slightly more common in blacks than in whites (Canadian Cancer Statistics 2010). Worldwide, OSCC is a major health-care problem, with more than 274,000 new cases in 2002, and is the most frequently diagnosed cancer in some countries (Parkin 2005). Great improvements in surgical techniques, radiotherapy, and chemotherapy have been achieved (Cooper 2004), but the 5-year survival rate for OSCC is still between 40% and 60%, and has not greatly improved over the last 30 years (Fonseca 2009)

Oral squamous cell carcinoma is a malignant tumor that commonly invades the jaw bone. Treatment often requires a surgical resection that compromises the patient's quality of life, function, and esthetic. Intraorally, it occurs most commonly in the tongue (20-30%), floor of the mouth (15-20%), retromolar and tonsillar pillar areas (15%), soft palate (10-15%), buccal mucosa (10%), gingiva (10%), alveolar bone (10%), and maxillary sinus (5-10%) (Marx 2003). About 40% of head and neck SCC mortality is due to locoregional recurrence, and about

30% develop a distant metastasis in the 5-year period following the diagnosis (de Bree 2000).

## **1.2 Environmental Risk Factors**

Multiple factors are involved in the etiology of OSCC, including a variety of toxic, viral, radiation, and genetic factors. It is related to the type of carcinogen (e.g, cigarette smoking, alcohol, and radiation), its dose, frequency, and application, and also is related to combining multiple carcinogens, which has an additive effect (e.g., combining cigarette smoking and alcohol has a higher carcinogenic effect than cigarette smoking alone). It is also affected by the host's susceptibility, and the duration in which the carcinogen was in contact with the body (Marx 2003).

### **1.2.1 Tobacco**

Large case-control studies in different countries show a strong correlation between tobacco use and the risk of oral cavity cancer, and smoking cessation reduced the risk of developing an oral cancer (McFarlane 1995). Another case-control study was conducted in Spain, and showed that tobacco smoking is an independent risk factor, and that the higher dose of cigarette smoking is associated with a higher risk of oral cancer development (Moreno-Lopez 2000). Tobacco contains more than 300 chemicals that are carcinogenic. Different forms of tobacco are used in different countries. For example, high incidence rates of oral cancer are associated with betel quid chewing in India and with smoking tobacco and alcohol use in western and southern Europe (Parkin 2005). Smokeless tobacco has also been associated with an increased risk of oral cancer. A recent meta

analysis of the relationship between smokeless tobacco and oral cancer found only a slight increase in oral cancer incidence among users of smokeless tobacco (Weitkunat 2007).

### **1.2.2 Alcohol**

There is a strong relationship between high alcohol consumption, especially for hard liquors (e.g., whisky) and the risk of oral cancer development. A study conducted in Swedish men aged 40-79 years showed that moderate alcohol consumption alone (10-19g/day) did not increase risk significantly for non-smokers, but higher alcohol intake (>50 g/day) was an independent risk factor with a relative risk of 5.5%. Additionally, the researchers found that combining alcohol with smoking has an additive increased risk, with a relative risk of 22.1% compared to 6.5% from smoking alone (Lewin 1998).

It has been observed that chronic alcohol consumption is related to the development of head and neck cancers through a different mechanism that includes DNA mutation, immune system suppression, catabolism, and microsomal enzymes induction (Riedel 2003).

### **1.2.3 Viruses**

A number of viruses have been considered in the etiology of OSCC. The association between oral cancer and viral infection with human papillomaviruses (HPVs), especially HPV 16, has been observed (Lee 2010). Other viruses such as herpes simplex virus (HSV) and Epstein Barr virus (EBV) have been associated with premalignant and malignant oral lesions (Jalouli 2010). A case-control study for 201 patients supported a strong association between human papillomavirus

(HPV) and peritonsillar cancers (base of the tongue and palatine tonsils). HPV DNA was detected in 19% of the total cases and 5% of controls, but in 43% of peritonsillar cancers (Pintos 2008).

### **1.3 Genetic Risk Factors**

The study of genetics and molecular oncology has advanced in the past decade. The genetic susceptibility of the individual such as decreased ability to repair DNA or to inactivate the carcinogens will lead to unregulated cell growth and function and increase the risk of cancer (Jefferies 2001). “601 genes were found to be significantly regulated in cancer tissue compared to adjacent intra-individual mucosa controls, and 25 genes that are differently regulated in early stage cancer samples compared to advanced disease” (Bagan 2009).

#### **1.3.1 Oncogenes and Tumor Suppressor Genes**

Damaged or altered sequence of DNA, which can be caused by tobacco, alcohol, irradiation or viruses, is called oncogenes, and tumor suppressor genes such as p53 suppress tumor development by DNA repair and thus play a major role in protection against carcinogenesis (Marx 2003)

“Transforming growth factor alpha (TGF- $\alpha$ ) has always been expressed in OSCC, and the simultaneous expression of TGF- $\alpha$  and Epidermal Growth Factor Receptor (EGFR) by the carcinoma cells is thought to result in an uncontrolled proliferation” (Wong 1993). Loss of tumor suppressor gene function leads to inactivation of the critical function of that gene and thus to tumor development (Jefferies 2001). Gene therapy trials that target specific genes that are upregulated in cancer could be the future for cancer treatment.

### **1.3.2 Epidermal Growth Factor Receptor (EGFR)**

An epidermal growth factor receptor is a receptor that binds by EGF (epidermal growth factor) and transforming growth factor- $\alpha$  (TGF- $\alpha$ ). Activation of this receptor leads to changes in cell growth regulation pathways (Fonsica 2009). TGF- $\alpha$  and EGFR are over-expressed in 92% of head and neck SCC, and the more advanced stages of cancer will have a higher expression (Grandis 1993).

Over-expression of EGFR has been associated with bone invasion and distant metastasis observed with HNSCC and thus with poor prognosis (P Oc 2000).

### **1.3.3 Nuclear Factor-Kappa B**

Nuclear Factor-Kappa B (NF- $\kappa$ B) is a transcription factor for inflammatory and immune response regulation that serves as a cytokine, chemokine, and enzyme modulator (Ghosh 2002). NF- $\kappa$ B is involved in many disease pathophysiologies, including cancer in which it is activated by a number of carcinogens as well as the tumor necrosis factor (TNF) superfamily. It promotes tumorigenesis, bone invasion, metastasis, and chemotherapeutic-resistant agents (Garg 2002). Immunohistochemistry studies of biopsy specimens for SCC and dysplasia showed that oral cancer specimens expressed higher levels of NF- $\kappa$ B compared to normal and dysplastic tissue and was associated with higher invasive behavior of the tumor (Nakayama 2001)

#### **1.4 Bone invasion by OSCC**

As tumor cells grow and mitosis increases, they invade the basement membrane, destroy the surrounding tissue locoregionally, resist the immune system, and secrete certain proteins and angiogenic factors that will facilitate lymphovascular invasion and metastasize regionally or distantly (Fonseca 2009).

OSCC tends to invade the adjacent bone due to its close anatomical proximity, so higher bone invasion will occur in the OSCC that lies in direct contact with the bone. In OSCC with deeper invasion into soft tissue, bone invasion will follow the least resistant route. Consequently, in the dentate patient, OSCC will invade bone through the attached gingiva, while in the edentulous patient, the tumor often invades the bone through the area that lacks or has a very thin cortical bone, which is usually the occlusal surface (Brown 2002). The size and proximity of the primary tumor to the jaw bone will determine the degree of bone invasion (Slootweg and Mueller 1989; Mueller and Slootweg 1990; Tsue 1994). Several studies showed that OSCC bone invasion will not necessarily predict poor survival rate and outcome (Dubner 1993, O'Brien 1986, Ash 2000, and Werning 2001). However, at least one study has found that OSCC bone invasion is a poor prognostic factor associated with a decreased survival rate in that the 5-year survival rate was 25% for patients with bone invasion, but 53% for those without such invasion (Jones 1997).

Prognosis is affected by the pattern of bone invasion, which could be either an erosive or infiltrative as described by Slootweg and Muller (1989). In the infiltrative pattern, OSCC invades the medullary spaces and forms multiple islands of cells without a separating layer of connective tissue and has a few active

osteoclasts, while in the erosive pattern, the tumor invades/erodes the cortical layer of bone in a broad form, with a connective tissue layer and active osteoclasts isolating the tumor from bone (Shaw 2004). Shaw also believed that soft tissue invasion is the most important factor in tumor prognosis even in the presence of bone invasion, and that tumor size, soft tissue invasive pattern, positive neck, extracapsular nodal spread, perineural invasion, depth of invasion, and involved margins demonstrate highly prognostic factors with local recurrence and disease-specific survival. Shaw concluded that infiltrative bone invasion is a sign of aggressive tumor behavior and is highly predictive of recurrence and survival. Similarly, Wong et al. (2000) found that the infiltrative pattern of bone invasion is associated with a higher recurrence rate of about 53% compared with the erosive pattern, which was about 17%. They recommended that pathologists should routinely comment on the bone invasion pattern of the tumor because this could be valuable prognostic information.

Presence of OSCC bone invasion is a major consideration in the treatment plan, and often complicates the patient's quality of life esthetically and functionally. Historically and prior to the introduction of Vascularised Free Fibula Bone Graft (VFFBG), segmental bony resection were left unreconstructed and resulted in major cosmetic and functional deformity, which often resulted in dental malocclusion, mandibular swing, temporomandibular joint disorders, and a fluid to soft type of diet. Patients might be lifelong tracheostomy dependent to relieve their upper airway obstruction and may require a feeding tube for nutrition. All of these postoperative morbidities have prompted many surgeons to consider a marginal mandibulectomy in cases in which the medullary canal was free from gross

invasion. Unlike the segmental mandibulectomy, a marginal resection preserves the inferior mandibular border and its architecture and some of its muscular attachments, resulting in less functional and cosmetic deformity. On the other hand, the VFFBG is considered as a first line of treatment after segmental mandibulectomy due to composite reconstruction of both hard and soft tissue defects. However, the procedure has an extended operative time and some postoperative morbidity, which raises the debate regarding the indications for a segmental versus a marginal resection (Genden 2005).

Namaki (2004) assessed the functional outcome after different types of surgical techniques used for oral cancer treatment objectively and subjectively using the adenosine triphosphate (ATP) absorption method and a questionnaire, respectively. The study included three treatment groups (a glossectomy group, a marginal mandibulectomy group, and a segmental mandibulectomy group) and two controls (a group with a complete set of natural teeth and a unilateral occlusion group). Twelve months postoperatively, the masticatory efficiency was higher (but not significantly), and self-assessed eating ability was significantly higher in the marginal mandibulectomy group than in the segmental mandibulectomy group. The researchers concluded that segmental mandibulectomy will limit the patient's diet to non-chewing type, which compromises the patient's quality of life more than the marginal mandibulectomy.

Petruzzelli et al. (2003) studied 152 patients retrospectively who had either segmental resection, marginal resection, or mandibulotomy between 1996 and 2002. Functional outcomes were assessed using the University of Washington Quality of Life instrument at 12 months. All patients had difficulty in chewing, but

they maintained a reasonable oral intake and sufficient nutritional status, and none required a feeding tube. The researchers concluded that posterior mandibular ostectomy did not affect the patient's esthetic significantly and that the patients were still able to maintain adequate oral intake.

## **1.5 Bone Microenvironment**

### **1.5.1 Osteoclasts**

Osteoclasts are large multinucleated short-lived cells that originate in the bone marrow and derive from hematopoietic stem cells via monocytes. Their main function is to resorb bone and maintain calcium balance (Ganong 2001). These cells resorb bone by forming a sealing zone and acidifying the area adjacent to the bone to pH of about 4, which dissolves the hydroxyapatite component of the bone. In addition, the acid proteases secreted by the osteoclasts break down collagen fibers, forming a shallow depression in the bone (Ganong 2001). Osteoclasts are terminally differentiated giant cells. In the absence of supporting factors such as M-CSF, IL-1, RANKL or TNF- $\alpha$ , they undergo rapid apoptosis (Miyazaki 2000).

Osteoclasts play a major role in bone remodelling and maintaining the balance between bone formation and resorption. This balance is well controlled in humans below the age of 50 years, but after that age, bone mass declines at an annual rate of approximately 1%. This loss of bone mass is exaggerated in women after menopause due to hormone deficiencies (Suda 2008).

Characteristic features of osteoclasts (Suda 2008) are:

- 1) multinucleated;
- 2) pleomorphic mitochondria;

- 3) cytoplasm rich in vacuoles that contain acid phosphatase;
- 4) extensive Golgi complex; and
- 5) special cell membrane surface with ruffled borders to increase the surface area in contact with bone.

Osteoclasts stain positive for tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor, and form resorption pits on bone and dentine slices (Suda 2008).

Osteoclasts seem not to work independently. They may play a role in osteoclastic bone resorption. Rodan and Martin (1981) demonstrated that parathyroid hormone (PTH) and some cytokines like interleukin 1 (IL-1) and 1 alpha, 25-dihydroxyvitamin D<sub>3</sub> ( $1\alpha,25(\text{OH})_2\text{D}_3$ ) have receptors in osteoblasts. McSheehy (1986) showed that PTH did not cause bone resorption in the presence of either osteoclasts or osteoblasts alone, but it resorbs bone when osteoblasts and osteoclasts are present together. He proposed that an osteoclast-activating factor (OAF) is produced by osteoblasts in response to bone-resorbing hormones that stimulate osteoclasts activation.

### **1.5.2 OPG/RANK/RANKL and their Roles in Bone Homeostasis**

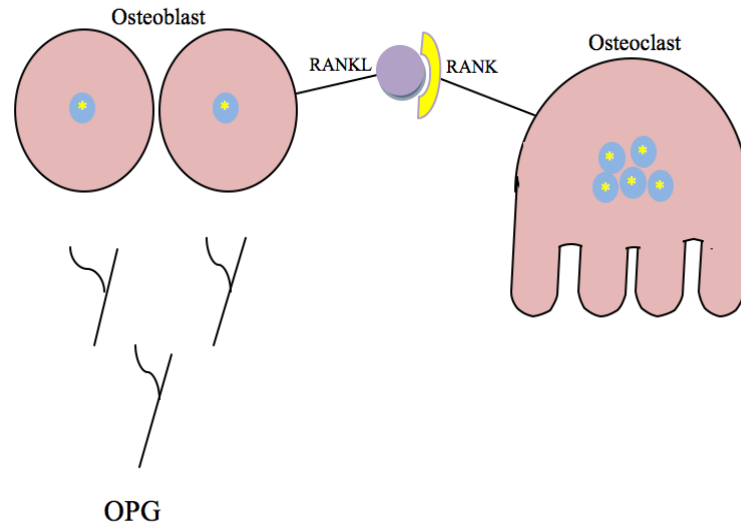
RANKL is a membrane factor that stimulates osteoclasts and is produced by osteoblasts. RANK is the RANKL receptor present on osteoclasts, and OPG is the bone protector protein that is a soluble decoy receptor also produced by osteoblasts. It binds to RANKL and prevents it from binding to its normal receptor RANK (Suda 2008).

The RANKL/RANK signaling pathway activates osteoclastic bone resorption and regulates osteoclast differentiation from osteoclast precursors together with macrophage colony-stimulating factor (M-CSF), which is an important factor in osteoclasts function (Yasuda 1998; Lacey 1998).

#### **1.5.2.1 OPG**

Osteoprotegerin (OPG; called osteoclastogenesis inhibitory factor (OCIF)), a protector of the bone, was the first of three bone regulators discovered. It inhibits osteoclastogenesis in vitro and in vivo. It is a member of the tumor necrosis factor (TNF) receptor superfamily. It lacks a transmembrane domain and is secreted by osteoblasts as a soluble protein (Suda 2008). “This secretory protein contained four cysteine-rich domains and two death domain homologous regions, which mediate apoptotic signals” (Suda 2008). OPG was first discovered as an osteoclasts inhibitor (Tsuda 1997; Simonet 1997). Transgenic mice over-expressing OPG and mice treated with recombinant OPG both had increases in bone density and osteopetrosis (Simonet 1997). In contrast, deletion of OPG in mice resulted in osteoporosis (Bucay 1998; Mizuno 1998). At the same time and independently, Tsuda isolated a protein called osteoclastogenesis inhibitory factor (OCIF) as a heparin-binding glycoprotein from lung fibroblasts, which was identical to that of OPG (Tsuda 1997).

Figure1 : Osteoblast role in osteoclast activation



### **1.5.2.2 RANKL**

RANKL is formed as a transmembrane molecule that is released from the cell membrane after proteolysis by the metalloprotease desintegrin TNF- $\alpha$  convertase, and plays an important role in osteoclasts survival and activation (Lum 1999). RANKL is highly expressed on osteoblasts and undifferentiated mesenchymal cells (Suda 2008). This osteoclast-stimulating factor, which is inhibited by OPG, was identified as RANKL by Lacey (1998) and Yasuda (1998), with the following results:

- 1) RANKL specifically binds to OPG;
- 2) RANKL supports the differentiation of undifferentiated mesenchymal cells into osteoclasts in vitro, and that can be inhibited by OPG; and
- 3) RANKL activates mature osteoclasts to resorb formed bone in vitro and in vivo.

Various hormones and cytokines, such as PTHrP, Vit D3, IL-1 $\beta$ , and TNF- $\alpha$ , are all essential for normal osteoclastogenesis; however, only RANKL has proven to be a critical factor for osteoclast function in vivo, as shown by the complete absence of osteoclasts in RANKL and RANK knockout mice (Dougall 1999). Knocking out the RANKL gene in animal models resulted in severe osteopetrosis (Lum 1999). These animals lack osteoclastic activity, which can be reversed after the reimplantation of the RANKL gene into bone marrow progenitor cells (Li 2000).

### **1.5.2.3 RANK**

The receptor activator of nuclear factor kappa B (RANK) is a member of the TNF receptor superfamily expressed on the surface of osteoclasts, chondrocytes, and epithelial mammary gland cells. Mice that lack either RANKL or RANK during pregnancy fail to develop mammary glands, which results in the death of their newborns (Fata 2000). Binding RANK with RANKL is an essential step for osteoclasts development and activation (Hsu 1999). When RANK was deleted from the mice germline, they become severely osteopetrotic, had multiple impacted teeth, and lacked all osteoclastogenic activity (Li 2000). These experimental findings have established the critical role of RANKL-RANK interactions in bone resorption, balanced by OPG, which functions as a bone protector and opposes RANKL action.

## **1.6 Interactions of Cancer Cells with Bone Microenvironment**

Bone destruction associated with cancer invasion manifested by either local or distant metastasis has been shown to be mediated by osteoclasts rather

than directly by cancer cells. Thus, bone invasion by tumor cells could involve a mechanism by which these cells acquire the ability to promote differentiation and activation of osteoclasts (Hiraga 1998).

The exact mechanism of bone invasion and the interaction between OSCC cells and osteoclasts is not fully understood. The initial process of bone invasion, which is unique for malignant cells, start with margination and migration of OSCC cells. This is an active process involving release of proteolytic enzymes to degrade the underlying basement membrane, which is mainly made up of collagen (Erdem 2007). Some OSCC cell lines express collagen degrading enzymes, such as MMP-1, MMP-2, MMP-7, MMP-9, and urokinase plasminogen activator (Kawamata 1997). Enzyme activity depends on the presence of growth factors, especially EGF, and absence of these enzymes showed no bone invasion (Ziober 2000). Expression of EGFR and MMP-3 were correlated with advanced tumor stage and bone invasion (Kusukawa 1996). Cathepsins are collagen-degrading enzymes that are also involved in OSCC bone invasion and metastasis (Erdem 2007).

To invade the bone, tumor cells need to recruit osteoclasts to degrade bone; Tada et al. (2005) have shown that OSCC induce osteoclastogenesis by suppressing the expression of OPG in host cells. It has been reported that BHY cells, a human OSCC cell line, are capable of invading the mandibular bone of nude mice (Kawamata 1997). When BHY cells were cocultured with mouse bone marrow cells, few osteoclasts were formed. When BHY cells were co-cultured with mouse primary osteoblasts (pOBs) and bone marrow cells (BMCs), however, marked osteoclastogenesis was induced in the absence of osteotropic factors. In addition, osteoclastogenesis was significantly, but not completely, inhibited by

adding OPG (Tada 2005). In another study by Tada et al. (2009), both BHY cells and BHY-conditioned media (BHY-CM) supported osteoclasts survival by suppressing the expression of Bim, a pro-apoptotic protein. BHY-CM-induced survival of osteoclasts was shown to depend on the ERK, not the NF- $\kappa$ B pathway (Tada 2009).

Okamoto et al. (2000) demonstrated that BHY-CM induces osteoclastic bone resorption as inhibited by anti-IL-6 antibody, and that IL-6 plays an important role in OSCC bone invasion. In 2005, Shibahara et al. carried out an immunohistological analysis using specimens obtained from mandibular gingival oral squamous cell carcinoma following resection. The results of this analysis showed that the presence of the osteoclastic activation factors interleukin-6 (IL-6), interleukin-11 (IL-11), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and parathyroid hormone-related protein (PTHrP) are strongly involved in this process.

Komarova et al. have examined the effect of MDA-MB-231 human breast cancer cells on osteoclast formation from human and mouse osteoclast precursors (Guo 2008; Tiedemann 2009). The researchers found that factors produced by breast cancer cells did not induce osteoclasts formation. However, when these co-cultures were treated with RANKL for 1-3 days, marked osteoclastogenesis was induced in the absence of supporting cell types. They also found that when osteoclasts are exposed to the soluble factors released by cancer cells, they will secrete enzymes that are critical for bone resorption such as cathepsin K and matrix metalloproteinase 9. This effect of breast cancer factors on osteoclasts was not completely inhibited by the addition of OPG, suggesting that carcinoma cells may produce additional factors that are capable of osteoclasts stimulation. It was

concluded that during differentiation, osteoclast precursors acquired the competency to respond to factors secreted by breast cancer cells, which may serve to promote tumor growth at skeletal sites undergoing active bone turnover.

The objective of this study was to test if the ability to induce osteoclast differentiation from RANKL-primed precursors may be common to other cancer types that can invade bone tissue, such as OSCC.

## **2 MATERIALS AND METHODS**

### **2.1 Materials**

Culture media consisted of DMEM; we added 1.5 g/L sodium bicarbonate, 4.5 g/L glucose with glutamine (Wisent Inc. Cat No 319-020-CL), 1% sodium pyruvate (Wisent Inc. Cat No 600-110-EL), bactericidal agent 1% penicillin-streptomycin (Wisent Inc. Cat No 450-201-EL) and 10% fetal bovine serum supplement (Hyclone, SH 30396-03). For inhibitors experiments, various pharmacological inhibitors were used to pretreat the cells for 30–60 minutes. The pharmacological inhibitors were obtained from Calbiochem: MEK inhibitor PD98059 (100  $\mu$ M, Cat No 513001), PKC inhibitor GÖ6976 (1  $\mu$ M, Cat No 365250), p38 inhibitor (1  $\mu$ M, SB203580, Sulfone; Cat No 559399) or the inactive analogue of p38 (1  $\mu$ M, SB202474, Cat No 559387) or from Invitrogen: Rapamycin (100 nM, Cat No PHZ1233), AKT inhibitor (5  $\mu$ M, Cat No AHO1112), and PI3K inhibitor (10  $\mu$ M, Cat No PV5374). Then, the medium was replaced with fresh medium containing 50% BHY-CM with inhibitors at the same level as during pre-treatment (Guo 2008 and Tiedemann 2009).

### **2.2 OSCC human cell-lines**

The BHY and HN human SCC cell lines were obtained from the German Collection of Microorganisms and Cell Cultures: DSMZ no. ACC 404 and ACC 417, respectively. BHY is a human OSCC cell line, which was obtained from a well-differentiated OSCC of the lower alveolar bone of a 52-year-old Japanese

man. The tumor was invasive to the mandibular bone and sublingual muscles, but it did not metastasise to any distant organ; moreover, it was shown to be tumorigenic in nude mice when introduced into their masseter muscles (Kawamata 1997). HN is a human OSCC cell line that was obtained from a moderately differentiated OSCC of the soft palate of a 60-year-old Japanese man; the tumor was invasive to the muscles but not to the bone. Moreover, it had affinity for local and distant metastasis. HN cell-line was obtained from a cervical lymph node metastasis; the patient had multiple distant metastases several years after treatment of the primary tumor to the lungs and brain. It was also shown to be tumorigenic in nude mice when introduced into their masseter muscles (Kawamata 1997).

### **2.3 BHY and HN cultures**

BHY and HN cells were cultured as previously described and passaged every 3-5 days, when they reached approximately 90% confluence (Okamoto 2000, Tada 2005, and Erdem 2007). BHY and HN cells were plated in T-75 tissue culture flasks and cultured in the incubation medium, which was collected every 24 hours. The degree of cell confluence was recorded under the microscope. Conditioned medium was filtered through 0.22- $\mu$ m disposable filters, centrifuged at 1200 RPM for 4 minutes, divided, and frozen at  $-80^{\circ}\text{C}$ .

### **2.4 Osteoclast culture**

The RAW 264.7 mouse monocytic cell line (American Type Culture Collection) was cultured according to provider's specifications. As described previously (Guo 2008), RAW 264.7 cells were plated at a density of  $4 \times 10^3$  cells/cm<sup>2</sup>; 24 h later (day 1) recombinant GST-RANKL (50 ng/ml) was added. On

day 3–5, cultures were provided with fresh medium containing indicated additions; on day 5–7 samples were fixed with 4% paraformaldehyde (10 min), washed with phosphate buffered saline (PBS), and stained for tartrate-resistant acid phosphatase (TRAP) (Sigma, Cat No 387A).

## **2.5 Osteoclast identification and quantification**

TRAP positive multinucleated cells ( $\geq 3$  nuclei) were recognized and counted as osteoclasts; the surface area of osteoclasts as well as images were analyzed and taken by a digital camera linked to a PixeLINK Capture SE® program (PixeLINK, Ottawa).

## **2.6 RNA isolation and real time PCR**

For gene expression studies, RNA was isolated from: 1) RAW 264.7 cells (never exposed to RANKL); 2) RAW 264.7 cells primed with RANKL (50 ng/ml) for 2 days; 3) Osteoclasts derived from RAW 264.7 cells; 4) RANKL-primed RAW 264.7 cells treated with BHY-CM 50%; 5) RANKL-primed RAW 264.7 cells treated with HN-CM 50% using the RNeasy mini kit (Qiagen Sciences, Maryland, USA; Cat. 3 74106) and QIAshredder columns according to the manufacturer's protocol. In brief, cells were collected by cell scraper in RLT buffer and homogenized by centrifugation through a QIAshredder spin column. Homogenized lysate was mixed with equal volume of 70% ethanol and washed with RW1 then with RPE buffers through the RNeasy spin column. RNA was eluted from the column by adding nuclease-free water and spinning twice. RNA and DNA were quantified using the Quant-iT™ dsDNA BR and RNA assays with

the Qubit™ flurometer according to invitrogen Cat No Q32862 and Q32852, respectively.

Reverse transcription of the total RNA to generate single-stranded cDNA suitable for quantitative PCR applications and long-term storage were done according to Applied Biosystems High-Capacity cDNA Reverse Transcription kit (cat No 4368813 and 4368814). In brief, equal amounts of RNA and 2X RT master mix were incubated at 25 °C for 10 minutes, at 37 °C for 2 h, and the resulting cDNA was stored at -20 °C. The double-strength master mix consisted of Multiscribe reverse transcriptase enzyme, random primers, and dNTP mix in RT buffer.

Real-time PCR was performed using 7500 Applied Biosystems instrument, and the TaqMan gene expression assays for the following osteoclastic markers: NFATc1 (TaqMan Mm00479445-m1), RANK (Mm00437134-m1), Calcitonin receptor (Mm00432271-m1) and TRAP (Mm00475698-m1). Actin B was used as a housekeeping gene (Mm00607939-s1).

## **2.7 Statistics**

Data are presented as means  $\pm$  standard error of the mean for RAW264.7 cells with sample size (n) indicating the number of independent experiments. Differences were assessed by Student t-test, and accepted as statistically significant at  $P < 0.05$ .

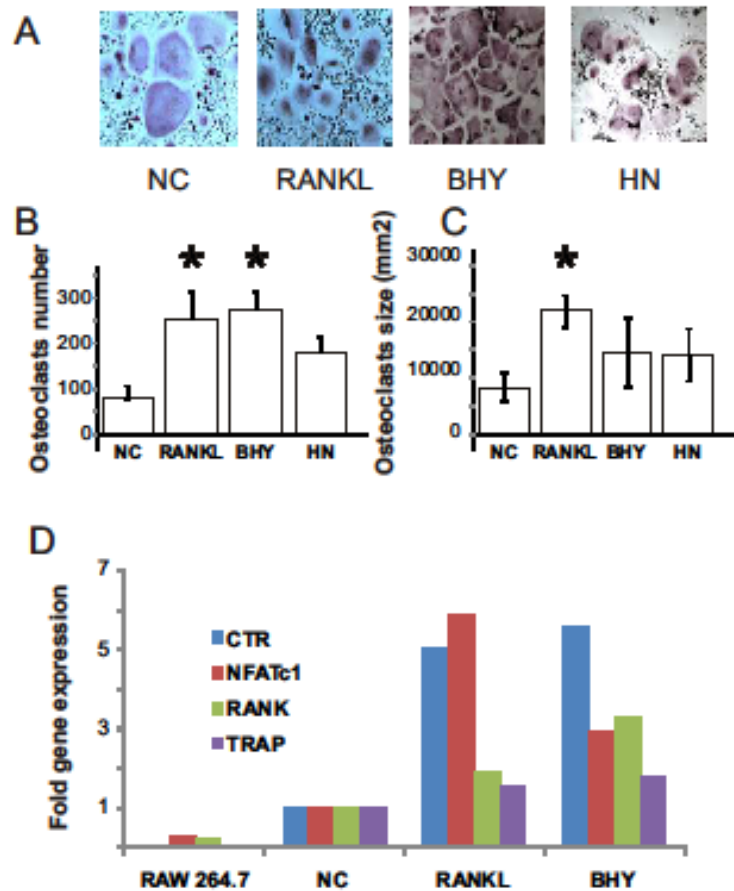
### **3 RESULTS**

#### **3.1 Soluble factors produced by squamous cancer cells stimulate osteoclast formation from RANKL-primed precursors**

To evaluate the effects of soluble factors released by BHY & HN SCC cell-lines on RANKL-primed osteoclast precursors, we treated RAW 264.7 cells with RANKL (50 ng/ml) for 2 days. The parallel samples were then divided into 4 groups and treated for additional 2 days as follows: negative control that was not treated further, positive control with continued treatment with RANKL, treated with BHY-CM (50%), and treated with HN-CM (50%) (Fig. 2). At the end of culture period, the samples were fixed, stained for TRAP and the number of multinucleated TRAP-positive osteoclasts were assessed. In the negative control group an average of  $81 \pm 31$  osteoclasts/cm<sup>2</sup> were formed; we found that adding BHY-CM stimulated osteoclast formation ( $286 \pm 40$  osteoclasts/cm<sup>2</sup>) to the same extent as in positive control, RANKL-treated cultures ( $267 \pm 60$  osteoclasts/cm<sup>2</sup>). In contrast, the effect of HN-CM on osteoclast formation was less evident and did not reach statistical significance, resulting in an average of  $188 \pm 36$  osteoclasts/cm<sup>2</sup> (Fig. 2B). It was previously shown that breast cancer-derived factors also significantly increase osteoclast size (Tiedemann 2009). In contrast, in BHY and HN CM-treated cultures, osteoclast size (estimated as cell planar area) did not significantly differ from the negative control  $P=0.4$  and  $0.31$ , respectively (Fig. 2C). We assessed the effect of BHY-CM on the expression of osteoclast

marker genes, calcitonin receptor (CTR), osteoclastogenic transcription factor NFATc1, RANK and TRAP. We found that BHY-CM induced osteoclastic genes as effectively as RANKL (Fig. 2D).

Figure2 : Effect of OSCC-derived factors on osteoclast formation



RAW 264.7 cells were cultured in the presence of RANKL for two days, then fresh medium alone (NC), fresh medium with RANKL, or with 50% of conditioned medium from BHY or HN culture was added for another two days.

A: Morphology of osteoclasts in different conditions.

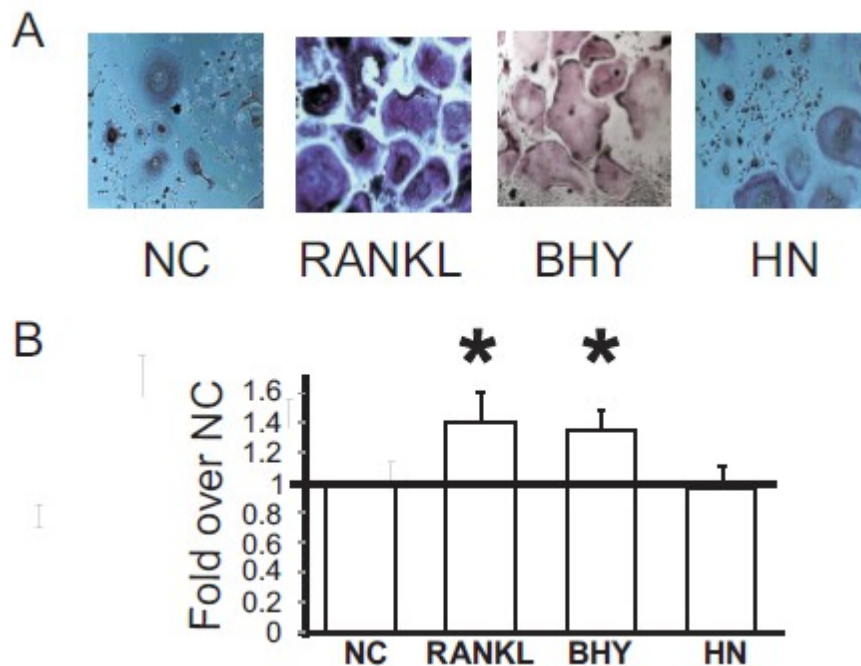
B: Osteoclast count normalized to NC. Data are means  $\pm$  SEM, n=5 independent experiments, statistical difference assessed using t-test, \*p<0.05.

C: Relative gene expression from cell lysate of a representative experiment normalized to negative control (NC).

### 3.2 Soluble Factors Released by BHY and HN cell lines Increase Osteoclasts Survival

To evaluate the effects of BHY and HN CM on osteoclast survival, we treated RAW 264.7 cells with RANKL (50 ng/ml) for 4 days, when mature osteoclasts are abundant. The parallel samples were then divided into 4 groups and treated for additional 2 days as follows: negative control that was not treated further, positive control with continued treatment with RANKL, treated with BHY-CM (50%), and treated with HN-CM (50%) (Fig. 3). We found that BHY-CM, similar to RANKL, promoted survival of mature osteoclasts. In contrast, HN-CM did not stimulate the survival of osteoclasts (Fig. 3B).

Figure3 : Effect of OSCC-derived factors on osteoclast survival



RAW 264.7 cells were cultured in the presence of RANKL for four days, then fresh medium alone (NC), fresh medium with RANKL, or with 50% of conditioned medium from BHY or HN culture was added for another two days.

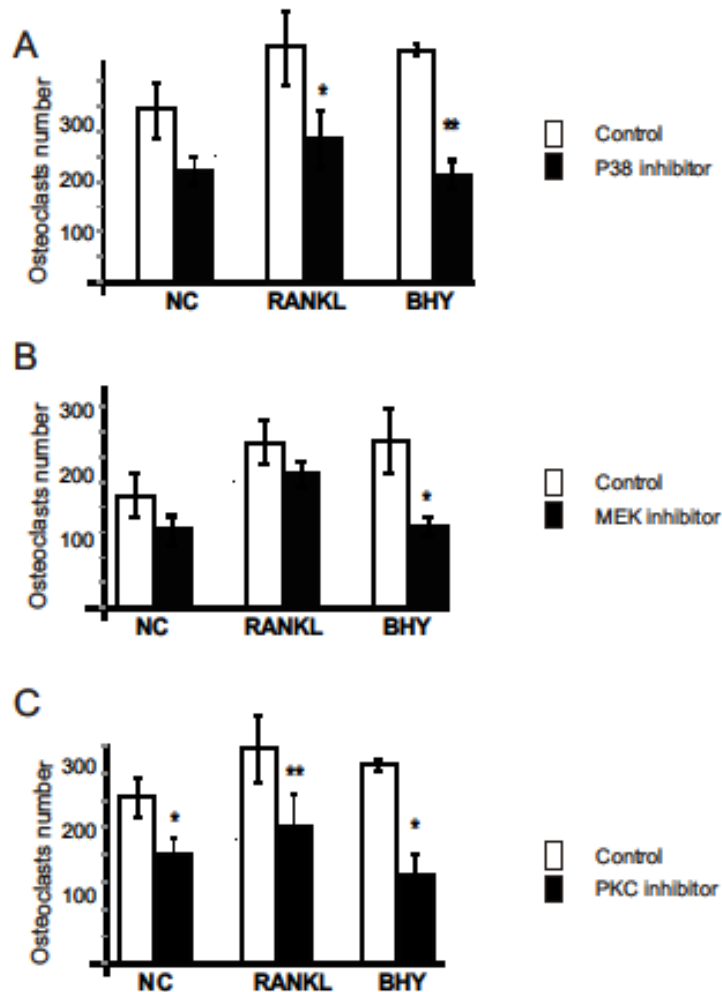
A: Morphology of osteoclasts in different conditions.

B: Osteoclast count normalized to NC. Data are means  $\pm$  SEM, n=5 independent experiments, statistical difference assessed using t-test, \*p<0.05.

### **3.3 Mechanism of Osteoclastogenic Effect of OSCC-Derived Factors**

It was previously shown that breast cancer cells stimulate osteoclast formation from RANKL-primed precursors through activation of PKC, ERK1/2 and p38 (Tiedemann 2009). To explore if ERK1/2, p38 and PKC mediate BHY-induced osteoclastogenesis, we used the following pharmacological inhibitors: MEK/ERK inhibitor PD98059 (100  $\mu$ M), PKC inhibitor GÖ6976 (1  $\mu$ M) and p38 inhibitor SB203580 (1  $\mu$ M) or the inactive analogue of p38 inhibitor SB202474 (1  $\mu$ M) (Fig. 4). We found that inhibition of p38 and PKC attenuated osteoclast formation in negative controls as well as RANKL- and BHY-CM-treated cultures. In contrast, the effect of MEK1/2 inhibitor was observed only in BHY-CM treated cultures, thus suggesting that activation of ERK is specific to OSCC-induced osteoclastogenesis (Fig. 4B).

Figure4 : Role of MAPK pathways on osteoclastogenic effects on OSCC-derived factors



RANKL-primed RAW 264.7 cells were cultured for 2 days untreated (NC), in the presence of RANKL (50 ng/ml, PC) or in the presence of BHY-CM (50%) combined with the following inhibitors:

A: p38 inhibitor, SB203580 (1  $\mu$ M) or inactive analog of p38 inhibitor SB202474 (1  $\mu$ M)

B: MEK1/2 inhibitor, PD98059 (100 $\mu$ M) or vehicle

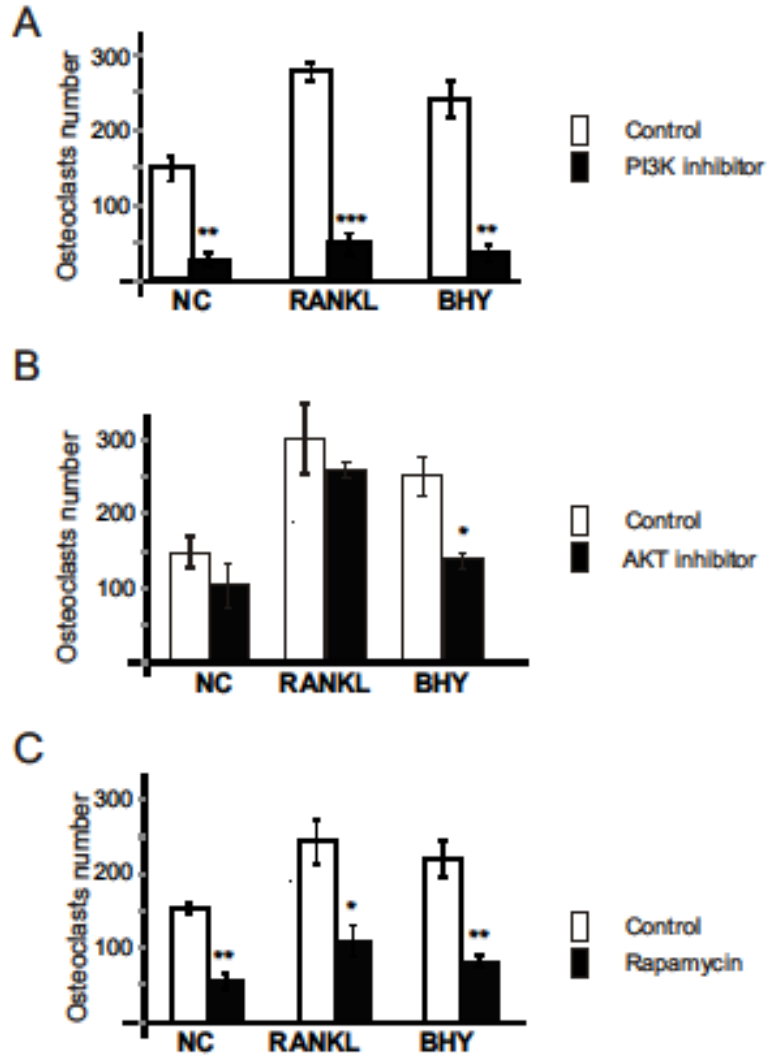
C: PKC inhibitor Go6976 (1  $\mu$ M)

The parallel samples were fixed, stained for TRAP, and average number of osteoclasts were analyzed, Data are means $\pm$ SEM, n=3 independent experiments, statistical difference assessed using t-test, \*p<0.05, \*\*p<0.01.

We have also considered whether PI3K/AKT/mTOR pathway may be involved in mediating osteoclastogenic effects of OSCC-derived factors. We have used the pharmacological inhibitors of PI3K (10  $\mu$ M), AKT (5  $\mu$ M) or mTOR inhibitor rapamycin (100 nM) (Fig. 5). We found that inhibition of PI3K almost completely prevents osteoclastogenesis. Inhibition of mTOR with rapamycin attenuated osteoclast in negative control, as well as RANKL- and BHY CM-treated cultures. However, inhibition of AKT specifically decreased osteoclast formation in BHY CM-treated cultures only (Fig. 5B).

Thus, we found that soluble factors produced by bone-invasive OSCC stimulate osteoclast formation both through activation of general osteoclastogenic pathways similar to those induced by RANKL, and through activation of ERK and AKT, which are not involved in RANKL-induced osteoclasts formation.

Figure5 : Role of PI3K/AKT/mTOR pathway on osteoclastogenic effects on OSCC-derived factors



RANKL-primed RAW 264.7 cells were cultured for 2 days untreated (NC), in the presence of RANKL (50 ng/ml, PC) or in the presence of BHY-CM (50%) combined with the following inhibitors:

A: PI3K inhibitor (10 μM), or vehicle

B: AKT inhibitor (5 μM), or vehicle

C: mTOR inhibitor rapamycin (100 nM),

The parallel samples were fixed, stained for TRAP, and average number of osteoclasts were analyzed, Data are means±SEM, n=3 independent experiments, statistical difference assessed using t-test, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

## **4 DISCUSSION AND CONCLUSION**

### **4.1 Osteoclastogenic effects of bone-invasive OSCC**

Bone invasion in malignancies have been shown to be mediated by osteoclasts rather than directly by carcinoma cells (Hiraga 1998). Therefore, tumor cells must establish productive interactions with osteoclasts. Both breast cancer cells and oral squamous carcinoma cells were previously shown to affect RANKL/OPG expression on supporting cells, and thus induce osteoclast formation indirectly (Park 2003 and Tada 2005). In addition, it was previously shown that breast cancer cells can also induce osteoclast formation directly from RANKL-primed osteoclast precursors (Guo 2008 and Tiedemann 2009). We found that similar to breast cancer cells, OSCC produce soluble factors, which can directly induce osteoclast formation from RANKL-primed precursors. Thus, the receptiveness of osteoclast precursors to signals produced by tumor cells can be altered by short exposure to RANKL, resulting in stimulation of osteoclastogenesis in the absence of supporting cell types.

In addition to the induction of osteoclast formation, we found that soluble factors produced by OSCC also increase the survival rate of mature osteoclasts. These data are in accordance with the previous study, which demonstrated that both BHY cells and BHY-CM supported the survival of osteoclasts by suppressing Bim expression (Tada 2009). The effects of BHY-CM on osteoclast formation and survival were similar in magnitude to those induced by RANKL. In addition,

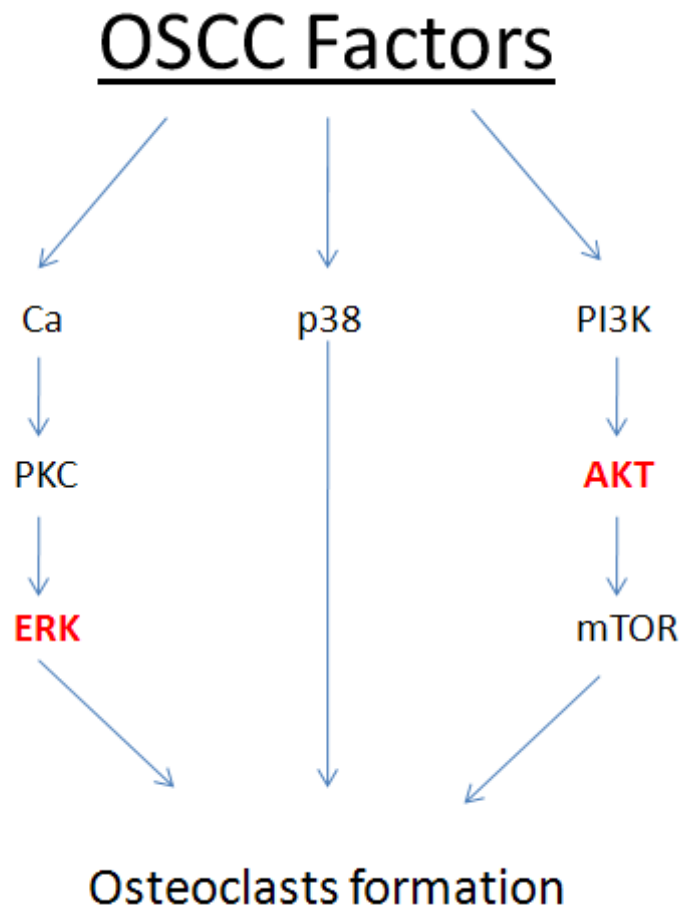
BHY-CM induced the expression of osteoclast markers genes, NFATc1, RANK, Calcitonin receptor and TRAP, to the same extent as RANKL.

Interestingly, bone-invasive OSCC (BHY) demonstrated significantly stronger osteoclastogenic capacity than generally metastatic but not bone-invasive OSCC (HN) with respect to both osteoclast formation and survival. This finding indicates that osteoclastogenic capacity of OSCC likely correlates with their bone-invasive ability. BHY and HN have been shown previously to express different proteases, which possibly confers differential invasiveness to these cell types (Kawamata 1997). However, these cell lines have been shown to exhibit similar migration and invasion *in vitro* (Uchida 2001). Therefore, it is possible that bone invasion is primarily dependent not on the migration or invasion ability of cancer cells themselves, but rather on their ability to induce osteoclast formation and maintain osteoclast survival.

## **4.2 Mechanism of osteoclastogenic action of OSCC**

We have investigated the involvement of several potential pathways in mediating osteoclastogenic effects of OSCC. At this time we cannot comment on the potential factors produced by OSCC or the receptors involved in cancer-induced signalling. Nevertheless, we have identified at least three different pathways stimulated in osteoclast precursors: PKC, MAPK and PI3K/AKT/mTOR (Fig. 6).

Figure6 : Suggested signaling pathways involved in mediating osteoclastogenic effects of OSCC



Calcium signalling is known to play a critical role in osteoclast formation (Takayanagi 2002) and survival (Komarova 2005). Breast cancer-derived factors were also shown to induce calcium oscillation in osteoclasts precursors, which were important for the osteoclastogenic effects (Tiedemann 2009). PKC is a calcium-dependent kinase that has been found to enhance both cancer-induced osteoclasts formation (Tiedemann 2009) and physiological osteoclast survival (Pereverzev 2008). In addition, PKC was implicated in regulating ERK in osteoclast precursors by breast cancer-derived factors (Tiedemann 2009). We

found that osteoclastogenic effects of OSCC-derived factors, similar to the effects of breast-cancer-derived factors, depend on the activation of PKC and ERK in osteoclast precursors. Interestingly, ERK was found to be uniquely involved in osteoclastogenic effects of OSCC but not in physiological RANKL-induced osteoclast formation.

Another member of MAPK signalling proteins, p38, has been shown to play a pivotal role in physiological osteoclast formation (Huang 2006), in inflammation-induced osteoclastogenesis (Mbalaviele 2006), as well as in mediating the breast cancer-induced osteoclast formation (Tiedemann 2009). We found that similar to breast cancer factors, OSCC-derived factors induced osteoclast formation in a p38-dependent manner.

Another pathway important in osteoclastogenesis is the PI3K/AKT/mTOR pathway. PI3K is a key mediator of RANKL-induced osteoclast formation (Saitoh 2007). AKT has been shown to play an important role in osteoclast survival (Kwak 2008). Mammalian target of rapamycin (mTOR) is a fundamental mediator of cell growth and hypertrophy that has been shown to be critical for osteoclast formation and survival downstream of the principal osteoclastogenic factors RANKL and M-CSF (Glantschnig 2003). Inhibition of PI3K completely blocked osteoclastogenesis in all conditions; therefore, it is impossible to comment on the involvement of this kinase in OSCC-induced osteoclast formation. In addition, we found that rapamycin inhibited OSCC- and RANKL- induced osteoclasts formation, indicating general importance of mTOR in osteoclastogenesis. Interestingly, inhibition of AKT specifically interfered with cancer-induced

osteoclast formation, but not with physiological, RANKL-induced osteoclastogenesis.

Thus, our data indicate that the bone-invasive oral carcinoma cell line BHY induced a multi-signalling program in osteoclast precursors that enhances osteoclasts formation and survival acting both through physiological osteoclastogenic program and by inducing cancer-specific signalling that involves activation of ERK and AKT.

### **4.3 Conclusion and Recommendation**

Thus, we demonstrated that bone-invasive squamous cell carcinoma cells produce soluble factors that stimulate osteoclast formation from RANKL-primed precursors in the absence of supporting cell types, acting through both physiological osteoclastogenic pathways and tumor-specific signaling. In the future, this study may help to develop prognostic markers for oral squamous cell carcinoma bone invasiveness, as well as new therapeutic treatments specifically targeting tumor-stimulated pathways in osteoclasts.

## 5 REFERENCES

1. Ash CS, Nason RW, Abdoh AA, Cohen MA. Prognostic implications of mandibular invasion in oral cancer. *Head Neck*. 2000 Dec;22(8):794-8.
2. Bagan JV, Scully C. Recent advances in Oral Oncology 2008; squamous cell carcinoma aetiopathogenesis and experimental studies. *Oral Oncol*. 2009 Jul;45(7):e45-8.
3. Brown JS, Lowe D, Kalavrezos N, D'Souza J, Magennis P, Woolgar J. Patterns of invasion and routes of tumor entry into the mandible by oral squamous cell carcinoma. *Head Neck*. 2002 Apr;24(4):370-83.
4. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, et al. osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev*. 1998 May 1;12(9):1260-8.
5. Canadian Cancer Society., National Cancer Institute of Canada. Advisory Committee on Records and Registries. Canadian cancer statistics. Toronto: Canadian Cancer Society; 2010. p. v
6. Cooper JS, Pajak TF, Forastiere AA, Jacobs J, Campbell BH, Saxman SB, et al. Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. *N Engl J Med*. 2004 May 6;350(19):1937-44.
7. de Bree R, Deurloo EE, Snow GB, Leemans CR. Screening for distant metastases in patients with head and neck cancer. *Laryngoscope*. 2000 Mar;110(3 Pt 1):397-401.

8. Dougall WC, Glaccum M, Charrier K, Rohrbach K, Brasel K, De Smedt T, et al. RANK is essential for osteoclast and lymph node development. *Genes Dev.* 1999 Sep 15;13(18):2412-24.
9. Dubner S, Heller KS. Local control of squamous cell carcinoma following marginal and segmental mandibulectomy. *Head Neck.* 1993 Jan-Feb;15(1):29-32.
10. Erdem NF, Carlson ER, Gerard DA, Ichiki AT. Characterization of 3 oral squamous cell carcinoma cell lines with different invasion and/or metastatic potentials. *J Oral Maxillofac Surg.* 2007 Sep;65(9):1725-33
11. Fata JE, Kong YY, Li J, Sasaki T, Irie-Sasaki J, Moorehead RA, et al. The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell.* 2000 Sep 29;103(1):41-50.
12. Fonseca, Marciani, RD, Turvey, Oral and maxillofacial surgery, 2<sup>nd</sup> ed. St. Louis (MO), Saunders, Elsevier; 2009.
13. Ganong WF, Review of medical physiology, 20<sup>th</sup> ed. New York; McGraw-Hill companies Inc.; 2001.
14. Garg A, Aggarwal BB. Nuclear transcription factor-kappaB as a target for cancer drug development. *Leukemia.* 2002 Jun;16(6):1053-68.
15. Genden EM, Rinaldo A, Shaha AR, Clayman GL, Werner JA, Suarez C, et al. Treatment considerations for head and neck cancer in the elderly. *J Laryngol Otol.* 2005 Mar;119(3):169-74.
16. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. *Cell.* 2002 Apr;109 Suppl:S81-96.

17. Glantschnig H, Fisher JE, Wesolowski G, Rodan GA, Reszka AA. M-CSF, TNFalpha and RANK ligand promote osteoclast survival by signaling through mTOR/S6 kinase. *Cell Death Differ*. 2003 Oct;10(10):1165-77.
18. Grandis JR, Tweardy DJ. Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. *Cancer Res*. 1993 Aug 1;53(15):3579-84.
19. Guo Y, Tiedemann K, Khalil JA, Russo C, Siegel PM, Komarova SV. Osteoclast precursors acquire sensitivity to breast cancer derived factors early in differentiation. *Bone*. 2008 Aug;43(2):386-93.
20. Hiraga T, Tanaka S, Ikegame M, Koizumi M, Iguchi H, Nakajima T, et al. Morphology of bone metastasis. *Eur J Cancer*. 1998 Feb;34(2):230-9.
21. Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, et al. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci U S A*. 1999 Mar 30;96(7):3540-5.
22. Huang H, Chang EJ, Ryu J, Lee ZH, Lee Y, Kim HH. Induction of c-Fos and NFATc1 during RANKL-stimulated osteoclast differentiation is mediated by the p38 signaling pathway. *Biochem Biophys Res Commun*. 2006 Dec 8;351(1):99-105.
23. Jalouli J, Ibrahim SO, Mehrotra R, Jalouli MM, Sapkota D, Larsson PA, et al. Prevalence of viral (HPV, EBV, HSV) infections in oral submucous fibrosis and oral cancer from India. *Acta Otolaryngol*. 2010 Nov;130(11):1306-11.
24. Jefferies S, Foulkes WD. Genetic mechanisms in squamous cell carcinoma of the head and neck. *Oral Oncol*. 2001 Feb;37(2):115-26.

25. Jones AS, England J, Hamilton J, Helliwell TR, Field J, Gerlinger I, et al. Mandibular invasion in patients with oral and oropharyngeal squamous carcinoma. *Clin Otolaryngol Allied Sci.* 1997 Jun;22(3):239-45.
26. Kawamata H, Nakashiro K, Uchida D, Harada K, Yoshida H, Sato M. Possible contribution of active MMP2 to lymph-node metastasis and secreted cathepsin L to bone invasion of newly established human oral-squamous-cancer cell lines. *Int J Cancer.* 1997 Jan 6;70(1):120-7.
27. Komarova SV, Pereverzev A, Shum JW, Sims SM, Dixon SJ. Convergent signaling by acidosis and receptor activator of NF-kappaB ligand (RANKL) on the calcium/calcieneurin/NFAT pathway in osteoclasts. *Proc Natl Acad Sci U S A.* 2005 Feb 15;102(7):2643-8.
28. Kusakawa J, Harada H, Shima I, Sasaguri Y, Kameyama T, Morimatsu M. The significance of epidermal growth factor receptor and matrix metalloproteinase-3 in squamous cell carcinoma of the oral cavity. *Eur J Cancer B Oral Oncol.* 1996 Jul;32B(4):217-21.
29. Kwak HB, Sun HM, Ha H, Lee JH, Kim HN, Lee ZH. AG490, a Jak2-specific inhibitor, induces osteoclast survival by activating the Akt and ERK signaling pathways. *Mol Cells.* 2008 Nov 30;26(5):436-42.
30. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell.* 1998 Apr 17;93(2):165-76.
31. Lee SY, Cho NH, Choi EC, Baek SJ, Kim WS, Shin DH, et al. Relevance of human papilloma virus (HPV) infection to carcinogenesis of oral tongue cancer. *Int J Oral Maxillofac Surg.* 2010 Jul;39(7):678-83.

32. Lewin F, Norell SE, Johansson H, Gustavsson P, Wennerberg J, Biorklund A, et al. Smoking tobacco, oral snuff, and alcohol in the etiology of squamous cell carcinoma of the head and neck: a population-based case-referent study in Sweden. *Cancer*. 1998 Apr 1;82(7):1367-75.
33. Li J, Sarosi I, Yan XQ, Morony S, Capparelli C, Tan HL, et al. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. *Proc Natl Acad Sci U S A*. 2000 Feb 15;97(4):1566-71.
34. Lum L, Wong BR, Josien R, Becherer JD, Erdjument-Bromage H, Schlondorff J, et al. Evidence for a role of a tumor necrosis factor-alpha (TNF-alpha)-converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival. *J Biol Chem*. 1999 May 7;274(19):13613-8.
35. Macfarlane GJ, Zheng T, Marshall JR, Boffetta P, Niu S, Brasure J, et al. Alcohol, tobacco, diet and the risk of oral cancer: a pooled analysis of three case-control studies. *Eur J Cancer B Oral Oncol*. 1995 May;31B(3):181-7.
36. Marx RE, Stern D., *Oral and maxillofacial pathology: a rationale for diagnosis and treatment*. Chicago (IL), Quintessence publishing Co. Inc.; 2003 p. 284-290
37. Mbalaviele G, Anderson G, Jones A, De Ciechi P, Settle S, Mnich S, et al. Inhibition of p38 mitogen-activated protein kinase prevents inflammatory bone destruction. *J Pharmacol Exp Ther*. 2006 Jun;317(3):1044-53.
38. McSheehy PM, Chambers TJ. Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone. *Endocrinology*. 1986 Feb;118(2):824-8.

39. Miyazaki T, Katagiri H, Kanegae Y, Takayanagi H, Sawada Y, Yamamoto A, et al. Reciprocal role of ERK and NF-kappaB pathways in survival and activation of osteoclasts. *J Cell Biol.* 2000 Jan 24;148(2):333-42.
40. Mizuno A, Amizuka N, Irie K, Murakami A, Fujise N, Kanno T, et al. Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. *Biochem Biophys Res Commun.* 1998 Jun 29;247(3):610-5.
41. Moreno-Lopez LA, Esparza-Gomez GC, Gonzalez-Navarro A, Cerero-Lapiedra R, Gonzalez-Hernandez MJ, Dominguez-Rojas V. Risk of oral cancer associated with tobacco smoking, alcohol consumption and oral hygiene: a case-control study in Madrid, Spain. *Oral Oncol.* 2000 Mar;36(2):170-4.
42. Muller H, Slootweg PJ. Mandibular invasion by oral squamous cell carcinoma. Clinical aspects. *J Craniomaxillofac Surg.* 1990 Feb;18(2):80-4.
43. Nakayama H, Ikebe T, Beppu M, Shirasuna K. High expression levels of nuclear factor kappaB, IkappaB kinase alpha and Akt kinase in squamous cell carcinoma of the oral cavity. *Cancer.* 2001 Dec 15;92(12):3037-44.
44. Namaki S, Matsumoto M, Ohba H, Tanaka H, Koshikawa N, Shinohara M. Masticatory efficiency before and after surgery in oral cancer patients: comparative study of glossectomy, marginal mandibulectomy and segmental mandibulectomy. *J Oral Sci.* 2004 Jun;46(2):113-7.
45. O'Brien CJ, Carter RL, Soo KC, Barr LC, Hamlyn PJ, Shaw HJ. Invasion of the mandible by squamous carcinomas of the oral cavity and oropharynx. *Head Neck Surg.* 1986 Mar-Apr;8(4):247-56.

46. Okamoto M, Hiura K, Ohe G, Ohba Y, Terai K, Oshikawa T, et al. Mechanism for bone invasion of oral cancer cells mediated by interleukin-6 in vitro and in vivo. *Cancer*. 2000 Nov 1;89(9):1966-75
47. P Oc, Modjtahedi H, Rhys-Evans P, Court WJ, Box GM, Eccles SA. Epidermal growth factor-like ligands differentially up-regulate matrix metalloproteinase 9 in head and neck squamous carcinoma cells. *Cancer Res*. 2000 Feb 15;60(4):1121-8.
48. Park HR, Min SK, Cho HD, Kim DH, Shin HS, Park YE. Expression of osteoprotegerin and RANK ligand in breast cancer bone metastasis. *J Korean Med Sci*. 2003 Aug;18(4):541-6.
49. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin*. 2005 Mar-Apr;55(2):74-108.
50. Pereverzev A, Komarova SV, Korcok J, Armstrong S, Tremblay GB, Dixon SJ, et al. Extracellular acidification enhances osteoclast survival through an NFAT-independent, protein kinase C-dependent pathway. *Bone*. 2008 Jan;42(1):150-61.
51. Petruzzelli GJ, Knight FK, Vandevender D, Clark JI, Emami B. Posterior marginal mandibulectomy in the management of cancer of the oral cavity and oropharynx. *Otolaryngol Head Neck Surg*. 2003 Dec;129(6):713-9.
52. Pintos J, Black MJ, Sadeghi N, Ghadirian P, Zeitouni AG, Viscidi RP, et al. Human papillomavirus infection and oral cancer: a case-control study in Montreal, Canada. *Oral Oncol*. 2008 Mar;44(3):242-50.
53. Riedel F, Goessler U, Hormann K. Alcohol-related diseases of the mouth and throat. *Best Pract Res Clin Gastroenterol*. 2003 Aug;17(4):543-55.

54. Rodan GA, Martin TJ. Role of osteoblasts in hormonal control of bone resorption--a hypothesis. *Calcif Tissue Int.* 1981;33(4):349-51
55. Saitoh Y, Koizumi K, Sakurai H, Minami T, Saiki I. RANKL-induced down-regulation of CX3CR1 via PI3K/Akt signaling pathway suppresses Fractalkine/CX3CL1-induced cellular responses in RAW264.7 cells. *Biochem Biophys Res Commun.* 2007 Dec 21;364(3):417-22.
56. Shaw RJ, Brown JS, Woolgar JA, Lowe D, Rogers SN, Vaughan ED. The influence of the pattern of mandibular invasion on recurrence and survival in oral squamous cell carcinoma. *Head Neck.* 2004 Oct;26(10):861-9
57. Shibahara T, Nomura T, Cui NH, Noma H. A study of osteoclast-related cytokines in mandibular invasion by squamous cell carcinoma. *Int J Oral Maxillofac Surg.* 2005 Oct;34(7):789-93.
58. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell.* 1997 Apr 18;89(2):309-19.
59. Sloopweg PJ, Muller H. Mandibular invasion by oral squamous cell carcinoma. *J Craniomaxillofac Surg.* 1989 Feb;17(2):69-74.
60. Suda T, Takahashi N. Contributions to osteoclast biology from Japan. *Proc Jpn Acad Ser B Phys Biol Sci.* 2008;84(10):419-38.
61. Tada T, Jimi E, Okamoto M, Ozeki S, Okabe K. Oral squamous cell carcinoma cells induce osteoclast differentiation by suppression of osteoprotegerin expression in osteoblasts. *Int J Cancer.* 2005 Aug 20;116(2):253-62.

62. Tada T, Shin M, Fukushima H, Okabe K, Ozeki S, Okamoto M, et al. Oral squamous cell carcinoma cells modulate osteoclast function by RANKL-dependent and -independent mechanisms. *Cancer Lett.* 2009 Feb 8;274(1):126-31.
63. Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, et al. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell.* 2002 Dec;3(6):889-901.
64. Tiedemann K, Hussein O, Sadvakassova G, Guo Y, Siegel PM, Komarova SV. Breast cancer-derived factors stimulate osteoclastogenesis through the Ca<sup>2+</sup>/protein kinase C and transforming growth factor-beta/MAPK signaling pathways. *J Biol Chem.* 2009 Nov 27;284(48):33662-70.
65. Tsuda E, Goto M, Mochizuki S, Yano K, Kobayashi F, Morinaga T, et al. Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochem Biophys Res Commun.* 1997 May 8;234(1):137-42.
66. Tsue TT, McCulloch TM, Girod DA, Couper DJ, Weymuller EA, Jr., Glenn MG. Predictors of carcinomatous invasion of the mandible. *Head Neck.* 1994 Mar-Apr;16(2):116-26
67. Uchida D, Kawamata H, Omotehara F, Nakashiro K, Kimura-Yanagawa T, Hino S, et al. Role of HGF/c-met system in invasion and metastasis of oral squamous cell carcinoma cells in vitro and its clinical significance. *Int J Cancer.* 2001 Aug 15;93(4):489-96.
68. Weitkunat R, Sanders E, Lee PN. Meta-analysis of the relation between European and American smokeless tobacco and oral cancer. *BMC Public Health.* 2007;7:334.

69. Werning JW, Byers RM, Novas MA, Roberts D. Preoperative assessment for and outcomes of mandibular conservation surgery. *Head Neck*. 2001 Dec;23(12):1024-3
70. Wong DT. TGF-alpha and oral carcinogenesis. *Eur J Cancer B Oral Oncol*. 1993 Jan;29B(1):3-7.
71. Wong RJ, Keel SB, Glynn RJ, Varvares MA. Histological pattern of mandibular invasion by oral squamous cell carcinoma. *Laryngoscope*. 2000 Jan;110(1):65-72.
72. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A*. 1998 Mar 31;95(7):3597-602
73. Ziober BL, Turner MA, Palefsky JM, Banda MJ, Kramer RH. Type I collagen degradation by invasive oral squamous cell carcinoma. *Oral Oncol*. 2000 Jul;36(4):365-72.